Patógenos entéricos en camarones peneidos
Principal amenaza sanitaria en la industria del camarón en Latinoamérica

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OIE Expert at the OIE Reference Laboratory for *H. penaei* (NHP)

Marzo 22 del 2022

Aquaculture Pathology Laboratory
OIE Reference Laboratory
USDA-APHIS Approved & ISO 17025, 17043 Accredited Laboratory
School of Animal & Comparative Biomedical Science
The University of Arizona, Tucson, Arizona, USA

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Agenda:

- Hora Tema
- 9:00 AM Introduccion de enfermedades de camaron y signos clinicos
- 10:00 AM Sindrome de la mortalidad temprana (EMS/AHPND)
- 11:00 AM Hepatopancreatitis necrotizante (NHP)
- 12:00 PM Microsporidiosis, Enterocytozoon hepatopenaei (EHP)
- 1:00 PM Almuerzo
- 2:00 PM Practica: Toma de muestra para analisis de laboratorio
- 3:00 PM Practica: Analisis en fresco: hepatopancreas, branquias, contenido intestinal, H&E
- 4:00 PM Practica: Analisis en fresco: hepatopancreas, branquias, contenido intestinal, H&E
- 5:00 PM Bioseguridad en el cultivo de camaron
- 6:00 PM Clausura

2

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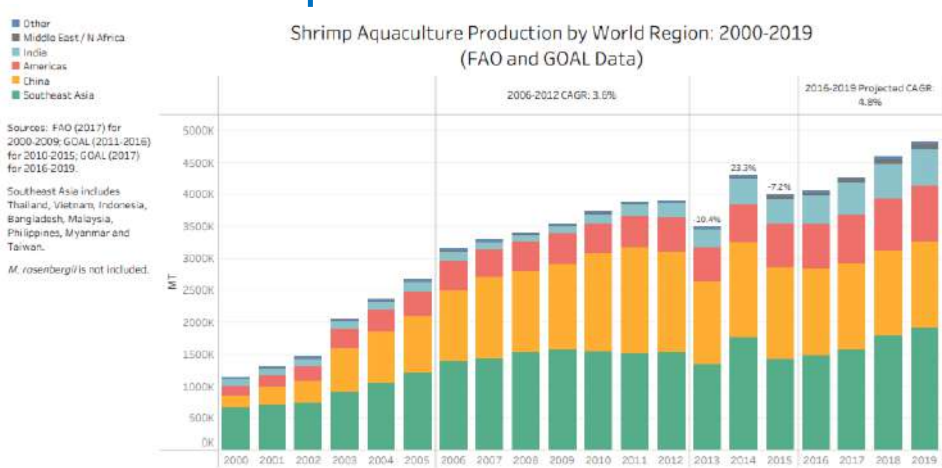
Introduction
Gross Anatomy and Normal Histology of Penaeid Shrimp

3

Global Trends in Shrimp Aquaculture



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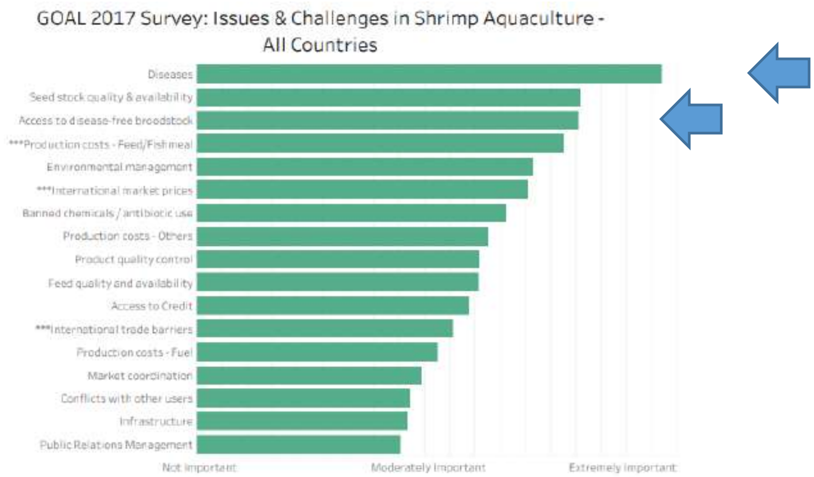

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What is a disease?

- ***Any adverse alteration in the health or culture performance of individuals or populations of shrimp.***

Types of Diseases:

Infectious - caused by transmittable pathogens:

viruses	rickettsia
bacteria	protozoa
fungi	metazoan parasites

Noninfectious diseases - non-transmittable agents:

nutritional	environmental/physical
toxic	neoplastic
genetic	

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OIE-Listed Crustacean Pathogens-2022

https://www.oie.int/en/what-we-do/standards/codes-and-manuals/aquatic-code-online-access/?id=169&L=1&htmfile=chapitre_diseases_listed.htm

Viral Diseases

•Infectious hypodermal and haematopoietic necrosis
•Infectious myonecrosis
•Taura syndrome
•White spot disease
•White tail disease
•Yellow head virus
•Infection with decapod iridescent virus 1

Bacterial Diseases

Acute hepatopancreatic necrosis disease
Necrotising hepatopancreatitis (Hepatobacter penaei)

Fungal Diseases

Crayfish plague (*Aphanomyces astaci*)

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Estimated losses caused by OIE-listed shrimp virus diseases since their emergence

Virus – region	Year of emergence	Product loss to industry
IHHNV – Americas[a]	1981	$0.5–1 billion
YHV – Asia	1991	$0.5 billion
TSV – Americas	1991/92	$1–2 billion
TSV – Asia	1999	$0.5–1 billion
WSSV – Asia	1992/93	$6 billion
WSSV – Americas	1999	$1–2 billion
IMNV – Americas	2004	$100–200 million
IMNV – Asia	2006	$1 billion (estimated)

Lightner et al., 2012. J. Inv. Pathol. 110: 174-183

***Acute Hepatopancreatic Necrosis Disease (AHPND)/ Early mortality syndrome (EMS), resulted ~$7.5 billion losses in Southeast Asia and a loss of 100,000 jobs since 2011.**

*Davies & Shinn, 2016 ttps://www.undercurrentnews.com/2016/09/09/disease-has-cost-asia-shrimp-sector-over-20bn/

In 2018-2019, WSD and EHP have caused US$ 238.33 Million and US$ 567.62 M, respectively (Patil et al., 2021).

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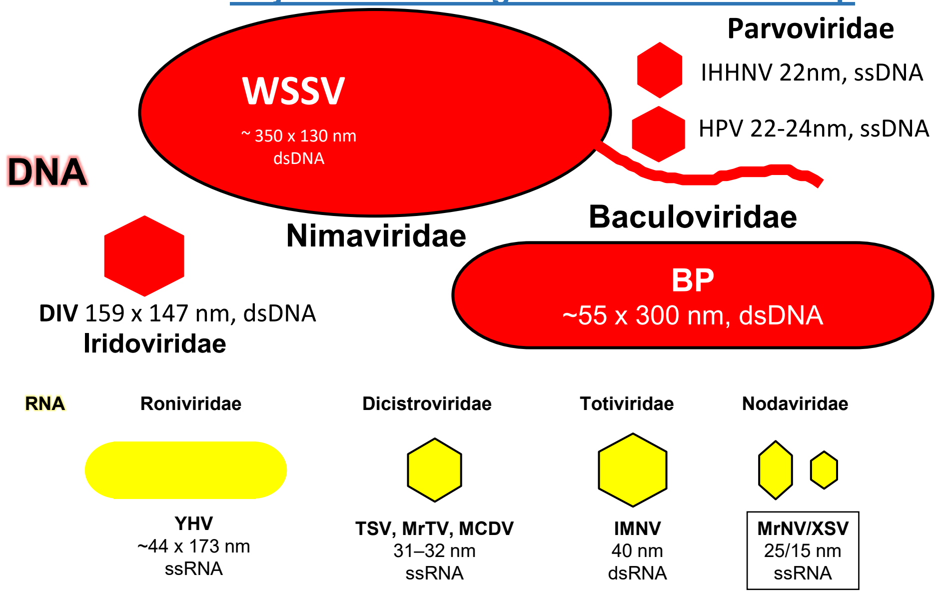
Major Viral Pathogens of Penaeid Shrimp



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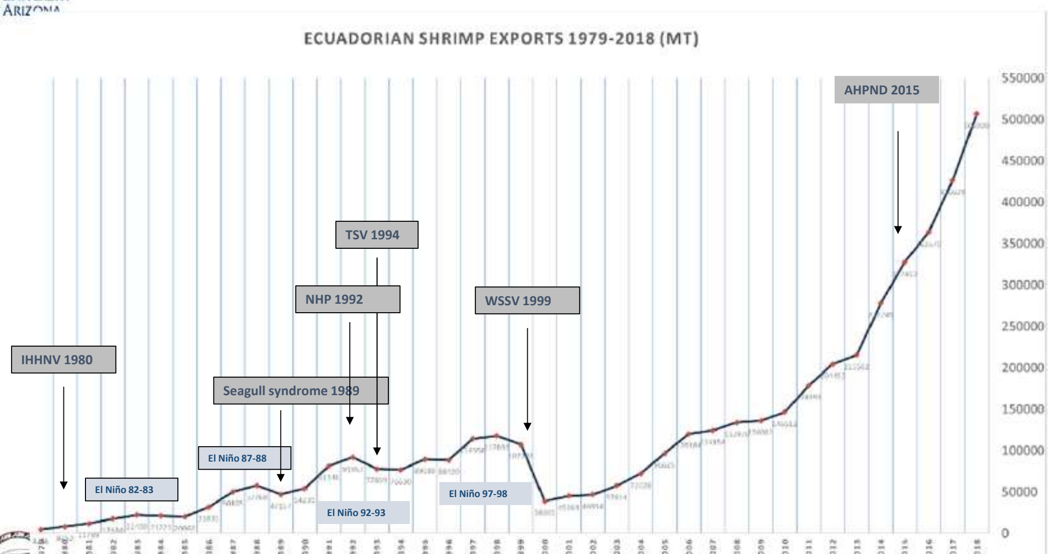
Pathogens and shrimp production



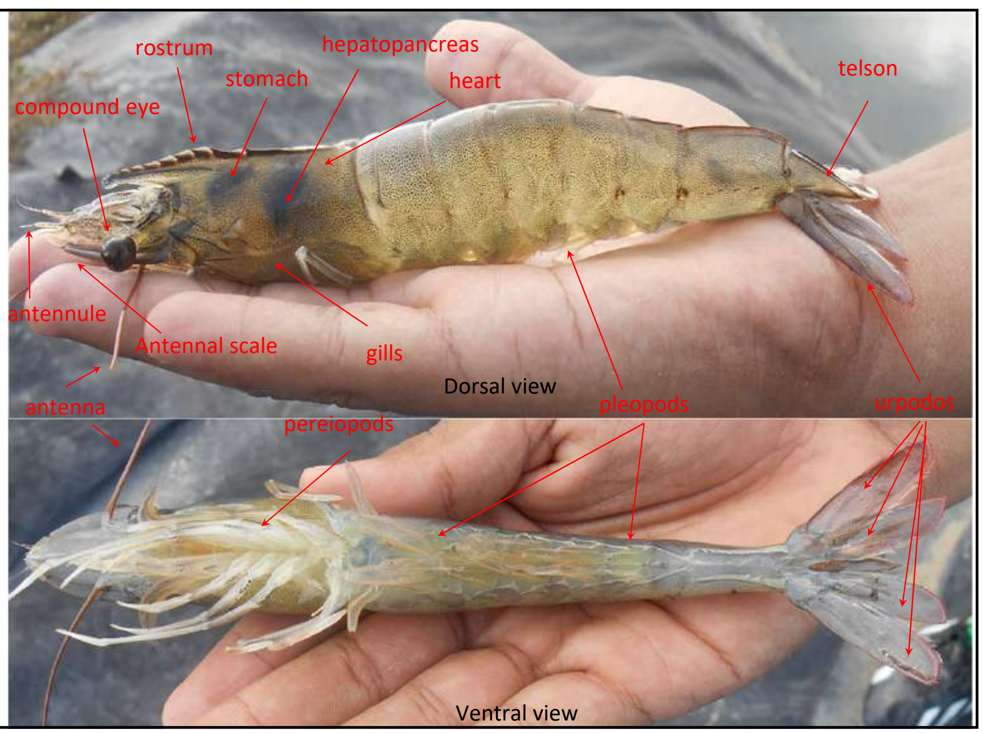
Year	Increase %
2001	14.24
2002	3.14
2003	18.71
2004	20.01
2005	25.46
2006	19.59
2007	3.21
2008	7.33
2009	1.54
2010	7.13
2011	17.87
2012	12.75
2013	5.15
2014	22.39
2015	15.17
2016	9.95
2017	14.78
2018	15.84

Adapted from Piedrahita et al., 2019

10


شرمب
أناتومي
SHRIMP
ANATOMY

11


rostrum
hepatopancreas
stomach
heart
telson
compound eye
antennule
Antennal scale
gills
antenna
Dorsal view
pleopods
urpodos
pereiopods
Ventral view

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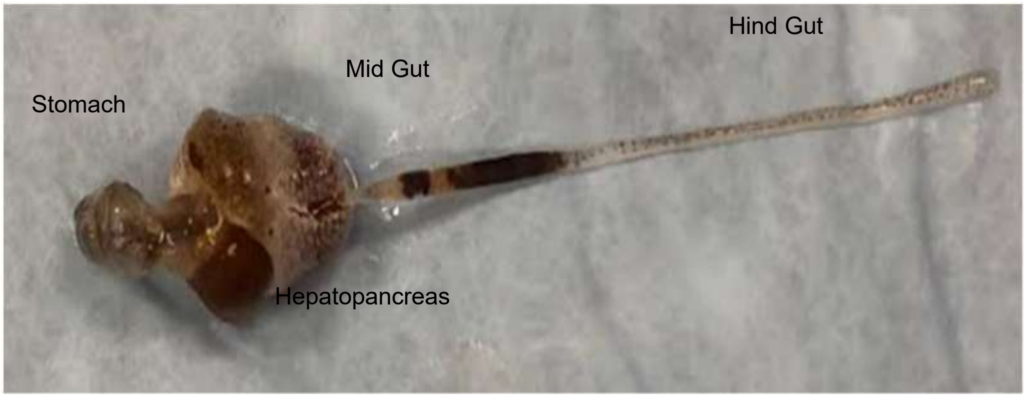
Anatomy



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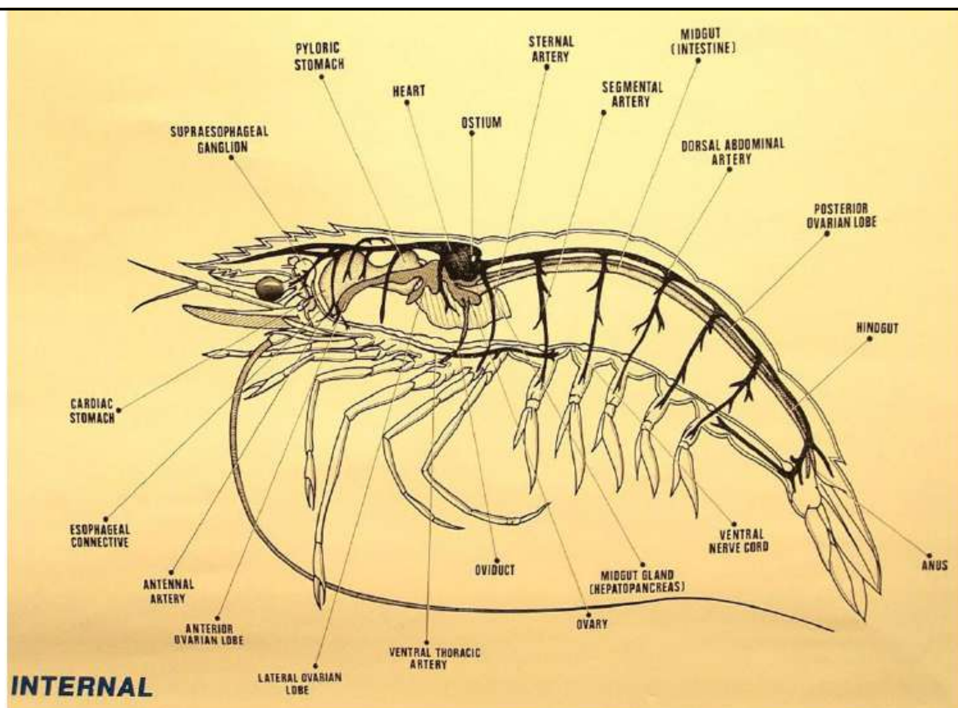

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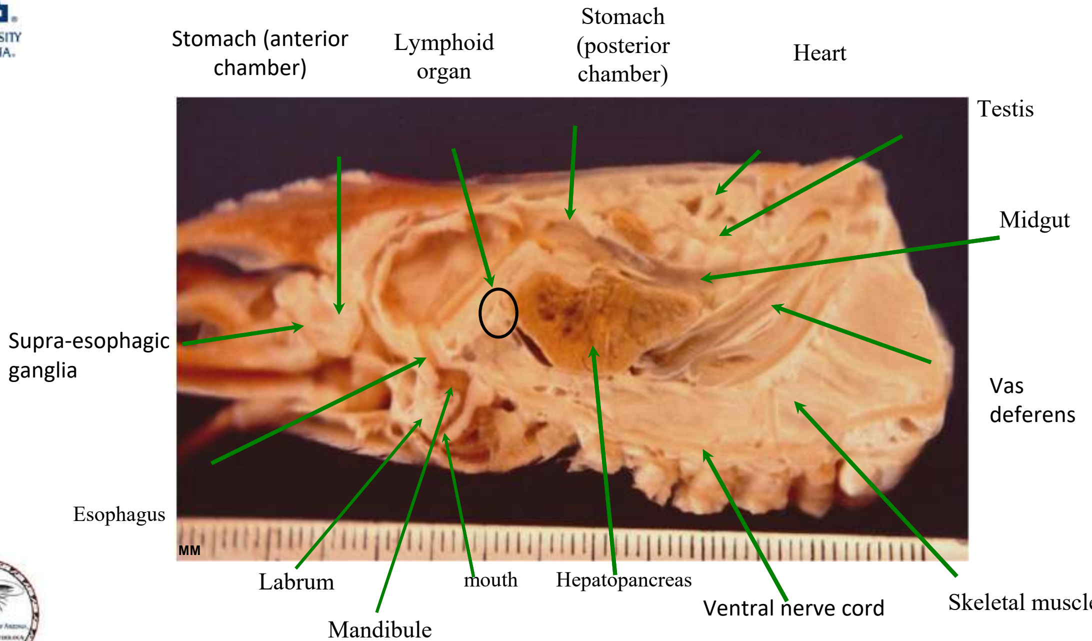
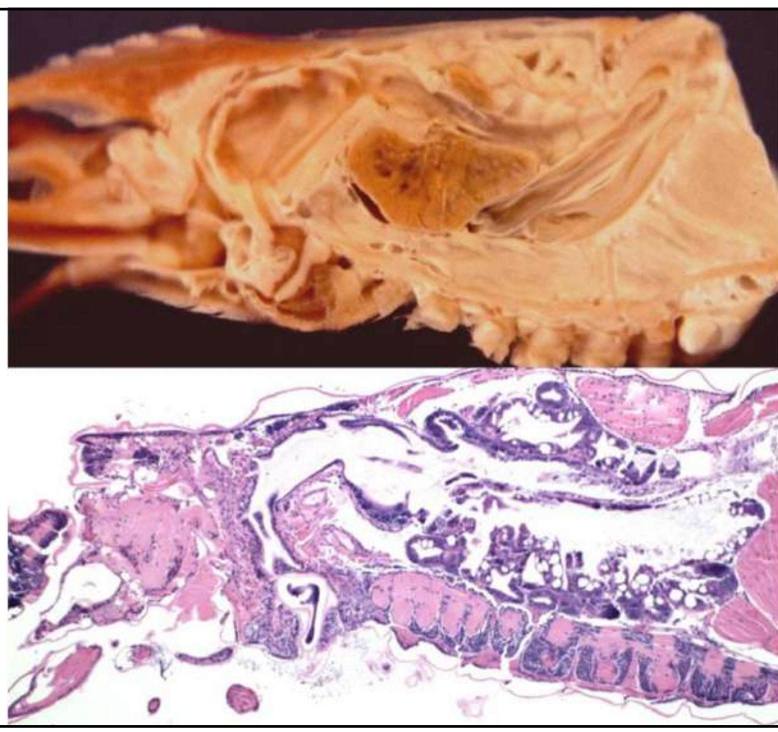

Stomach (anterior chamber)
Lymphoid organ
Stomach (posterior chamber)
Heart
Testis
Midgut
Supra-esophagic ganglia
Vas deferens
Esophagus
MM
Labrum
mouth
Hepatopancreas
Ventral nerve cord
Skeletal muscle
Mandibule

15



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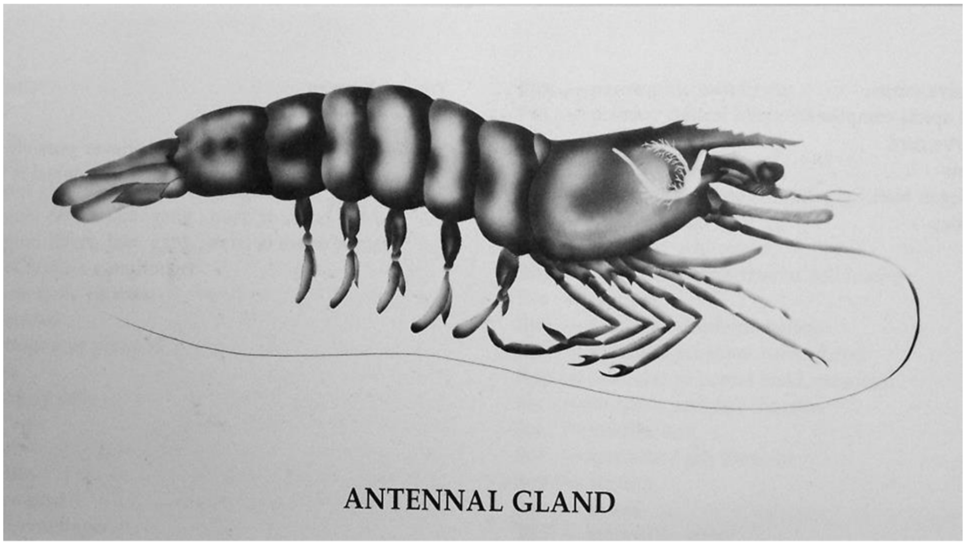

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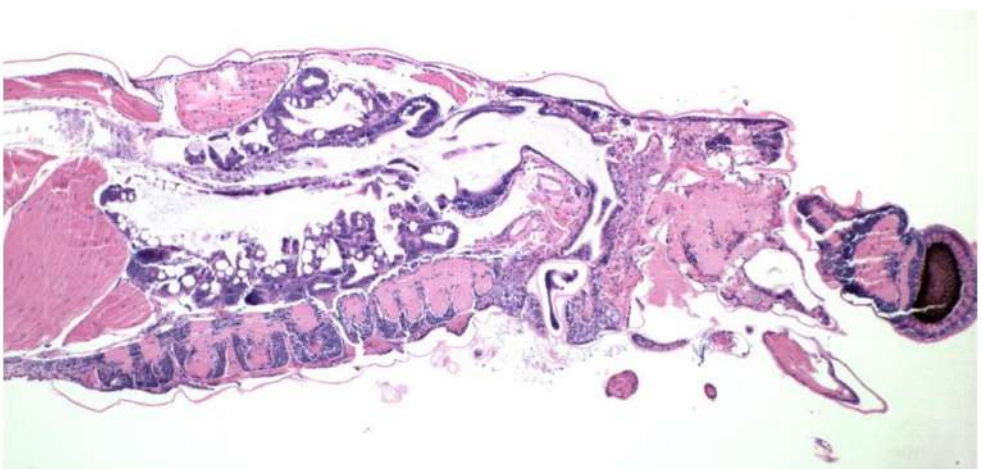

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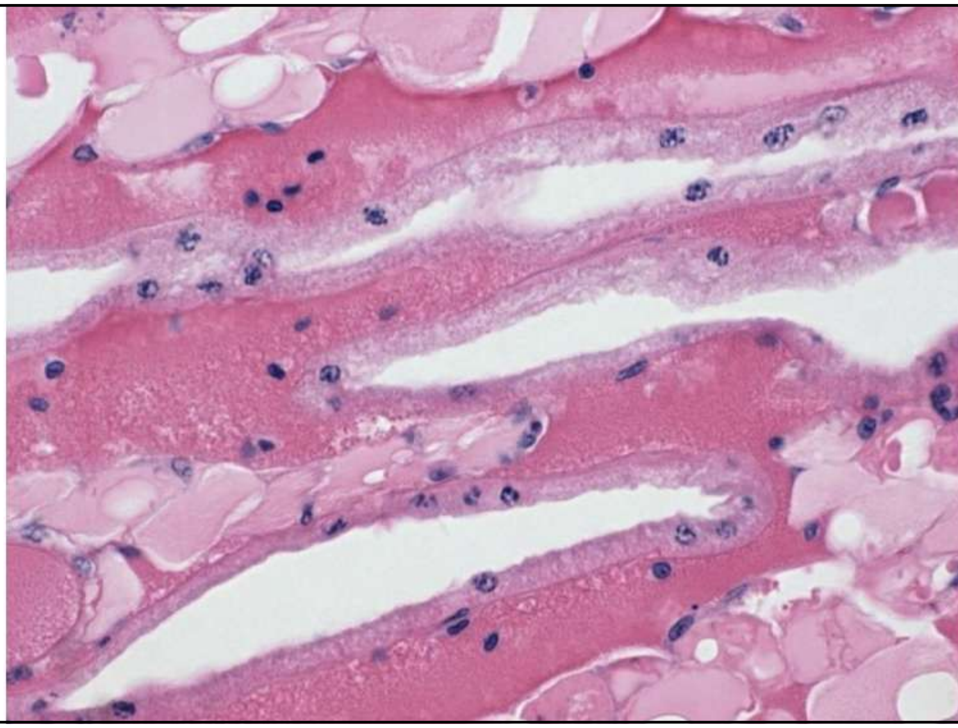

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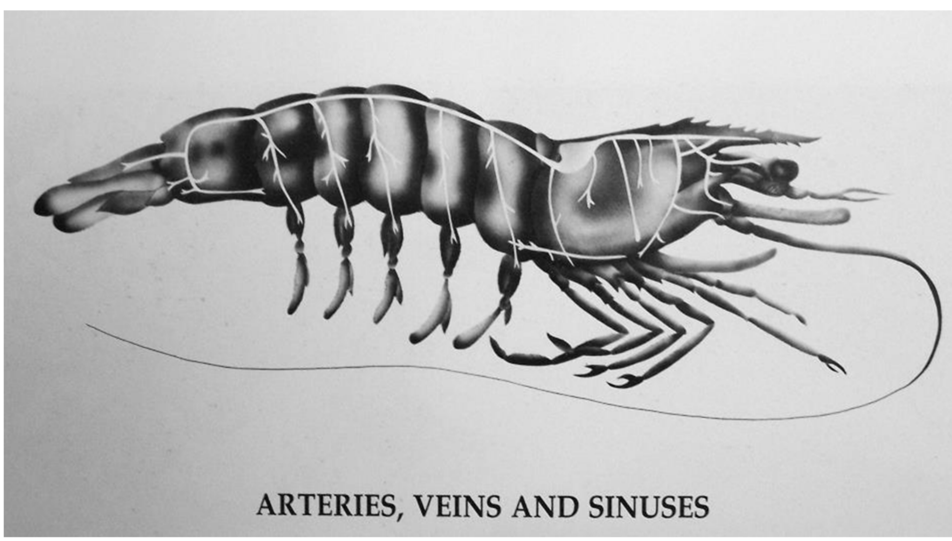

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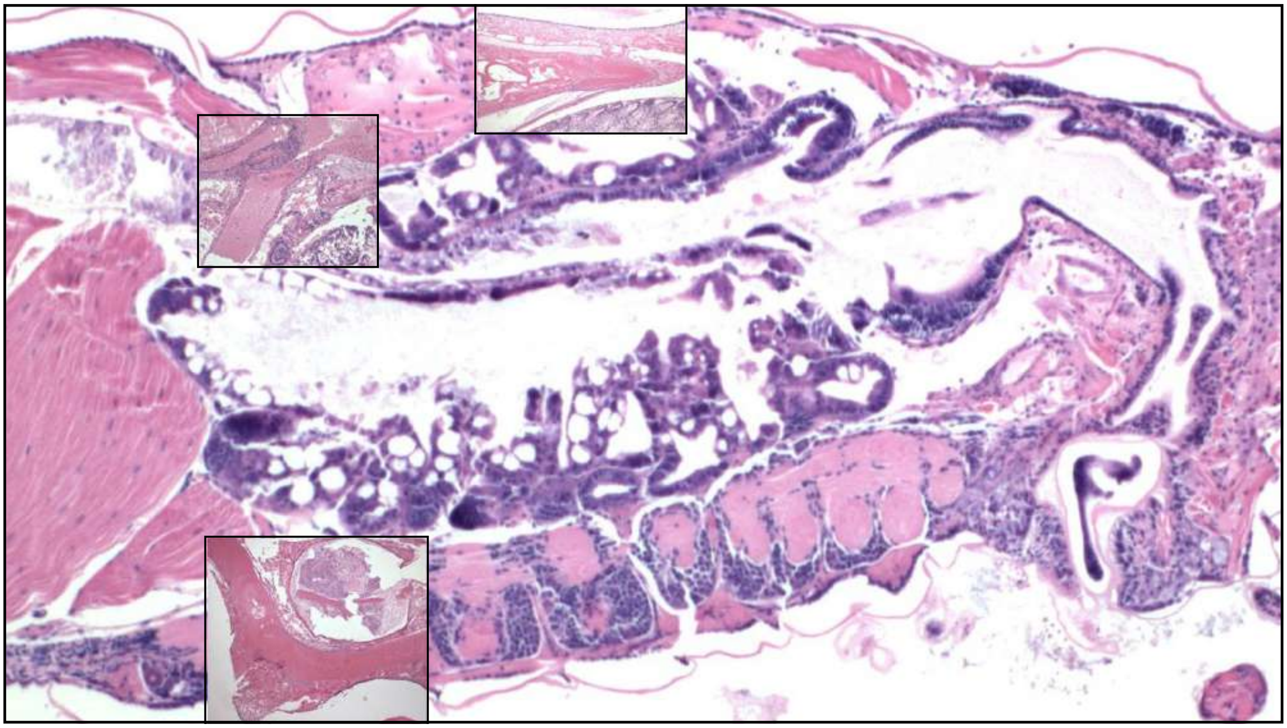

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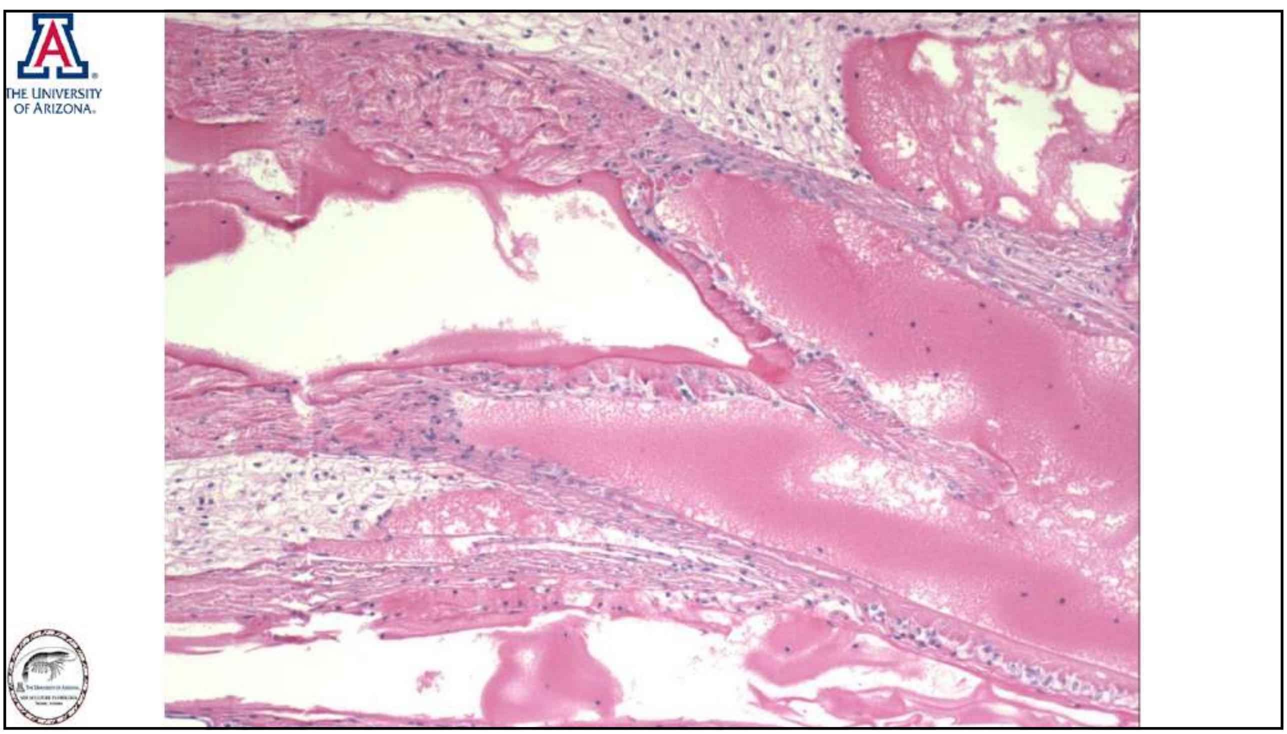

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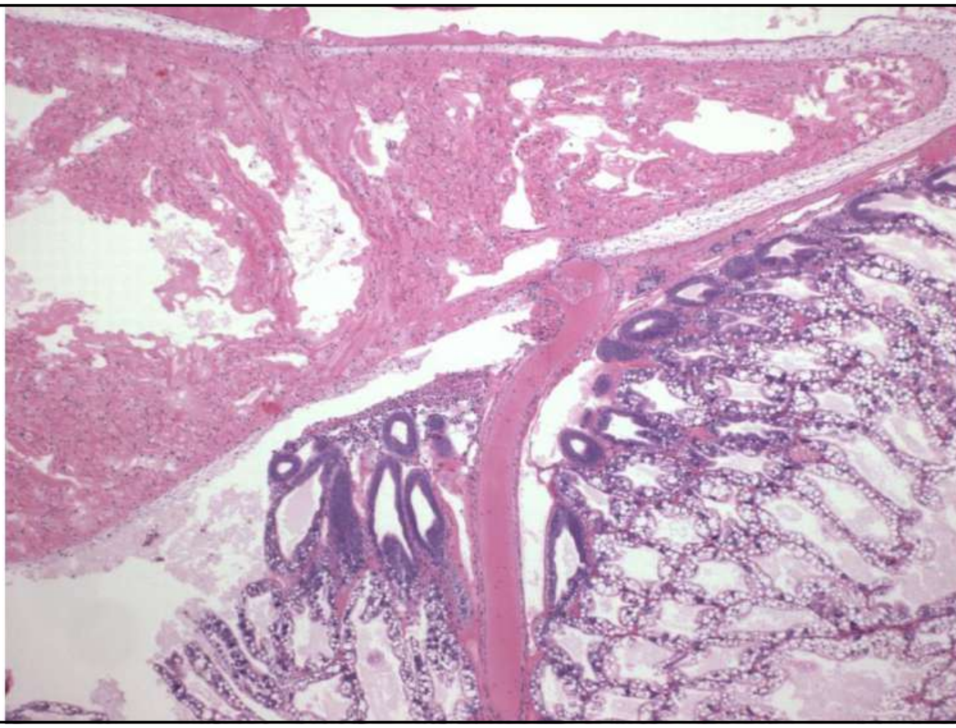

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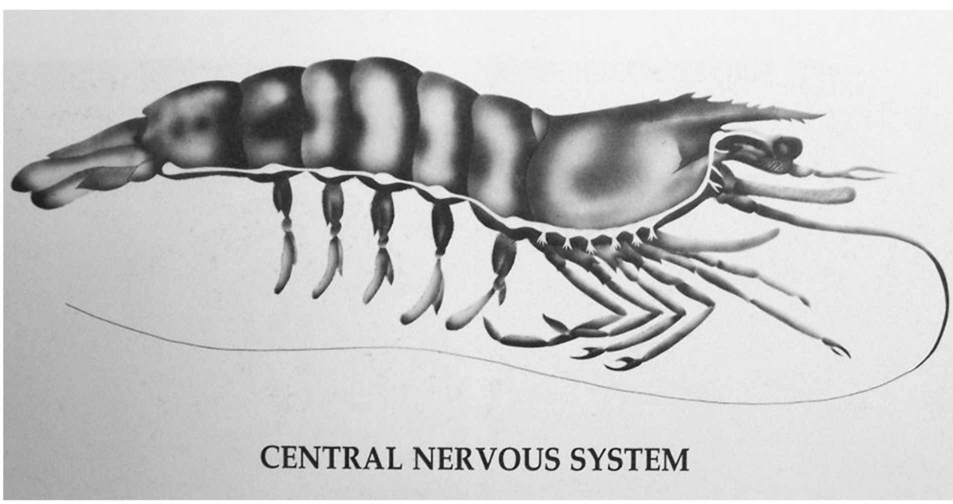

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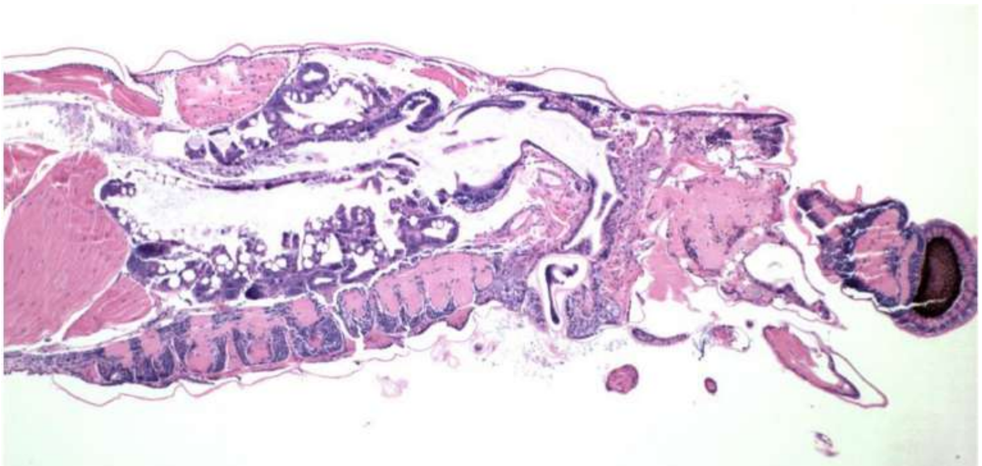

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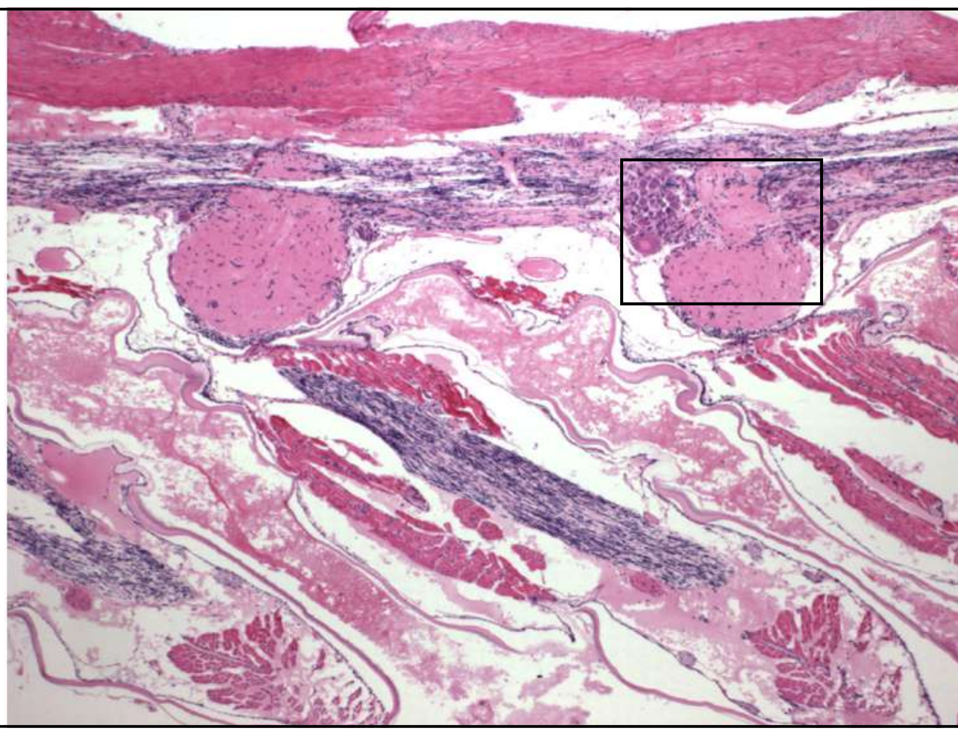

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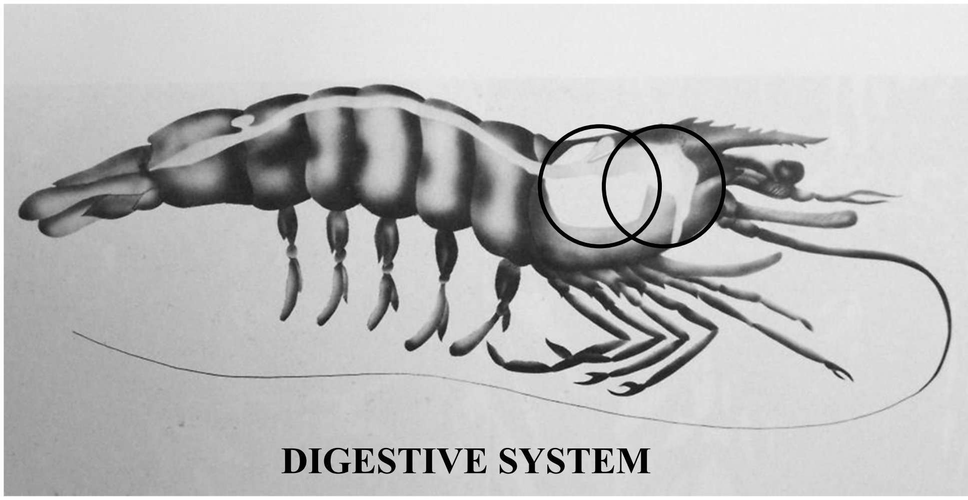

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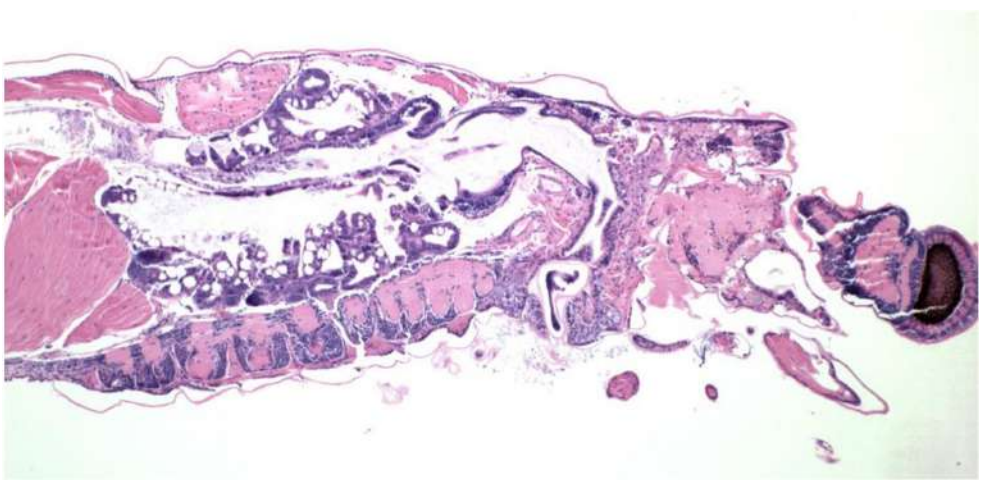

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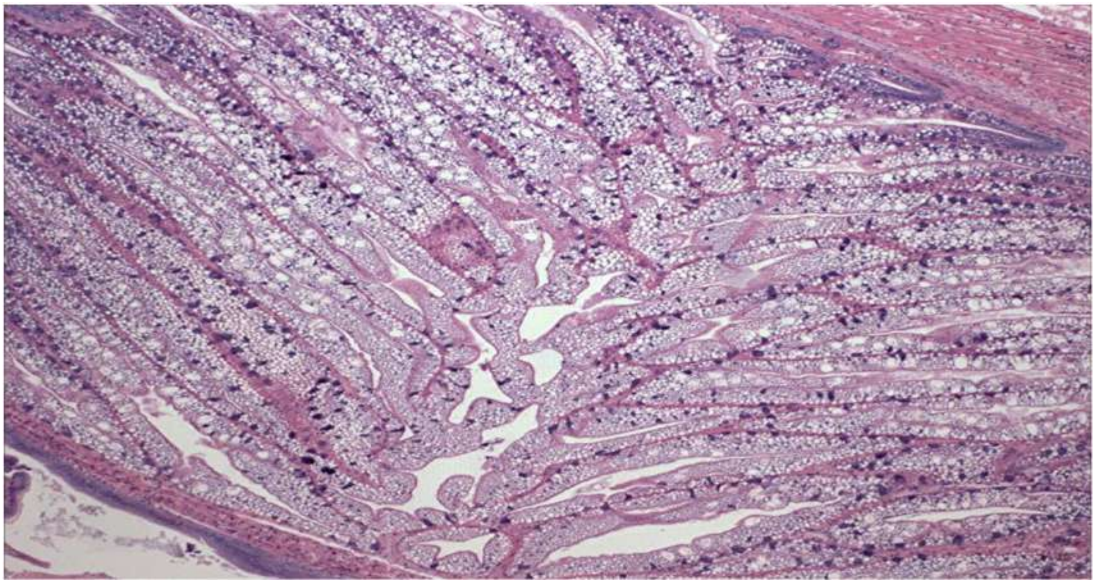

Normal hepatopancreas (HP)

29

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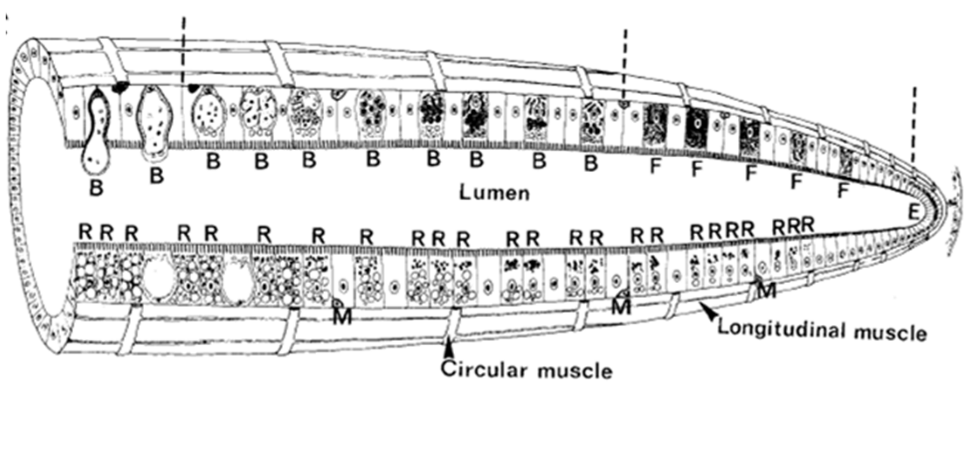

Al-Mohana & Nott. 1989. Functional cytology of the hepatopancreas of *Penaeus semisulcatus* (Crustacea: Decapoda) during the moulting cycle. Marine Biology (101) 535-544.

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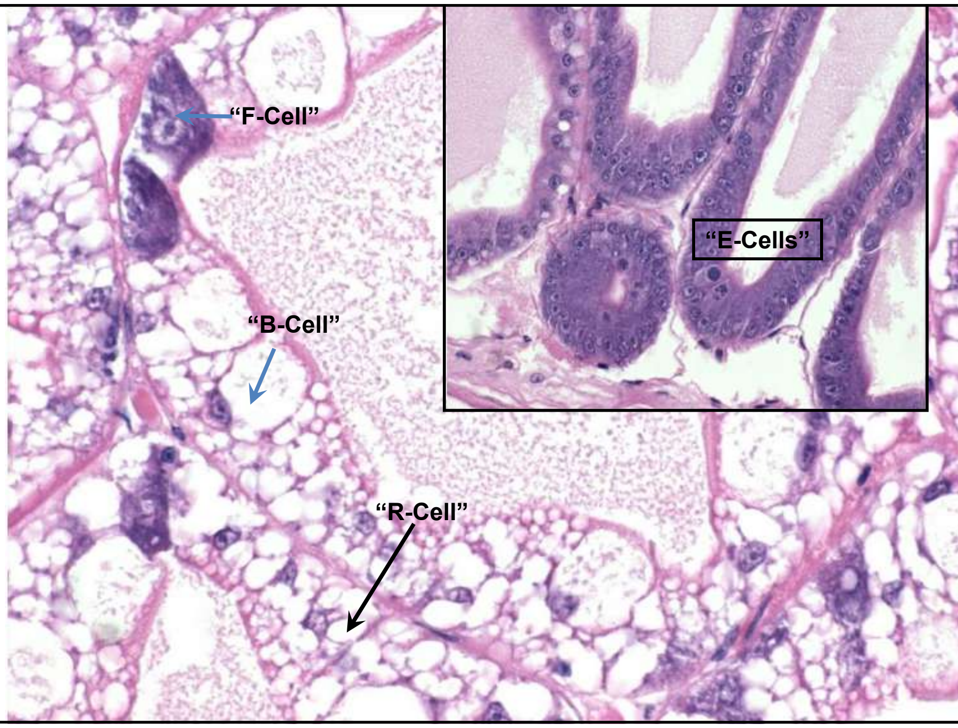

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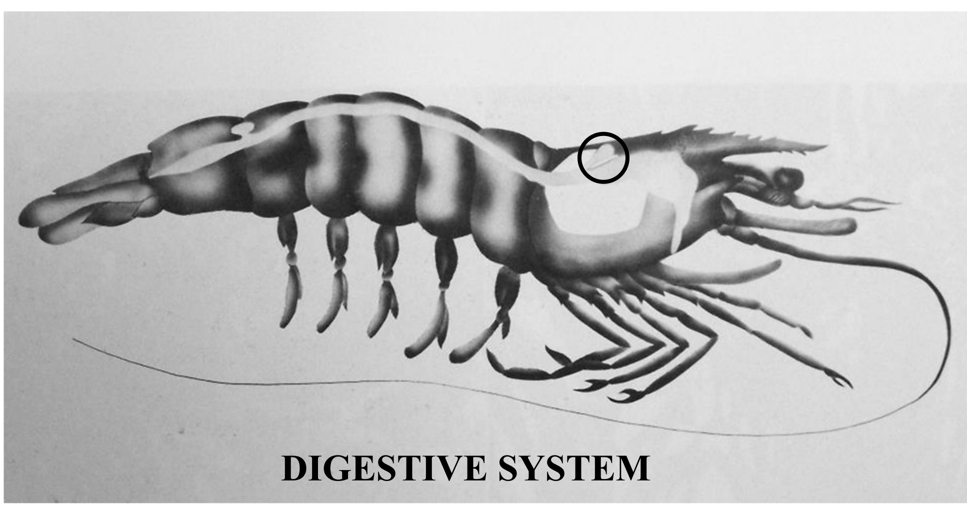

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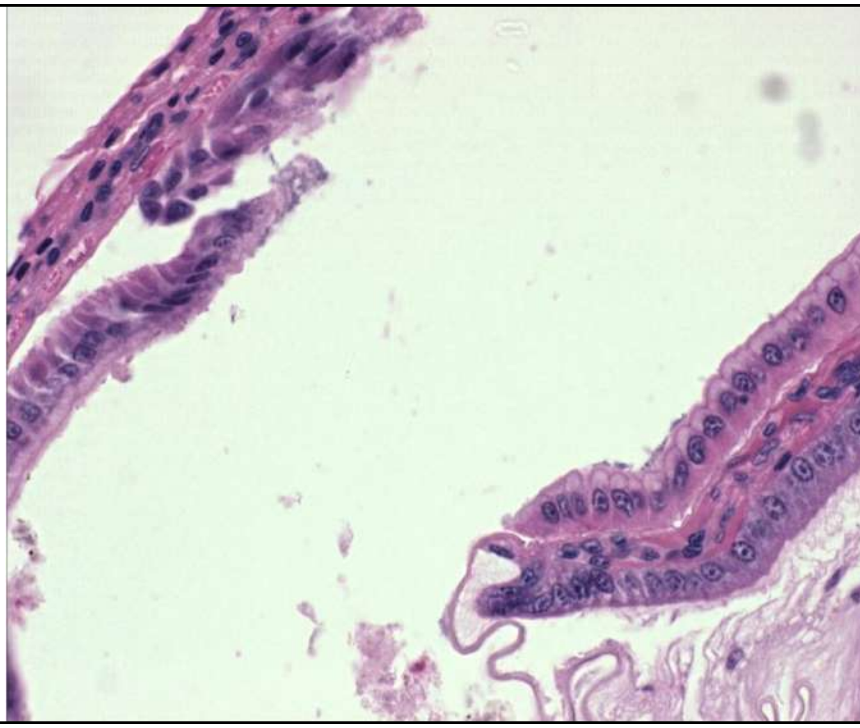

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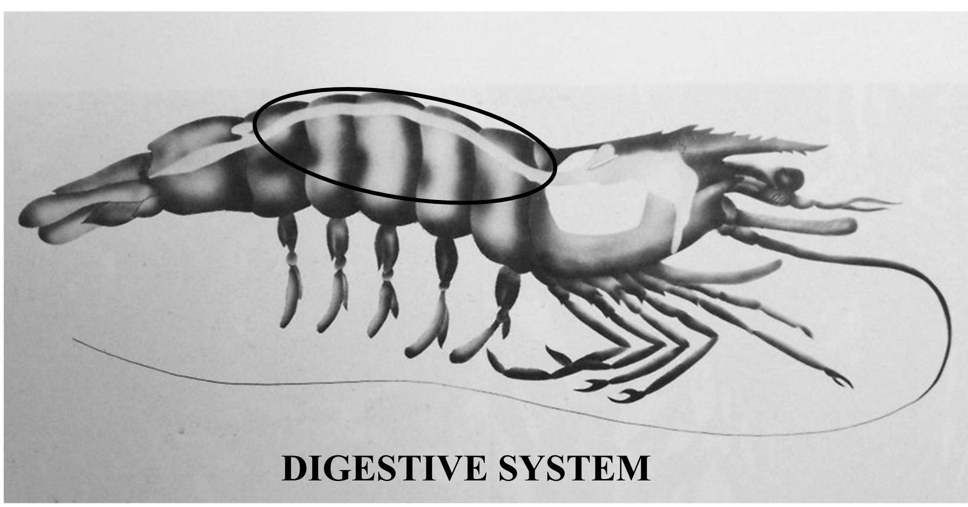

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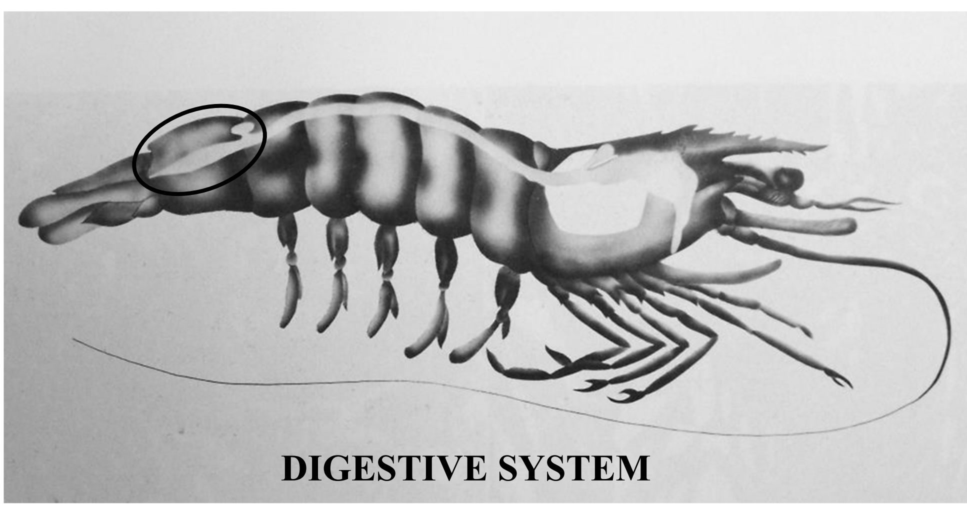

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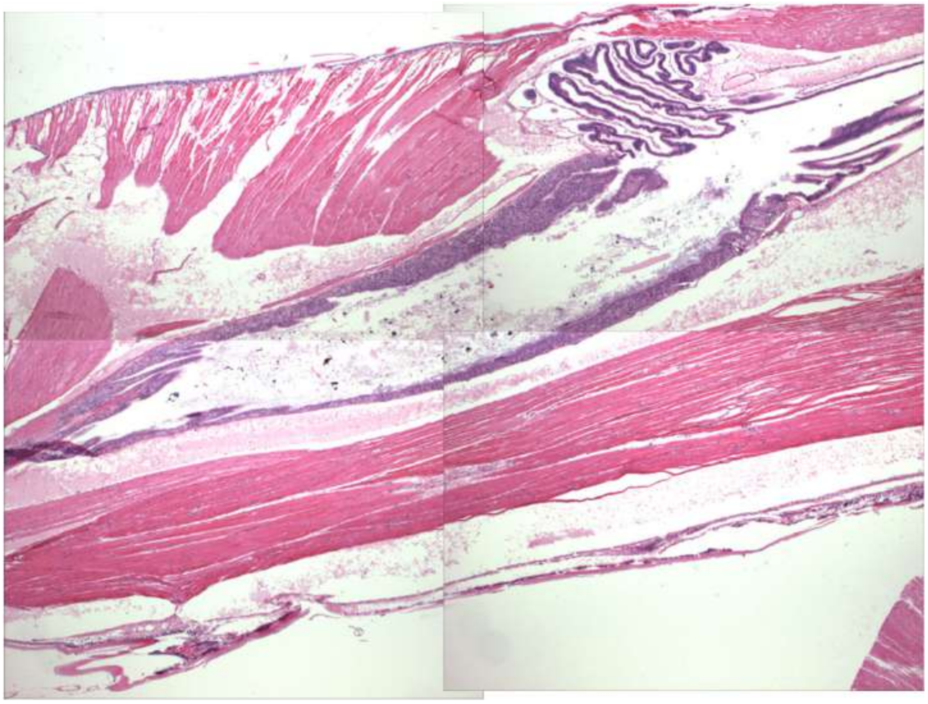

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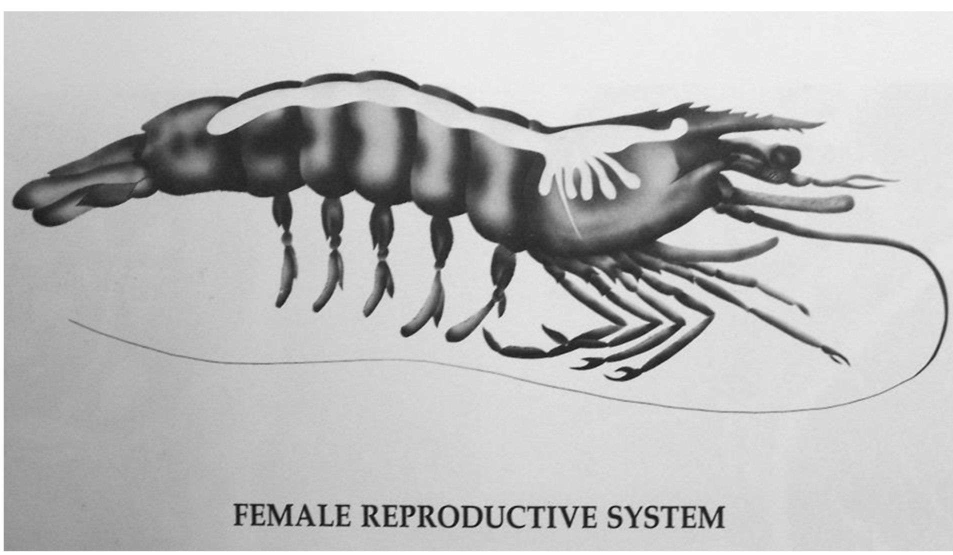

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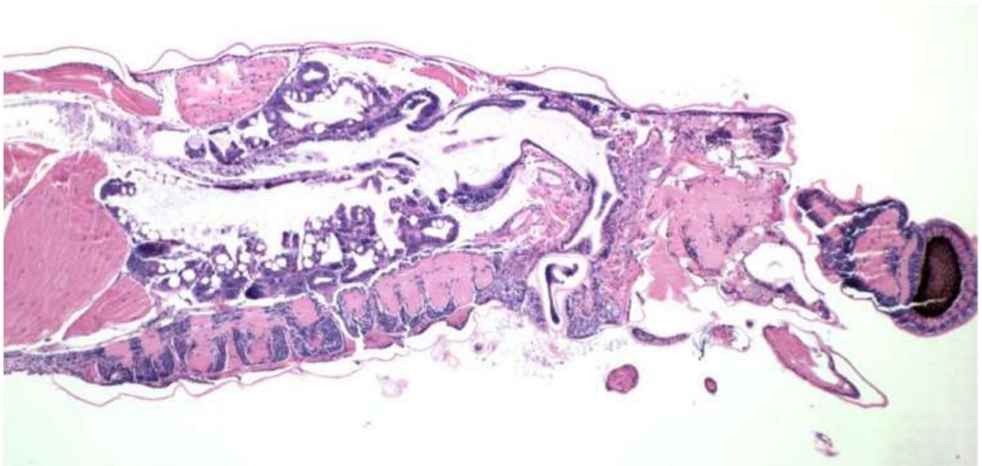

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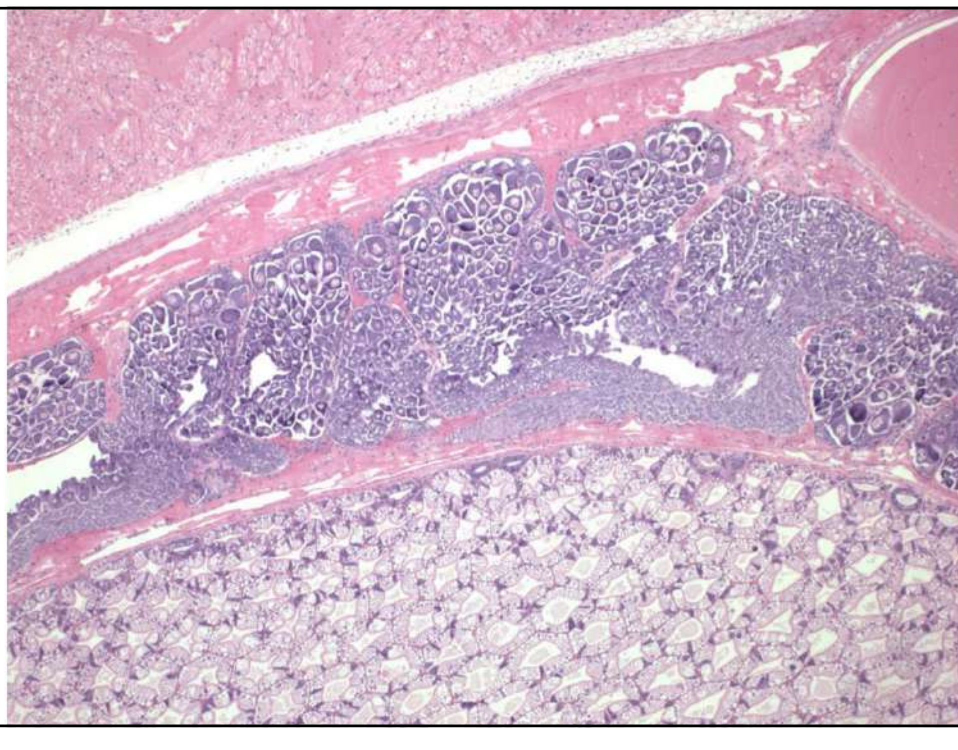

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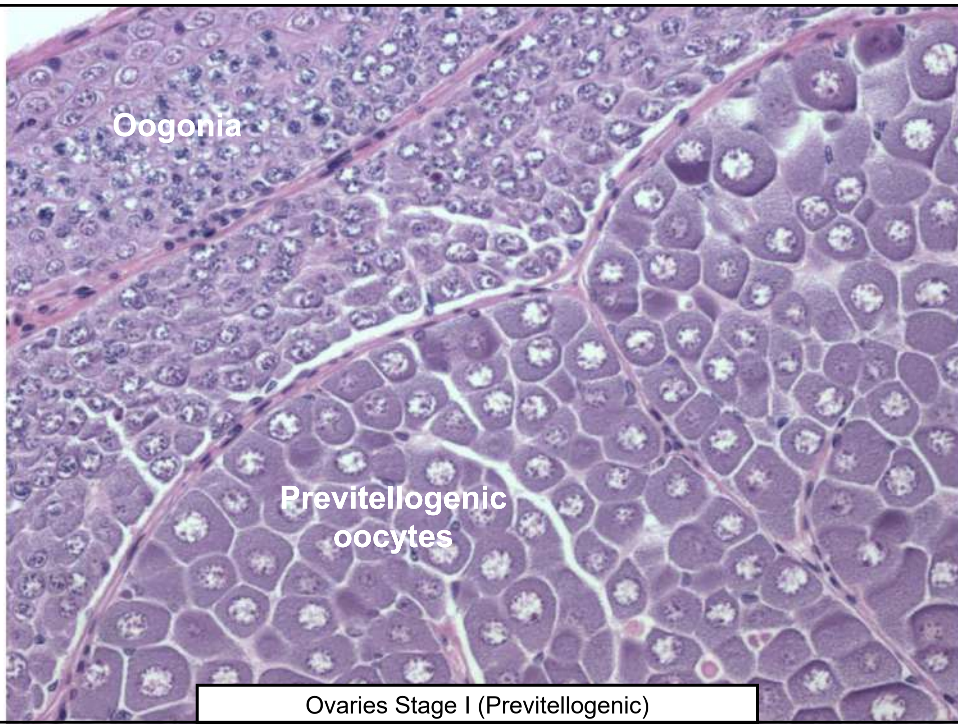

Ovaries Stage I (Previtellogenic)

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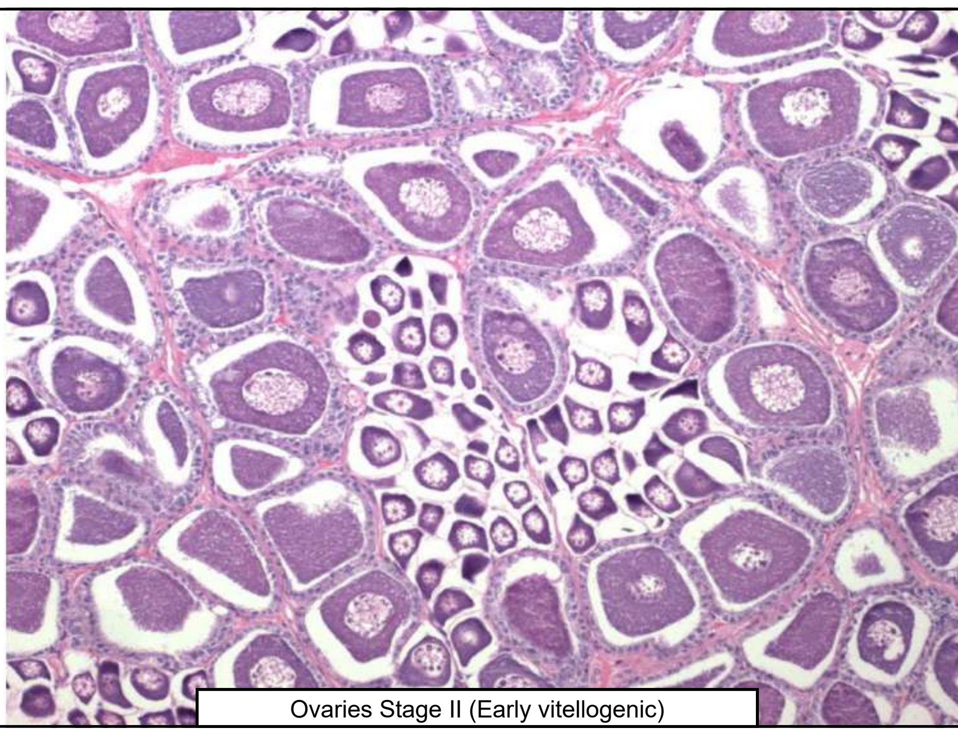

Ovaries Stage II (Early vitellogenic)

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Ovaries Stage III (Late vitellogenic)

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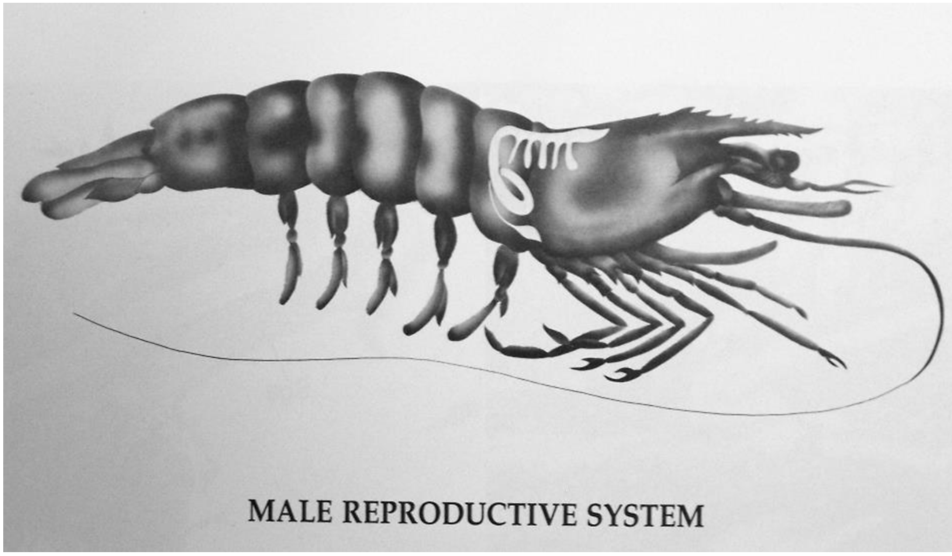
Ovaries Stage IV (Mature)

46



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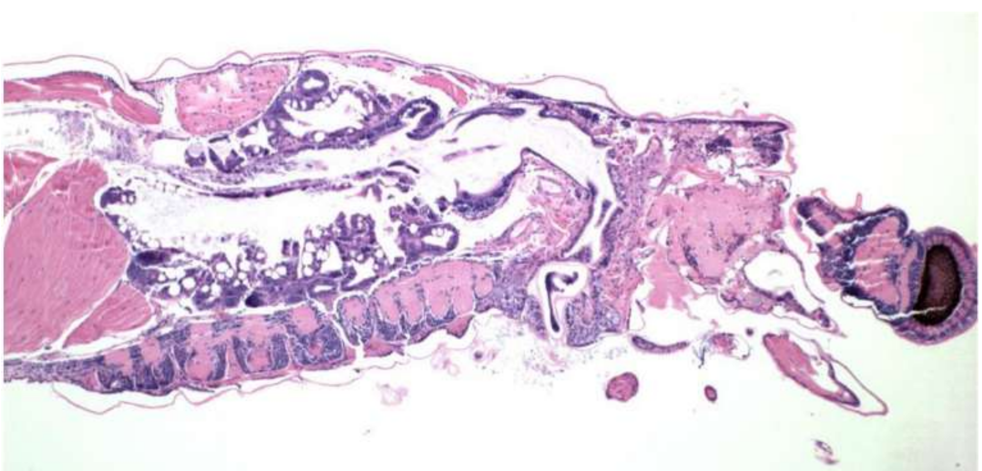

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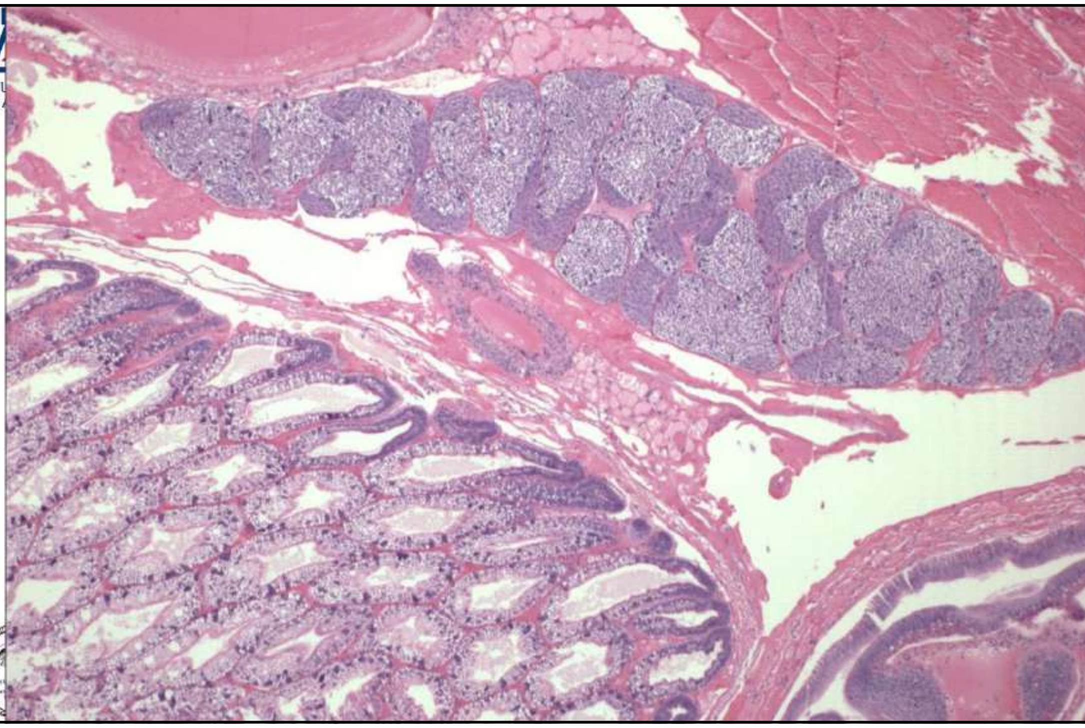

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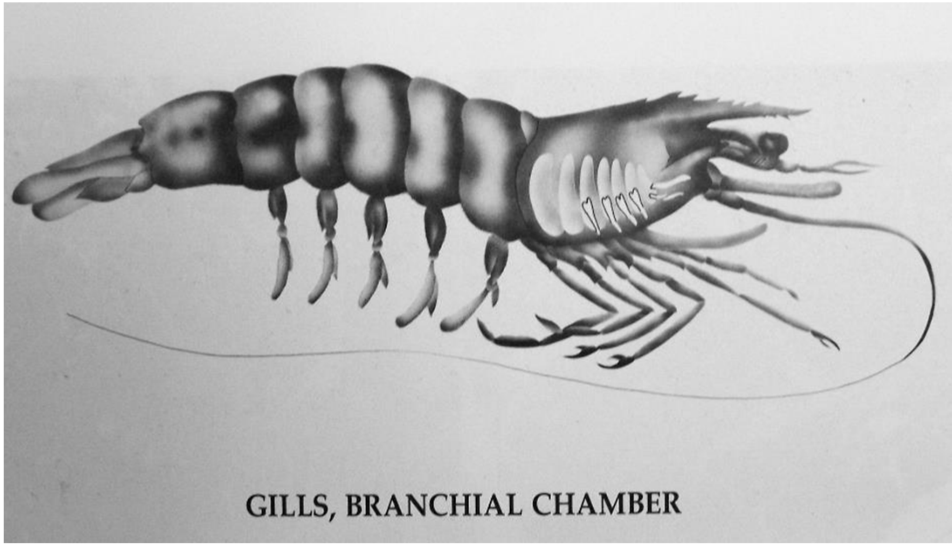

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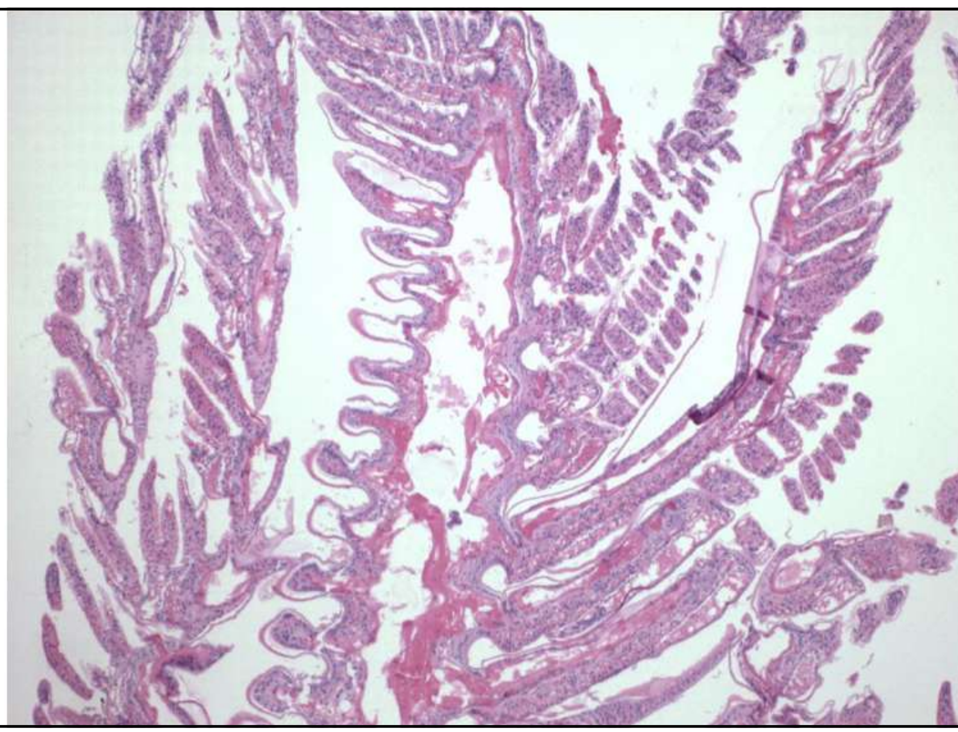

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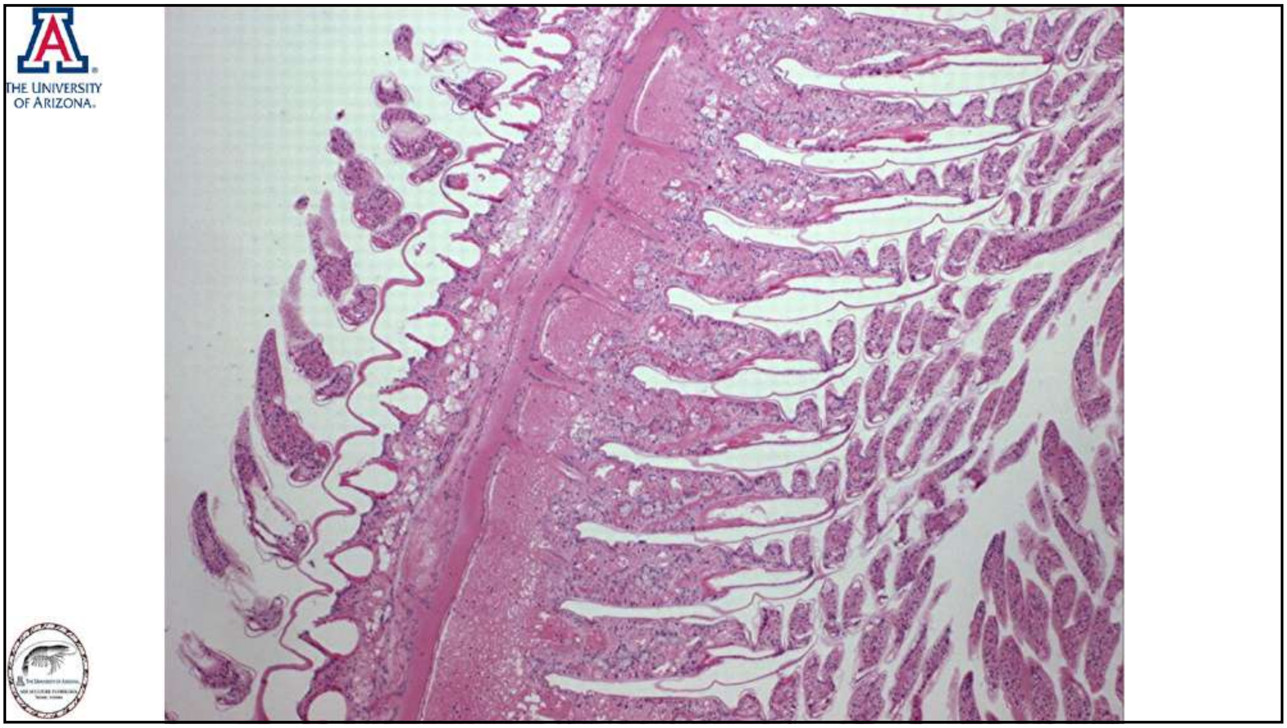

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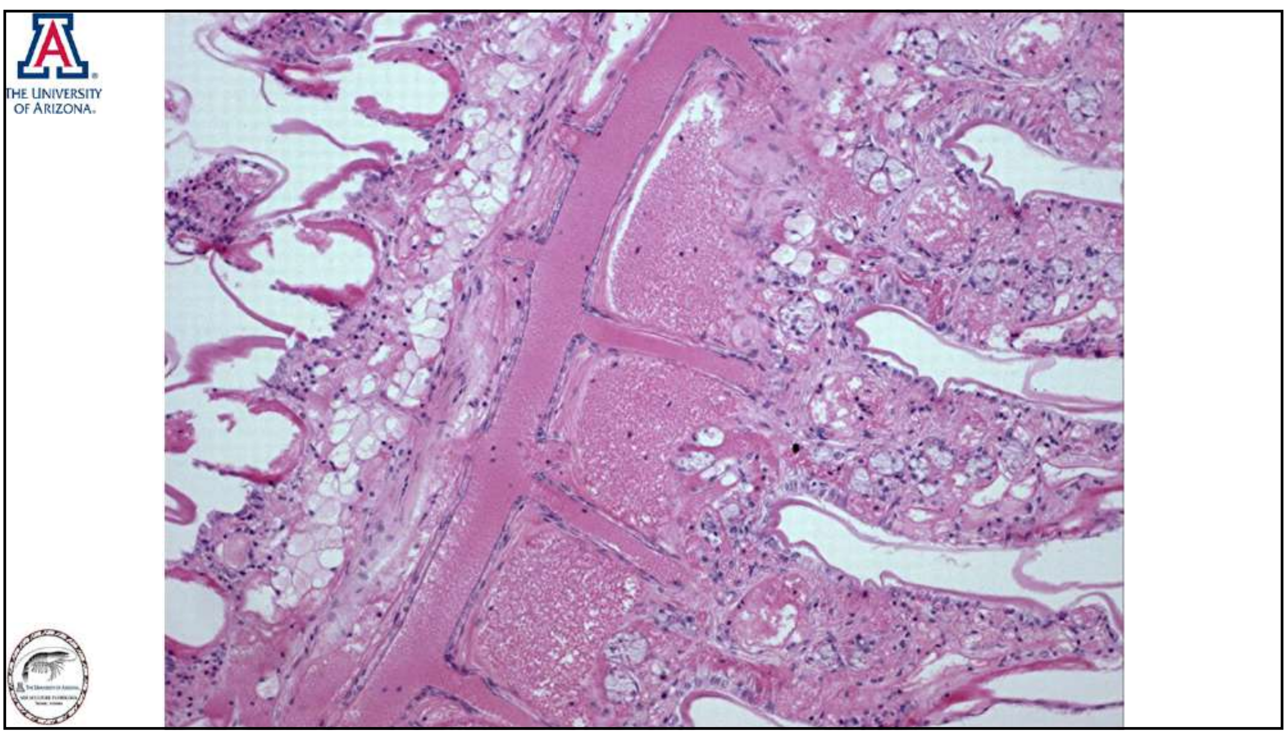

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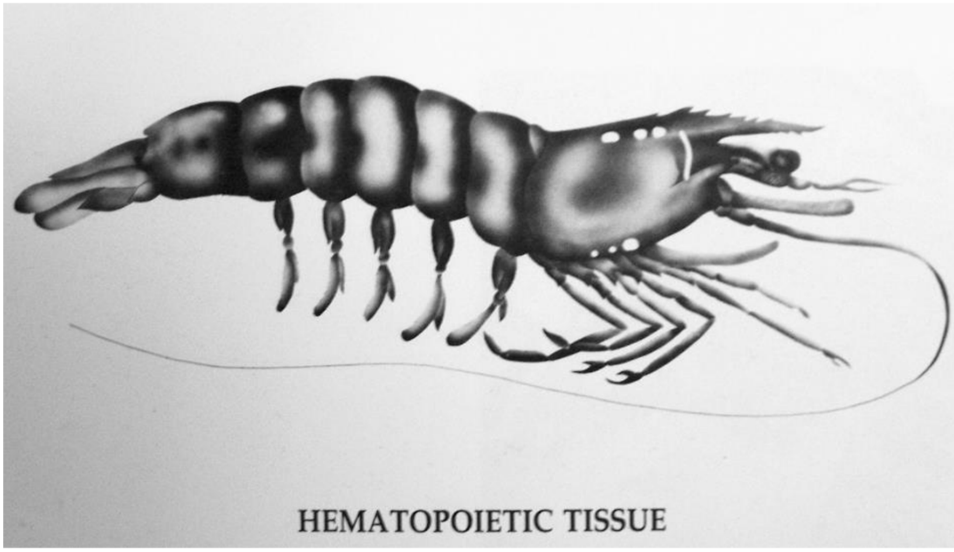

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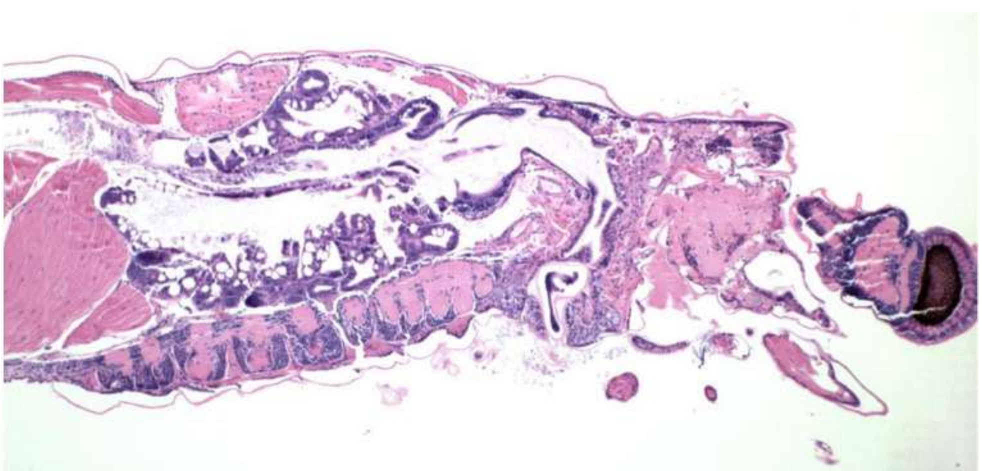

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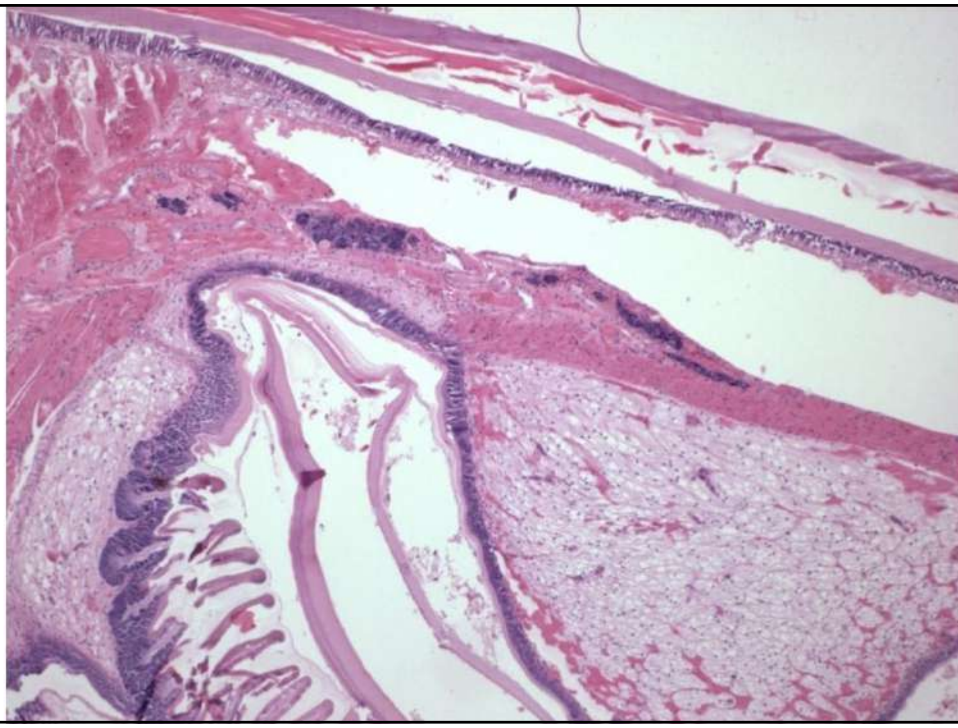

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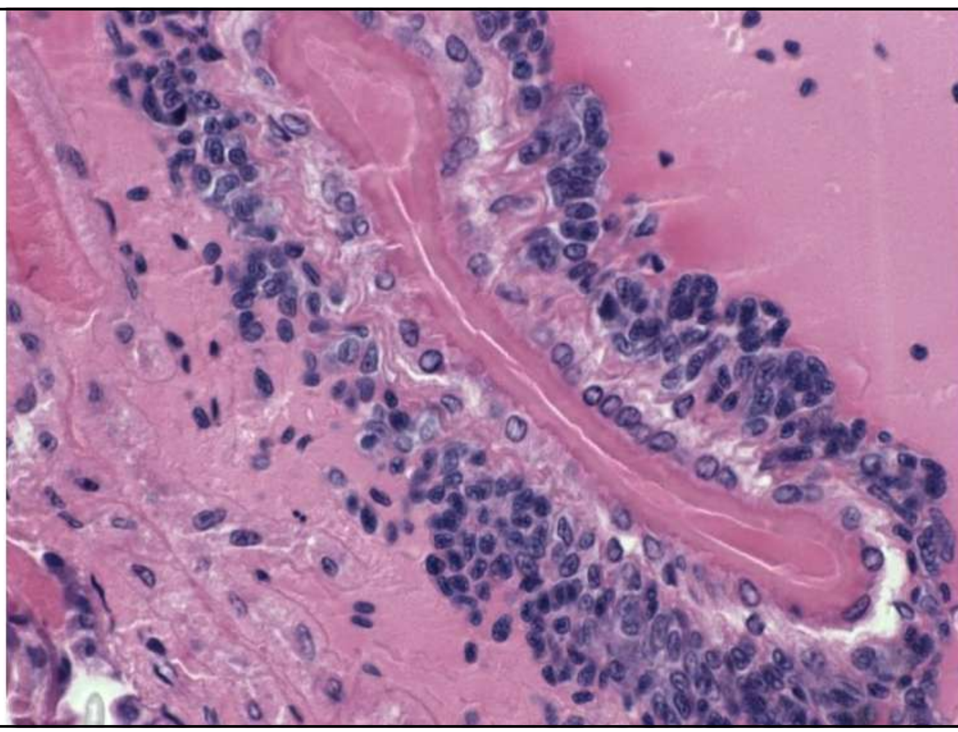

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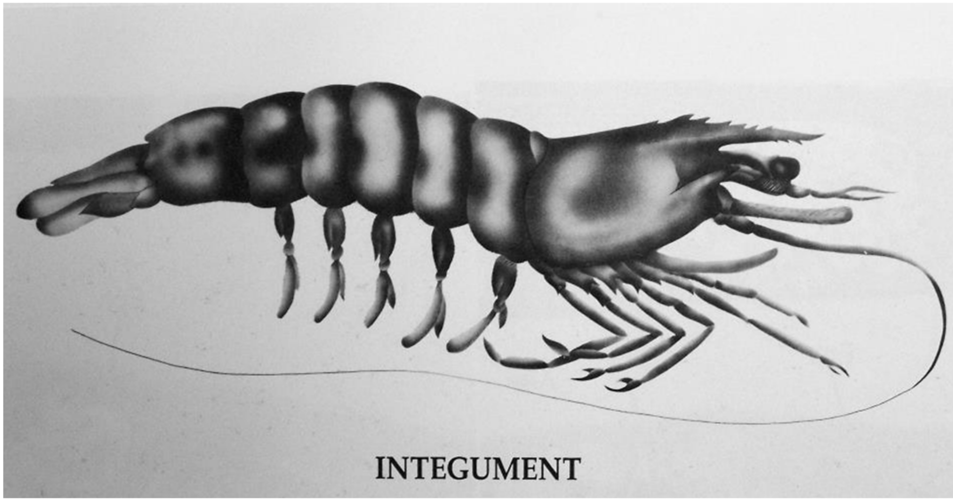
INTEGUMENT

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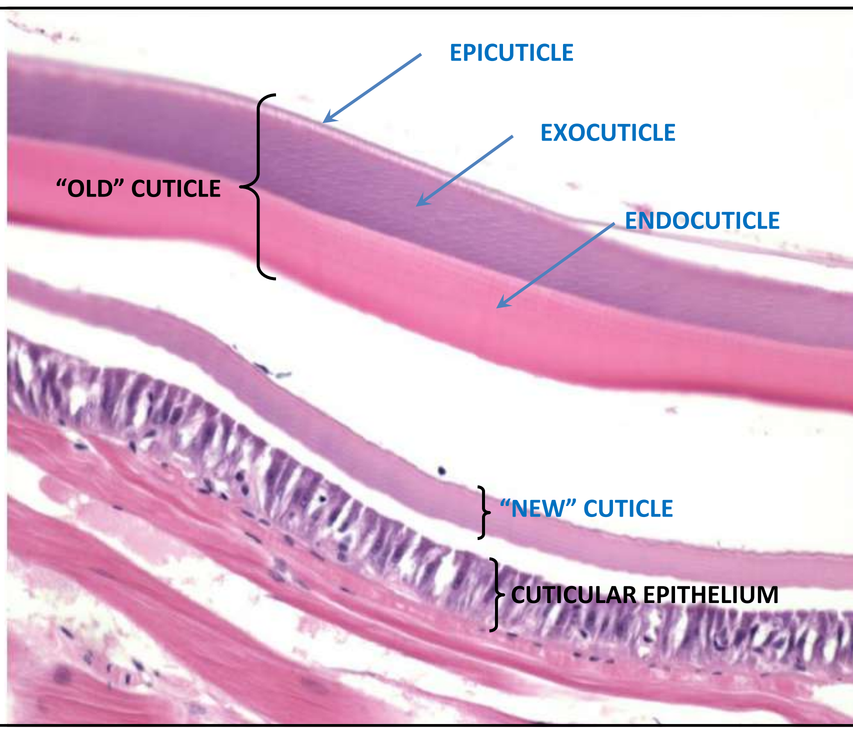
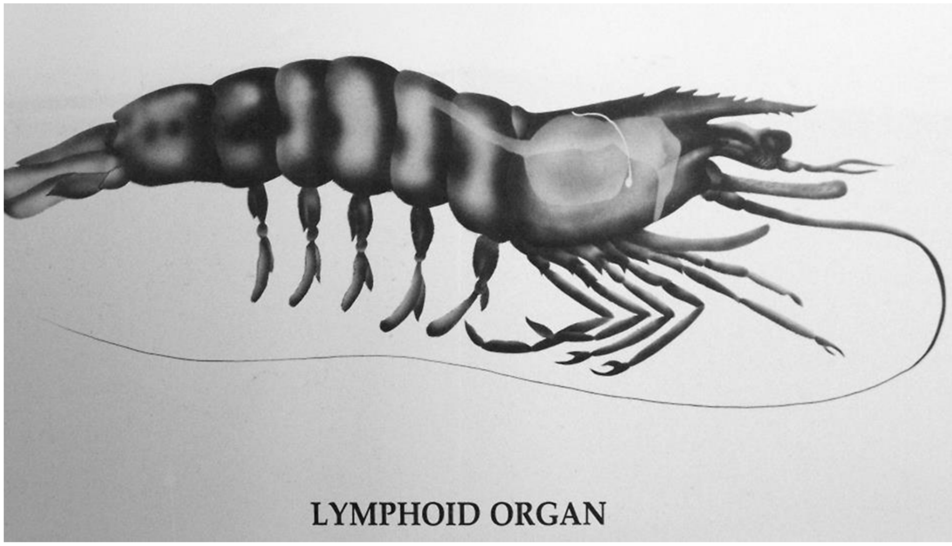

EPICUTICLE
EXOCUTICLE
"OLD" CUTICLE
ENDOCUTICLE
"NEW" CUTICLE
CUTICULAR EPITHELIUM

60



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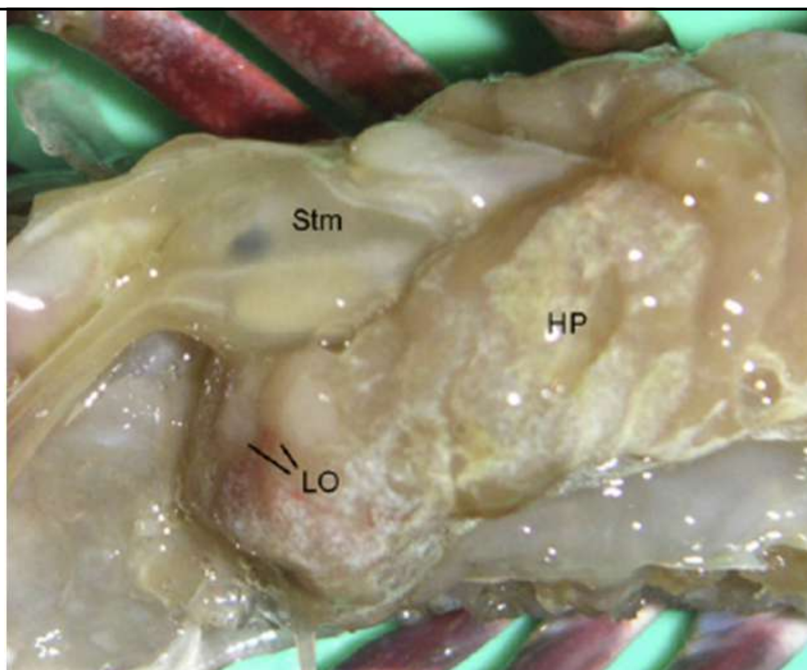

Duangsuwan, P., et al. (2008). Histological and three dimensional organizations of lymphoid organ tubules in normal lymphoid organ of *Penaeus monodon*. Fish & Shellfish Immunology 24(426-435).

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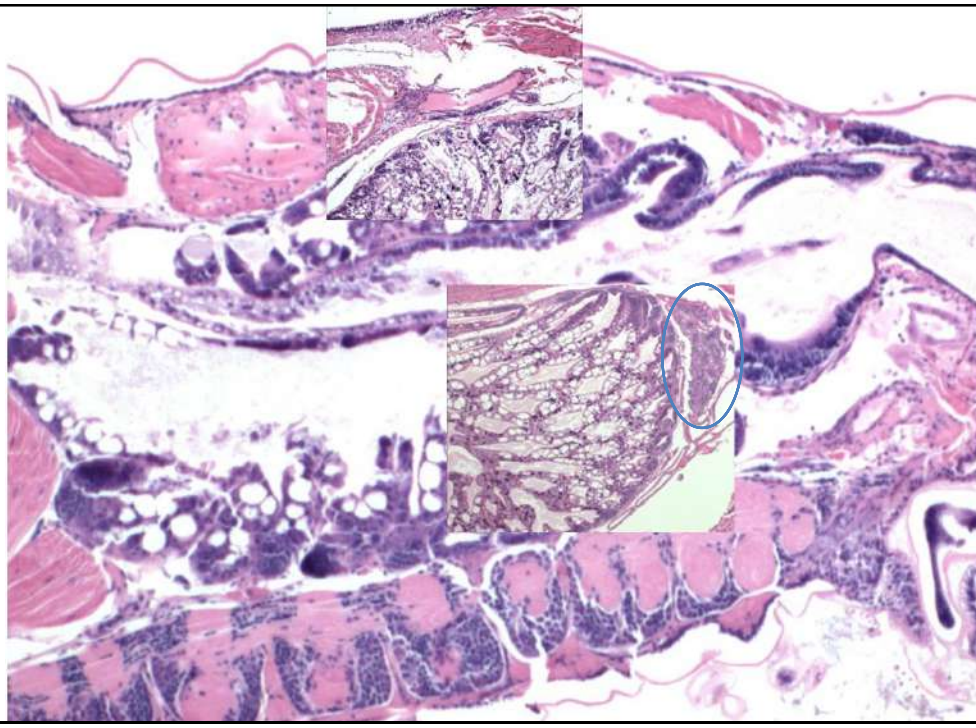

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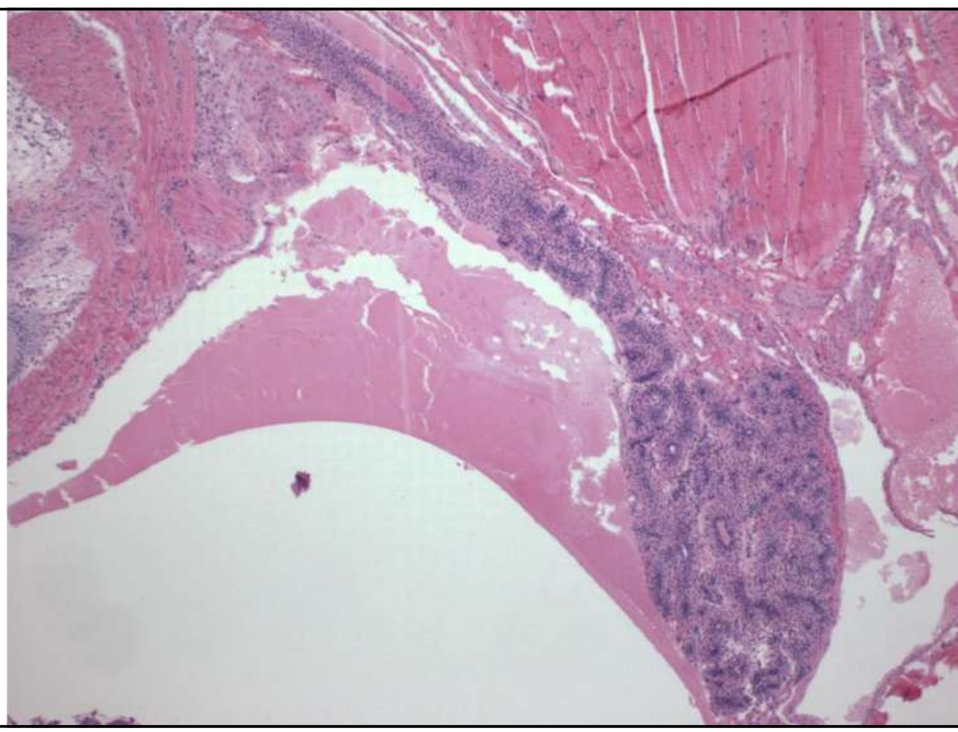

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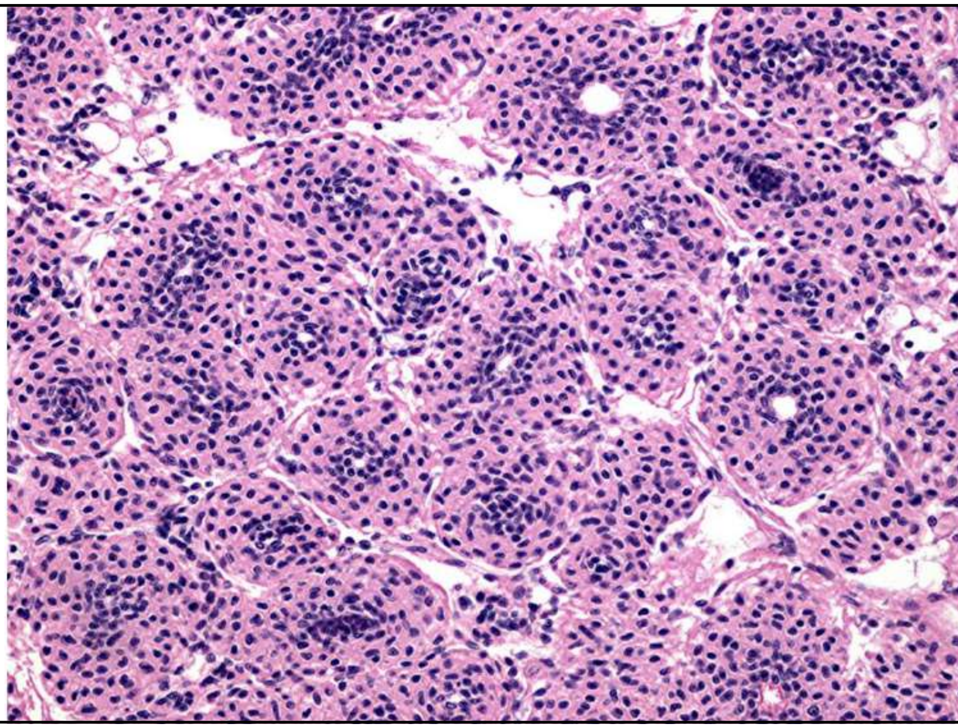

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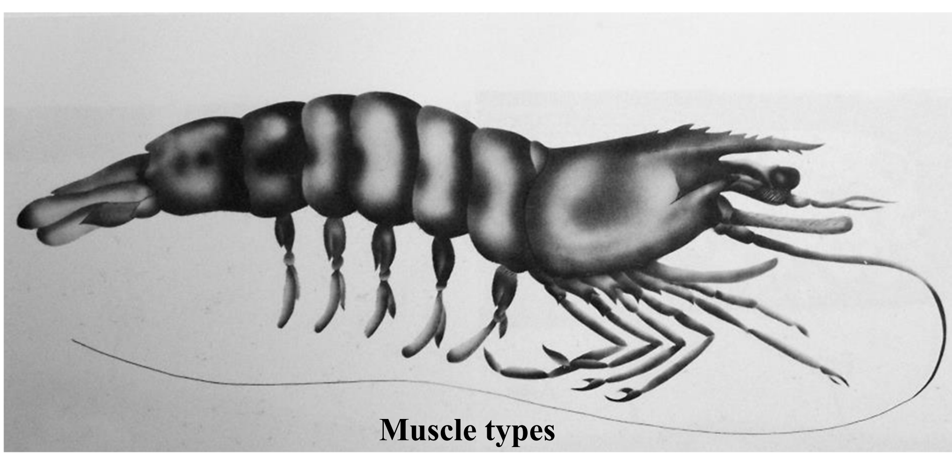

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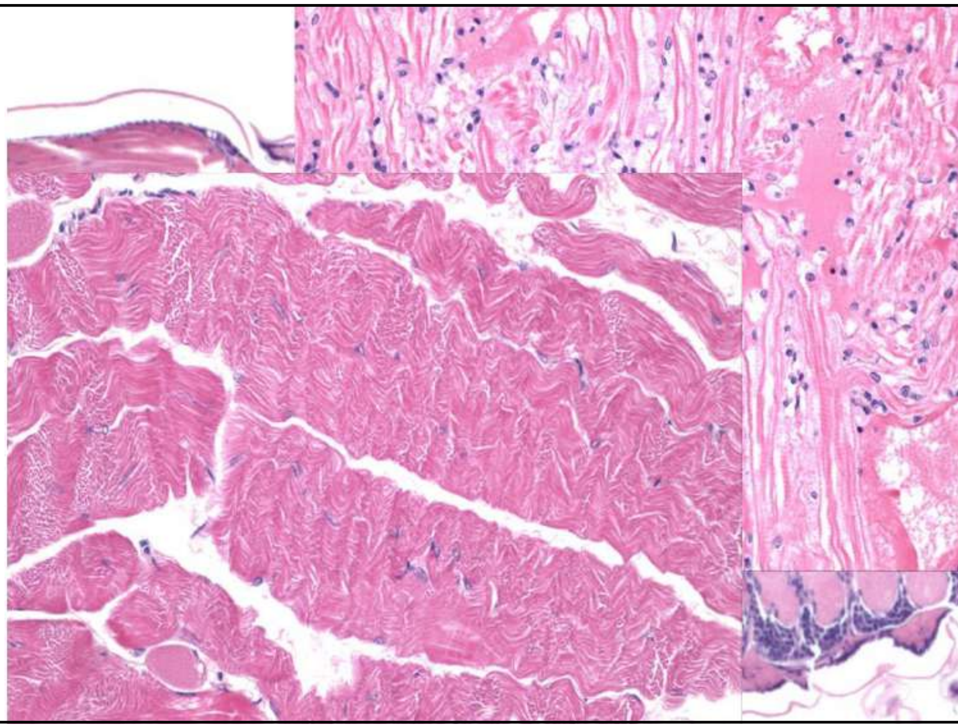

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Shrimp sampling for pathogen detection

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One of the most critical aspects to detect a pathogen when this is present, is based on a good sampling technique

- Gross signs
- Shrimp/pond behavior

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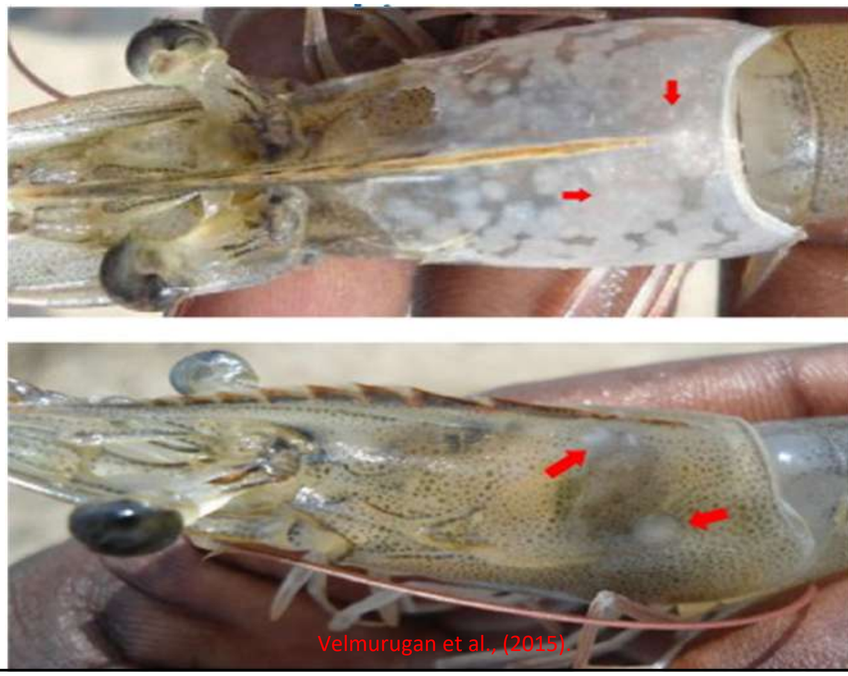
Gross signs

70

71

Erosive areas/white spots

72



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Chromatophores expansion

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Soft shell

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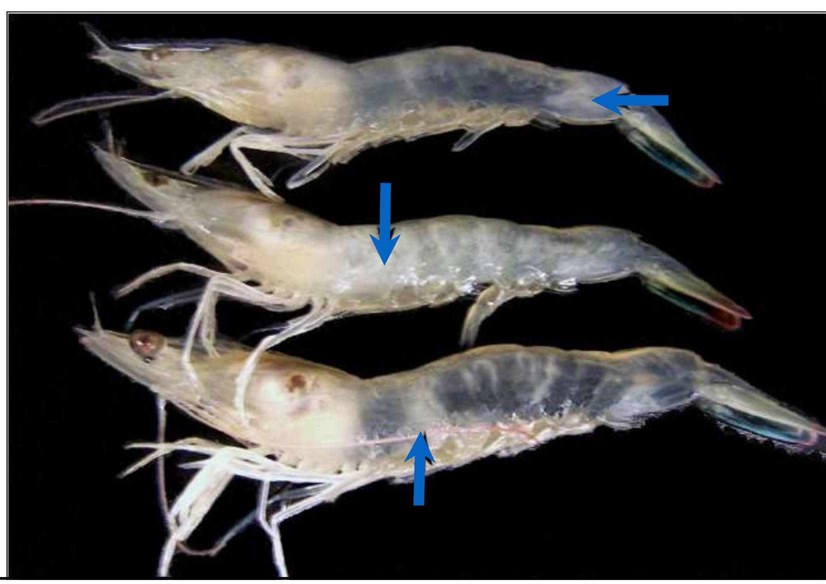
deformity

78

Black/brown gills

79

White muscle



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83

Muscle cramp

84

Necrosis spermatophore

85

Red discoloration

86

Size disparity

87

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Molting problems?

Kummari et al., (2018).

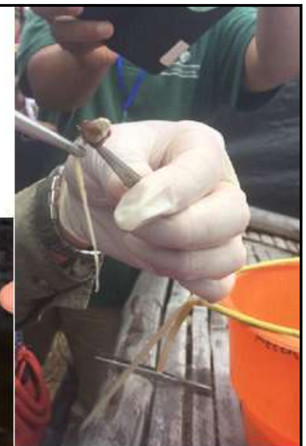
89

Discoloration in hepatopancreas

V.P R13 R14 A3

90

White faeces syndrome



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Unusual discoloration in exoskeleton

92

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Shrimp behavior

- Slow growth
- Poor feed consumption
- High Feed Conversion Rate
- Presence of birds
- Weakness
- Vulnerability to predators
- Erratic swimming
- Mortalities:
  - Chronic
  - Acute

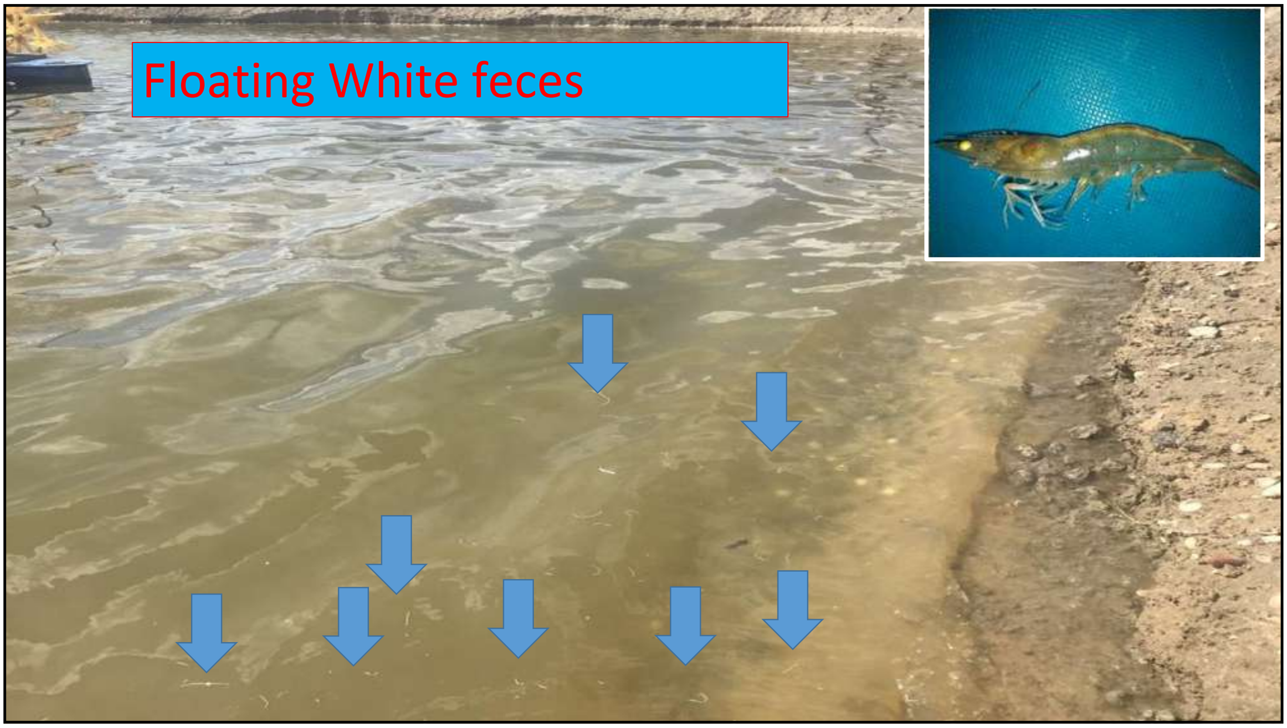
94

Bird presence

95

Erratic swimming

96



97

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Unusual mortalities

98

Sampling

99

Sampling

100

Formula for Successful Histological Sections:

PROPER SAMPLE OF LIVING SHRIMP + CORRECT FIXATIVE & METHOD + SUFFICIENT FIXATION TIME =

GOOD SECTIONS FOR HISTOLOGICAL STUDY

101

Thank you for your attention!

102

Acute Hepatopancreatic Necrosis Disease (AHPND)

Early Mortality Syndrome (EMS)

Luis Fernando Aranguren Caro, Ph.D

March 22, 2022

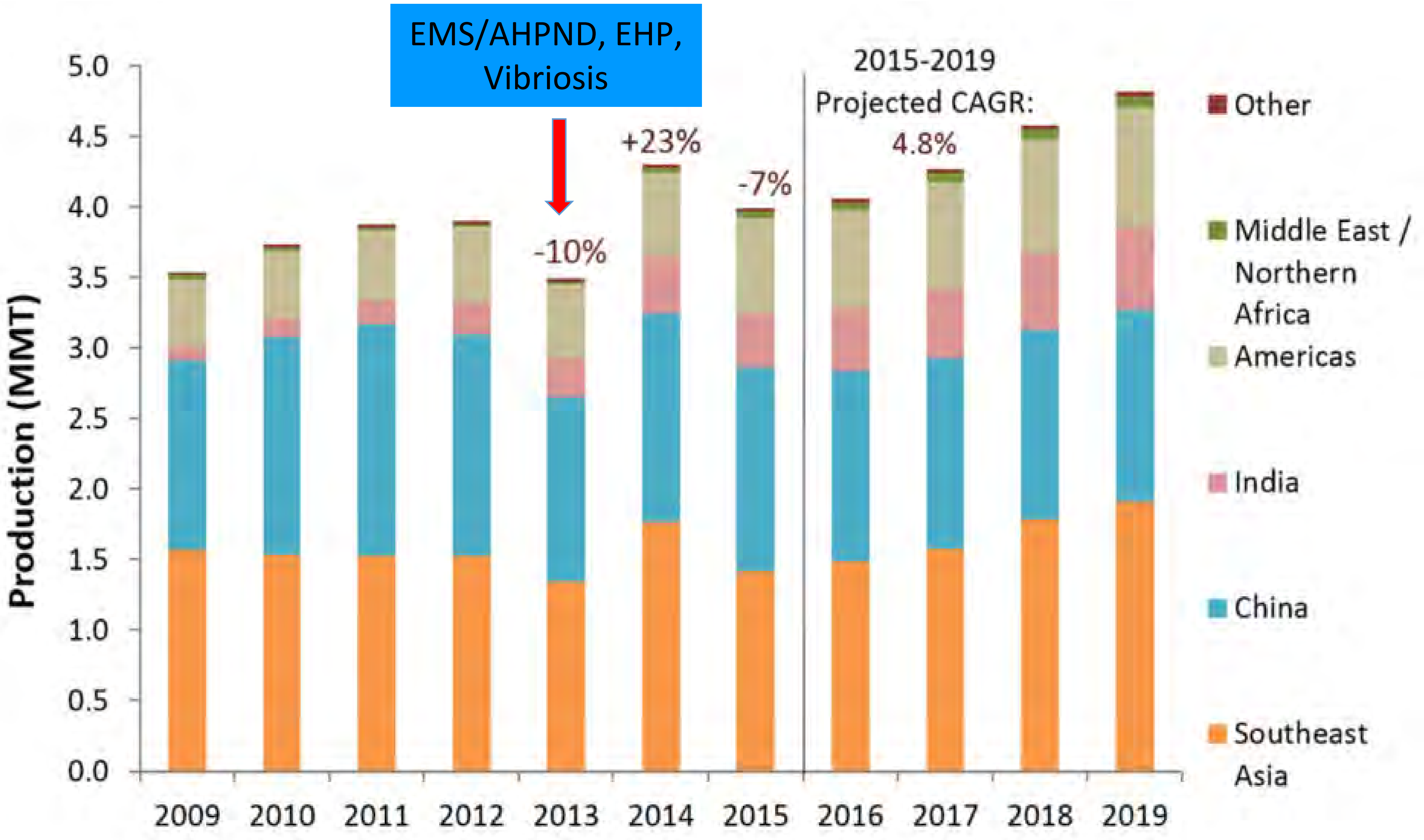
Aquaculture Pathology Laboratory
OIE Reference Laboratory
USDA-APHIS Approved & ISO 17025, 17043 Accredited Laboratory
School of Animal & Comparative Biomedical Science
The University of Arizona, Tucson, Arizona, USA

OIE (World Organisation for Animal Health)

Crustacean diseases

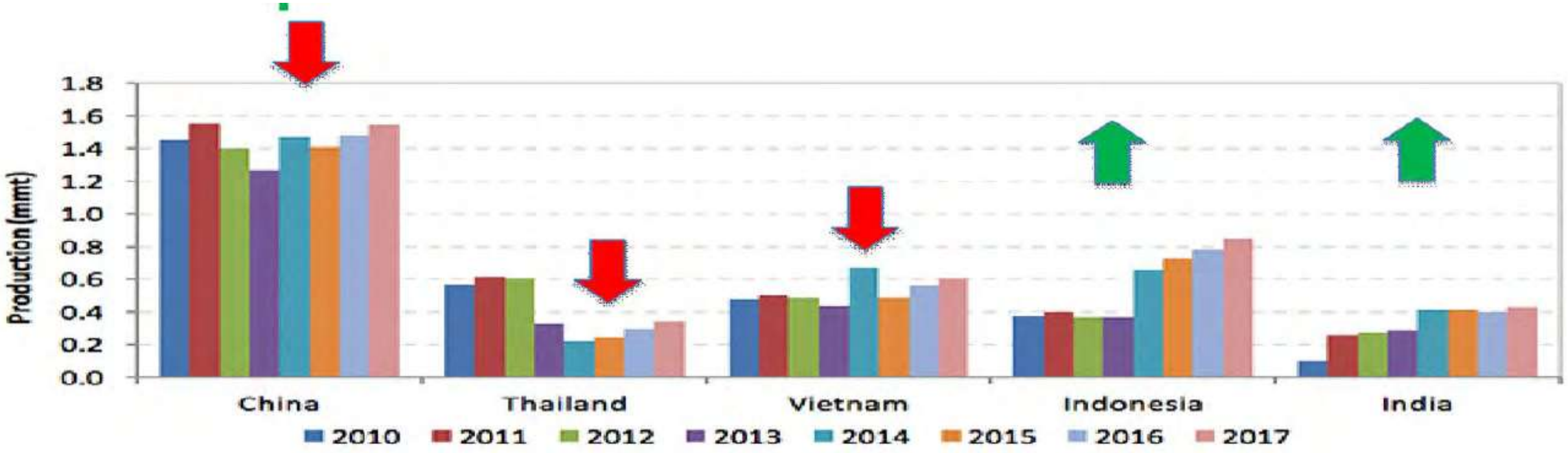
- Crayfish plague (*Aphanomyces astaci*)
- Infection with Yellow head virus (YHV)
- Infectious hypodermal and haematopoietic necrosis (IHHNV)
- Infectious myonecrosis (IMNV)
- Necrotising hepatopancreatitis (NHP)
- Taura syndrome (TSV)
- White spot disease (WSSV)
- White tail disease
- Decapod Iridescent virus (DIV1)
- + Acute Hepatopancreatic necrosis Disease (AHPND)

World Shrimp Aquaculture 2009-2019 (E)

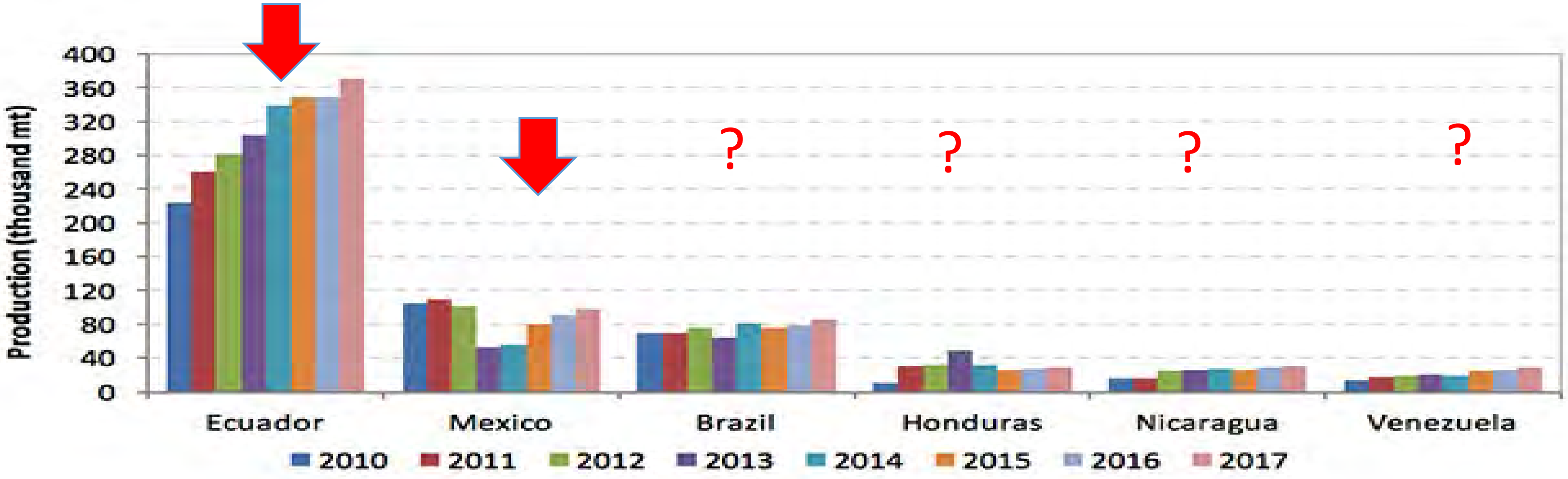


Sources: FAO (2016) for 1995-2011; FAO (2016) and GOAL (2017) for 2012-2018.

Shrimp Production & AHPND Emergence: Asia & The Americas



- Nunan et al., 2014
- Restrepo et al., 2016
- Jun et al., 2016
- Han et al., 2017
- Ahn et al., 2017
- Cuellar & Brock et al., 2018
- Restrepo et al., 2018
- Kanrar & Dhar 2018



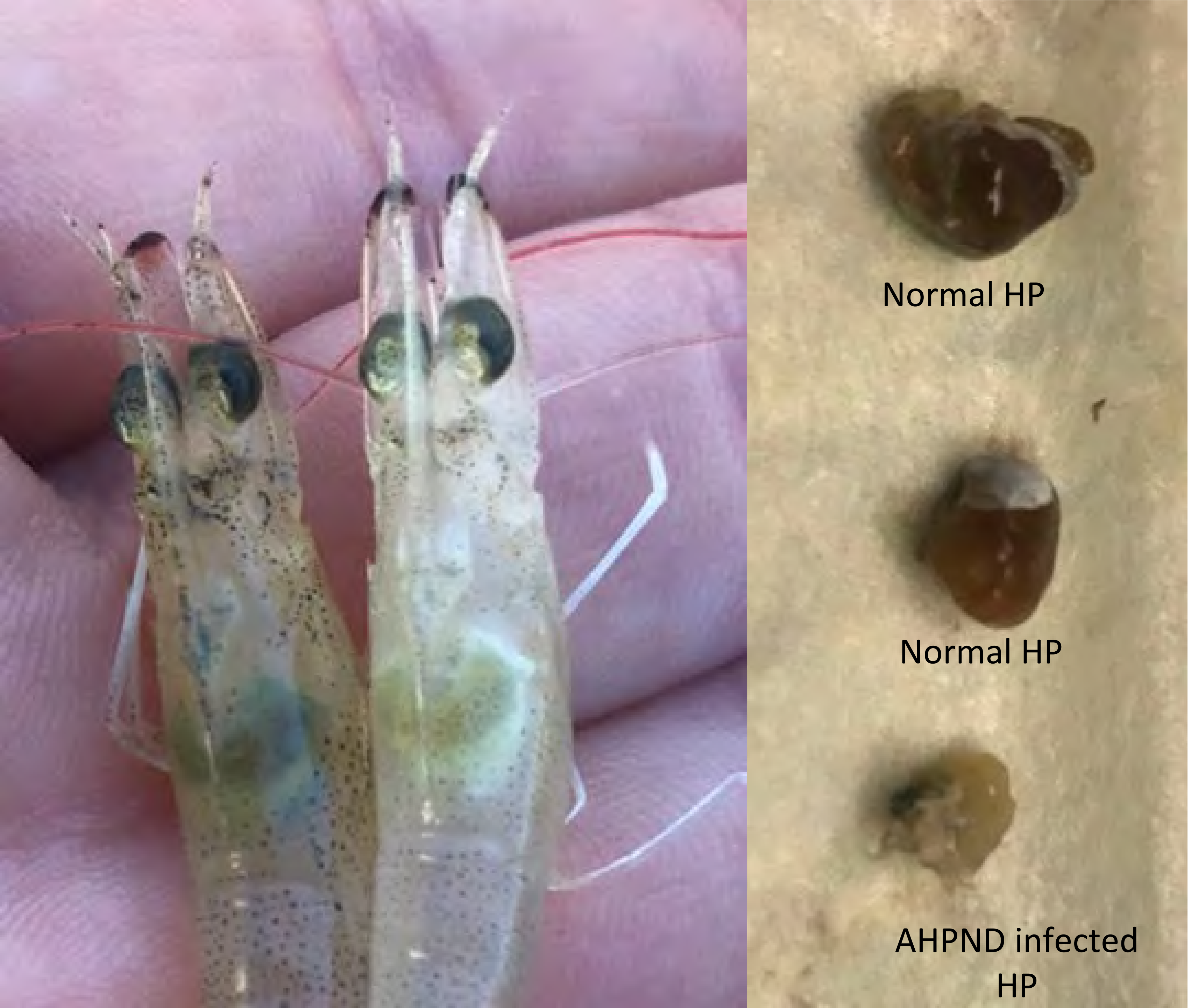
Sources: FAO (2013); GOAL (2016)

Evolution of Acute Hepatopancreatic Necrosis Disease "AHPND"

Unusual mortalities	2009
EMS	Lightner, 2011
AHPNS	NACA, 2012
AHPND	Tran et al., 2013
Plasmid from *V. parahaemolyticus*	Han et al., 2015
Toxin from plasmid in VP (*pir A pir B*)	Han et al., 2015 Lee et al., 2015
Toxin from plasmid in *V. parahaemolyticus, V. harveyi, V campbelli, V. owensii* and *V. punensis, Vibrio* spp.	Lieu et al., 2015; Dong et al., 2017 Liu et al., 2018, Restrepo et al., 2018

Spread of EMS/AHPND in East and SE Asia

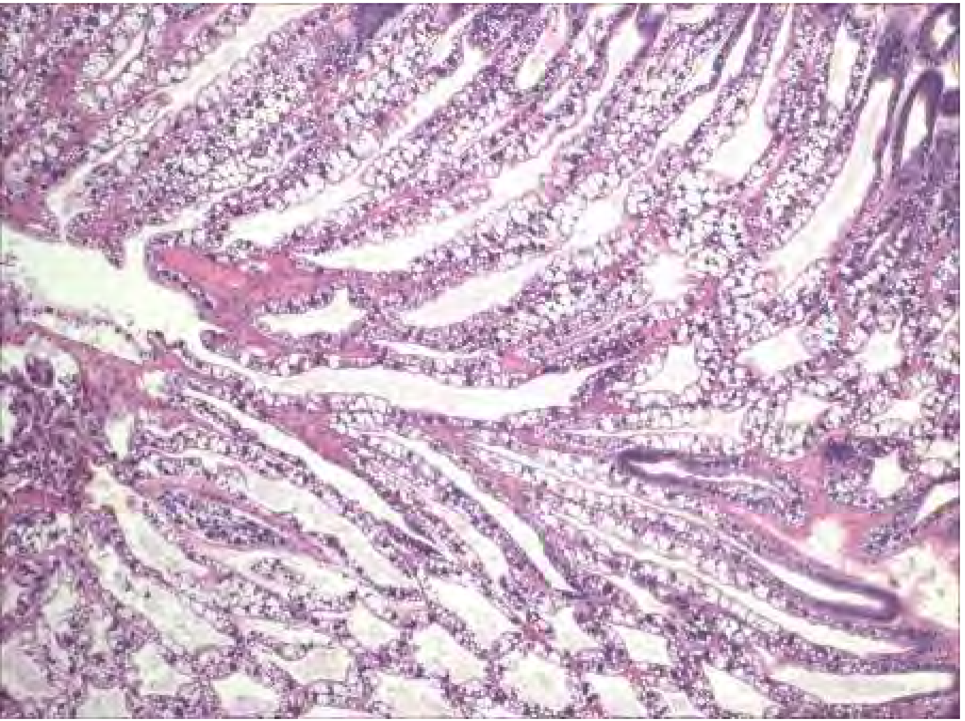
EMS in China – Photos courtesy of Mr. Luyi

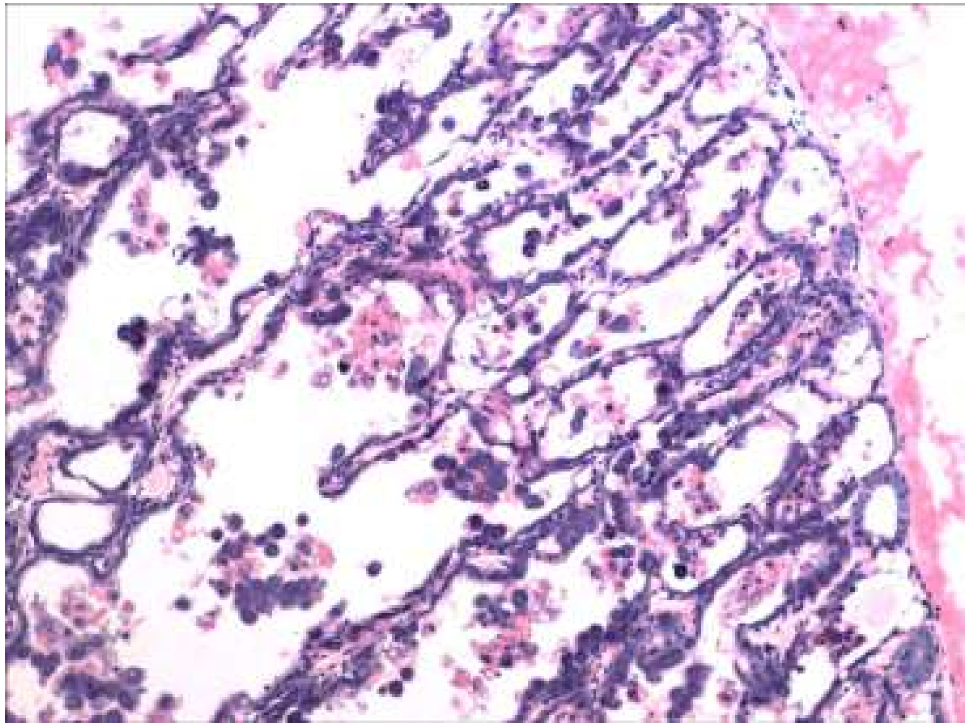


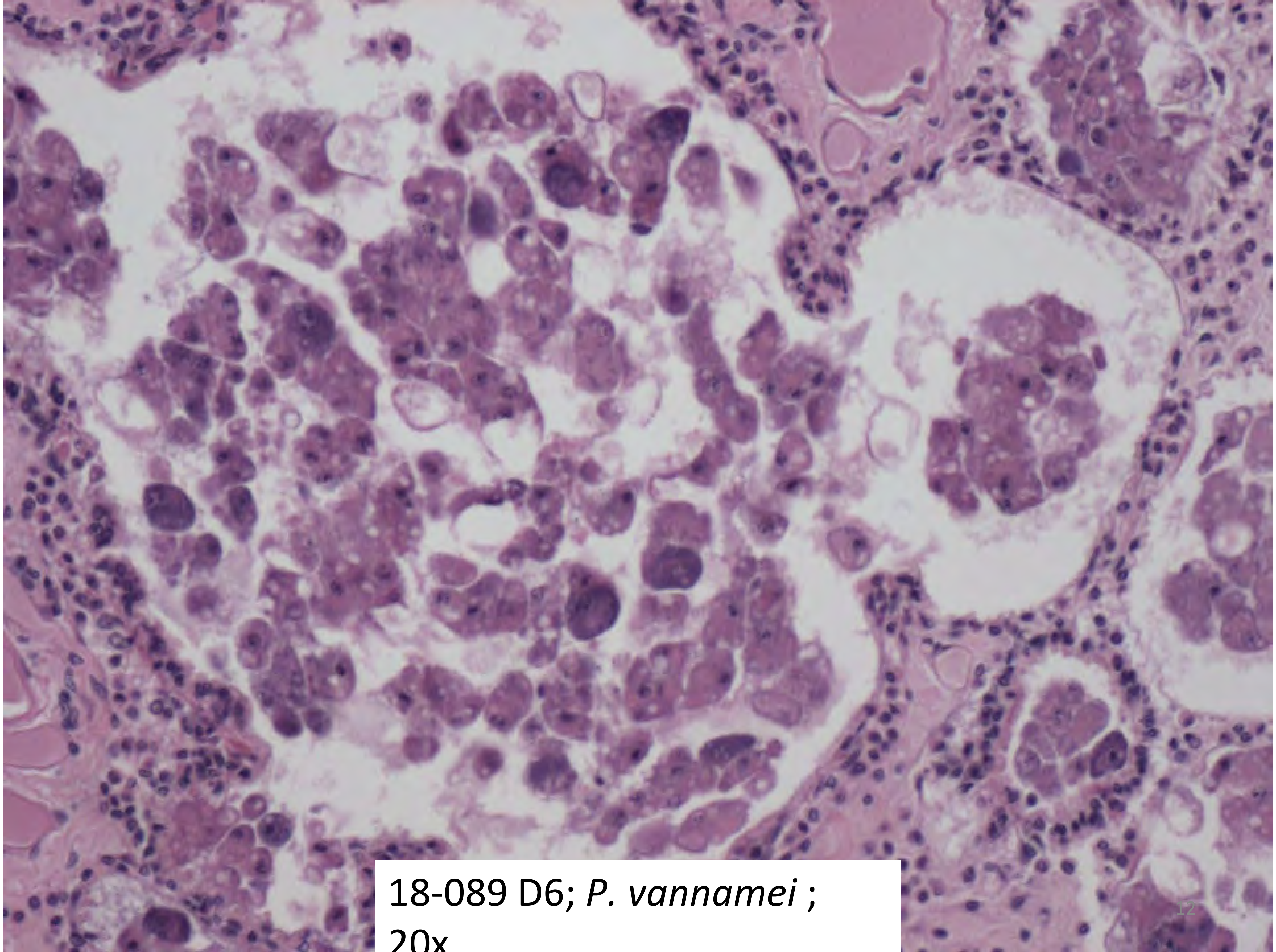
Juvenile *Penaeus vannamei*
- right with AHPND
- left appears normal

AHPND

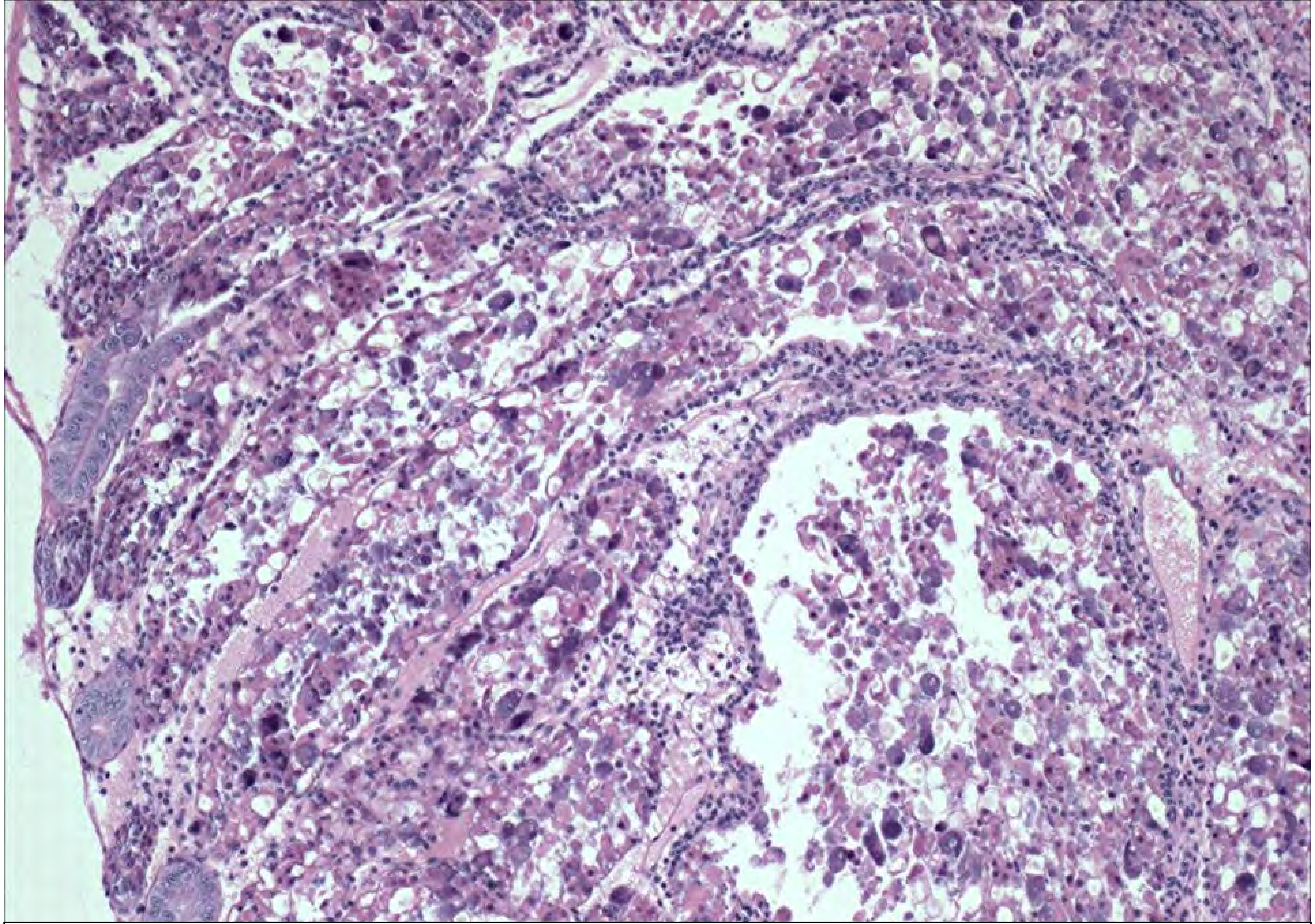
- **An acute phase**
  - HP tubule cells (R, B, F & later E-cells) show acute loss of function
  - Bacteria (of any kind) are not easily demonstrated by *in situ* hybridization with a 16S rRNA universal probe
  - Acute progressive degeneration of hepatopancreas (HP) from medial to distal with dysfunction of all HP cells, prominent necrosis, & sloughing of these tubule epithelial cells



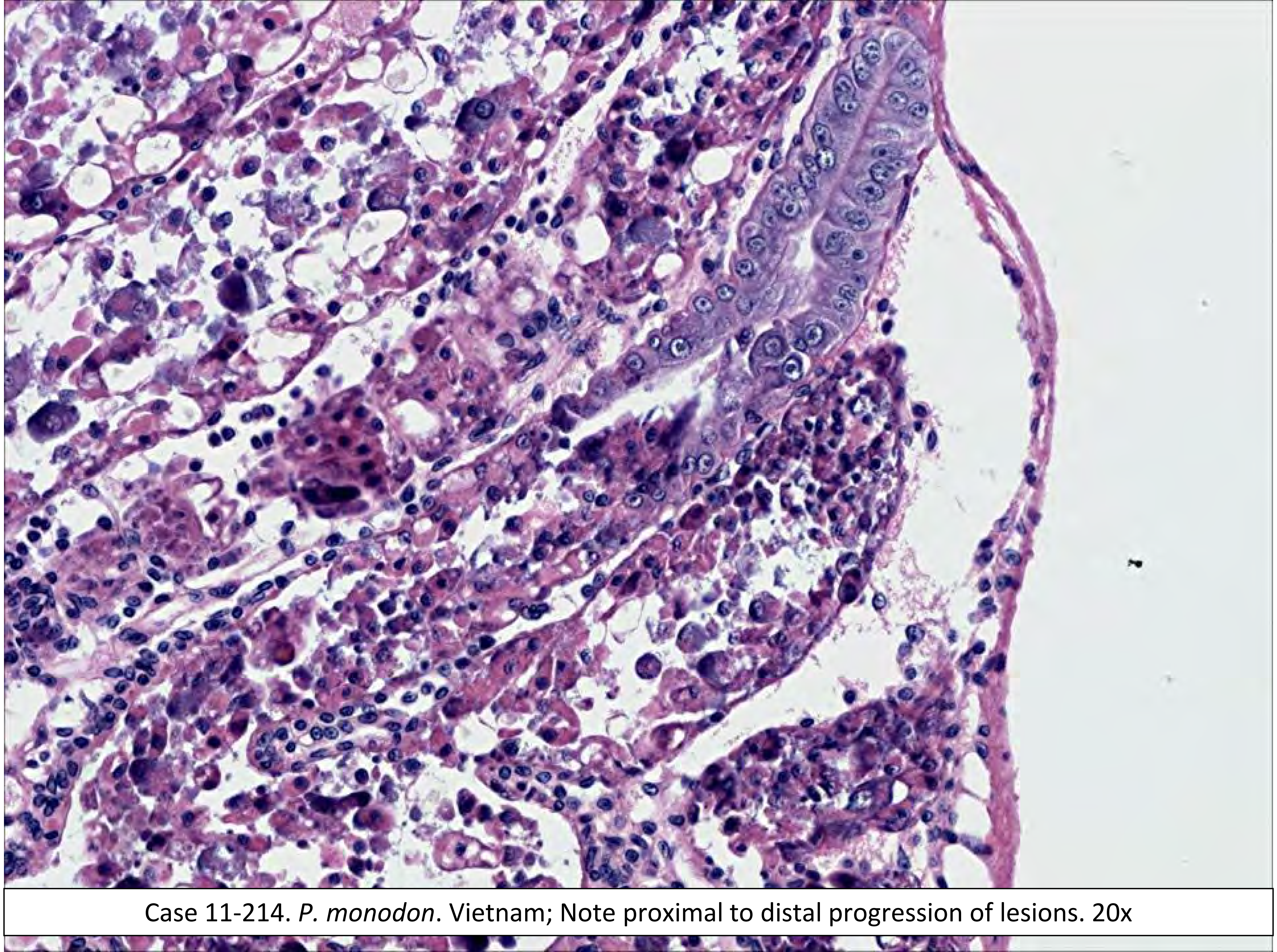




18-089 D6; *P. vannamei* ; 20x



Case 11-214. *P. monodon*. Vietnam; Severe sloughing & moderate inflammation



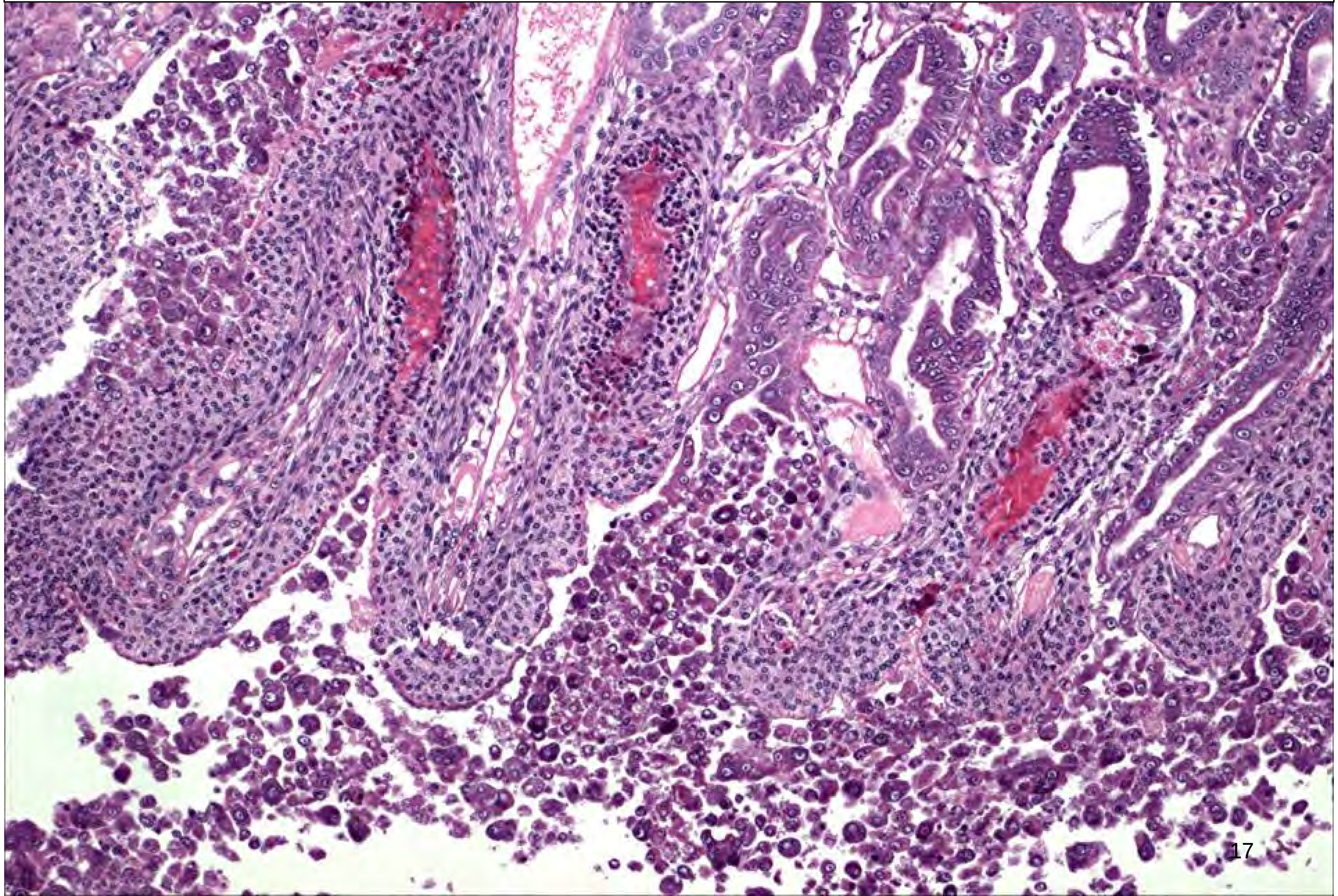
Case 11-214. *P. monodon*. Vietnam; Note proximal to distal progression of lesions. 20x

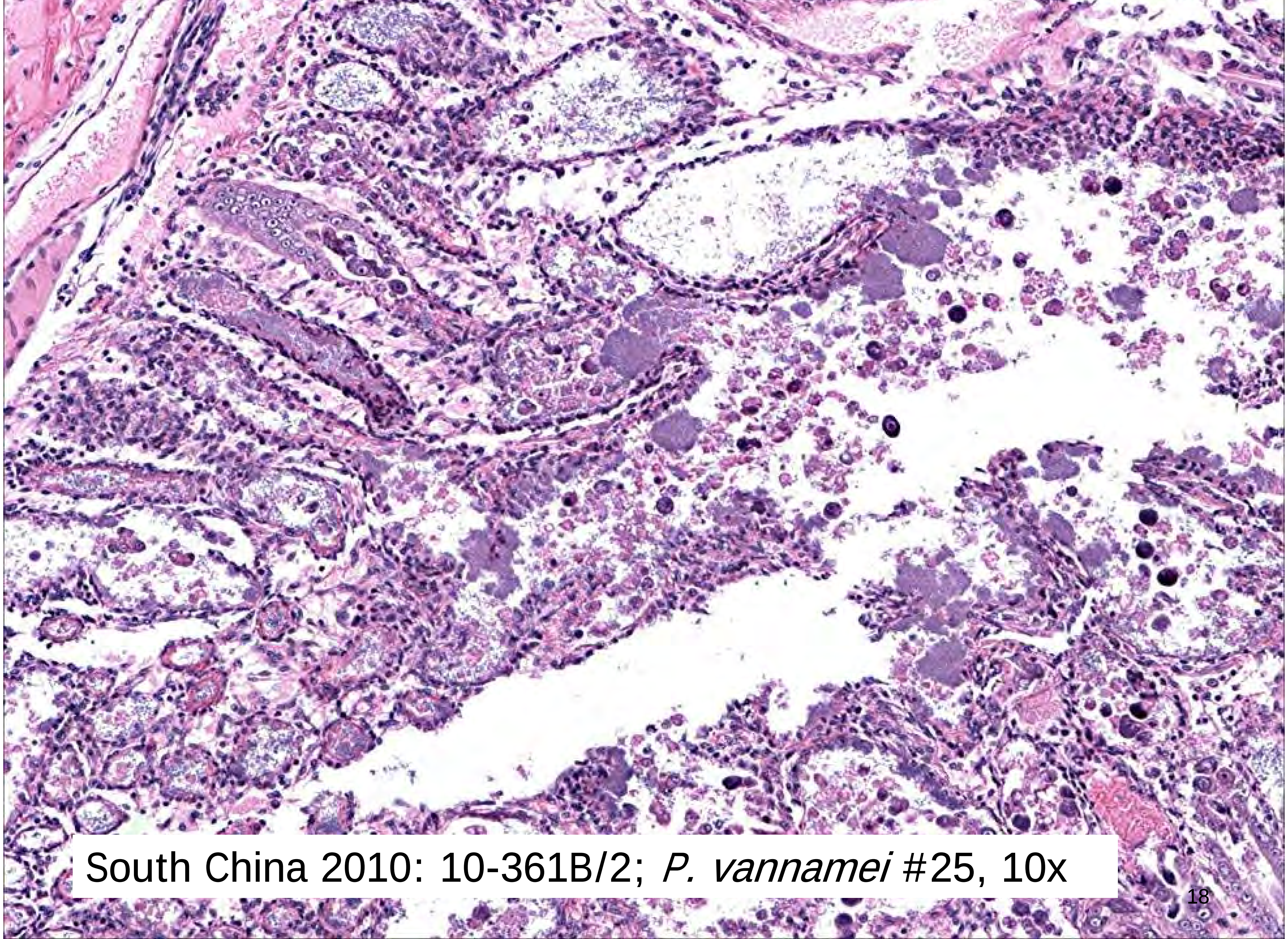
AHPND

Terminal phase

- shows marked inter- & intra-tubular hemocytic inflammation
- development of massive secondary bacterial infections that occur in association with necrotic & sloughed HP tubule cells

Case 11-254. *P. vannamei*. Vietnam; HP tubule epithelium sloughing, significant proximal hemocytic inflammation & some tubules with putative vibriosis; 10x





South China 2010: 10-361B/2; *P. vannamei* #25, 10x

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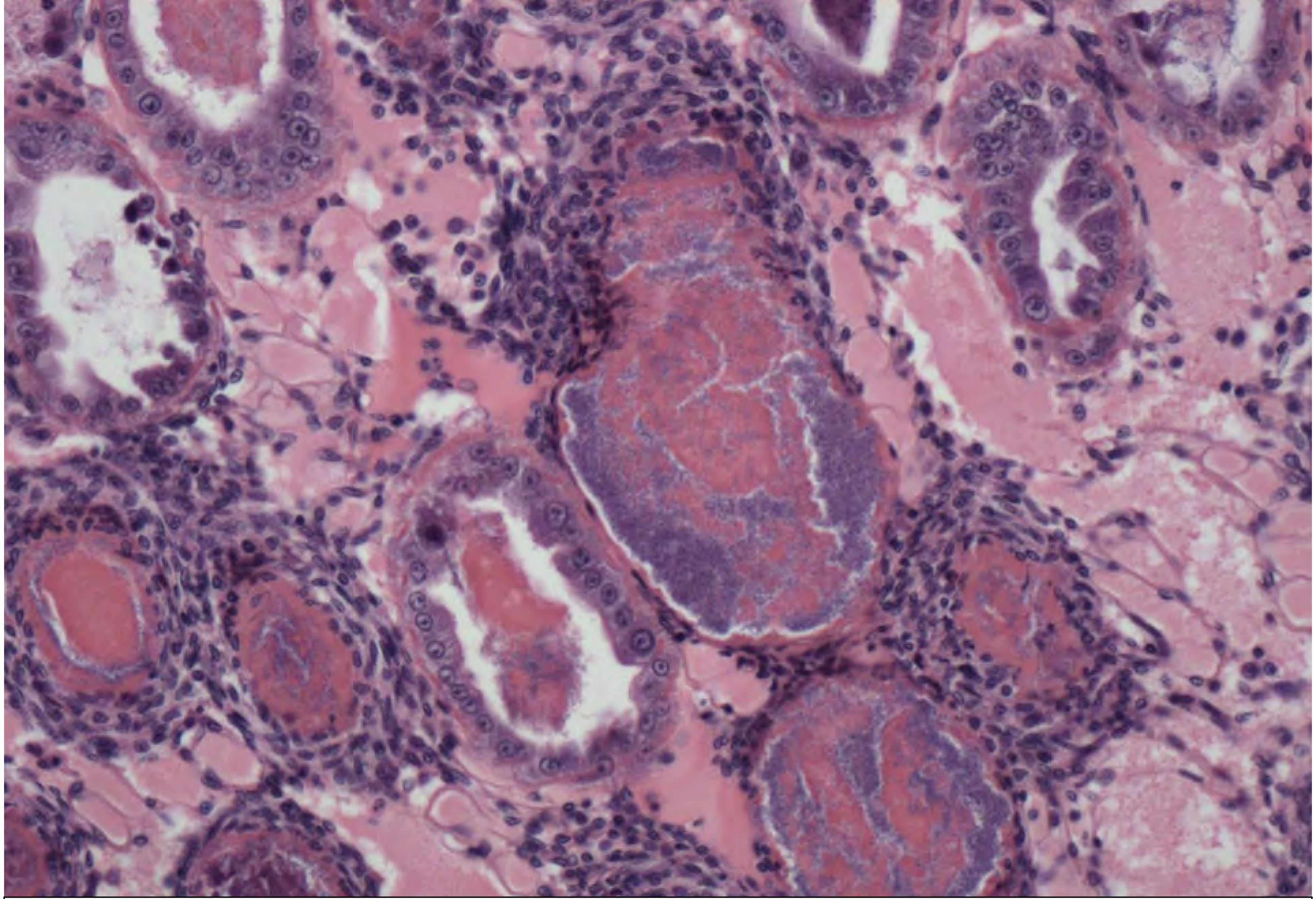

Case 17-648. *P. vannamei.*; Terminal phase of AHPND. Most HP tubules are destroyed. Massive bacterial infection by a probable *Vibrio* spp. 20x.

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AHPND

Chronic phase

- Granuloma
- Focal to multifocal melanization of HP tubules
- Low cytoplasmic lipid and atrophy of tubule epithelial cells
- Tubules with epithelial necrosis accompanied by bacteria and inflammation, which resembles to a septic hepatopancreatic necrosis (SHPN)

ELSEVIER

Journal of Invertebrate Pathology

Volume 174, July 2020, 107424

Acute hepatopancreatic necrosis disease (VP_{AHPND}), a chronic disease in shrimp (*Penaeus vannamei*) population raised in latin America

Luis Fernando Aranguren Caro, Hung N. Mai, Brenda Noble, Arun K. Dhar

Show more

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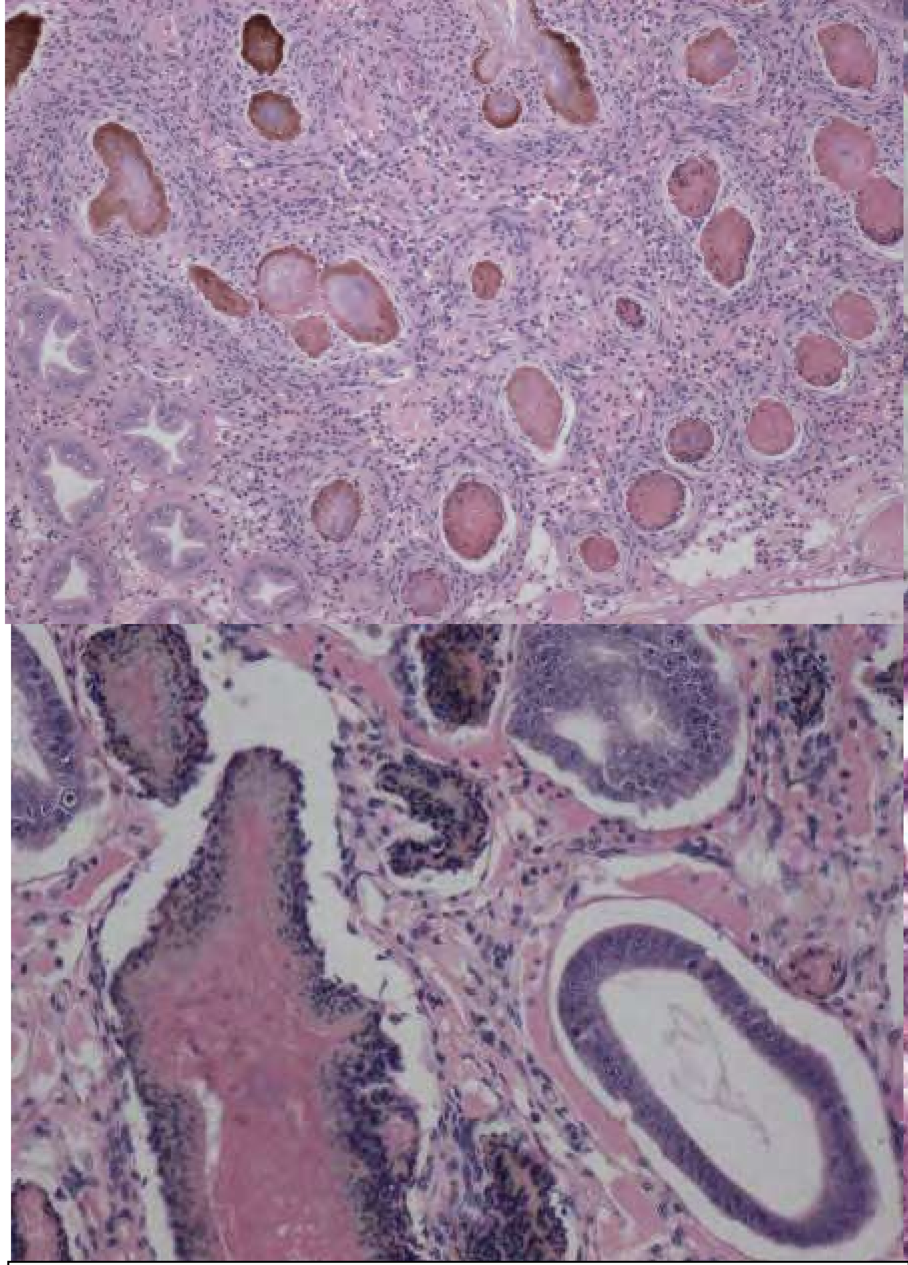
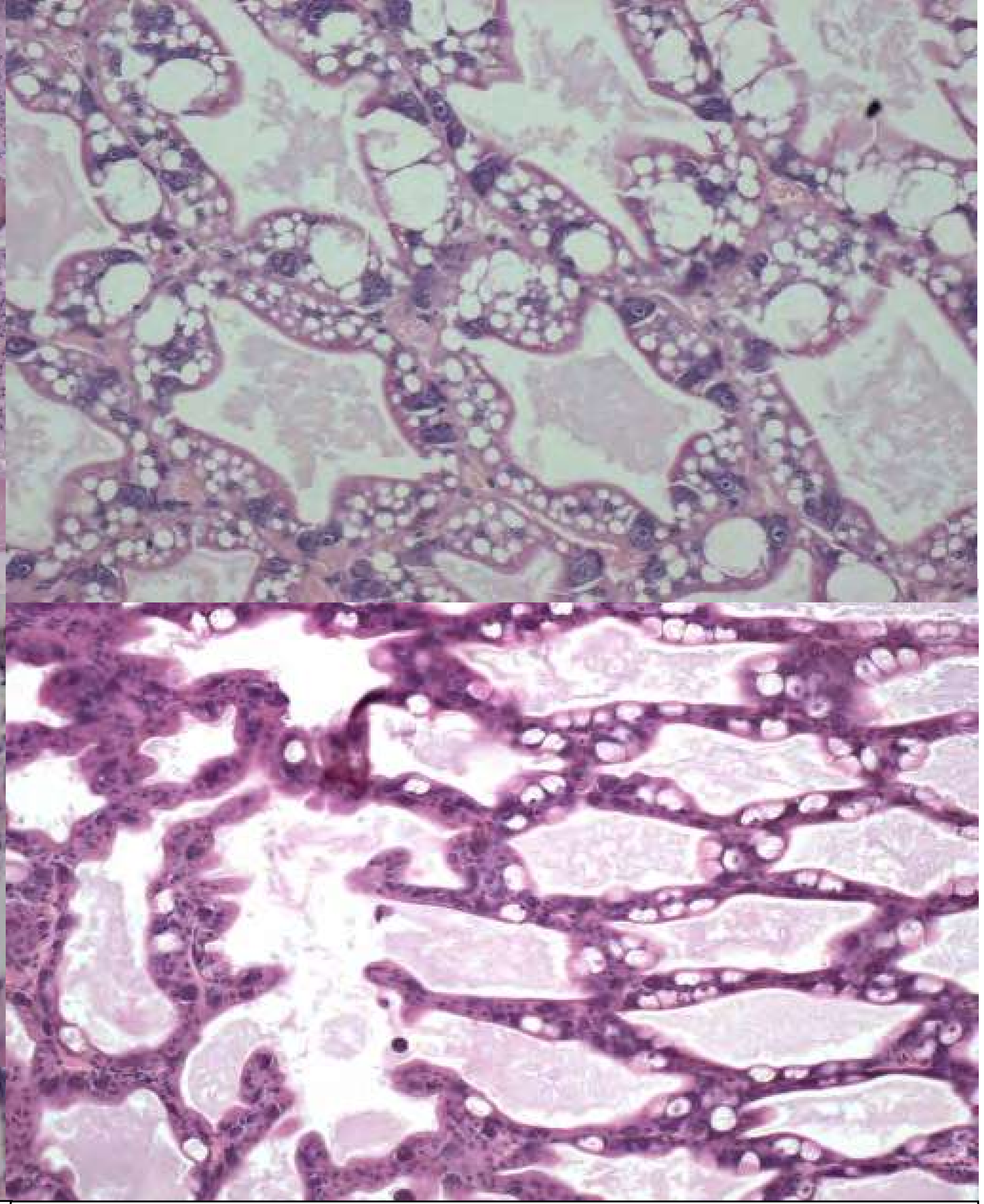


Case 17-648. *P. vannamei*.; Chronic phase of AHPND. Most HP tubules are melanized. bacterial infection by a probable *Vibrio* spp. 20x.



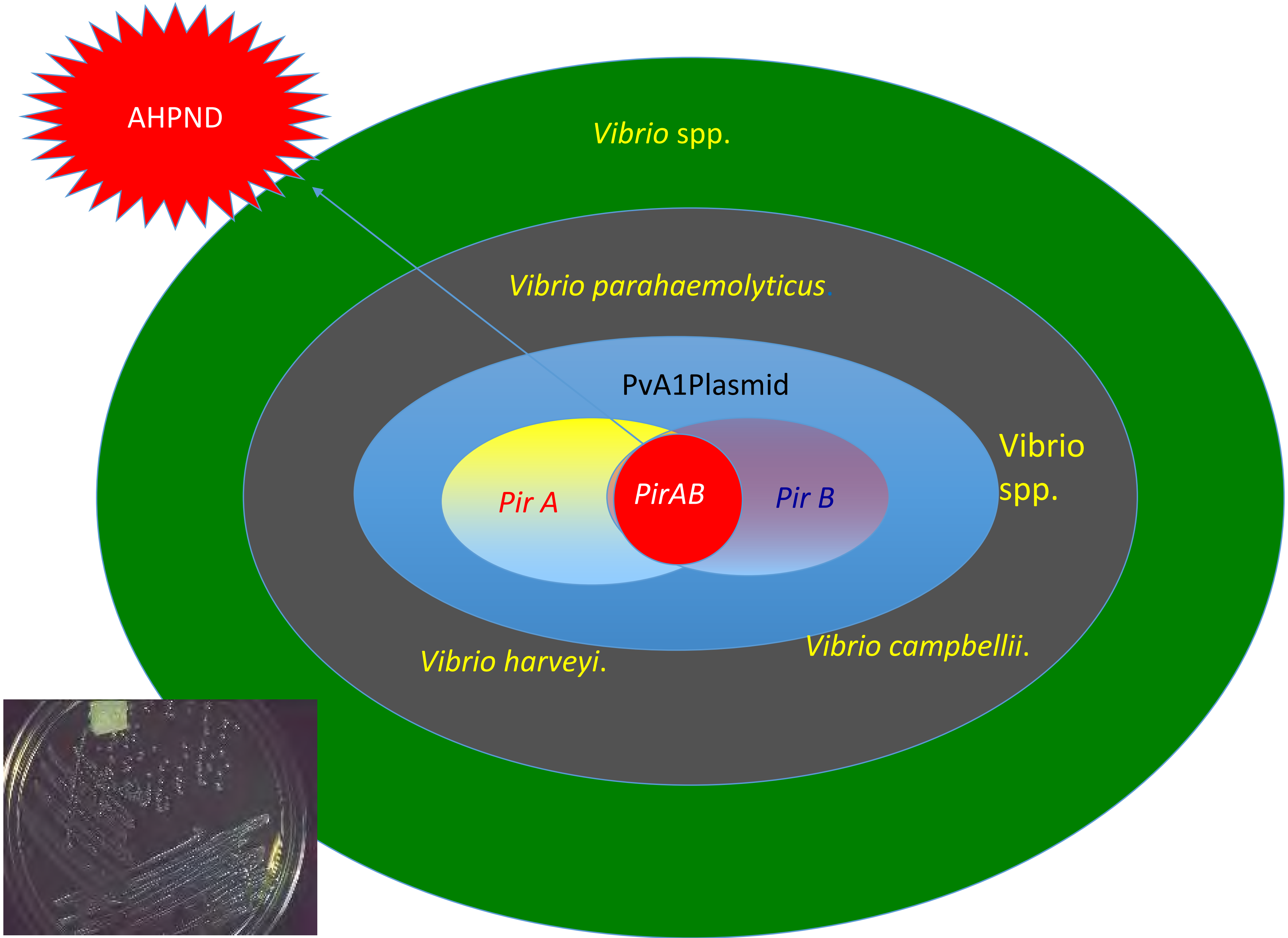
Case 18-089. *P. vannamei*.; Chronic phase of AHPND. Mild sloughing of cells. 20x.

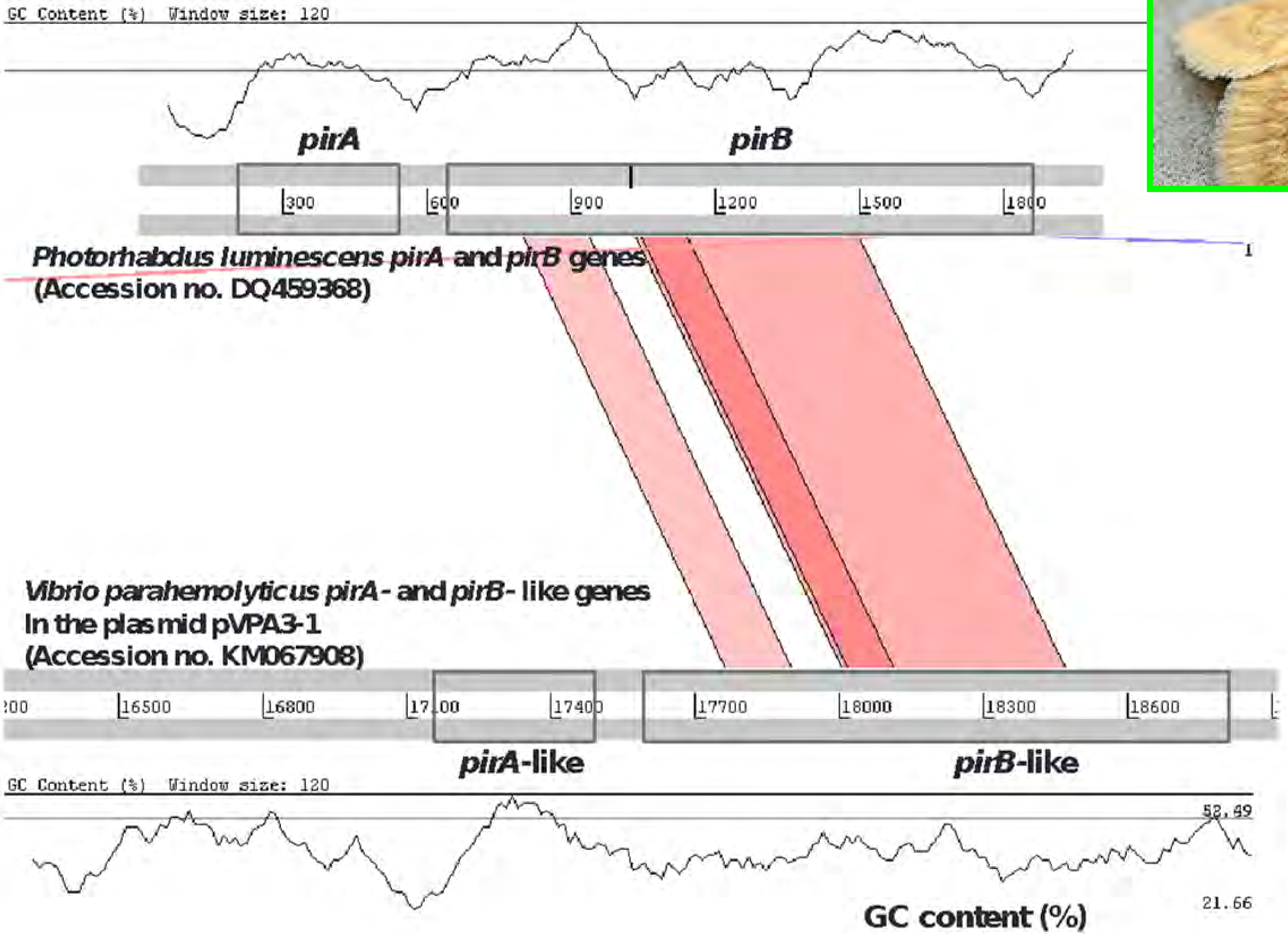
Causative agent of AHPND/EMS

- *V. parahaemolyticus, V. harveyi, V campbellii, V. owensii, and V. punensis, Vibrio spp.*
- Halophilic bacteria:
  - Common bacteria in marine /brackish water environments
  - Associated to shrimp farming. High bacterial count in HP & Gi tract
  - Facultative anaerobic
    - T°C : 25-37°C
    - Salinity: 10-40 ppt
  - High replication rate (higher than *E. coli)*
  - Sucrose +/- in TCBS

VIRULENCE FACTOR

Toxin Genes (*pirA* and *pirB*) in the Mobile Plasmid

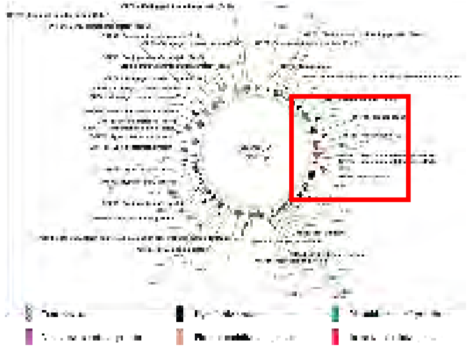

AHPND
Vibrio spp.
Vibrio parahaemolyticus.
PvA1Plasmid
Pir A
PirAB
Pir B
Vibrio spp.
Vibrio harveyi.
Vibrio campbellii.


GC Content (%) Window size: 120
pirA
pirB
300
600
900
1200
1500
1800
Photorhabdus luminescens pirA and pirB genes
(Accession no. DQ459368)
Vibrio parahemolyticus pirA- and pirB- like genes
In the plasmid pVPA3-1
(Accession no. KM067908)
16500
16800
17100
17400
17700
18000
18300
18600
pirA-like
pirB-like
GC Content (%) Window size: 120
52.49
21.66
GC content (%)


moth

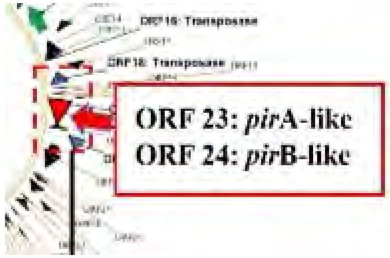
Evolution of AHPND Detection by PCR

V. parahaemolyticus

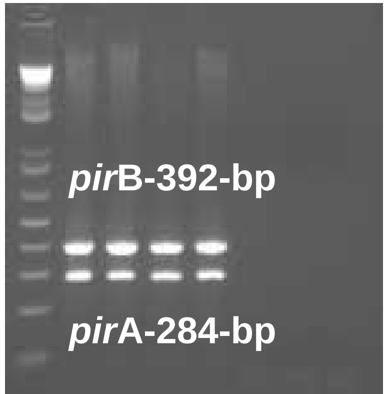


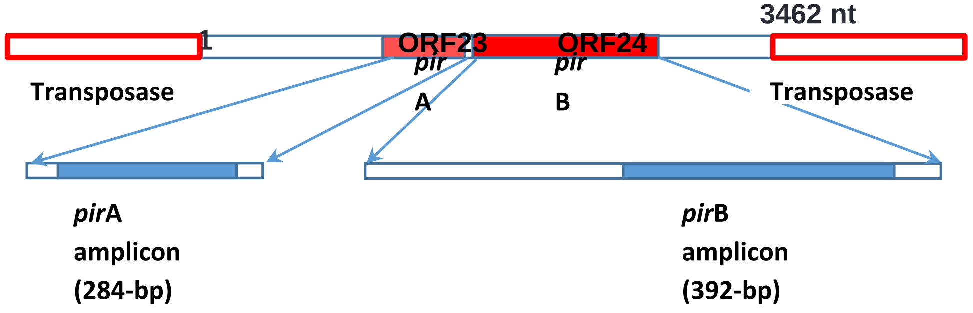
70 kb Plasmid

Pir A (AP 3)

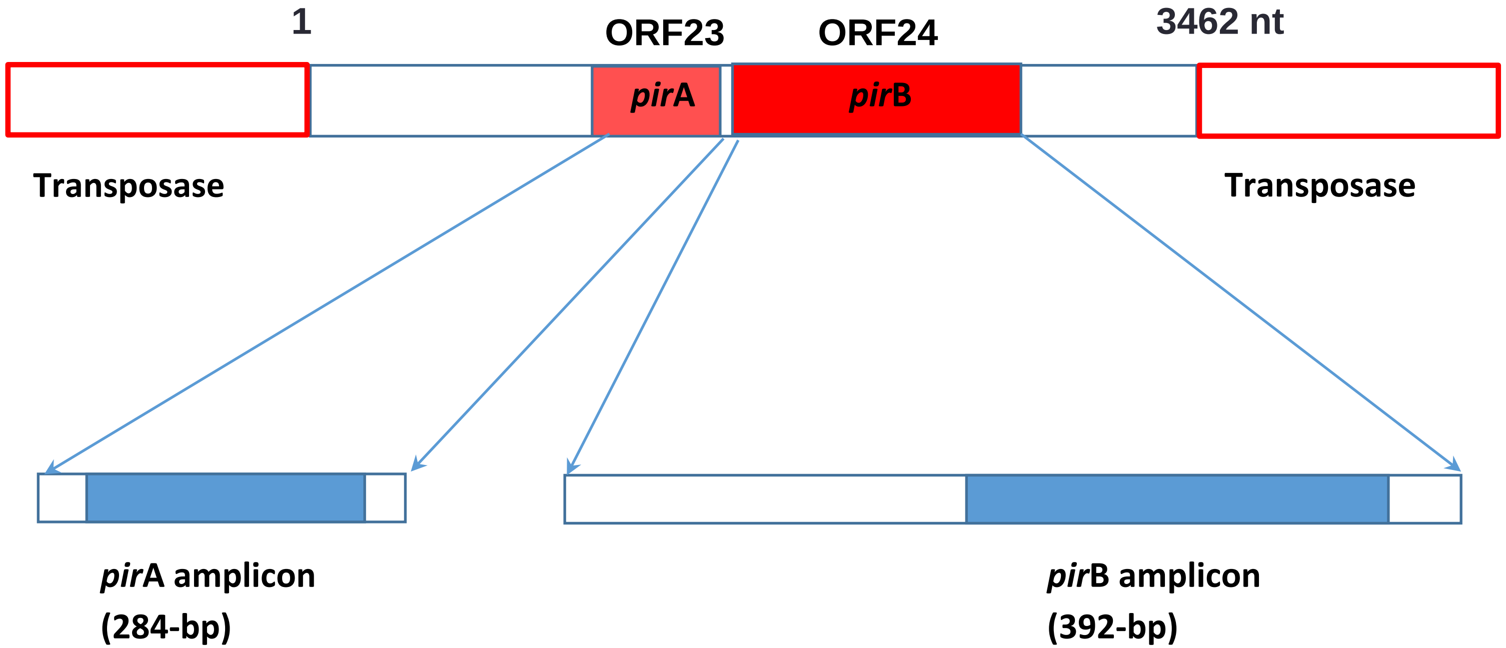


Pir AB (AP4)





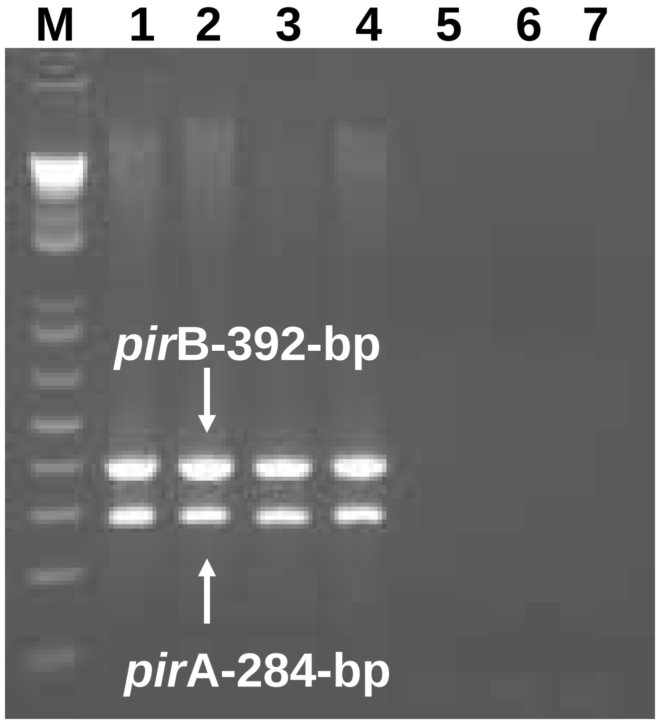
AHPND Detection PCR Assay (Duplex)



Primer	Sequence (5’ to 3’)	Amplicon size	Target
VpPirA-284F	TGACTATTCTCACGATTGGACTG	284-bp	Detection PCR (*pir*A)
VpPirA-284R	CACGACTAGCGCCATTGTTA		
VpPirB-392F	TGATGAAGTGATGGGTGCTC	392-bp	Detection PCR (*pir*B)
VpPirB-392R	TGTAAGCGCCGTTTAACTCA		

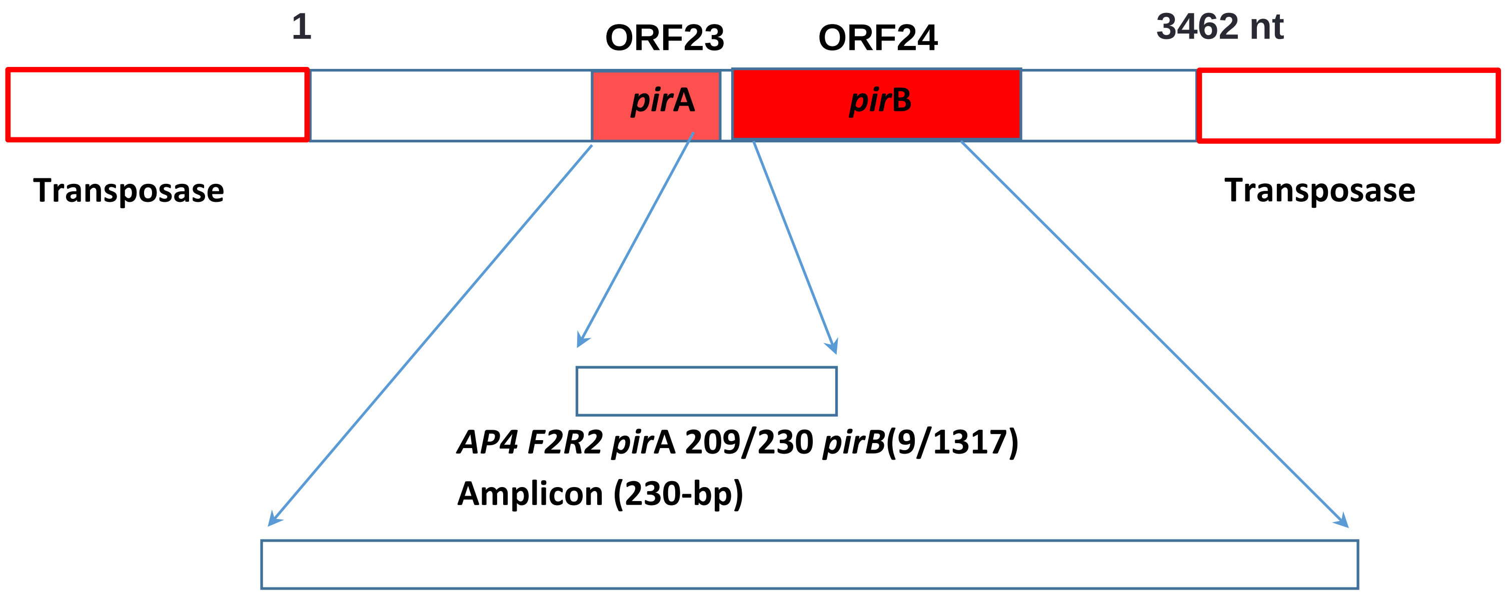
Han et al., (DAO, 2015)

AHPND Detection PCR Assay (Duplex)



Lane #	Strain	AHPND	Origin
1	13-511A/1	**Pos**	MX
2	A3	**Pos**	VN
3	13-306D/4	**Pos**	MX
4	12-194G	**Pos**	VN
5	A2	-	VN
6	13-488L	- (SHPN+)	India
7	13-431/1	-	US-TX

AHPND Detection PCR Assay (AP4)



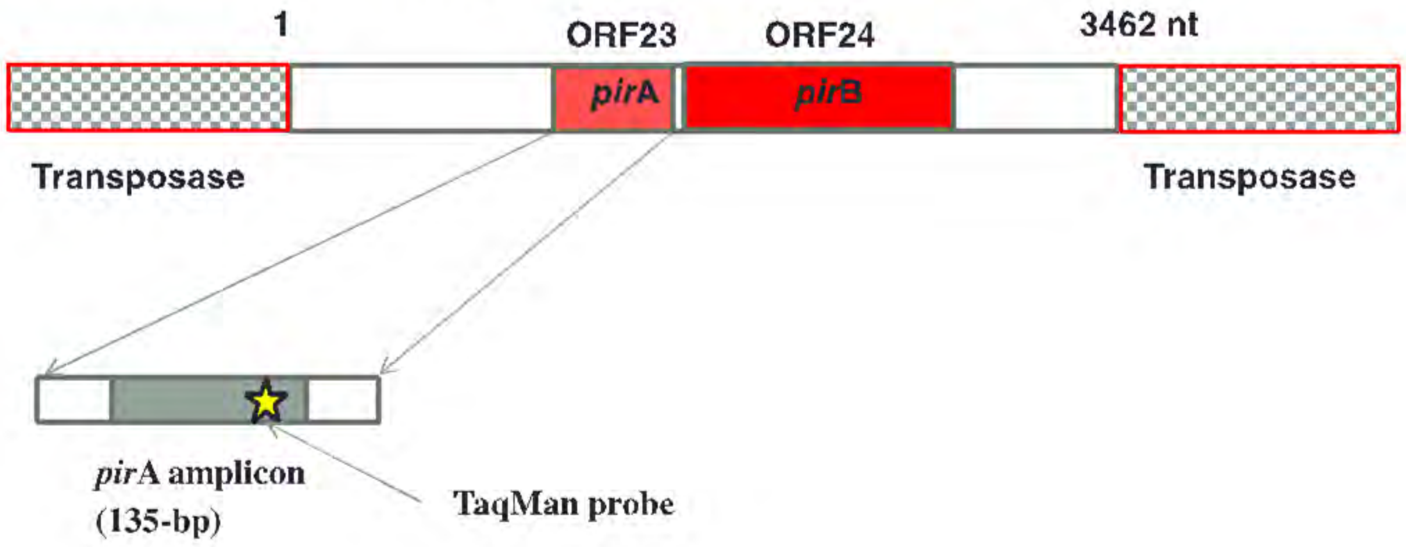
***AP4 F1 R1 pirA (whole) pir*B (921/1317) amplicon (1269-bp)**

Primer	Sequence (5' to 3')	Amplicon size	PCR
AP4-F1	ATG-AGT-AAC-AAT-ATA-AAA-CAT-GAA-AC	1269-bp	First step
AP4-R1	ACG-ATT-TCG-ACG-TTC-CCC-AA		
AP4-F2	TTG-AGA-ATA-CGG-GAC-GTG-GG	230-bp	Nested
AP4-R2	GTT-AGT-CAT-GTG-AGC-ACC-TTC		

Dangtip et al., 2015.

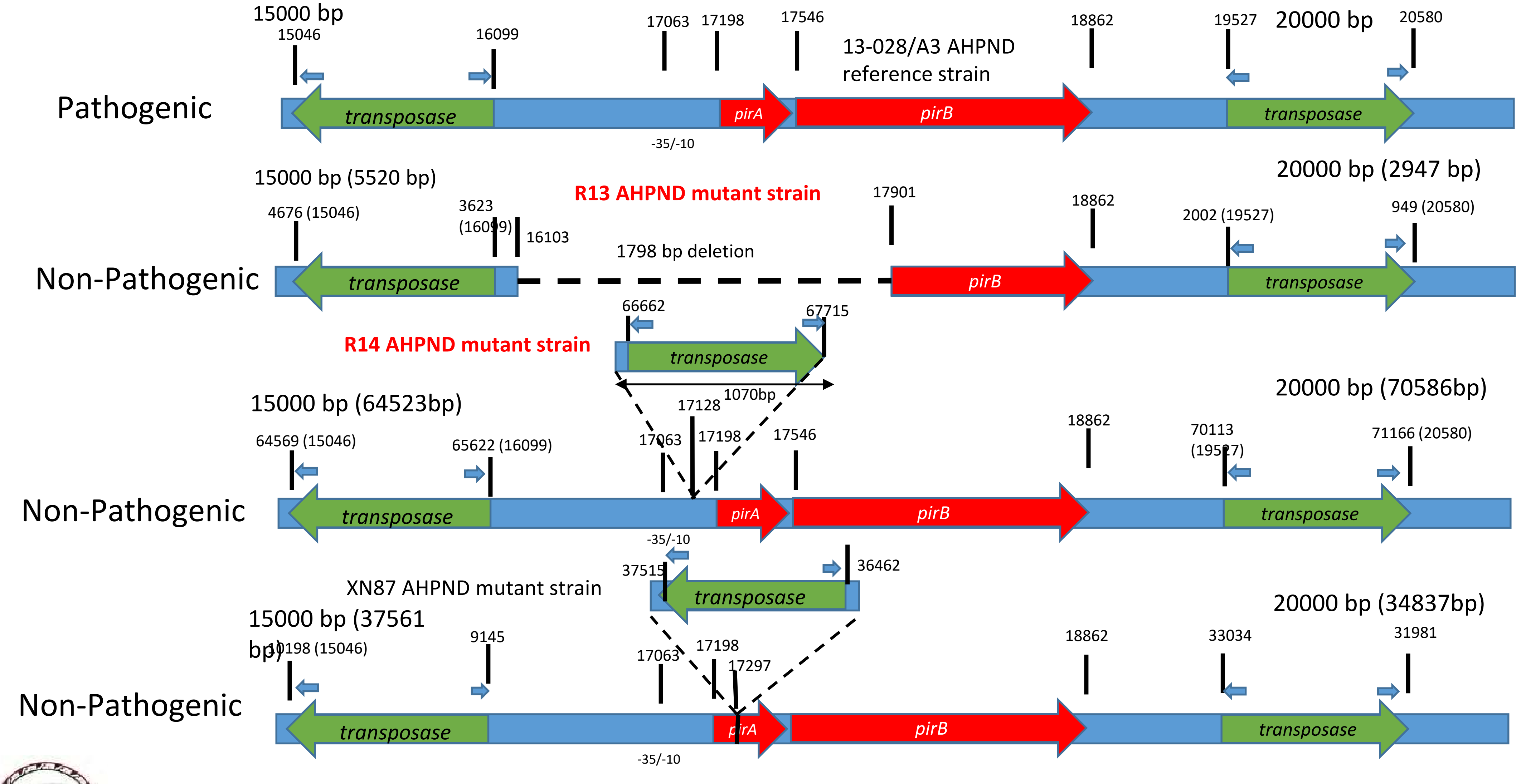
Real time PCR (qPCR) Assay

- AHPND detection and **quantification** method
- **Specific, Fast & Sensitive**
  - Within 30 min
  - Detection limit: <10 copies of virulence plasmid



Han et al., (Aquaculture, 2015)

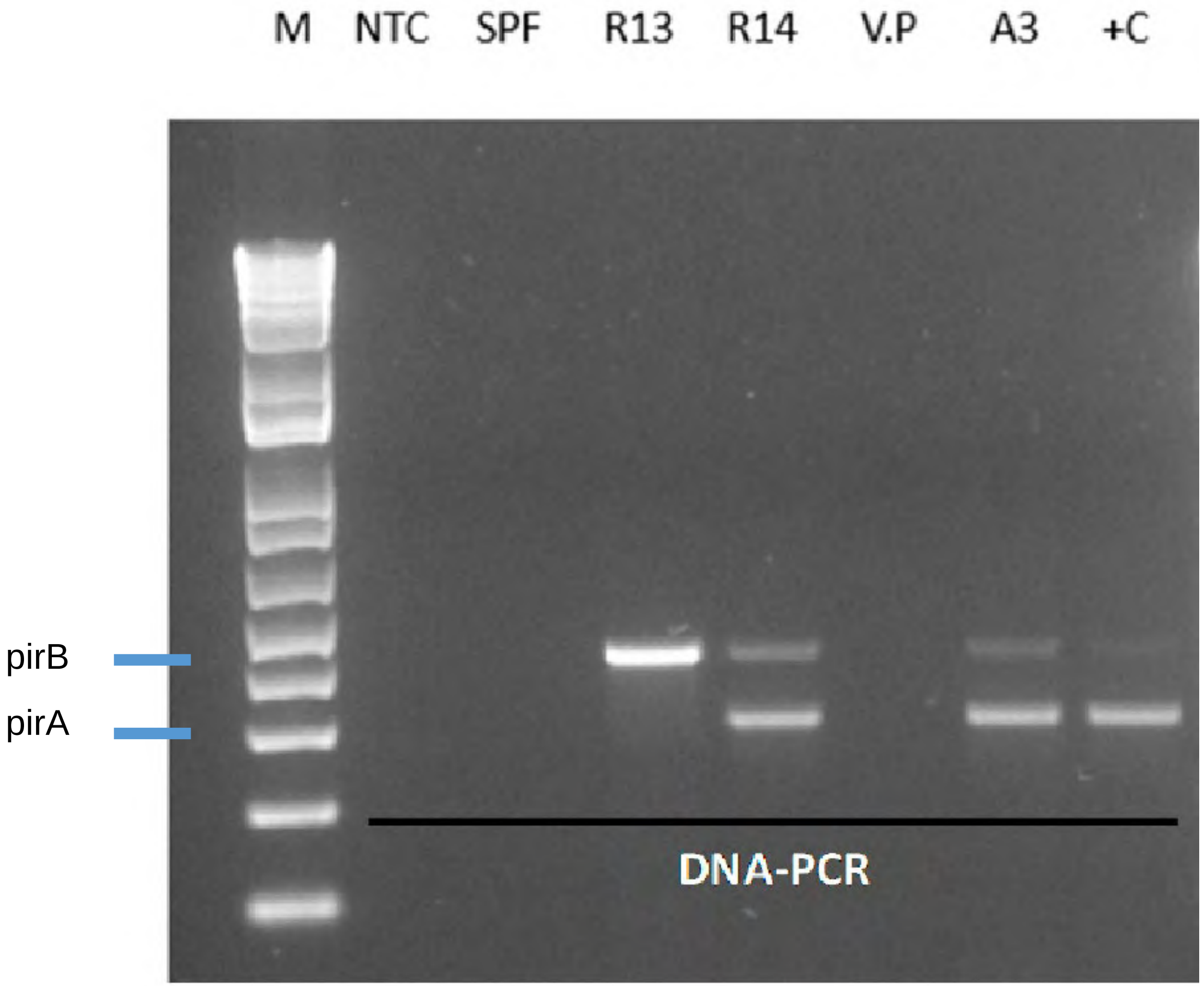
Transposition of *pir*AB genes: AHPND

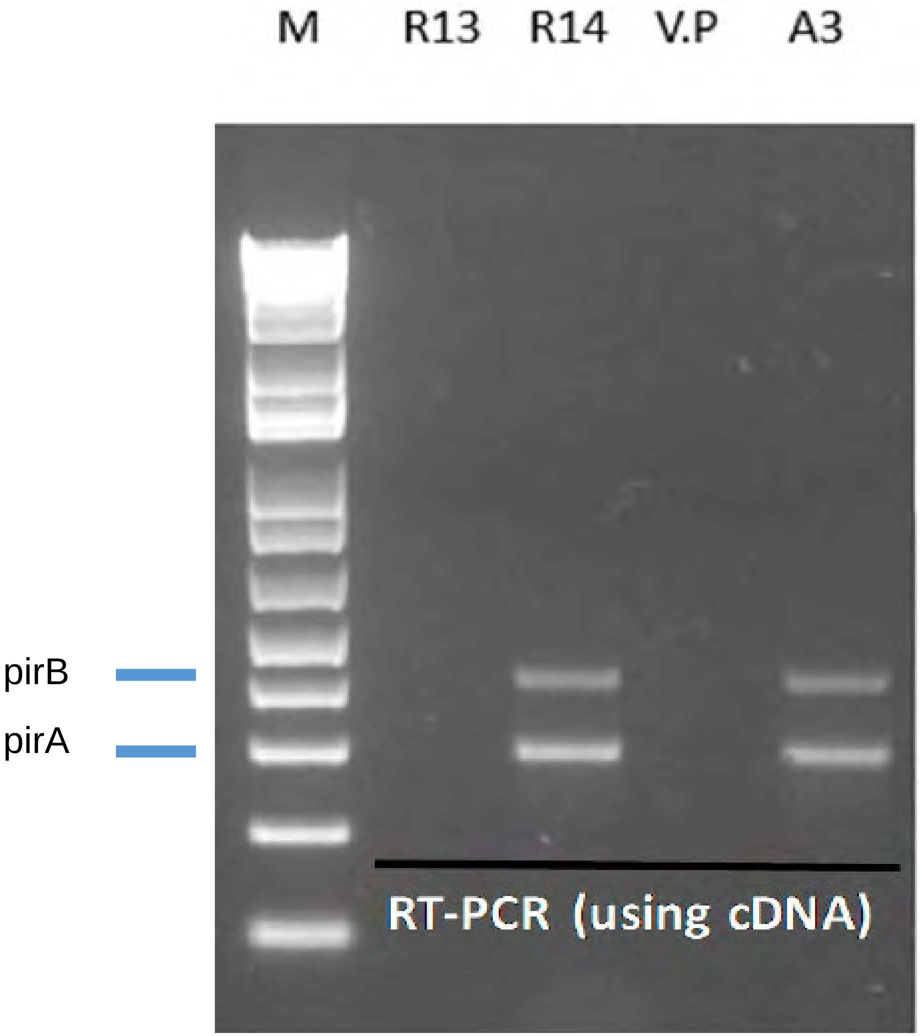


Aranguren et al., 2017.

Phiwsaiya et al., 2017.

Characterization of *V. parahaemolyticus* isolates R13 and R14

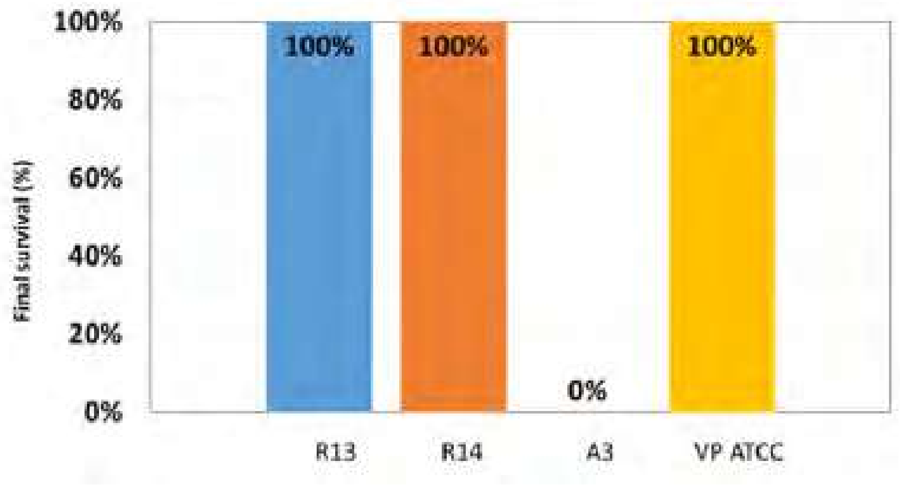




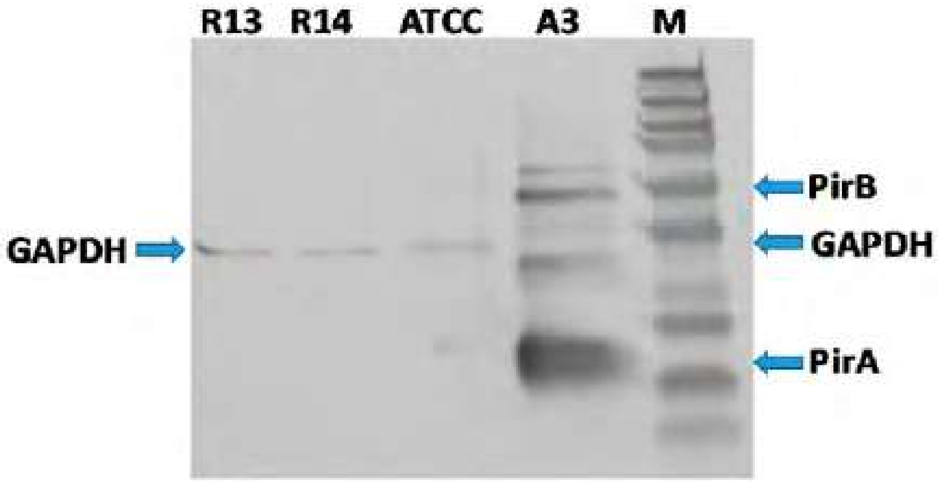
Aranguren et al., 2020.

Bioassay to characterize *V. parahaemolyticus* isolates R13 and R14

- SPF *P. vannamei* was challenged with Vp R13 and R13 isolates following an immersion challenge method
- *V. parahaemolyticus* isolate A3 isolate (Vietnam) and ATCC non-pathogenic culture of *V. parahaemolyticus* was used as a positive and negative controls, respectively



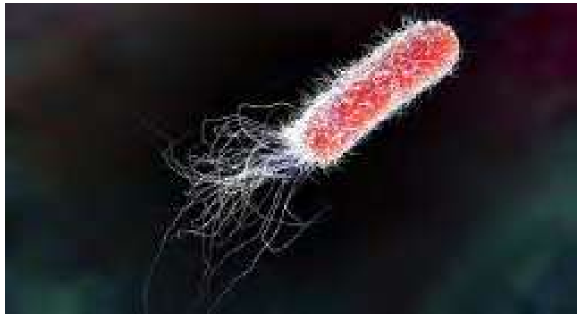
8B. Western blot analysis



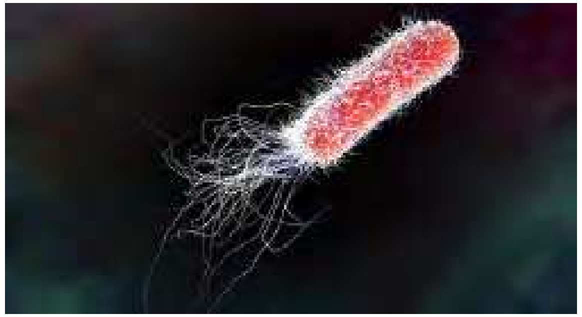
Results: Isolates R13, R14 and Vp ATCC did not cause any mortality in SPF shrimp, but the isolate A3 caused 100% mortality within 48 hr post-challenge

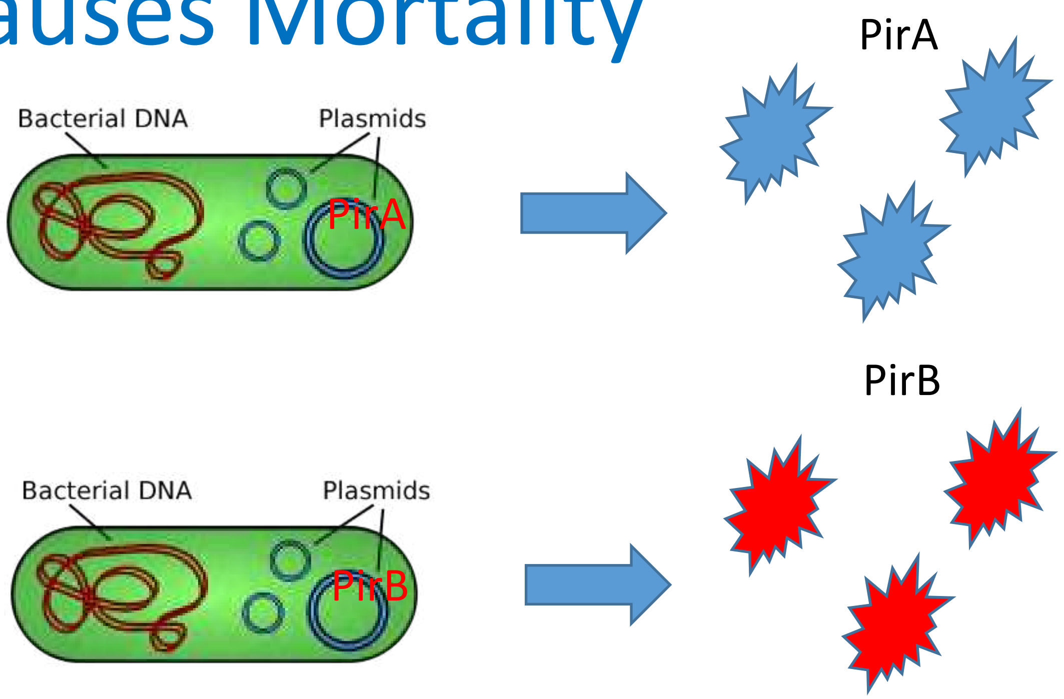
Aranguren et al., 2020.

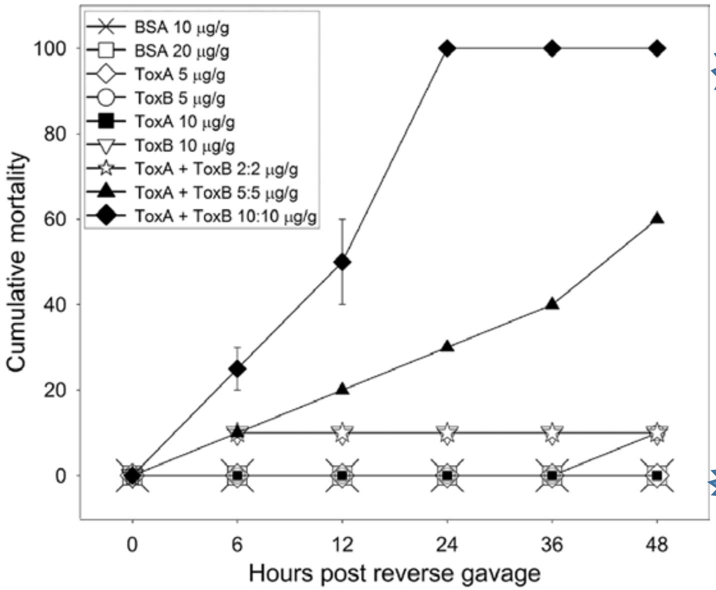
How AHPND Causes Mortality



E. coli







10 ug/g shrimp

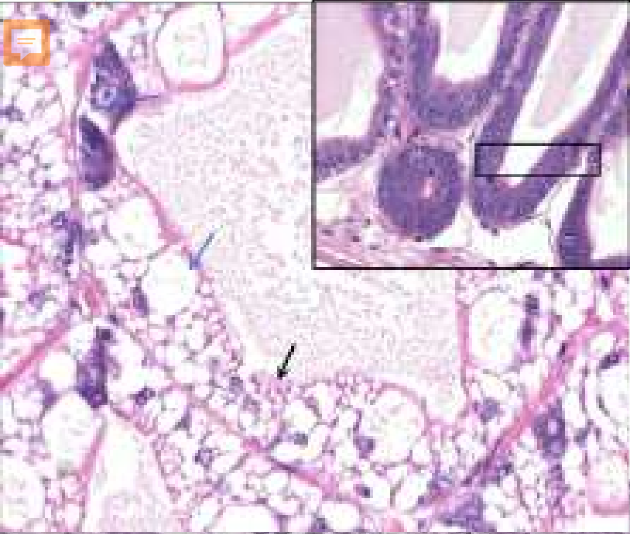
5 ug/g shrimp

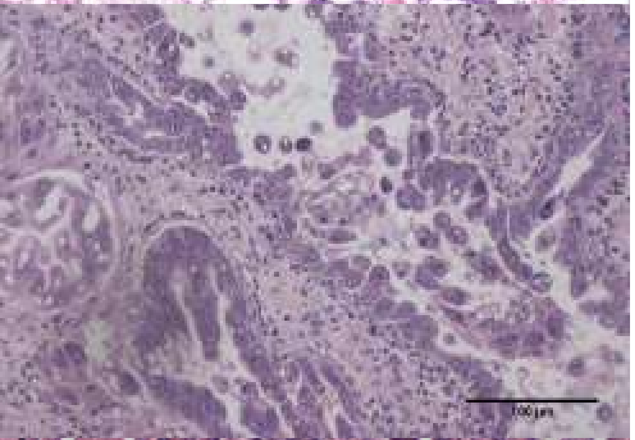
PirB

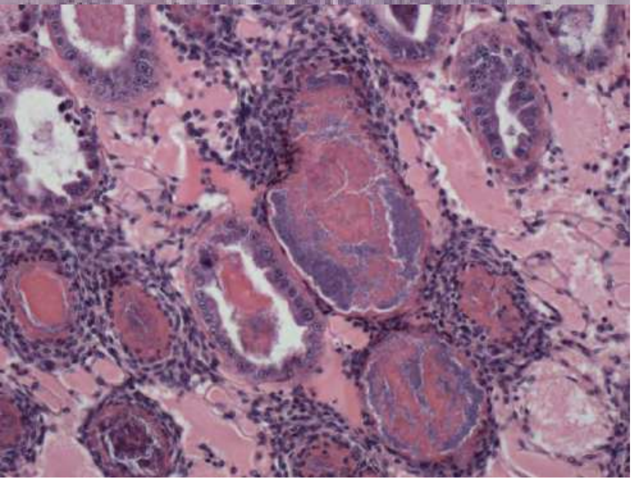
PirA

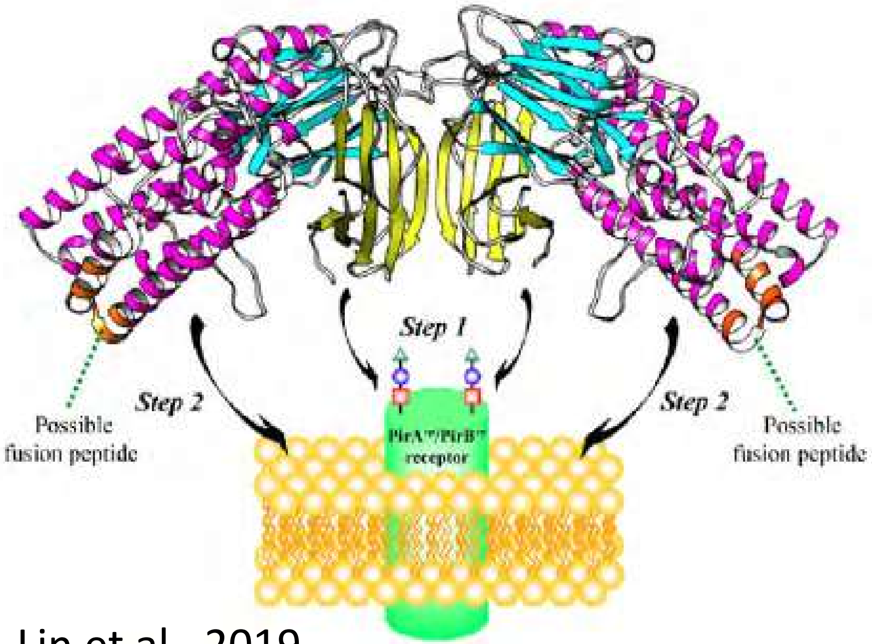
Sirikharin et al., 2015.

AHPND Possible pathogenicity model

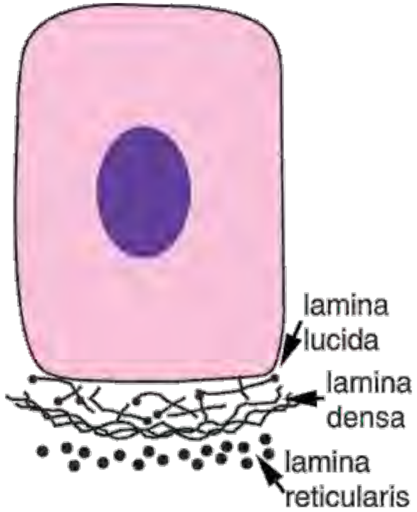








Lin et al., 2019

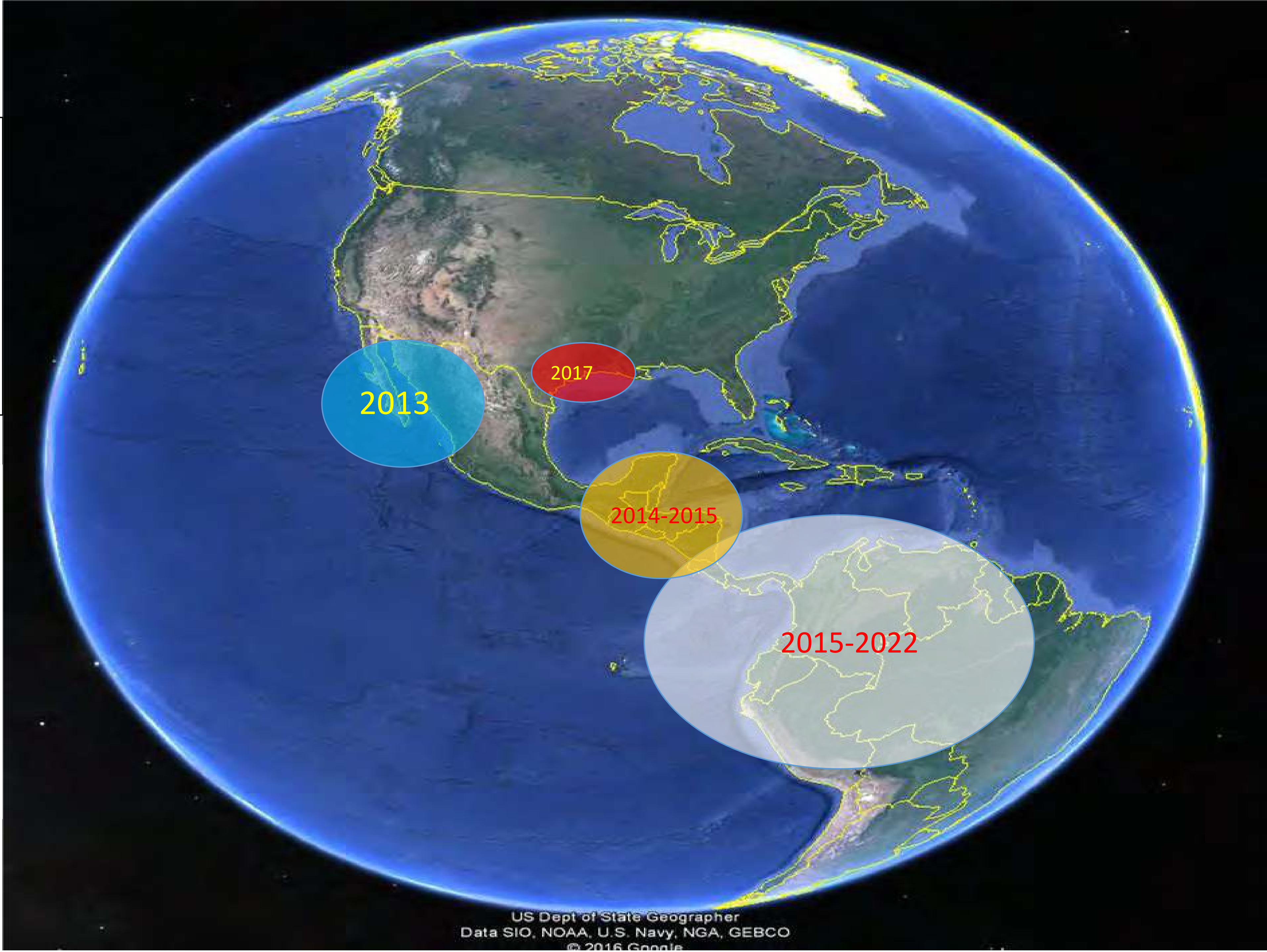


1. The PirA$_{vp}$/PirB$_{vp}$ heterotetramer complex
2. Uses PirA$_{vp}$ to recognize and bind with a receptor on the host cell membrane
3. The newly-exposed N-terminus region of PirB$_{vp}$ (orange) is pulled toward the cell membrane
4. N-terminus region of PirB$_{vp}$ inserts into the membrane using its a-helix and initiates the process of pore formation
5. PirA binds to cadherin which may trigger an alternative signal transduction pathway
6. This will destabilize the cytoskeleton and ion channels on the membrane, and induce cell death

Lin et al., 2019; Lee et al., 2015

Current status of AHPND in the Americas

- Nunan et al., 2014
- Restrepo et al., 2016
- Jun et al., 2016
- Han et al., 2017
- Ahn et al., 2017
- Cuellar & Brock et al., 2018
- Restrepo et al., 2018
- Kanrar & Dhar 2018
- Aranguren et al., 2020



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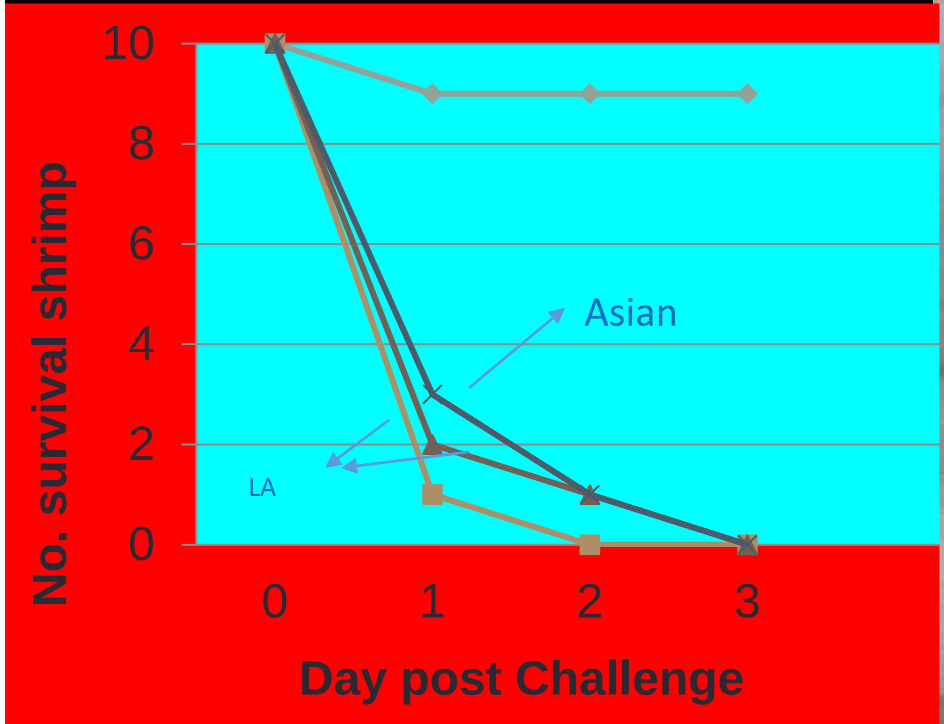
EMS in Sonora, Mexico – Photo by Ms. Silvia Gomez

AHPND Pathogenicity Asian vs Latin American type



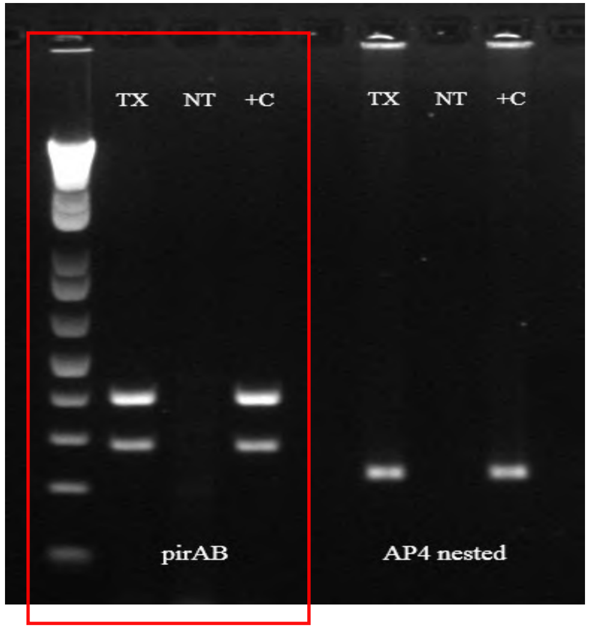
AHPND Detection in the US

- In June 2017, a shrimp disease diagnostic case (Case 17-327) was submitted submitted to the Aquaculture Pathology Laboratory, University of Arizona
- Samples were tested by PCR and histopathology
- PCR analysis revealed that the samples were infected with AHPND-causing *V. parahaemolyticus*

PCR Screening of Texas samples

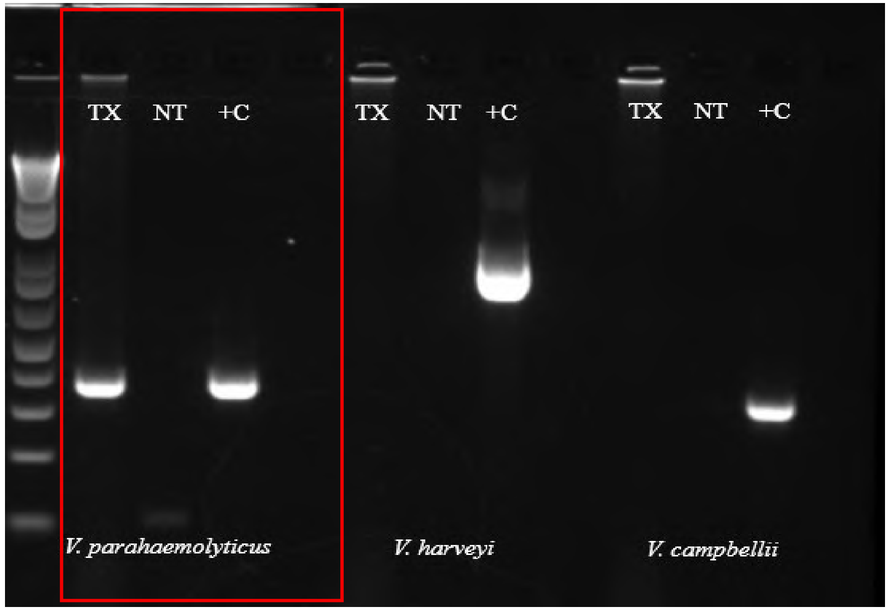
*pir*B 392 bp

*pir*A 284 bp



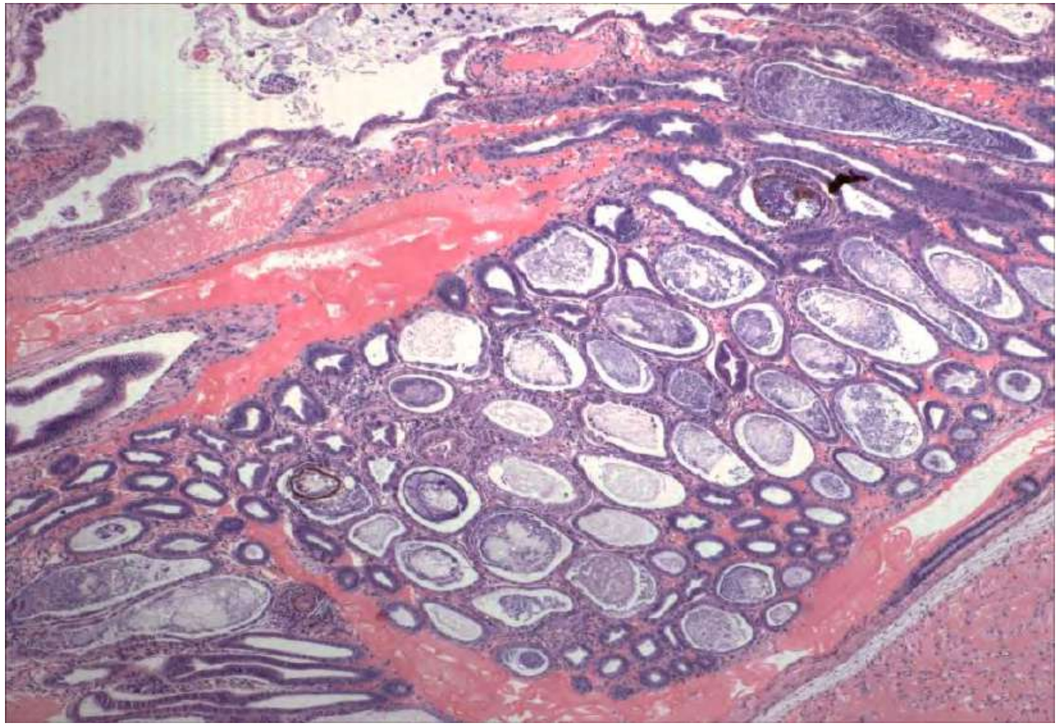
230 bp

Identification of *Vibrio sp. in* Texas samples



Dhar et al., 2019.

UAZ Case 17-327. Histopathology of *P. vannamei*, Texas, USA samples



Terminal phase of AHPND: Most HP tubules are destroyed. Massive bacterial infection, probably by *Vibrio* spp. 4x.

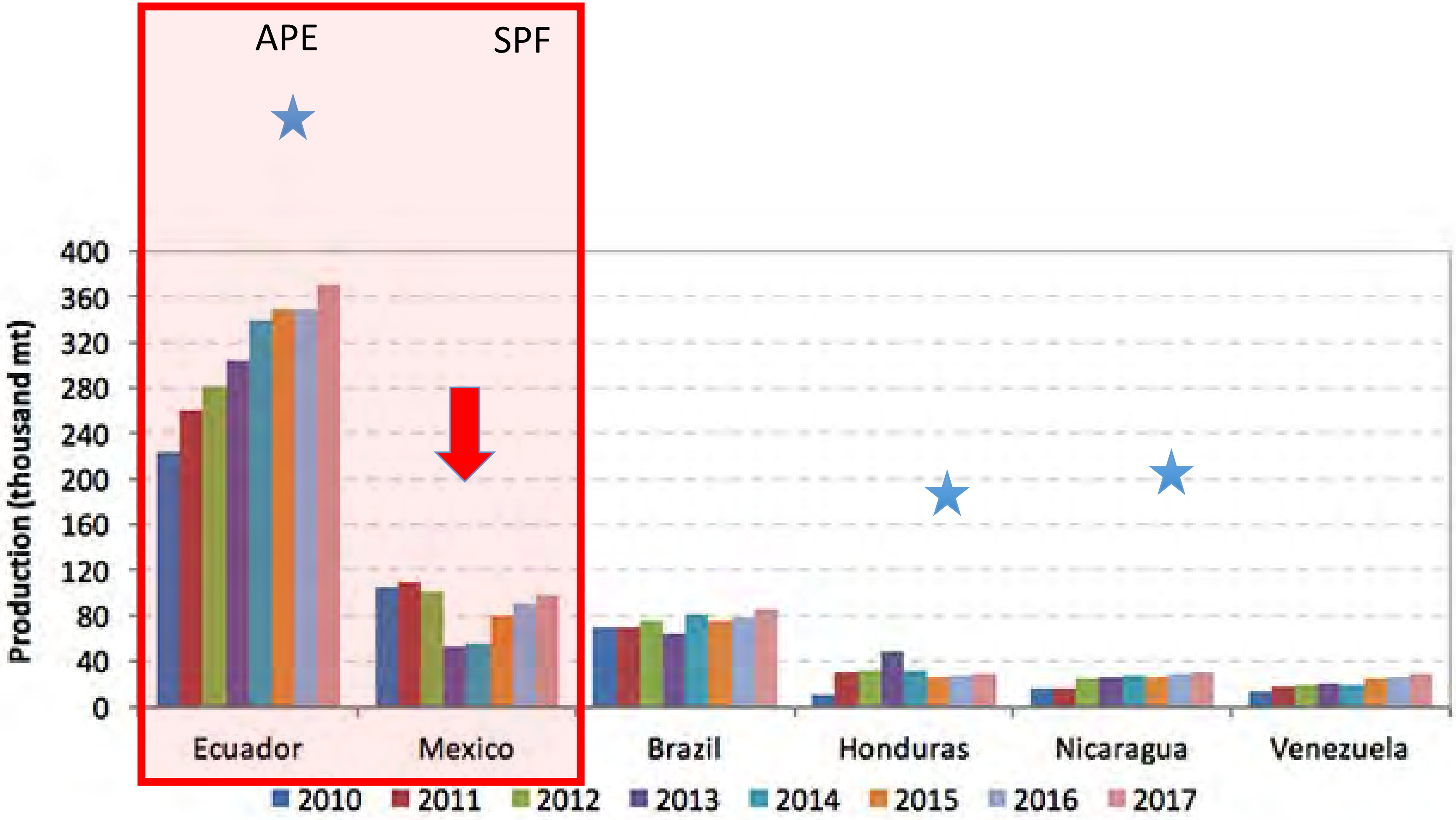
Conclusion: PCR and histopathology analysis of *P. vannamei* originated in Texas, USA clearly revealed the characteristics of AHPND.

Dhar et al., (2019)

Current status of AHPND in Latin-American

- No official report in many countries
- SPF populations are highly susceptible
- Latin American lines: More tolerant/resistant
- Interact with other enteric diseases
- High temperature favors its pathogenicity
- It is likely to be present in several Latin American countries
- Affect broodstock and PLs as well

Shrimp production in the American Countries



Sources: FAO (2013).; GOAL (2016).

Evolution of Acute Hepatopancreatic Necrosis Disease “AHPND” in SHRIMP Farming

Acute mortalities during the first 30 days of culture

SPF stocks

Acute mortalities during the cycle

Chronic mortalities in grow-out ponds

Chronic mortalities in maturation labs/broodstock

SPR/APE stocks

Acute mortalities in hatcheries /nurseries

AHPND in SE Asian shrimp culture

Breeding Centers
with F_N
SPF/SPR stocks

Imported stocks

Maturation/Nauplii
production units

Hatcheries
& nursery

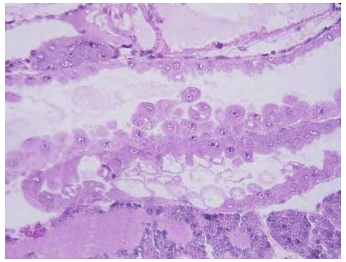
Pond management: High stocking density, DOC 90 days

Grow out ponds
Acute mortalities

AHPND: Latin-American shrimp culture

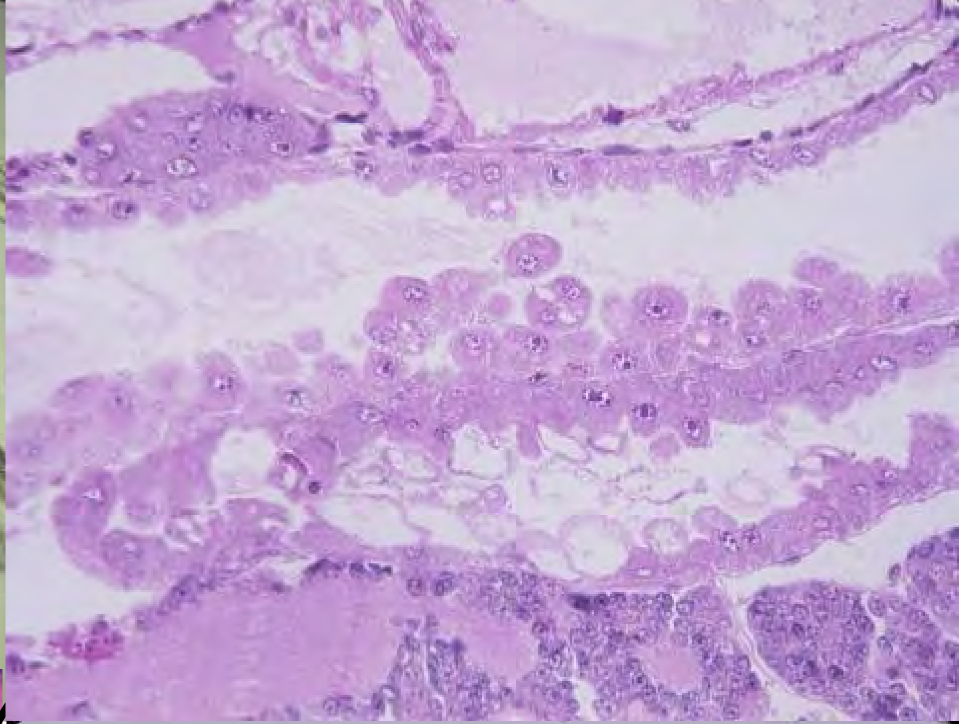
Maturation / Nauplii production units
Broodstock chronically infected

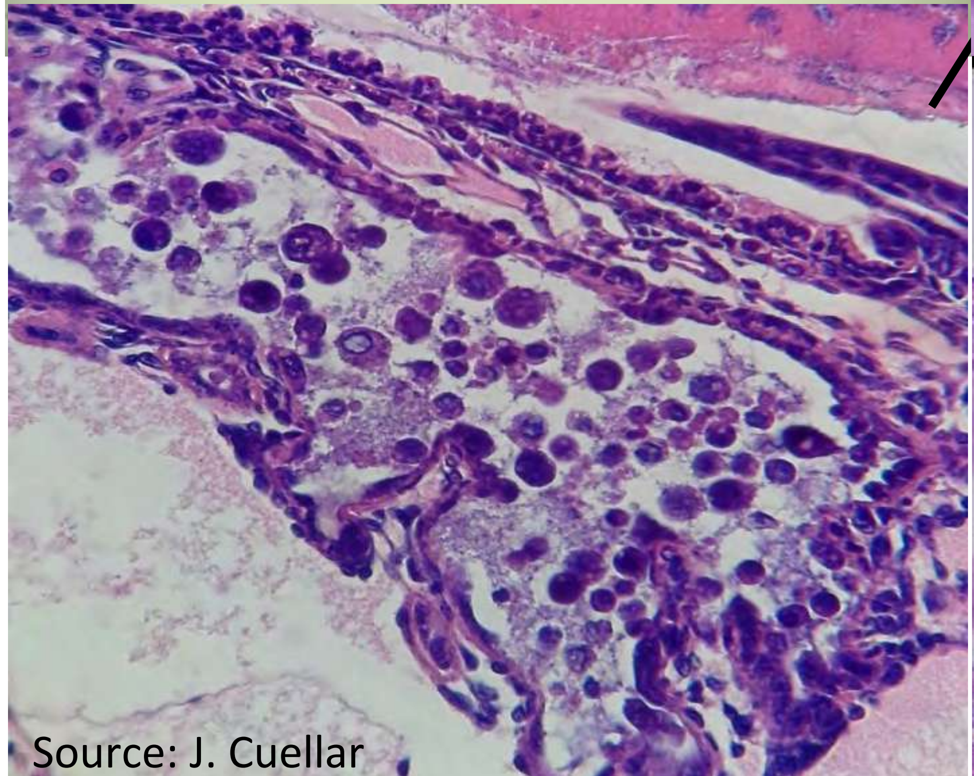
Hatcheries & nursery
Larva & PL infected: Acute mortalities

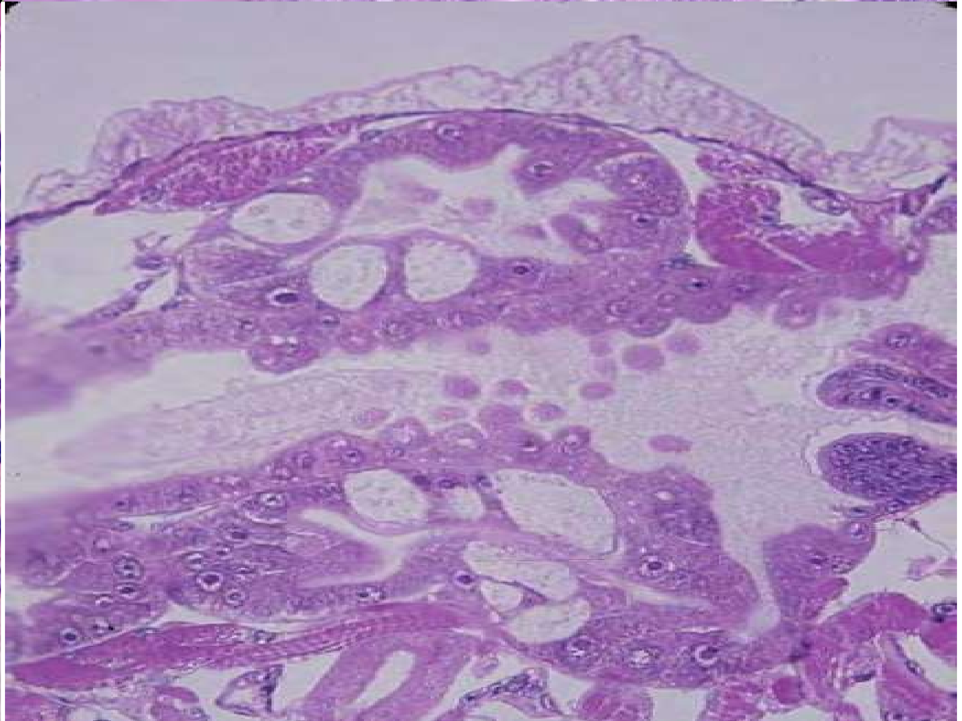


Broodstock ponds
Chronic mortalities

Grow out ponds
Chronic mortalities

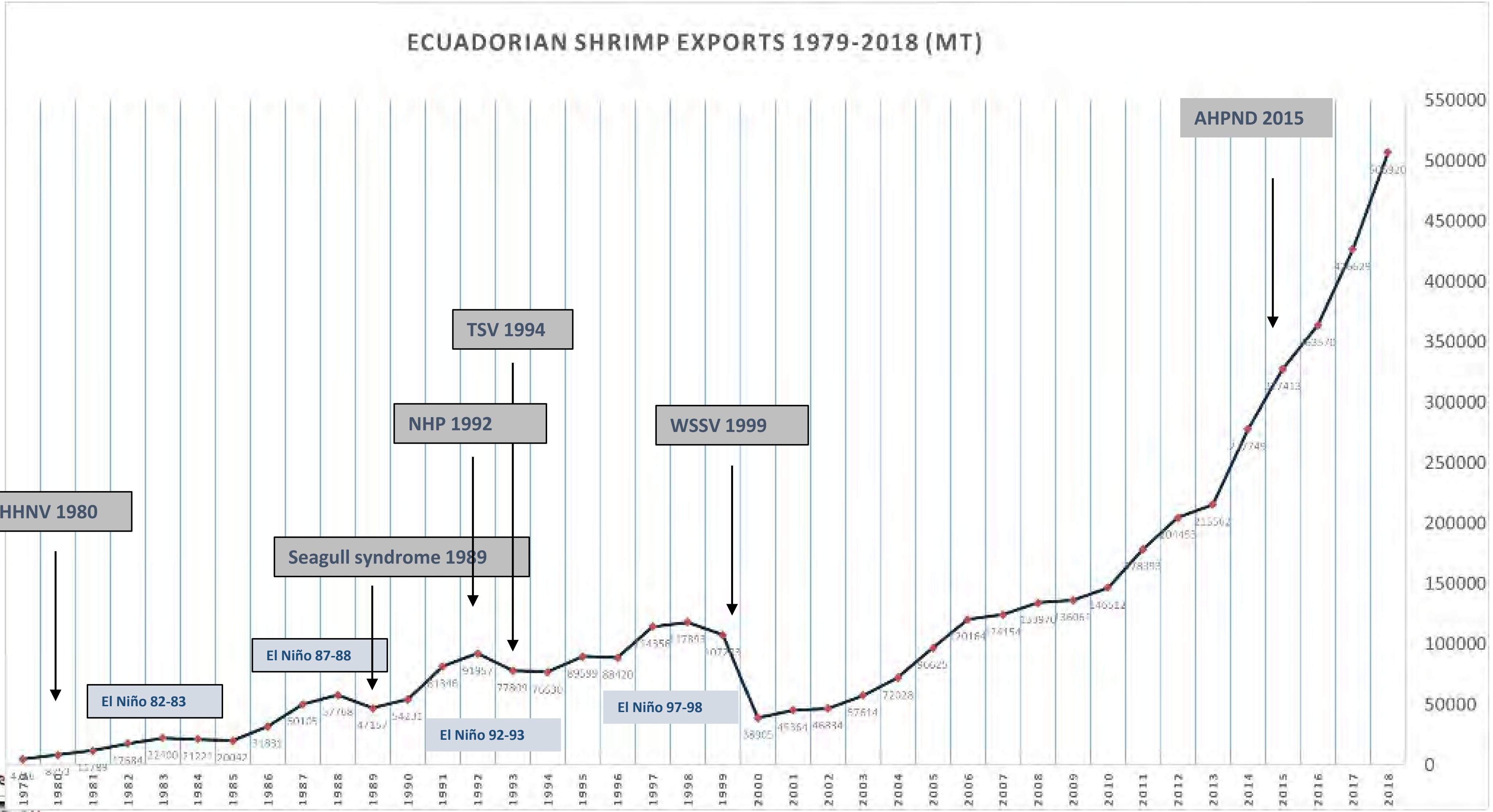






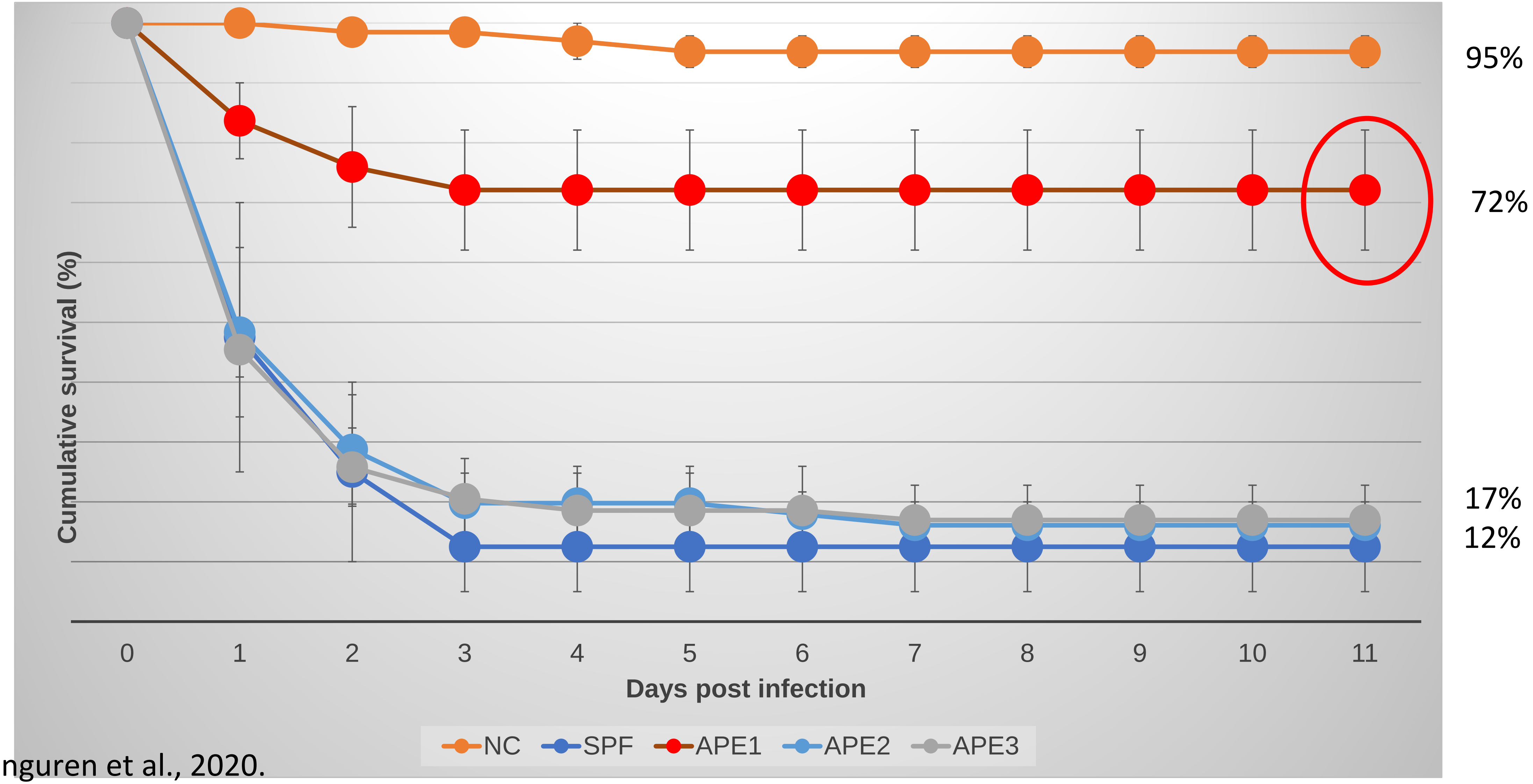
Source: J. Cuellar

AHPND: APE



Year	Increase %
2001	14.24
2002	3.14
2003	18.71
2004	20.01
2005	25.46
2006	19.59
2007	3.21
2008	7.33
2009	1.54
2010	7.13
2011	17.87
2012	12.75
2013	5.15
2014	22.39
2015	15.17
2016	9.95
2017	14.78
2018	15.84

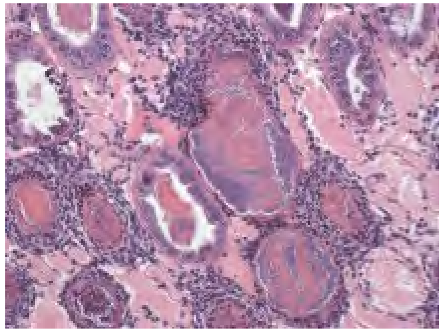
AHPND challenge test



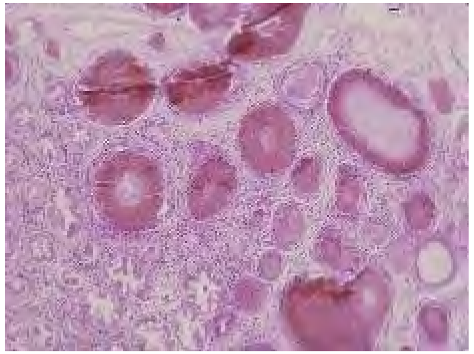
Aranguren et al., 2020.

Implication of the Chronicity of AHPND

- Chronic mortalities caused by opportunistic *Vibrio* spp. during the whole cycle

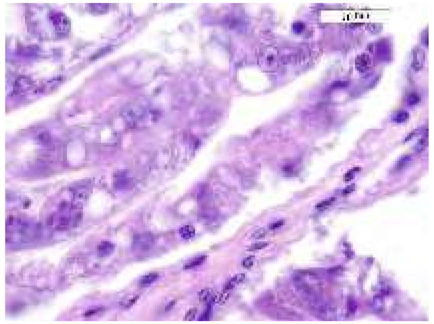


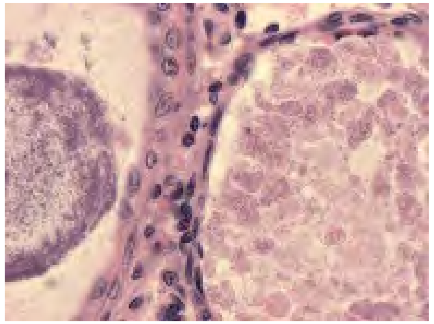
ISH for PirAB. Negative results (Cruz et al., unpublished)



Vibriosis in hepatopancreas (SHPN)

- Favors establishment of other enteric pathogens including *Vibrio* spp., NHP, & EHP





- Presence of chronic/acute mortalities in broodstock /post-larvas

Thank you for your attention!

Vibriosis & NHP

Luis Fernando Aranguren Caro, Ph.D
OIE Expert at the OIE Reference Laboratory for *H. penaei* (NHP)

Aquaculture Pathology Laboratory
OIE Reference Laboratory
USDA-APHIS Approved & ISO 17025, 17043 Accredited Laboratory
School of Animal & Comparative Biomedical Science
The University of Arizona, Tucson, Arizona, USA

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Primary and Secondary Infection by *Vibrio* spp.

VIBRIOSIS

- ✓ **Systemic infections**
- ✓ **Enteric & oral region infections**
- ✓ **Focal appendage necrosis**
- ✓ **Wound infections**
- ✓ **Shell disease**

2

VIBRIO - Agents

Disease Names (examples):

- Vibriosis, sea gull syndrome
- Septic hepatopancreatic necrosis (SHPN)
- Luminescent vibriosis, swollen hindgut
- Shell disease & appendage necrosis
- Acute hepatopancreatic necrosis disease (AHPND)

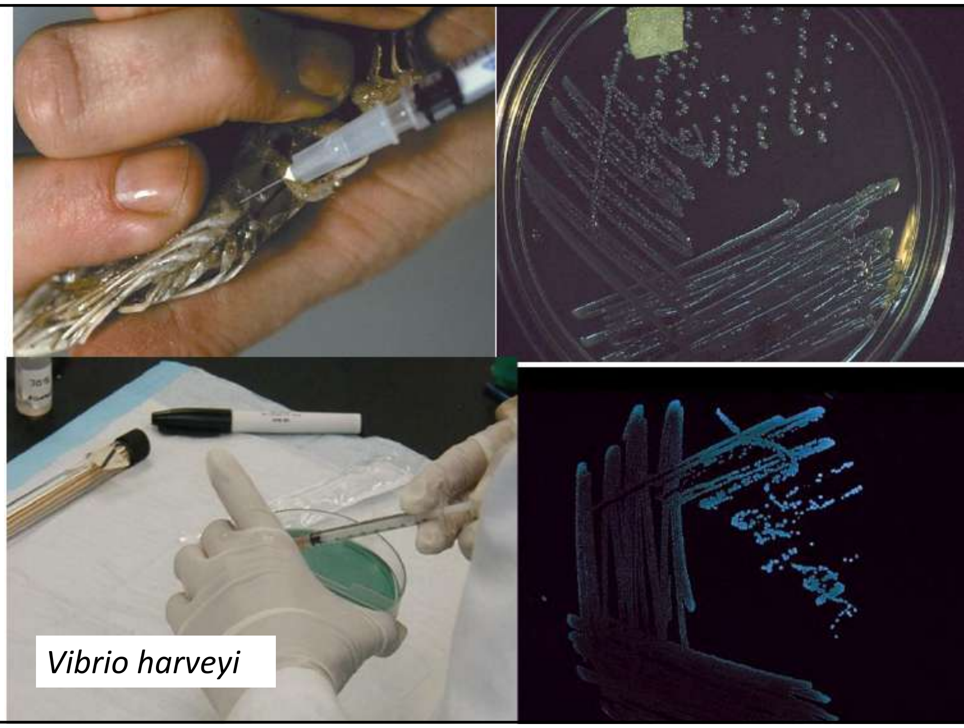
3

VIBRIO - Examples of Species

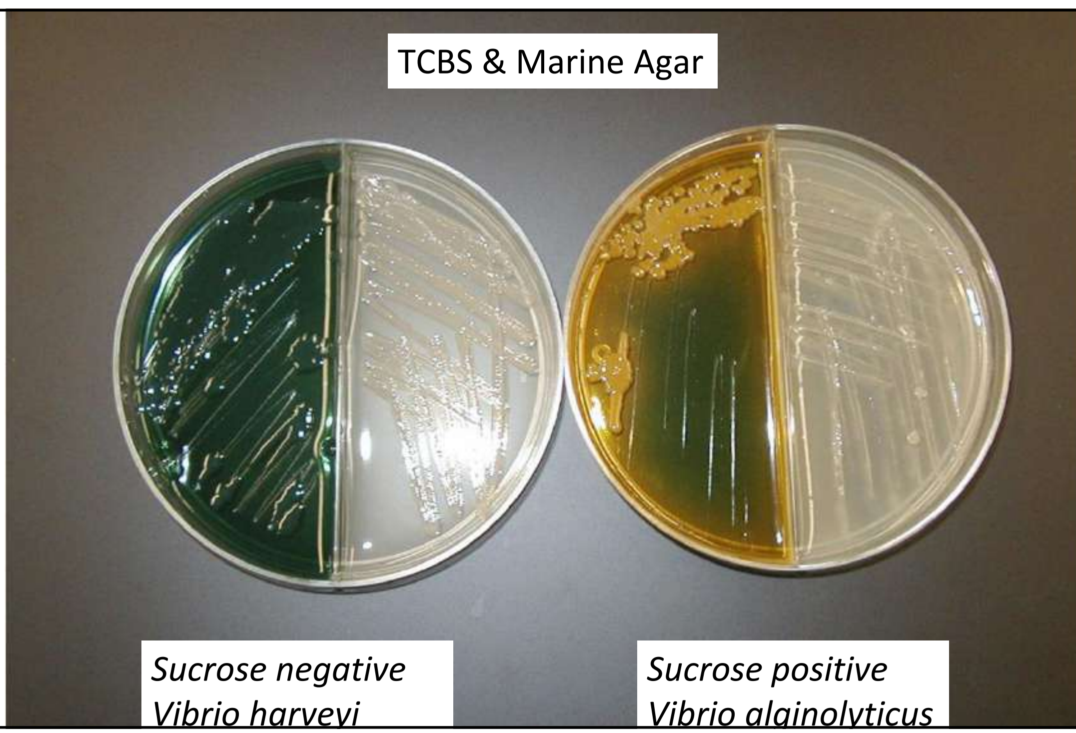
	Sucrose (TCBS)	Luminescence
*V. parahaemolyticus**	-	- / (+ rare)
*V. harveyi**	-	+ / (- rare)
*V. vulnificus**	-	+ / (- rare)
*V. penaeicida**	-	-
*V. nigripulchrituda**	-	-
*V. alginolyticus**	+	-
V. fluvialis	+	-

* Species with some pathogenic strains

4


Vibrio harveyi

5


TCBS & Marine Agar
Sucrose negative
Vibrio harveyi
Sucrose positive
Vibrio alginolyticus

6

VIBRIOSIS - Epizootiology

- All life stages affected (E, L, PL, J, A)
- Enteric, systemic, or external infections
- Antibiotic resistance easily developed
- Many vibrio infections are opportunistic or secondary
- Worldwide occurrence
- Some strains/species may be highly pathogenic. E.g AHPND causing-*Vibrio* spp.

7

VIBRIOSIS - Diagnosis in Hatchery

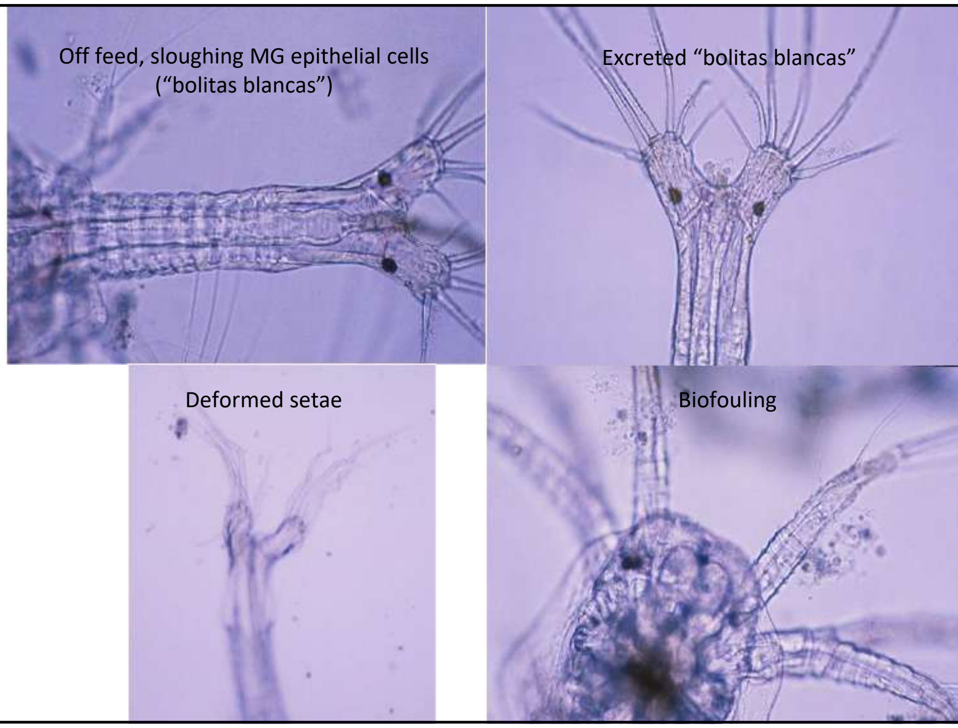
Presumptive Vibriosis:

- Wet-mounts - observation of bacteria, necrosis, inflammation & melanization, sloughing of HP, or MG epithelial cells
- Histology (H&E, Giemsa & tissue Gram stain):
  - External vibriosis: heavy cuticular bacterial colonization
  - Enteric vibriosis: colonization of internal cuticle (oral region, esophagus, stomach), HP/MG cell sloughing, necrosis, inflammation, & melanization
  - Systemic vibriosis: septicemia, HE nodules, muscle atrophy.

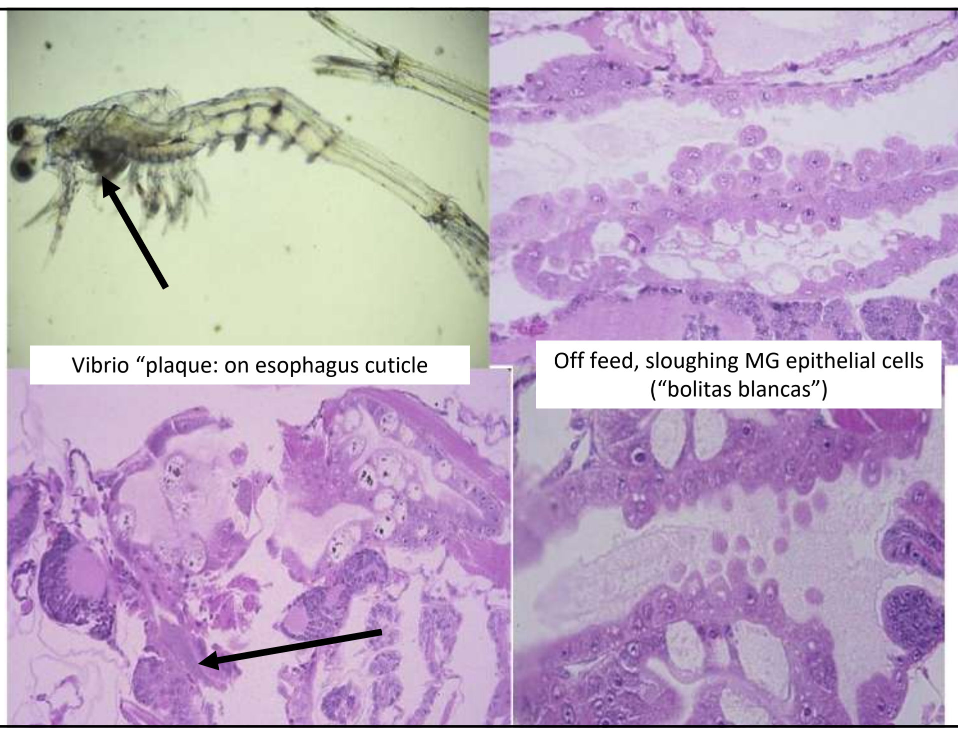
Confirmatory Vibriosis:

- Isolation, culture, & identification

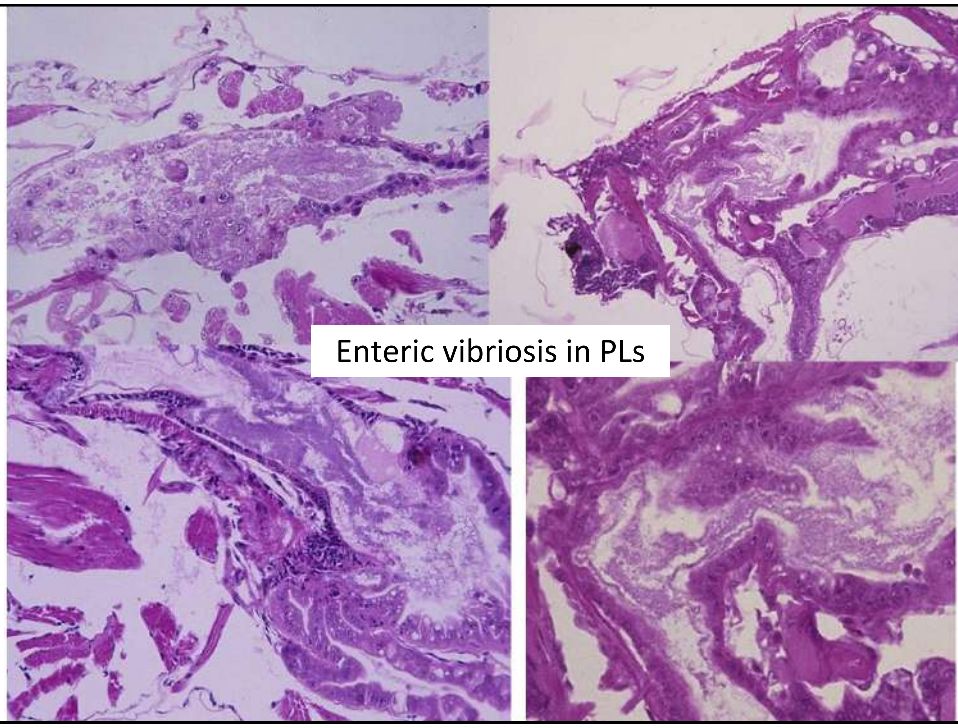
8


Off feed, sloughing MG epithelial cells
(“bolitas blancas”)
Excreted “bolitas blancas”
Deformed setae
Biofouling

9


Vibrio “plaque: on esophagus cuticle
Off feed, sloughing MG epithelial cells
(“bolitas blancas”)

10



Enteric vibriosis in PLs

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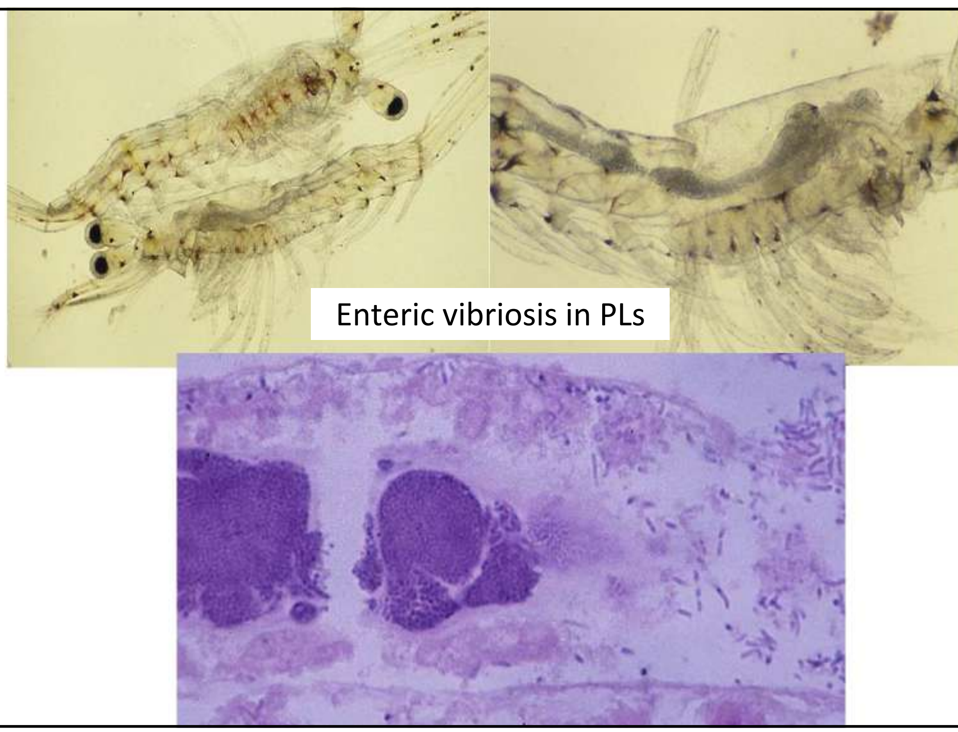
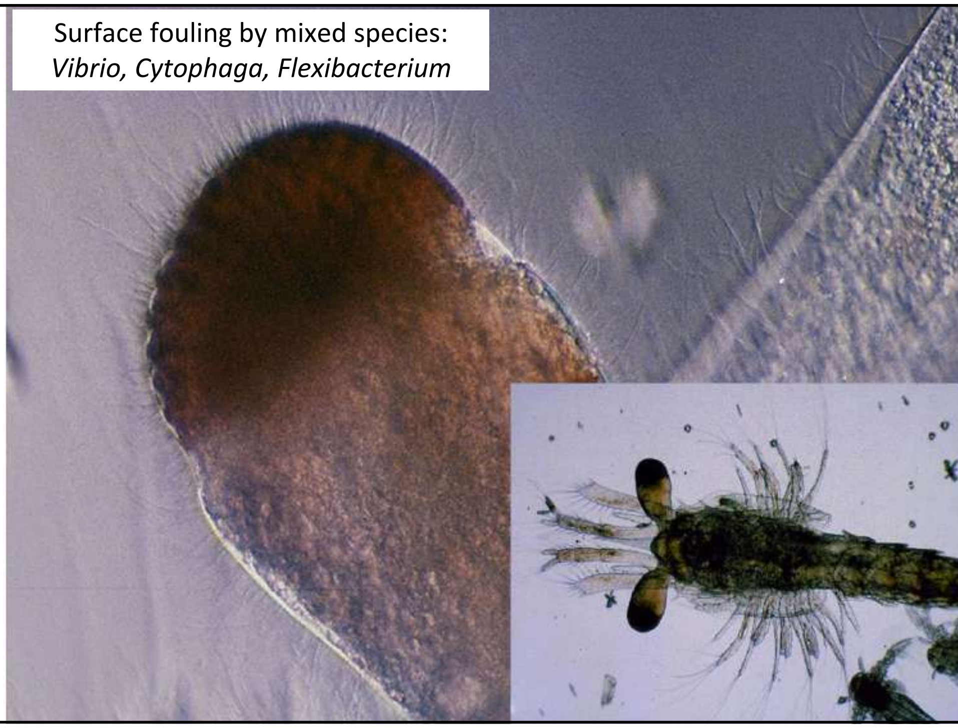
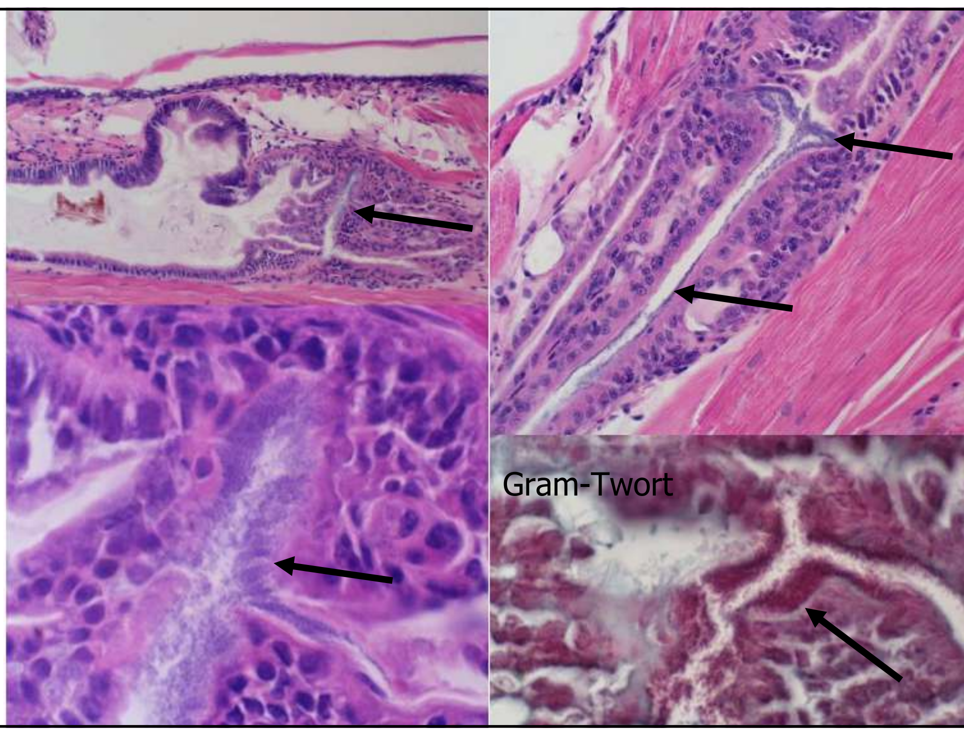


Enteric vibriosis in PLs

12


Surface fouling by mixed species:
Vibrio, Cytophaga, Flexibacterium

13


Gram-Twort

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VIBRIOSIS - Farm

Disease names: systemic or enteric vibriosis, Septic HP Necrosis (SHPN), 'sindrome gaviota' (sea gull syndrome), septic hemocytic enteritis, shell disease

Clinical signs:

- Reduced feeding
- High to severe mortalities
- Sea birds attracted to dead or moribund shrimp
- Hemolymph with increased clotting time (> 1-2 minutes)
- Melanized foci in/on cuticle, gills, or appendages
- Discolored atrophied HP (white, black streaks)

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VIBRIOSIS - Diagnosis in Juveniles/Adults

Presumptive Vibriosis:

- Hemolymph – increased clotting time (>1.0-2 min)
- Wet-mounts - observation of bacteria, necrosis, inflammation & melanization, sloughing of HP, or MG epithelial cells
- Histology (H&E, Giemsa & tissue Gram stain):
  - External vibriosis: heavy cuticular bacterial colonization of necrotic & inflamed lesions of cuticular or appendage lesions
  - Enteric vibriosis: colonization of cuticle foregut, colonization, & invasion of MG, HG
  - SHPN: heavy bacterial colonization of tubules, marked HP cell sloughing, necrosis, inflammation, & melanization
  - Systemic vibriosis: septicemia, bacterial micro-colonies in LO, hemocytic nodules w/bacteria in LO, heart, gills, CT, etc.

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‘SINDROME DE GAVIOTA’ (SEA GULL SYNDROME)

Example of a severe systemic, luminescent vibriosis epizootic in Ecuador from 1989-1990.

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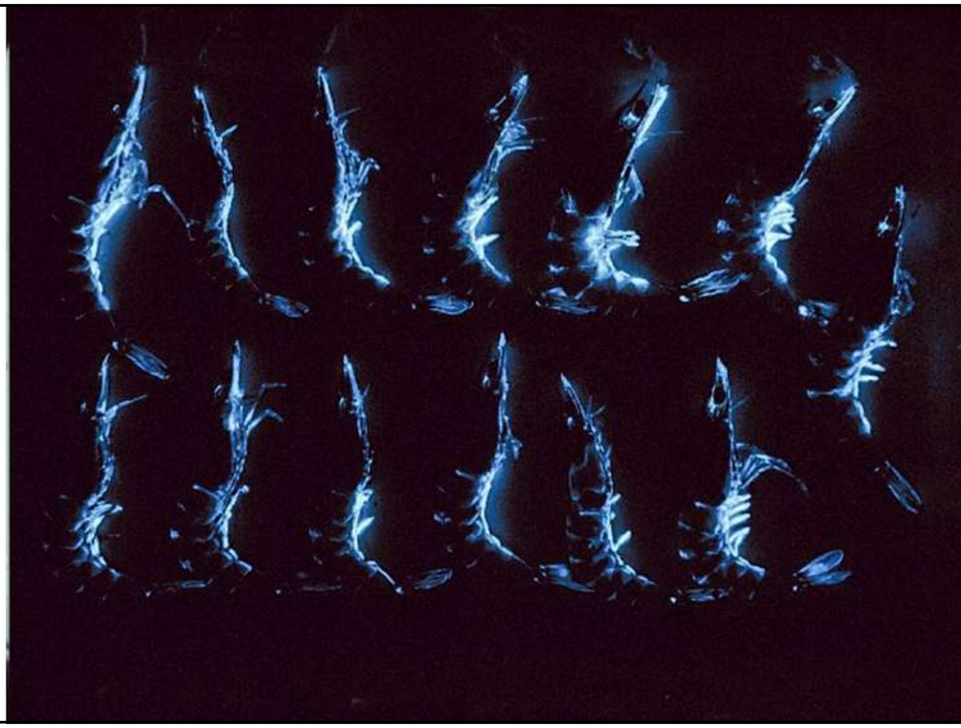

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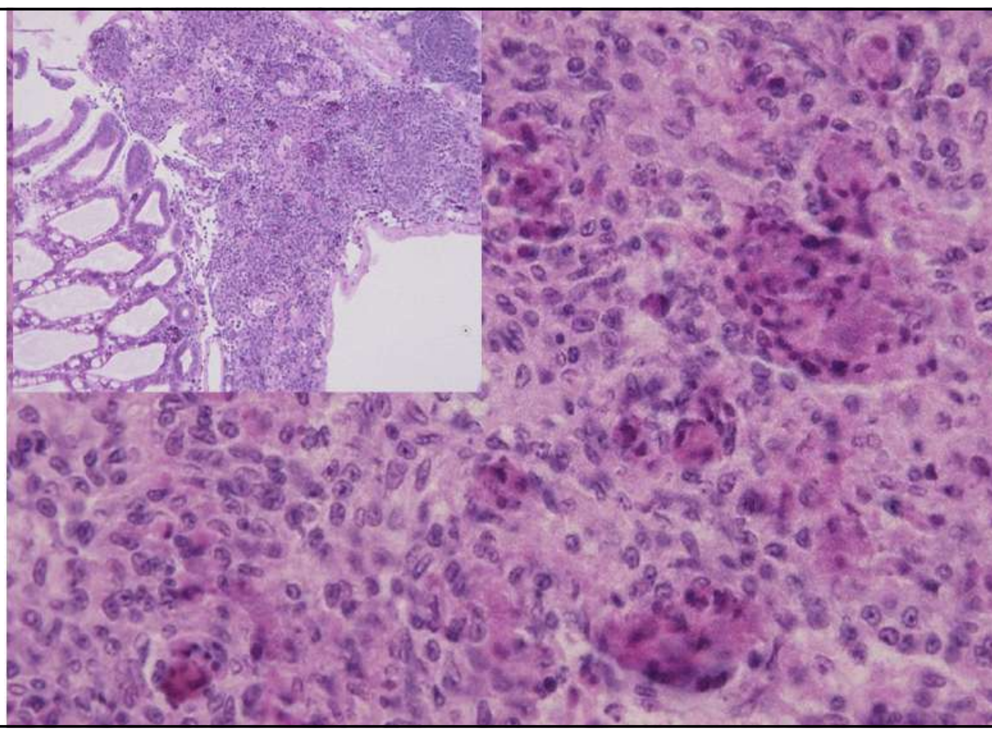

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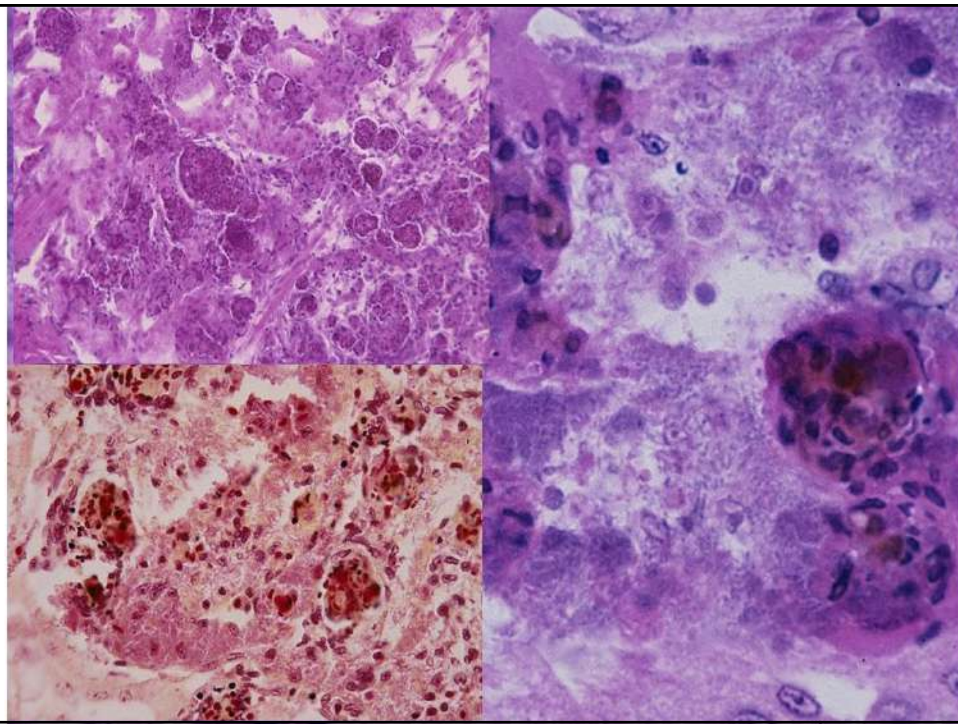

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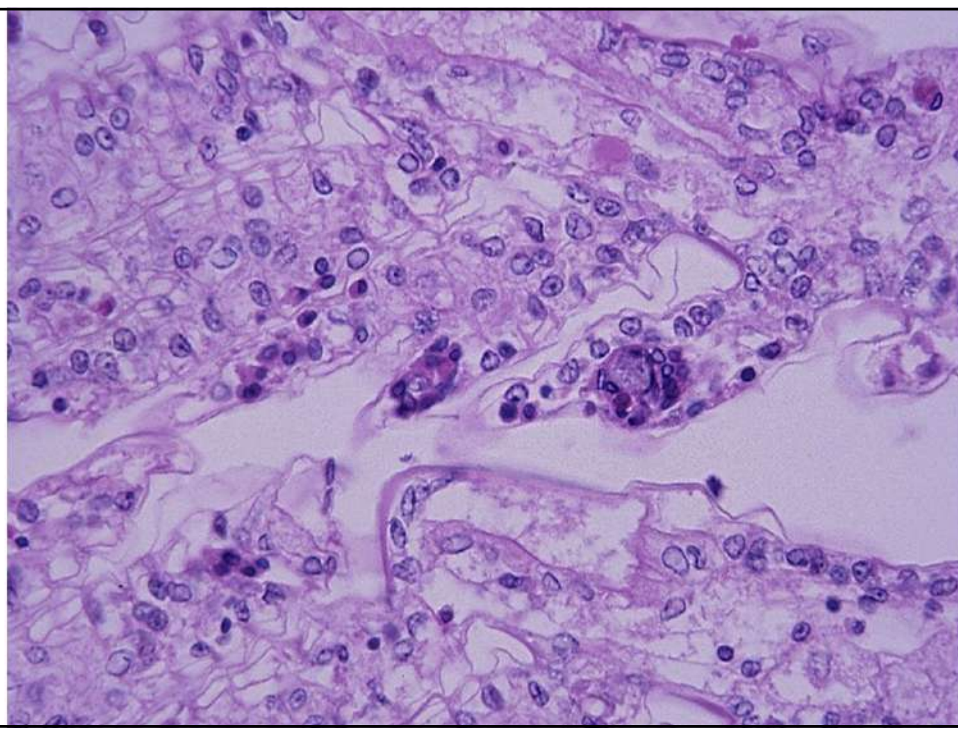

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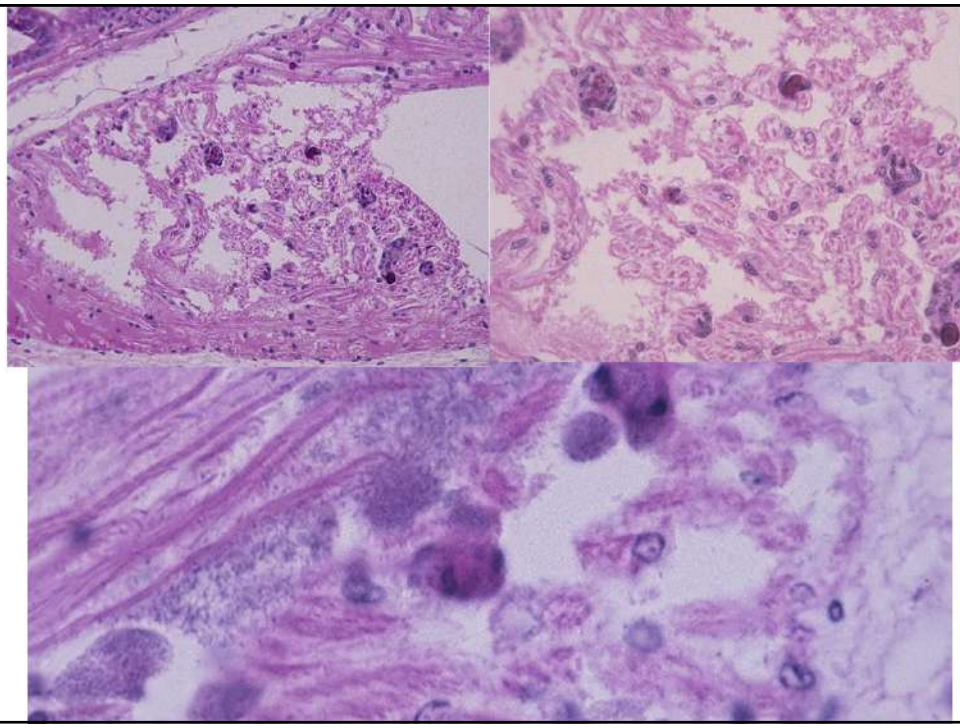

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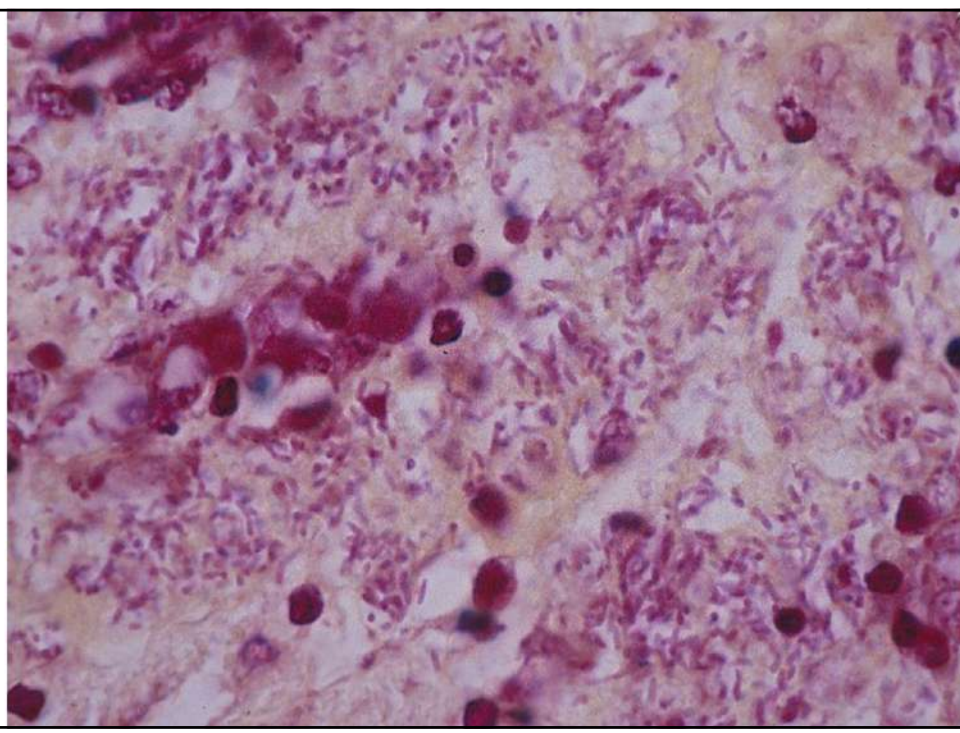

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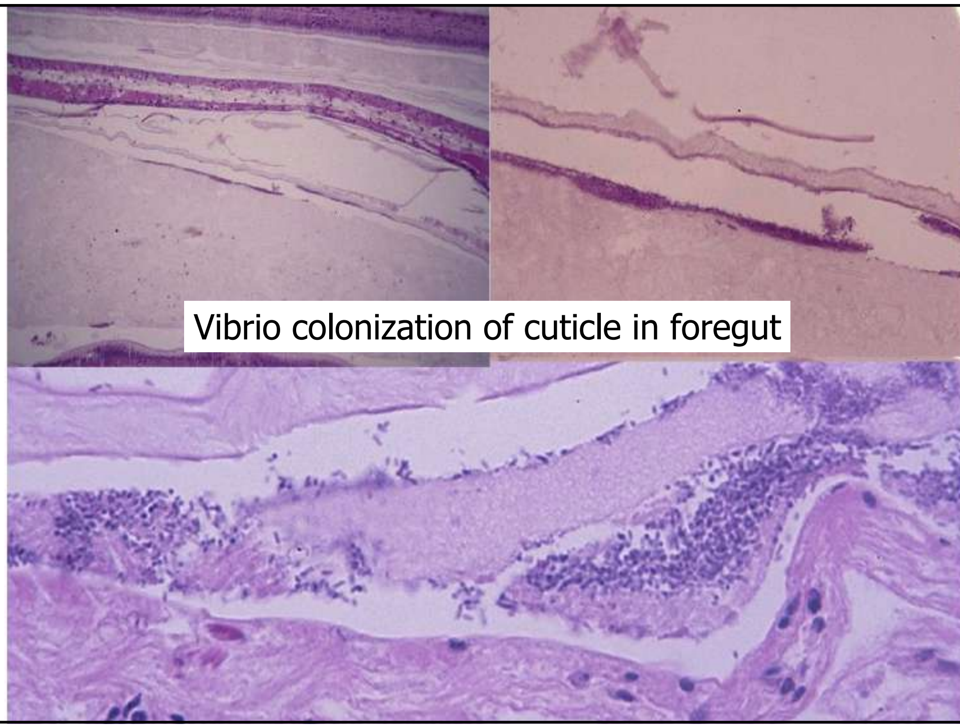

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SEPTIC HEPATOPANCREATIC NECROSIS (SHPN)

Clinical signs:

- Reduced feeding
- Extreme lethargy; accumulate on pond edges & surface.
- Increasing mortalities
- Muscle opacity & pigmentation changes
- HP atrophied, whitish, often with black spots, streaks
- Hemolymph with increased clotting time (> 1.0-2.0 minutes)

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SEPTIC HEPATOPANCREATIC NECROSIS (SHPN)

Histopathology:

- Sepsis of HP tubules
- Marked atrophy
- Low levels of lipid droplets & B-cell vacuoles
- Necrosis, inflammation, melanization of HP tubules

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SHPN – Vibriosis of HP

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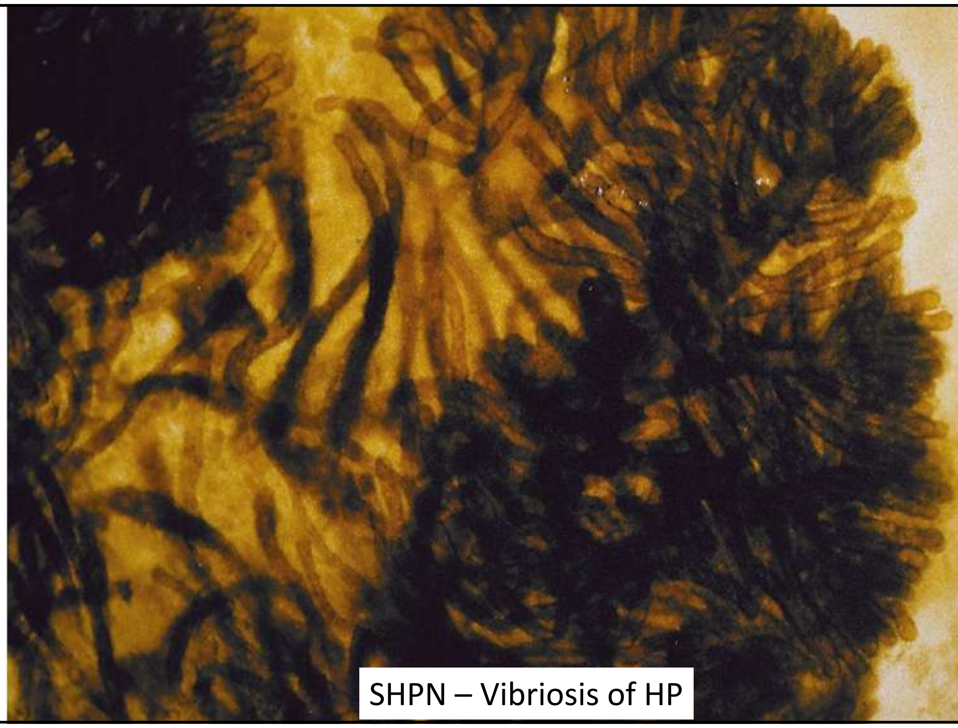

SHPN – Vibriosis of HP

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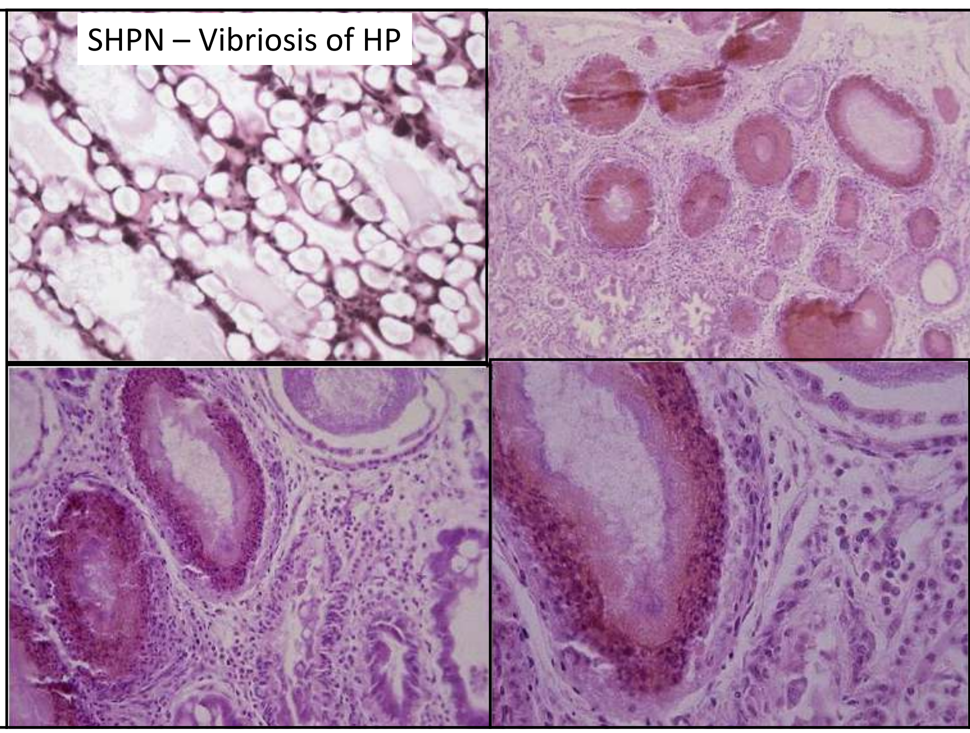

SHPN – Vibriosis of HP

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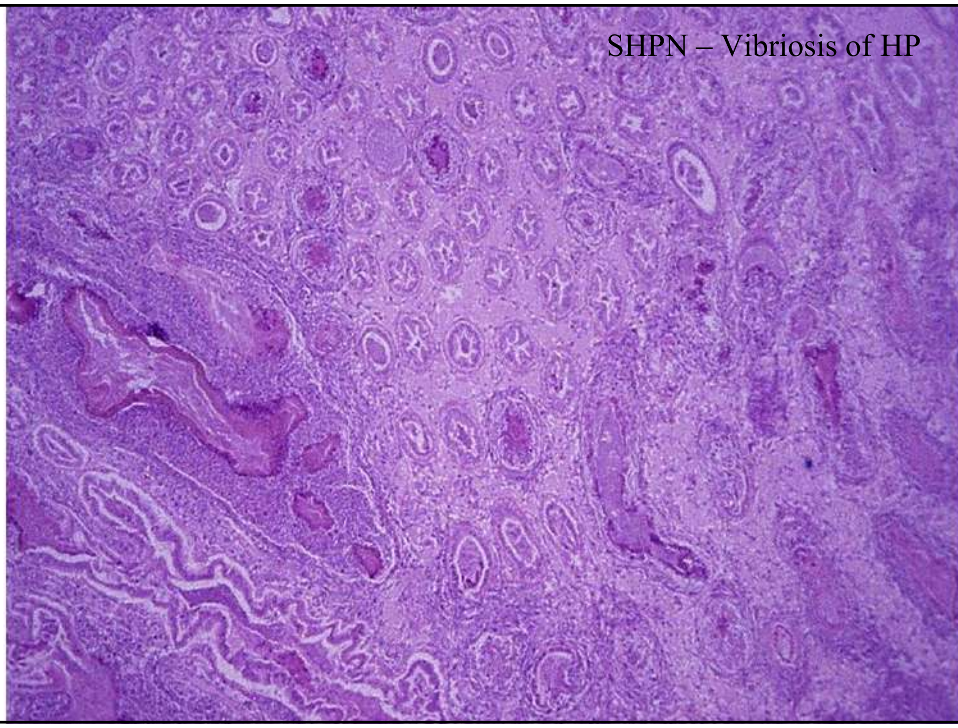

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Bacterial Shell Disease

- Cuticular erosion, ulceration, vesicles, & 'pustules'
- Appendage necrosis
- Black gills
- "Splinters"
- Causative bacteria possess enzymes:
  - chitinase, lipase, protease

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Shell Disease

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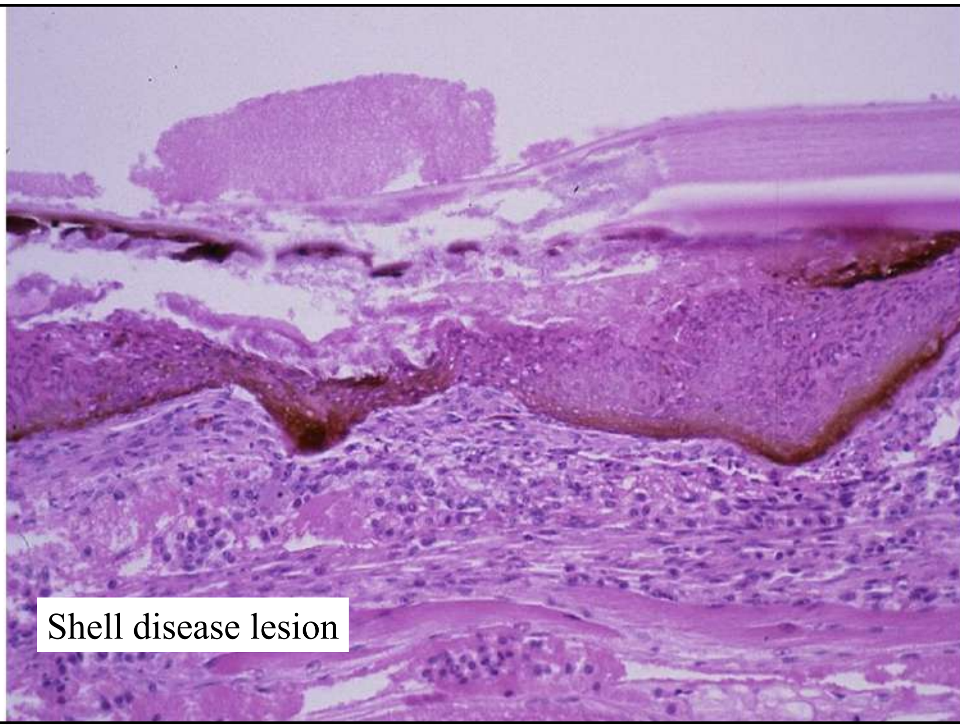

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VIBRIOSIS: Management in Hatchery & Farm

Hatchery:

- Batch culture with disinfection & dry-out between runs
- Disinfection of eggs, nauplii, artemia nauplii
- Use of good culture practices (feeding, density, water treatment)
- Use of probiotics
- Use of biofloc
- Therapeutic antibiotic use when required

Farm:

- Use of good culture practices (plankton blooms, optimum feeding, pond liming, fallowing, etc.)
- Maintain low level of organic matter in ponds
- Therapeutic use of medicated feeds when required

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Necrotising hepatopancreatitis (NHP)

Hepatobacter penaei

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OIE (Office International des Epizooties; World Organisation for Animal health)

- Crustacean diseases
  - Crayfish plague (*Aphanomyces astaci*)
  - Infection with Yellow head virus (YHV)
  - Infectious hypodermal and haematopoietic necrosis (IHHNV)
  - Infectious myonecrosis (IMNV)
  - Necrotizing hepatopancreatitis (NHP); *Hepatobacterium penaei.* (listed by OIE on 2010)
  - Taura syndrome (TSV)
  - White spot disease (WSSV)
  - Decapod Iridescent virus (DIV-1)
  - White tail disease
  - Acute Hepatopancreatic necrosis Disease (AHPND)

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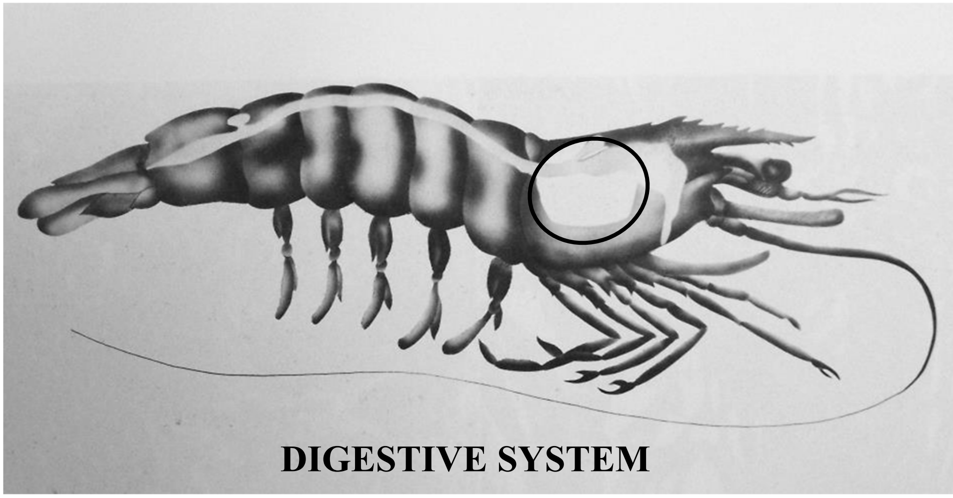
Target Organ: Hepatopancreas



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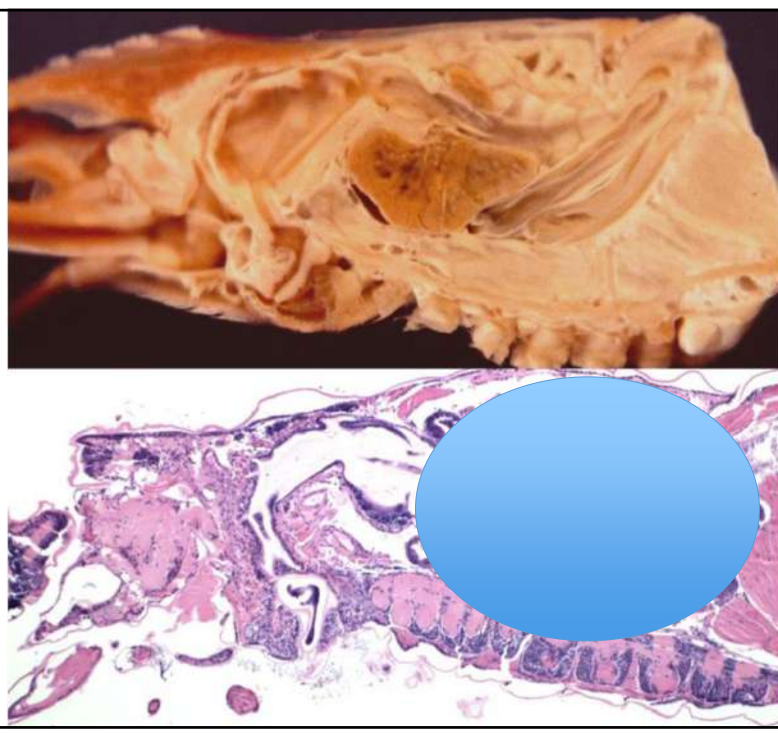


40

NHP Bacterium
Alpha-proteobacteria.
Orden: Rickettsiales (Nunan et al., 2012)/Halosporales (Szokoli et al., 2016.)

Morphology: Pleomorphic, rickettsia-like, gram negative

Rod: no flagella

Helix: 8 apical flagella

+ 2 flagella on the helix

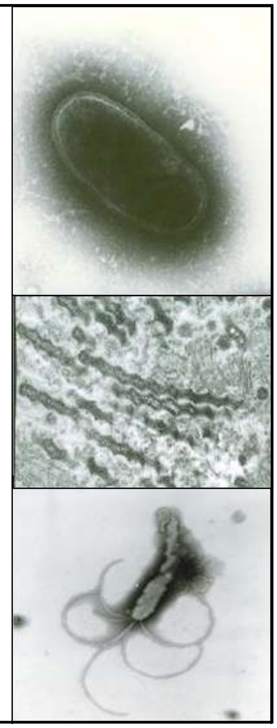
Dimensions: Rod: 0.25 x 0.90 µm

Helix: 0.25 x 2-3.5 µm

Replication: Cytoplasmic

Target Tissue: Hepatopancreas

Lightner et al., 1992.



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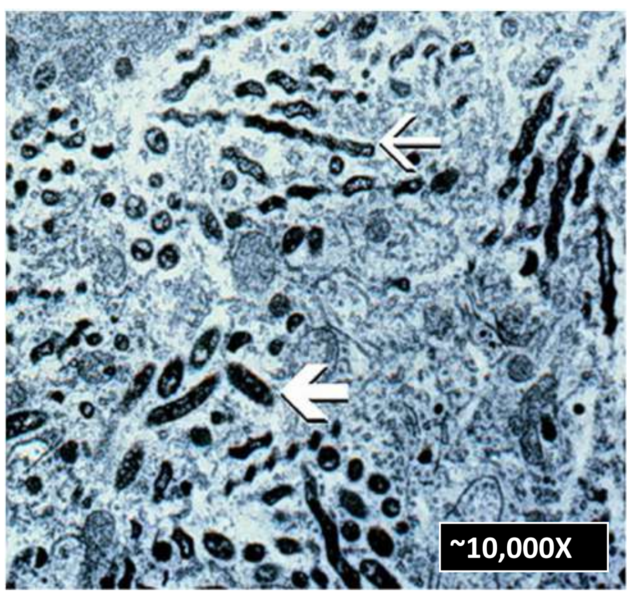
H. penaei

Low magnification TEM of a hepatocyte from a juvenile *P. vannamei* with NHP. Profiles of intracellular rod-shaped forms (thick arrow) and helical forms (thin arrow) of *H. penaei* are abundant in the cytoplasm.



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NHP: History & Epizootiology

- 1985: NHP syndrome was first recognized on Texas coast in cultured *Litopenaeus vannamei*; agent described in 1991
- Since 1993, NHP has been diagnosed in Peru, Ecuador, Colombia, Venezuela, Brazil, Mexico, Central America & NE Africa
- Histology: diagnostic HP granulomas & cells with intracellular bacteria
- Bacteriology often shows vibriosis
- Therapeutic treatment use of OTC medicated feed beneficial
- Disease tends to occur when: >30% & >28 °C

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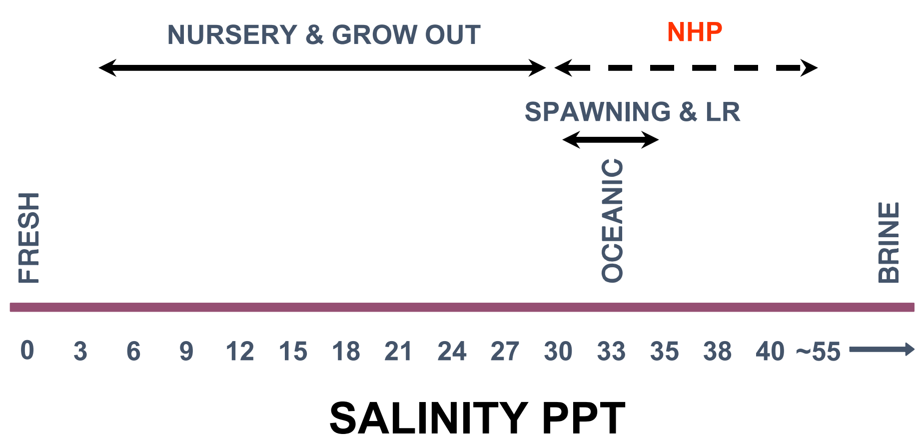
SALINITIES IN BRACKISHWATER & MARINE AQUACULTURE



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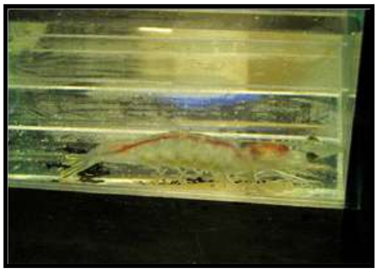
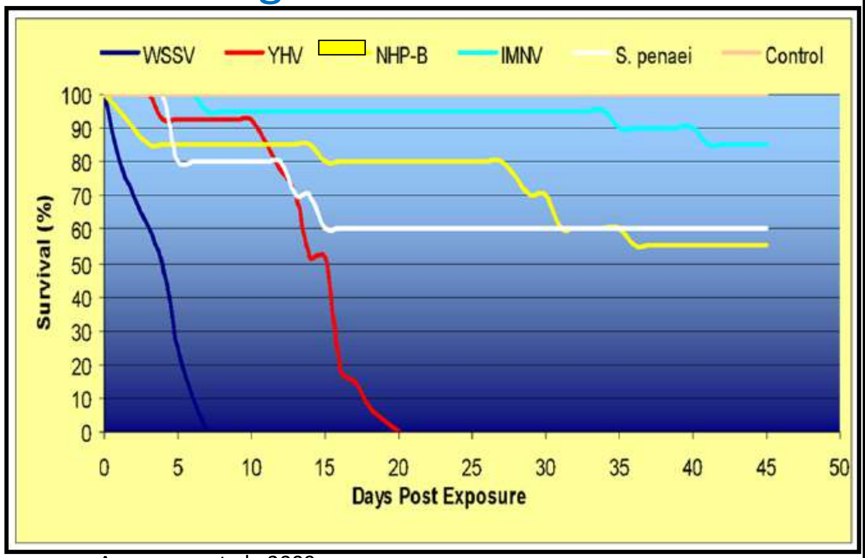
Comparative Survival of P. *vannamei* of Several Shrimp Pathogens





Aranguren et al., 2009.

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Infection by Reverse Gavage

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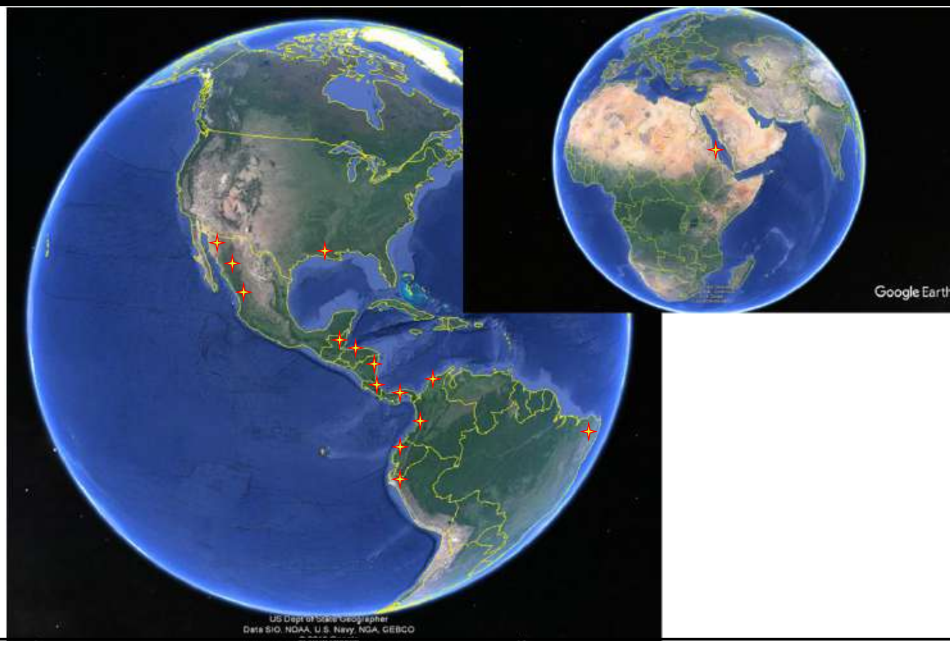
Presence of NHP (as 2021p)



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NHP GEOGRAPHIC DISTRIBUTION (AS OF 2021)

- NHP has not been reported (and confirmed) from the main shrimp growing countries or regions of SE & East Asia
- NHP has been found in NE Africa (Eritrea) & it was introduced with pond-reared *P. vannamei* broodstock from Mexico

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NHP: Epizootiology

- Stages affected:
  - Postlarvae
  - Juveniles and sub-adults
  - Chronic course of infection
  - Mortalities may range from 50 to 99%
- Clinical signs:
  - Reduced growth & mortalities
  - Soft cuticle, flaccid bodies
  - Epibiont fouling, black gills, & appendages
  - Black pleopods due to expansion of melanophores
  - HP atrophy, reduced lipid droplets, melanized tubules

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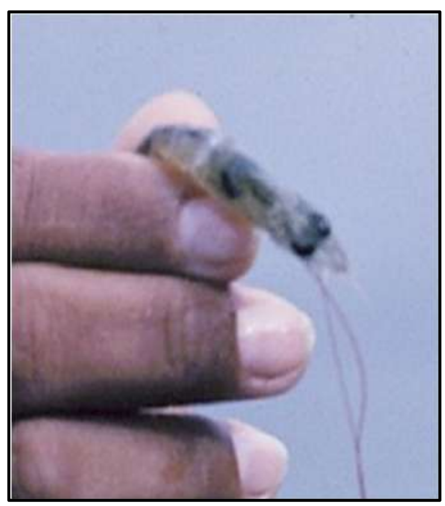

Peru, 1993

50

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Peru, 1993

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Peru, 2021

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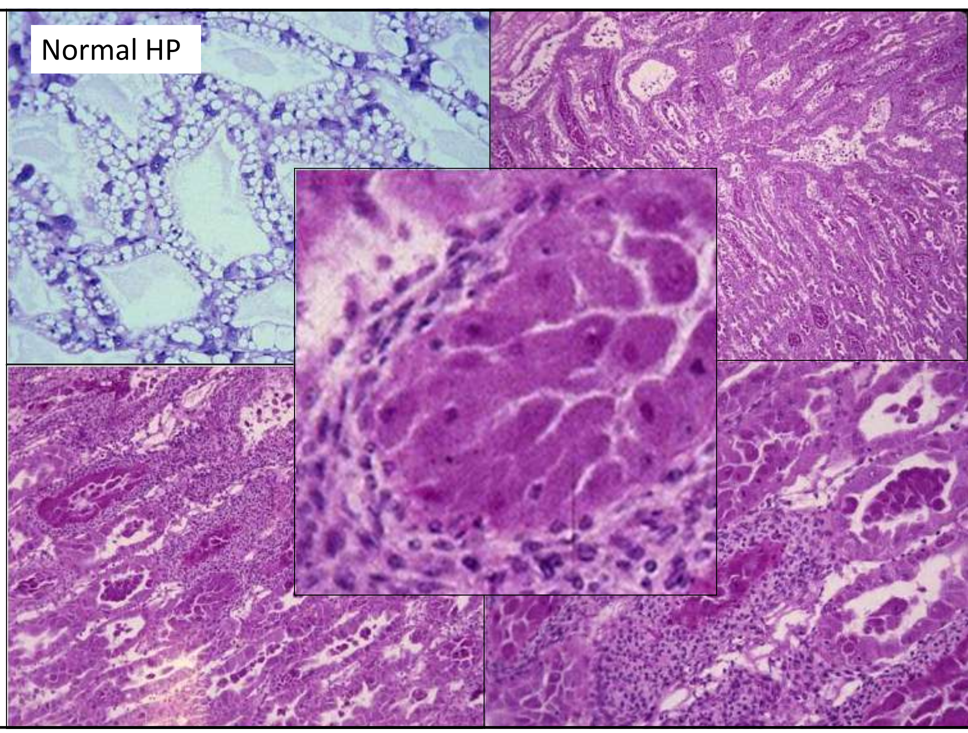

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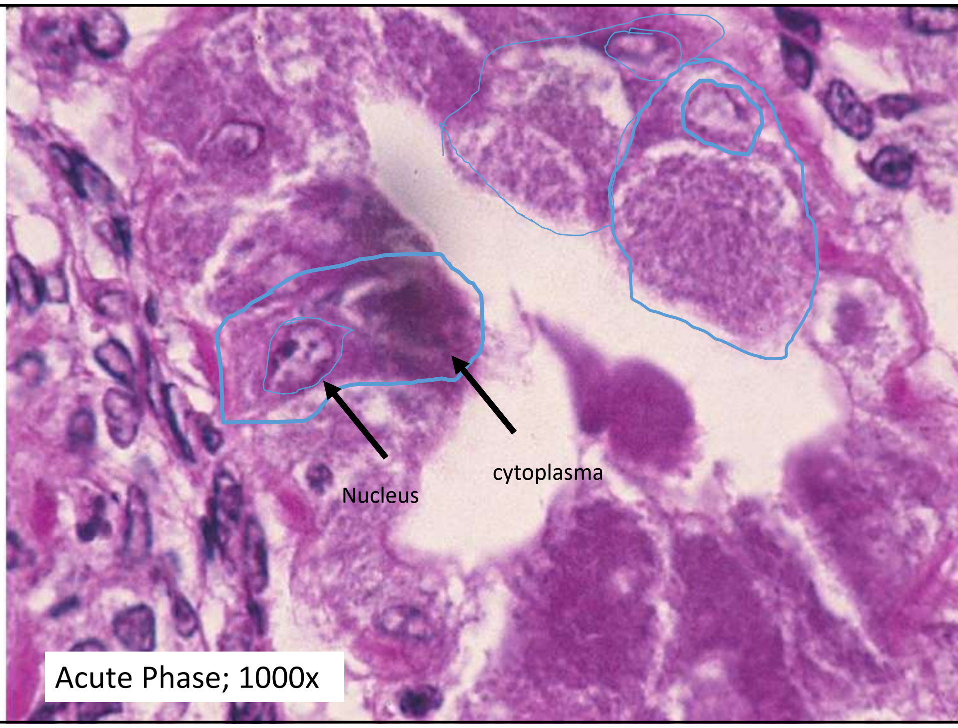

55

THE UNIVERSITY OF ARIZONA.

NHP: Pathology

✓Hepatopancreas lesions include (early in disease):
- ➢intense intratubular hemocytic response
- ➢a few too many HP tubules melanized
- ➢necrosis & sloughing HP tubule epithelial cells
- ➢markedly reduced levels of lipid droplets
- ➢cytoplasmic masses of very small intracellular bacteria
- ➢TEM shows rod & helical forms
- ➢secondary infections by *Vibrio* spp. are common

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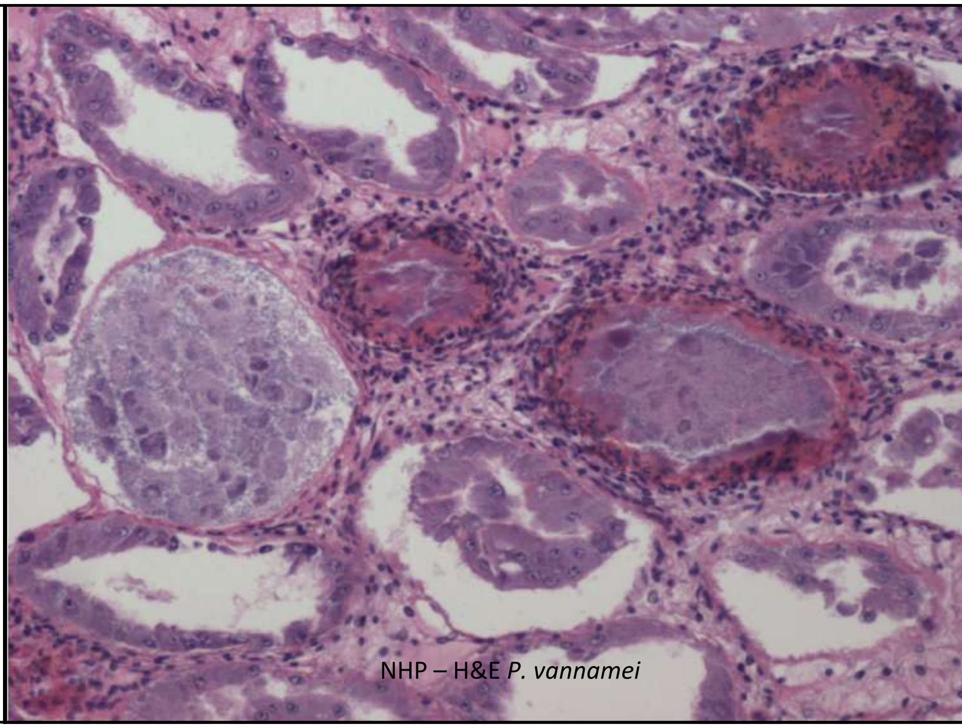

NHP – H&E *P. vannamei*

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NHP: Pathology

✓HP lesions in late chronic phase of disease:

- marked atrophy of tubules & reduced epithelial cell height
- low (L0-1) lipid storage
- intratubular edema
- granulomas may be reduced or absent
- some cells contain cytoplasmic intracellular bacteria

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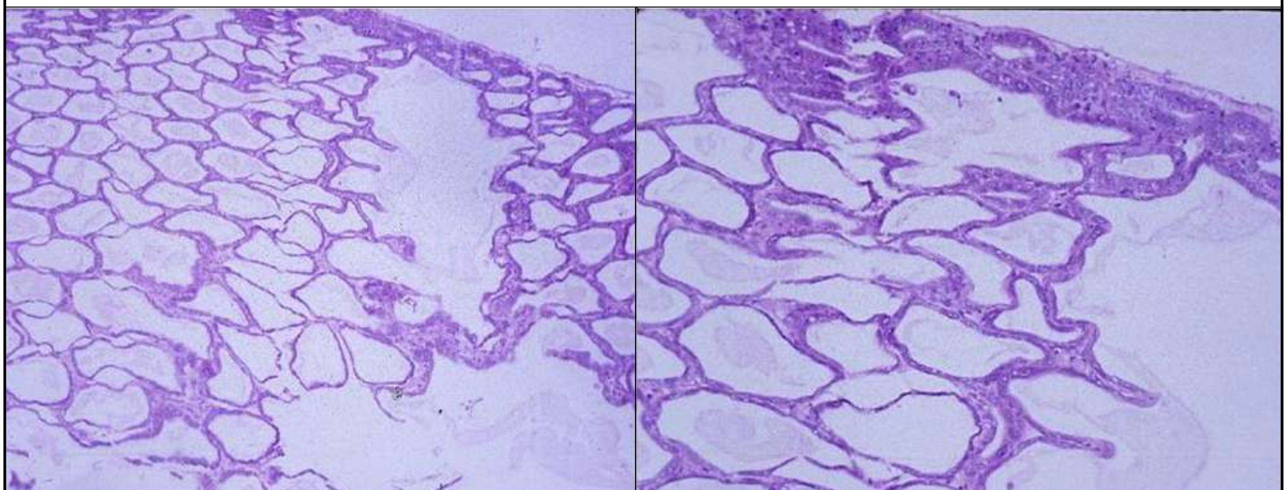
Chronic Phase



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NHP: Diagnosis

- ✓Presumptive diagnosis:
  - ➢history of elevated water temperature (>29-35ºC)
  - ➢salinity (> 20-40‰ or higher, but also seen < 10 ‰)
  - ➢soft shells, black spots, black edges of tail fan, pleopods
  - ➢HP atrophied, whitish, melanized, or fluid filled
  - ➢Wet mounts of HP may show lack of lipid droplets, atrophied tubule mucosal epithelium, necrosis, and melanized tubules
- ✓Confirmatory diagnosis
  - ➢Histology: H&E, Giemsa
  - ➢PCR & qPCR
  - ➢In situ hybridization (ISH) with specific DNA probes

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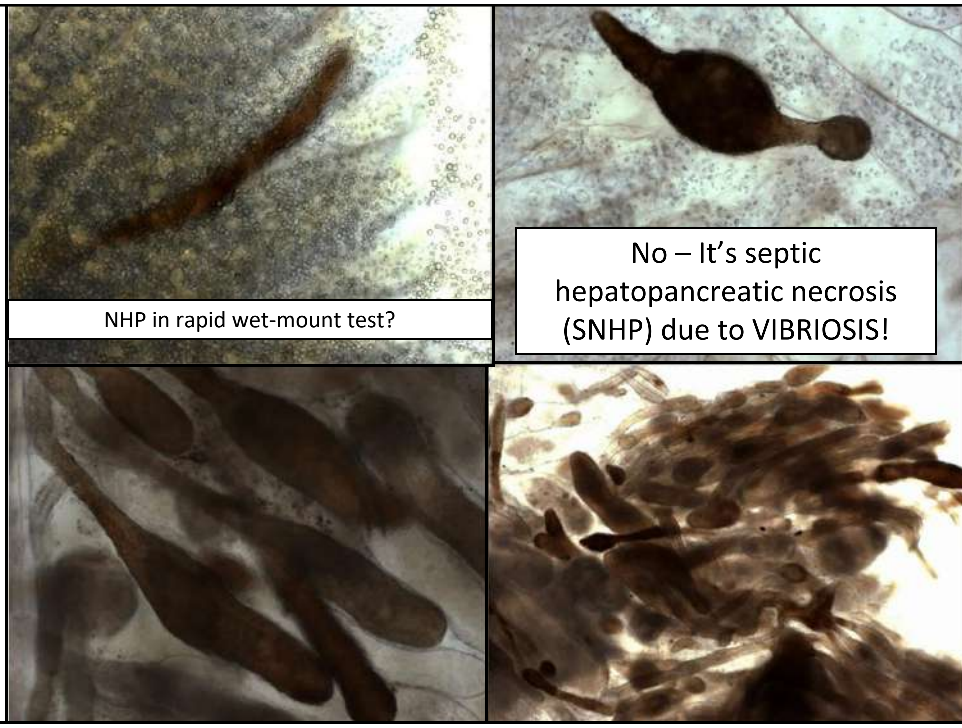

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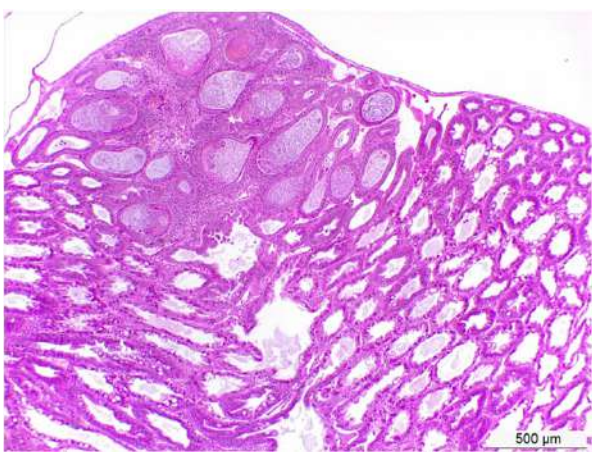
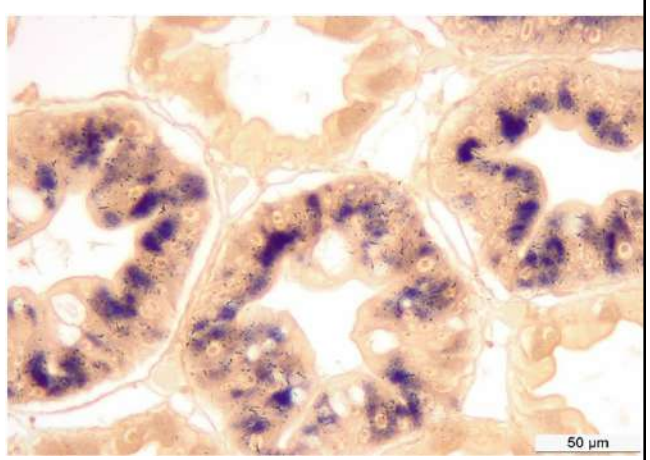
NHP: Diagnosis



Histology

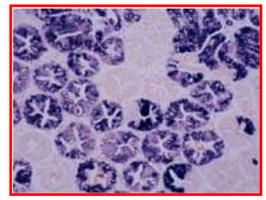


In-situ hybridization

62

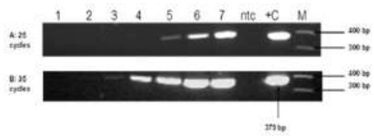
NHP: Diagnosis

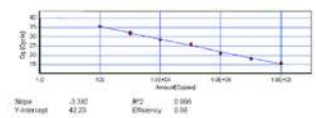
✓Confirmatory diagnosis
- ➢Histology: H&E,
- ➢In-situ hybridization (ISH) with specific DNA probes
- ➢PCR and qPCR



Nunan et al., 2008

Aranguren et al., 2010



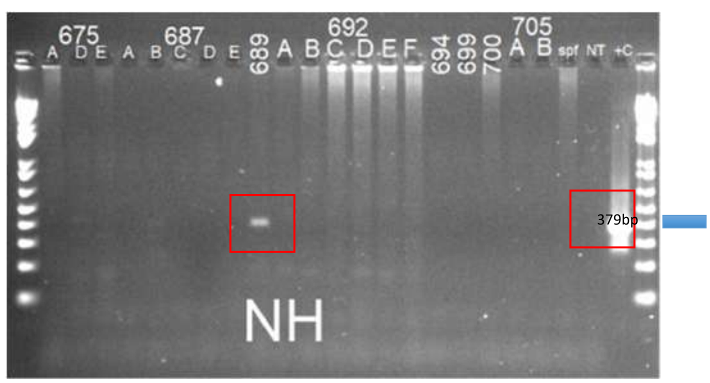


Aranguren et al., 2010

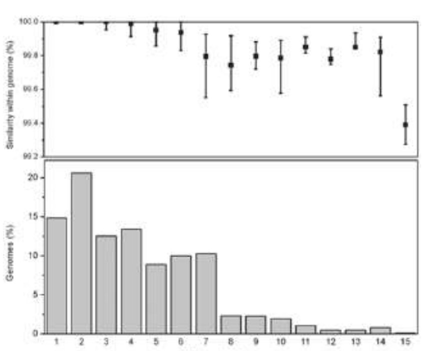
The only OIE detection method for NHPB by PCR and qPCR is by detecting a target region of the 16SrRNA gene.

63

16SrRNA is a Highly Conserved Gene



Artemia salina cysts, subphylum: crustacea



Vetrovsky & Baldrian 2013

64

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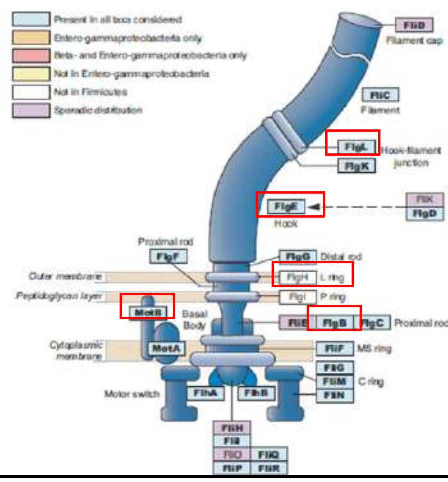
Target regions of DNA sequences involved in NHP-B motility included flagella genes were used. Some genes including *MotB, FlgH, FLbB, FlgL. FlgE*



Liu et al., 2007.

65

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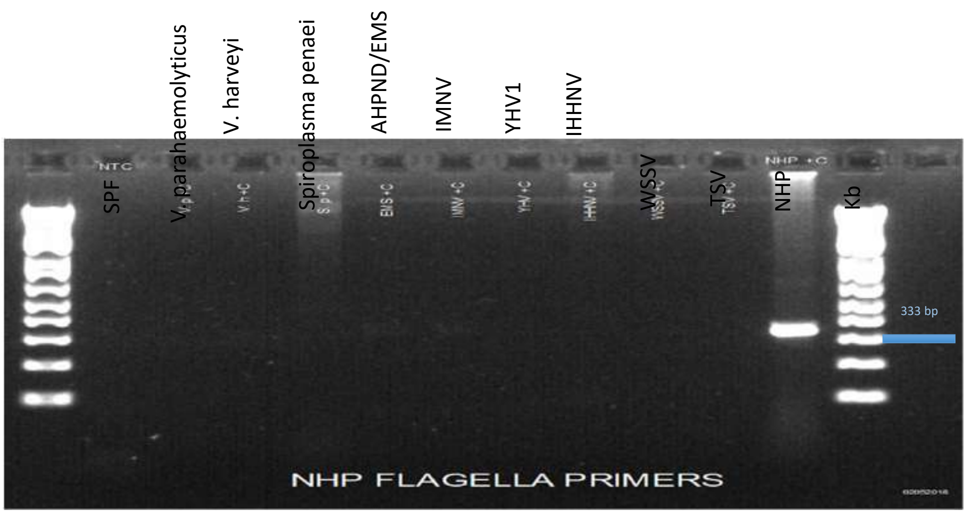
Specificity of the NHP Flg-E primers



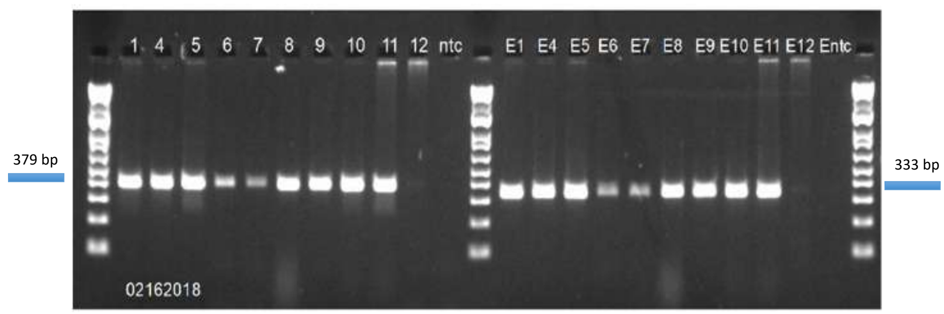
66

Aranguren et al., 2018.

66

Sensitivity of NHPB: Flagellar Hook Protein *flgE* (gene that encode for the hook subunit of the flagella) vs. 16S rRNA

16SrRNA Flg E

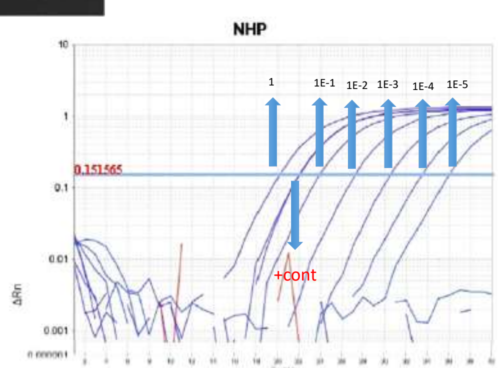


67

67

Samples analyzed using 16S rRNA

SAMPLES ANALYZED using by qPCR Flg E region



qPCR NHPB FlgE 10-100 times more sensitive than PCR NHPB 16srRNA

68

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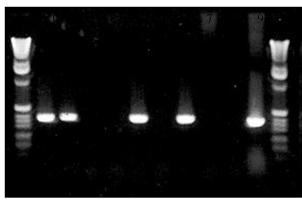
Non-Lethal Disease Screening by PCR

Live shrimp to be examined

Fecal sampling for detection of NHP by PCR



Visualization of a 379 bp PCR product in 1% agarose gel

(Aranguren & Brinez et al., 2006.)

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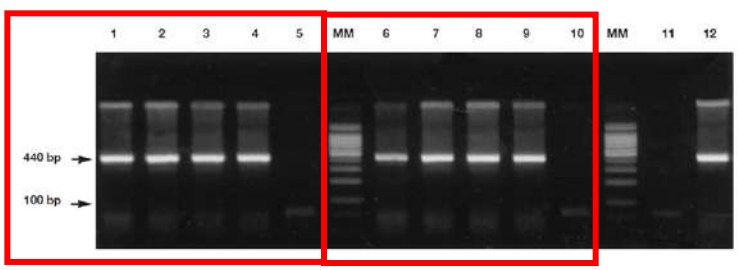
For DNA extraction from paraffin-embedded tissues:

- Sections of 10 µm were placed in sterile 1.5 ml tubes
- Deparaffinized with 1 ml of xylol for 5 min
- Washed in 100% ethanol
- Dried to remove the ethanol
- Pellet was resuspended in 50 µl of lysis buffer (10 mM Tris EDTA, 2% Tween-20, 500 µg ml–1 Proteinase K)
- incubated overnight at 60°C
- Inactivation of Proteinase K

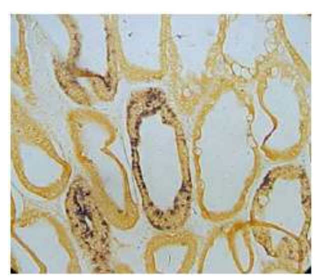


Tissue

Paraffin block

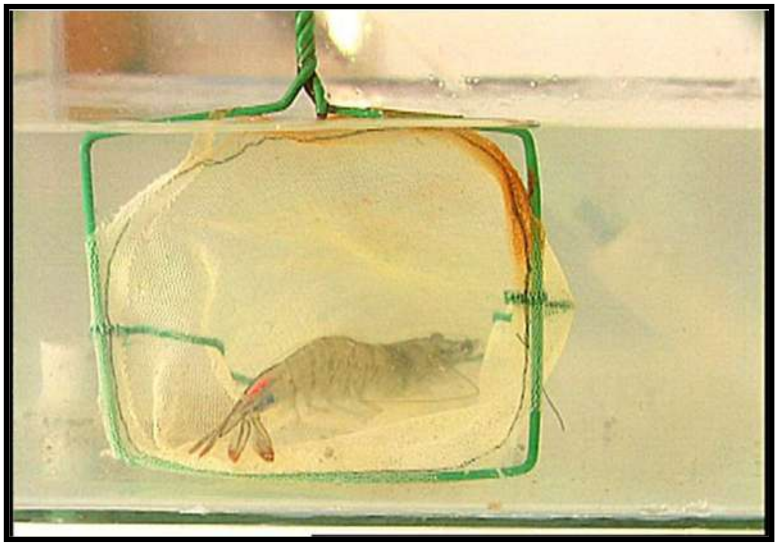
	In situ hybridization		
	Positive	Negative	Total
Fecal samples			
PCR positive	14	0	14
PCR negative	0	11	11
Total	14	11	25
Paraffin blocks[a]			
PCR positive	19	1	20
PCR negative	0	20	20
Total	19	22	40

[a]The 5 samples that did not amplify with the housekeeping gene are not included



70

NHP tolerance/Resistance



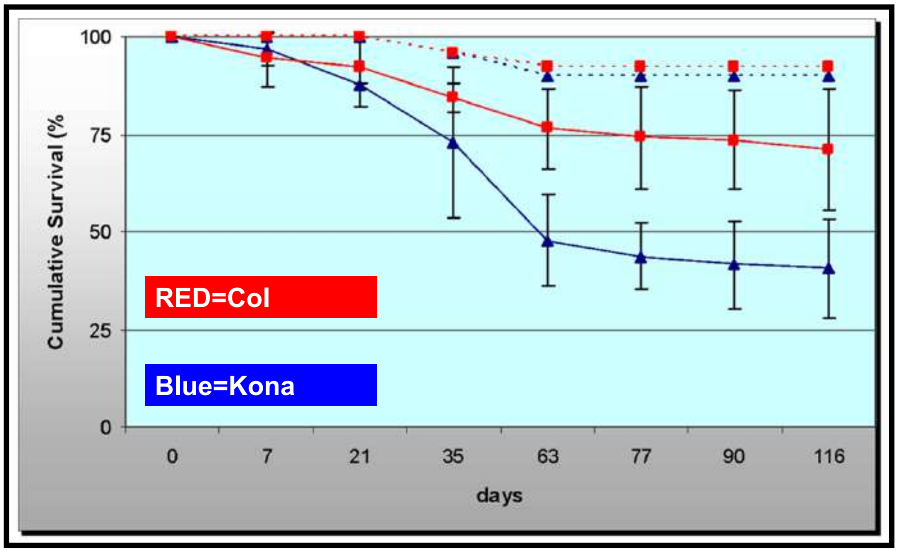
71

Sampling & Monitoring

- Shrimp were periodically counted from each tank to determine the number of surviving shrimp from each population
- Moribund shrimp were fixed in Davidson's (AFA) for histological analysis
- Dead shrimp were frozen at -70^{o}C for PCR analysis
- At the end of the challenge (116 PI), the HP from all survivors were preserved in 95% ethanol for qPCR

72

- Survival behavior



Kaplan Meier: Log Rank test (P=0.0002)

73

RESULTS : Challenge Test

Final Survival

Tank	1	2	Mean
COL	60%	82%	71%
KONA	32%	50%	41%
Difference of survival	-28	-32	-30

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Selection Process

TSV Survivors from a commercial grow out ponds

Larger and healthier shrimp are transfer to the next phase

Flaccid, moribund, soft shell, melanized shrimp are discarded

Larger and healthier shrimp are transfer to the next phase

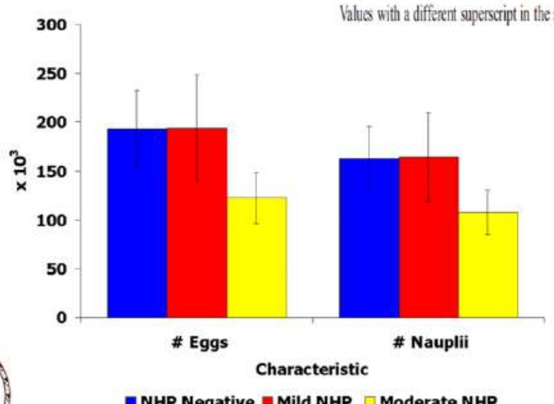
Aranguren et al., 2010.

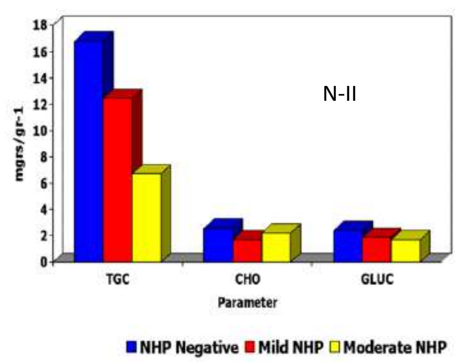
75

Broodstock performance based on the NHP status

Productive parameters	NHP negative, $n=23$	Mild NHP, $n=20$	Moderate NHP, $n=12$
Spawner's average weight (g)	47.0 ± 4.9^a	46.0 ± 6.0^a	44 ± 3.8^a
Eggs/female ($\times10^3$)	193.4 ± 80.4^a	183.4 ± 94.3^a	122.3 ± 75.1^b
Nauplii/female ($\times10^3$)	162.9 ± 61.1^a	164.1 ± 70.8^a	102.4 ± 41.1^b
Nauplii/eggs $\times$ 100	84.2 ± 9.1^a	84.9 ± 11.6^a	83.8 ± 12.4^a

Values with a different superscript in the same row are significantly different ($P<0.05$).

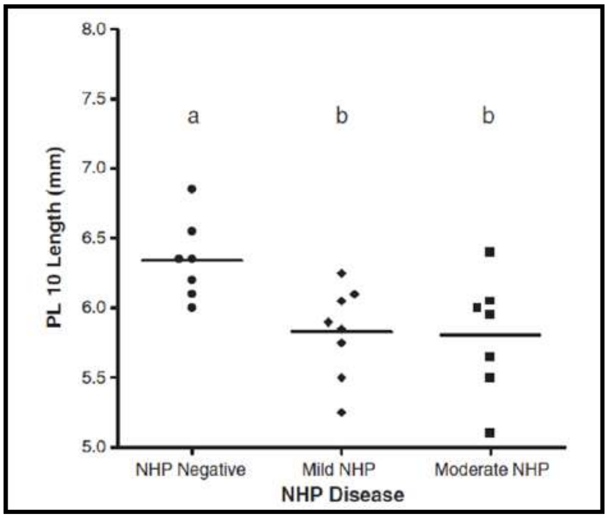




Aranguren et al., 2006

76

NHP vs. PL-10 Length



Aranguren et al., 2006.

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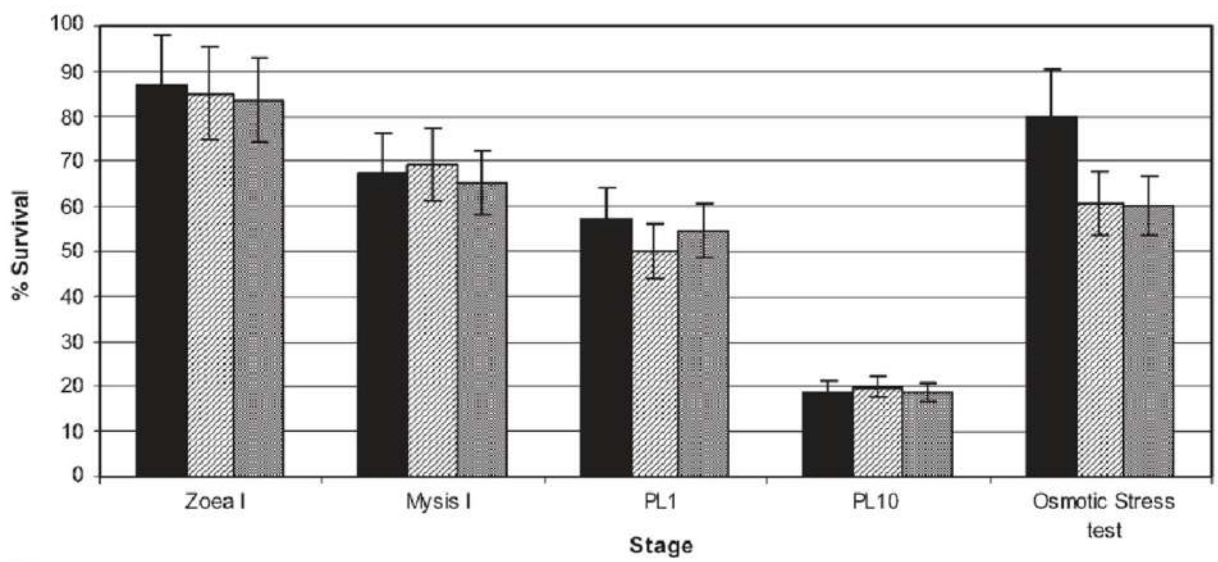
NHP vs. PL-10 Osmotic Stress Test



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Relationship Among the NHP Isolates

- 15-277/B1Ecuador
- 13-436/G1 Mexico
- 13-280/B Texas
- 2006 Texas
- 16-620 Honduras
- 2006 Texas
- 2013 texas
- 15-277/c1 Ecuador
- 11-171/3H Ecuador
- 2013 Texas

Aranguren et al., 2018.

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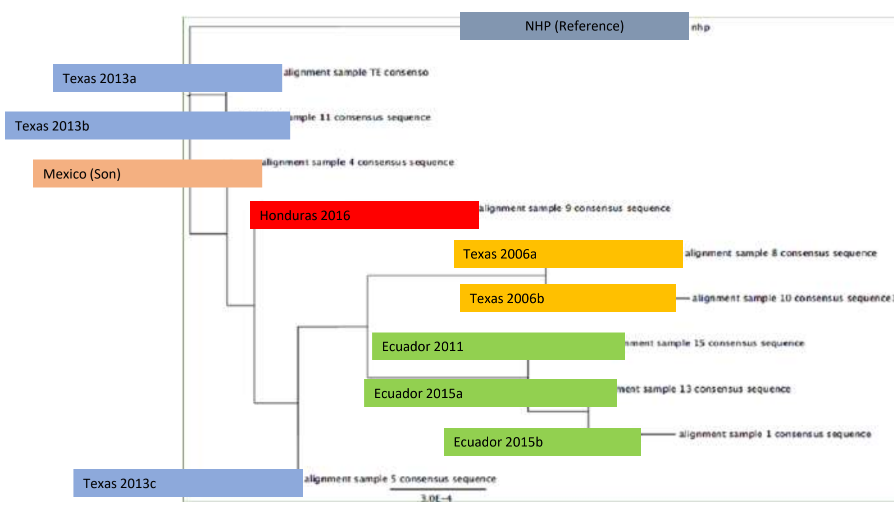
Phylogenetic Analysis 16S rRNA

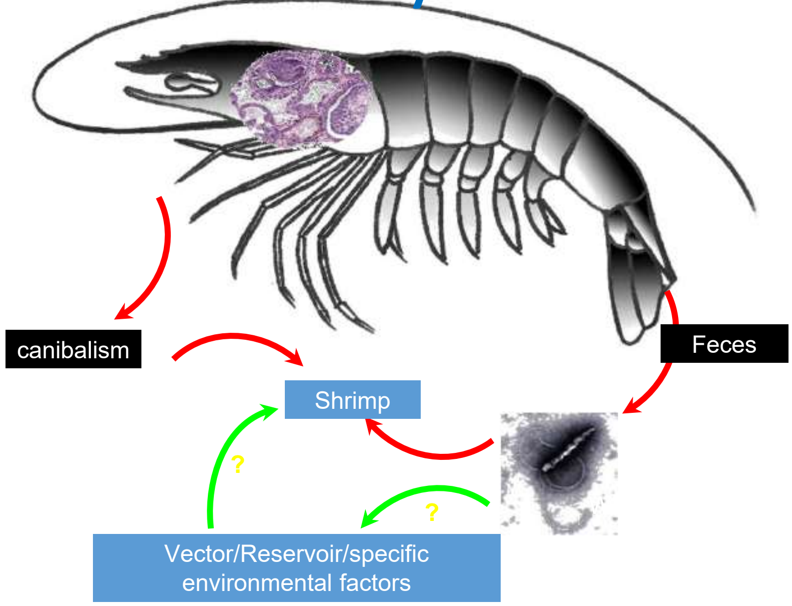


Neighbor-joining tree constructed from an alignment of the nucleotide sequence of. NHP
Genetic distance model: Tamura-nei

Aranguren et al., 2018.

80

Hypothetical Infection Cycle



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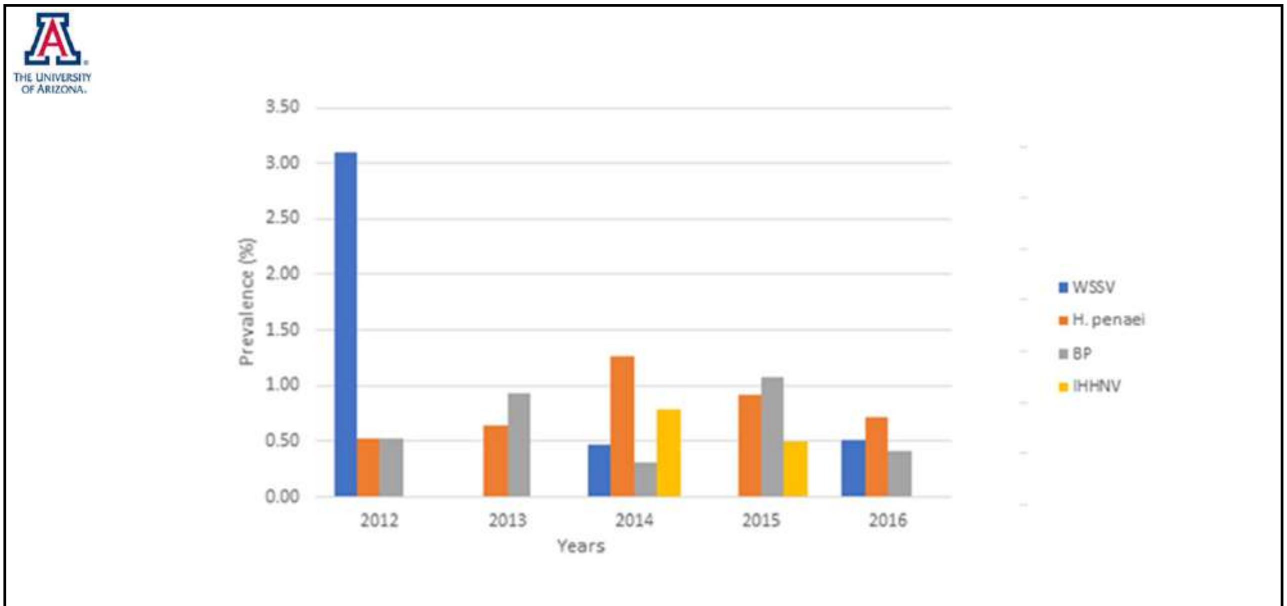
NHP in Wild Population

Ramirez, et al., 2020

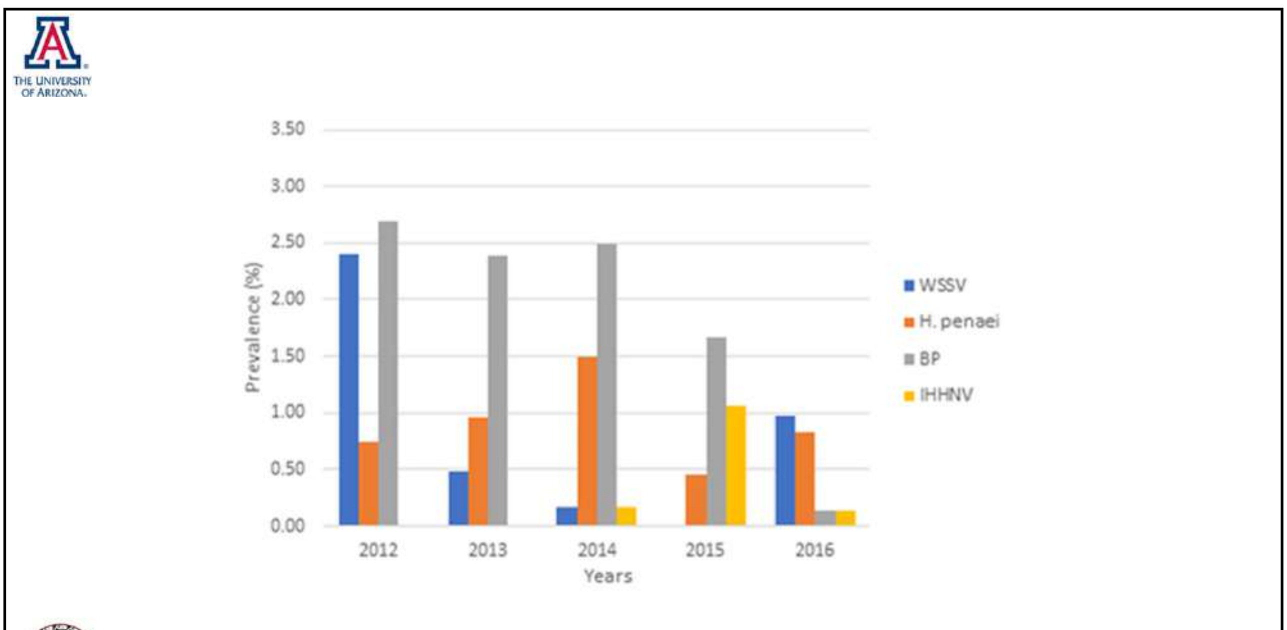
82



Samples of wild *P. stylirostris* nearby shrimp farming region

Ramirez, et al., 2020.

83



Samples of wild *P. vannamei* nearby shrimp farming region

Ramirez, et al., 2020.

84

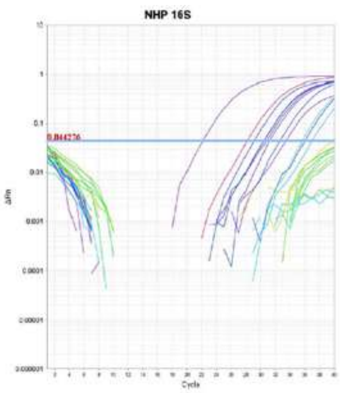
85

https://www.dreamstime.com/stock-photo-gull-eating-shrimp-laughing-found-nearby-salt-marsh-image61884296

86

Sample Information and Results

Amplification Plot (ΔRn vs. Cycle)



Aranguren et al., in prep

Sample Site	Sample Type	Pool ID	CT
Farm 1 Water pumping station	Water	21-882 A1	Undetermined
		21-882 A2	Undetermined
		21-882 A3	Undetermined
		21-882 A4	Undetermined
Farm 2 Water pumping station	Water	21-882 B1	Undetermined
		21-882 B2a	Undetermined
		21-882 B2b	Undetermined
Farm 1 Ponds and water inlet channels	Guano	21-882 E2a	30.51
		21-882 E2b	33.44
Farm 2 Ponds and water inlet channels	Guano	21-882 E1a	31.27
		21-882 E1b	22.16
Farm 1 Production ponds	Snails	21-882 C1	35.67
		21-882 C2	Undetermined
		21-882 C3	Undetermined
		21-882 C4	Undetermined
		21-882 C5	37.01
Farm 2 Production ponds	Snails	21-882 D5	36.05
	Mussels	21-882 D1	32.7
		21-882 D2	30.55
		21-882 D3	30.87
		21-882 D4	29.19

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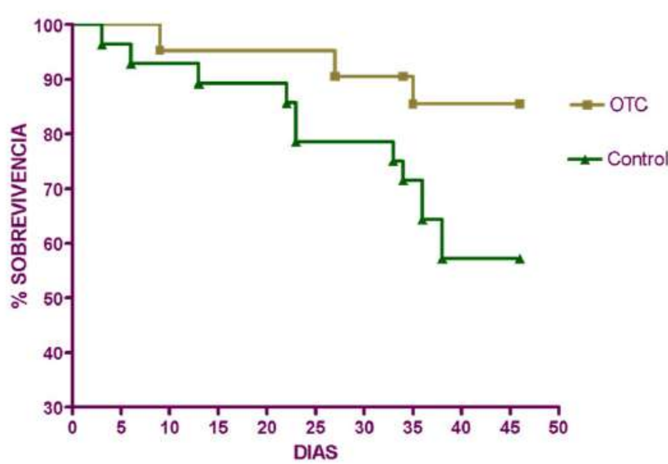
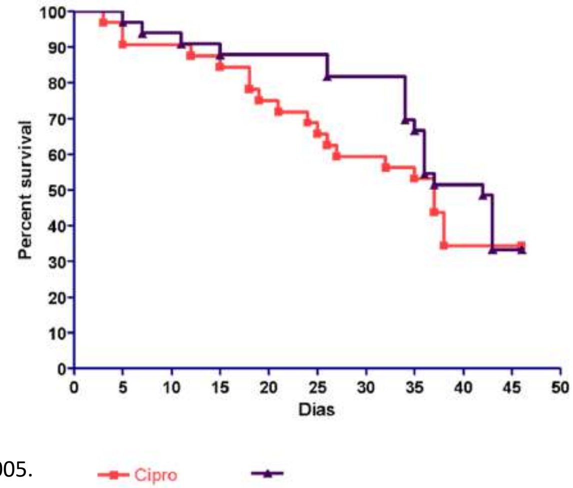
OTC Treatment



Aranguren et al., 2005.

88

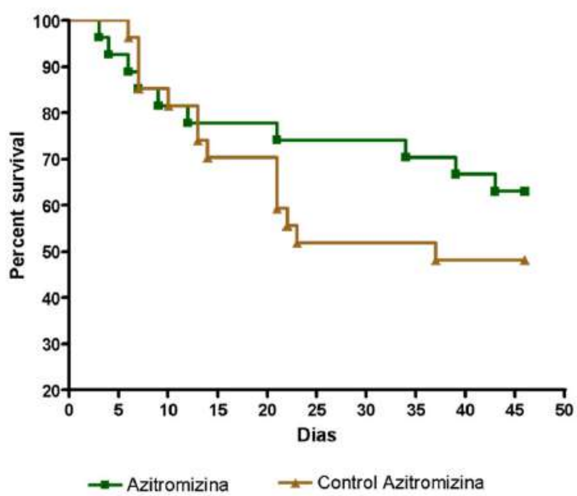
CIPROFLOXACINA Treatment



Aranguren et al., 2005.

89

Azithromycin Treatment



Aranguren et al., 2005.

90

NHP Management

- Use of clean broodstock
- Use of clean PL (SPF)
- Use of NHP resistant strain
- Farm designed with deeper ponds to mitigate high temperatures
- Water exchange to reduce salinity
- Therapeutic use of medicated feeds (OTC hydrochloride at 7-8 kg/t feed for 14 days)
- Observe areas based on farm history where NHP is likely to occur
- Have medicated feed available on short notice
- Monitor stocks & begin treatment at first sign of disease
- Florfenicol recently developed for NHP in Mexico (150 mg/Kg Biomass for 14 days)
- Use of lined ponds

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Thank you

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Hepatopancreatic Microsporidiosis
Enterocytozoon hepatopenaei (EHP)

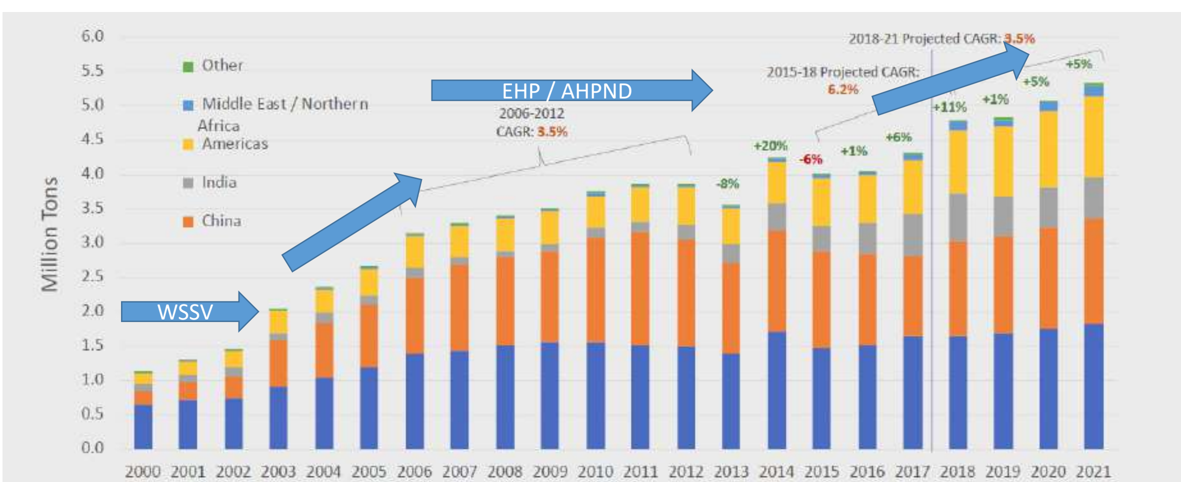
Luis Fernando Aranguren Caro, Ph.D

OIE Expert at the OIE Reference Laboratory for *H. penaei* (NHP)

Aquaculture Pathology Laboratory
OIE Reference Laboratory
USDA-APHIS Approved & ISO 17025, 17043 Accredited Laboratory
School of Animal & Comparative Biomedical Science
The University of Arizona, Tucson, Arizona, USA

1

Shrimp Aquaculture Production by World Region



Sources: FAO (2019) for 2000-2009; GOAL (2011-2018) for 2010-2017; GOAL (2019) for 2018-2021

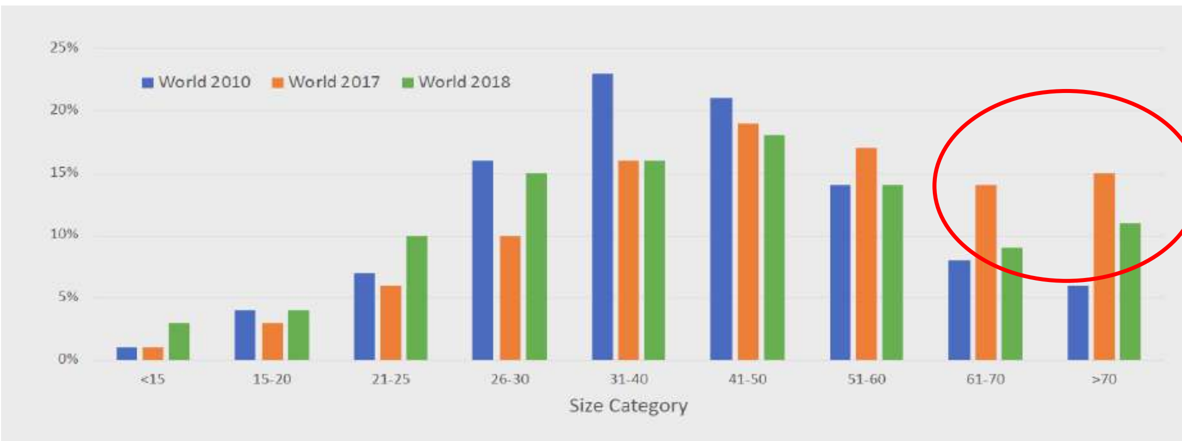
2

Enterocytozoon hepatopenaei (EHP)

- First described in *Penaeus monodon* from Thailand
- Impacting aquaculture production by severely retarding the growth of cultured shrimp
- It infects only the tubules of the hepatopancreas, which damages the ability of this critical organ to gain nutrition from feed
- High size variability
- Causes chronic mortality in severe cases
- Secondary vibriosis are common

3

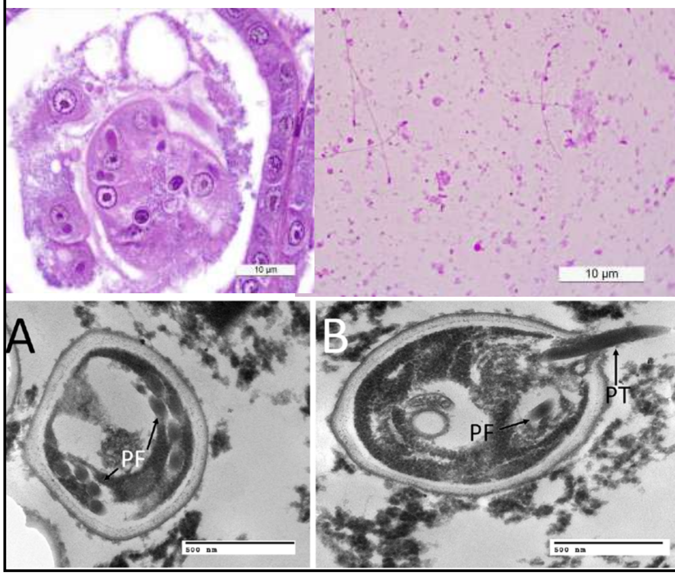
Composition of Shrimp Aquaculture by Size Categories World 2010 vs. World 2017 vs. World 2018



Sources: GOAL (2011, 2018, 2019).

4

Enterocytozoon hepatopenaei (EHP)



Family: *Enterocytozonidae*

Genus: *Enterocytozoon*

Oval shape (0.7 x 1.1 µm) containing 5-6 coils

Associated with White feces disease

Associated with slow growth syndrome

EHP infection causes a greater susceptibility to AHPND infection

5

EHP GEOGRAPHIC DISTRIBUTION

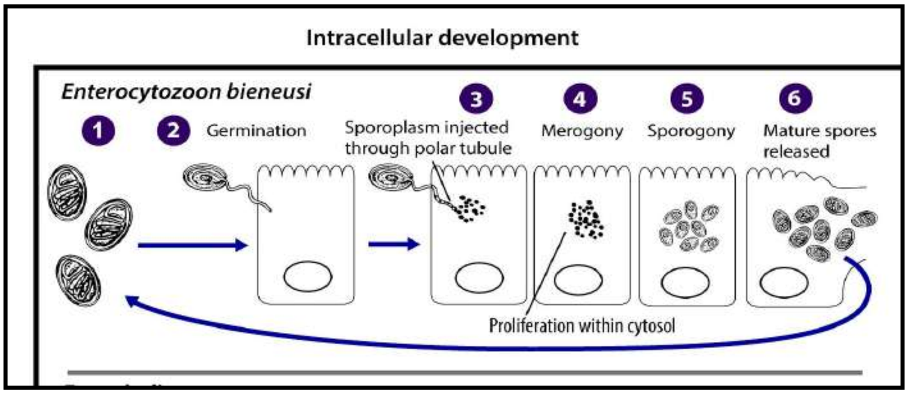
Confirmed cases

Suspected cases

6

EHP Life Cycle

EHP does not require an intermedia host



CDC website. www.cdc.gov

7

Diagnosis of EHP

Light microscopy

- Demonstration of spores in wet mount preparations of hepatopancreatic tissue
- Histopathological demonstration of characteristic lesions and life stages of the parasite

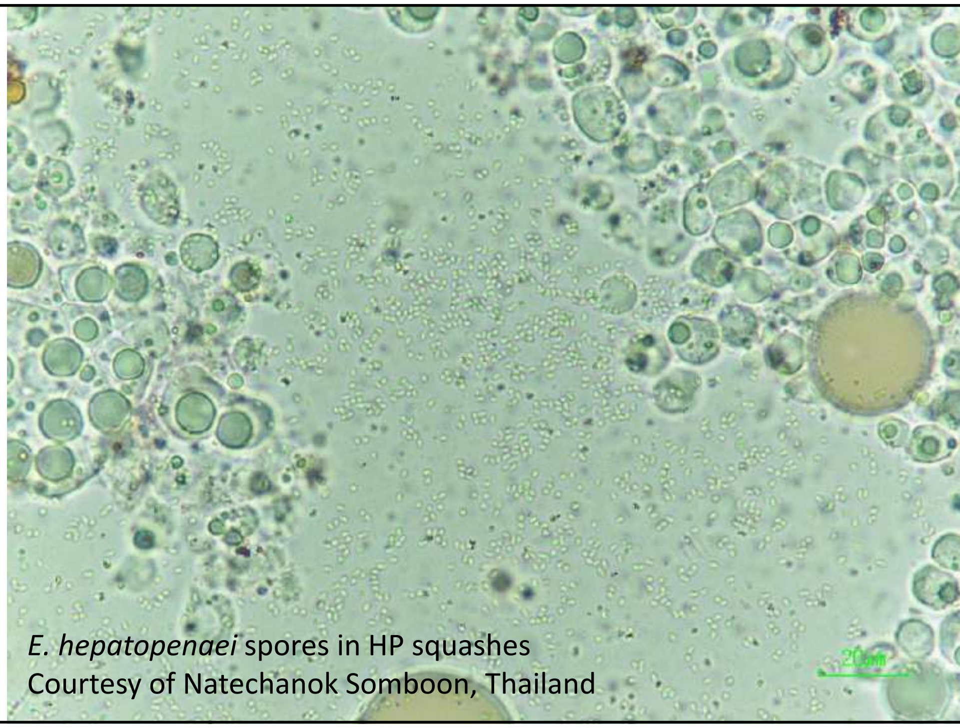
Electron microscopy(less sensitive)

- Observation of the ultrastructure of the pathogen

Molecular methods (highly sensitive and specific)

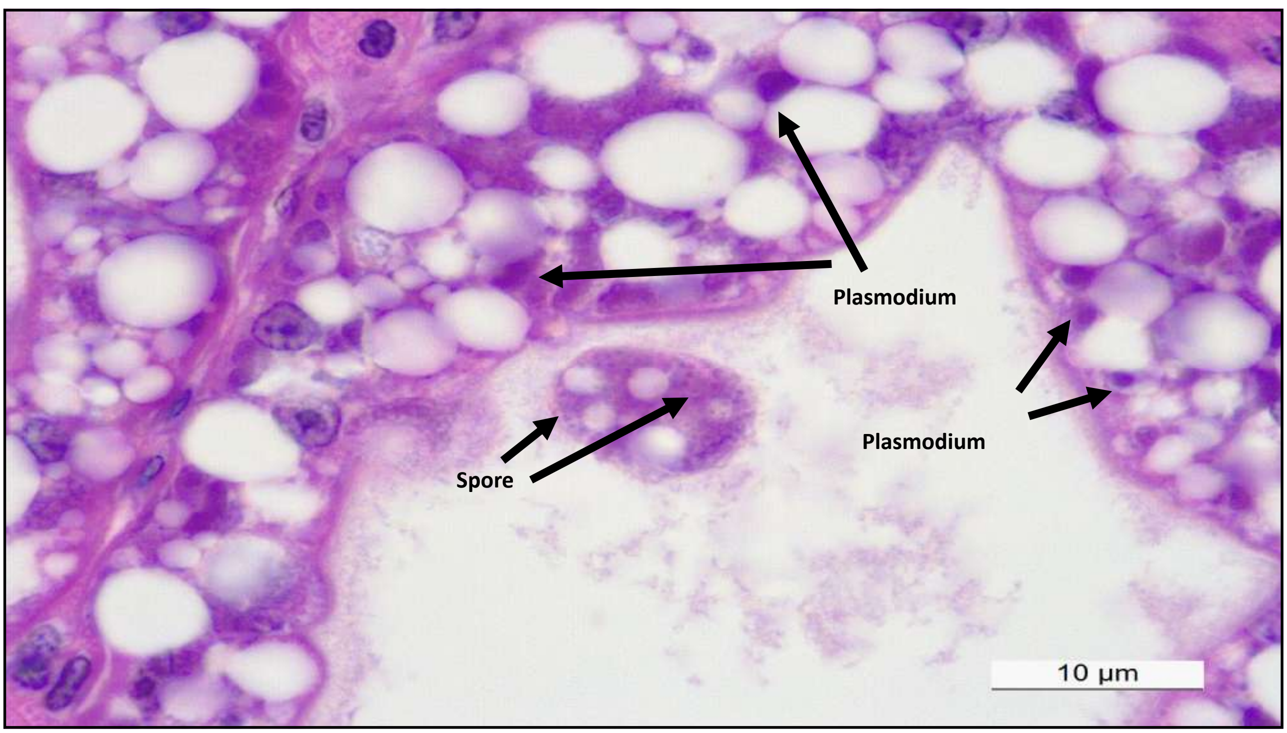
- PCR
- In situ hybridization (ISH)

8

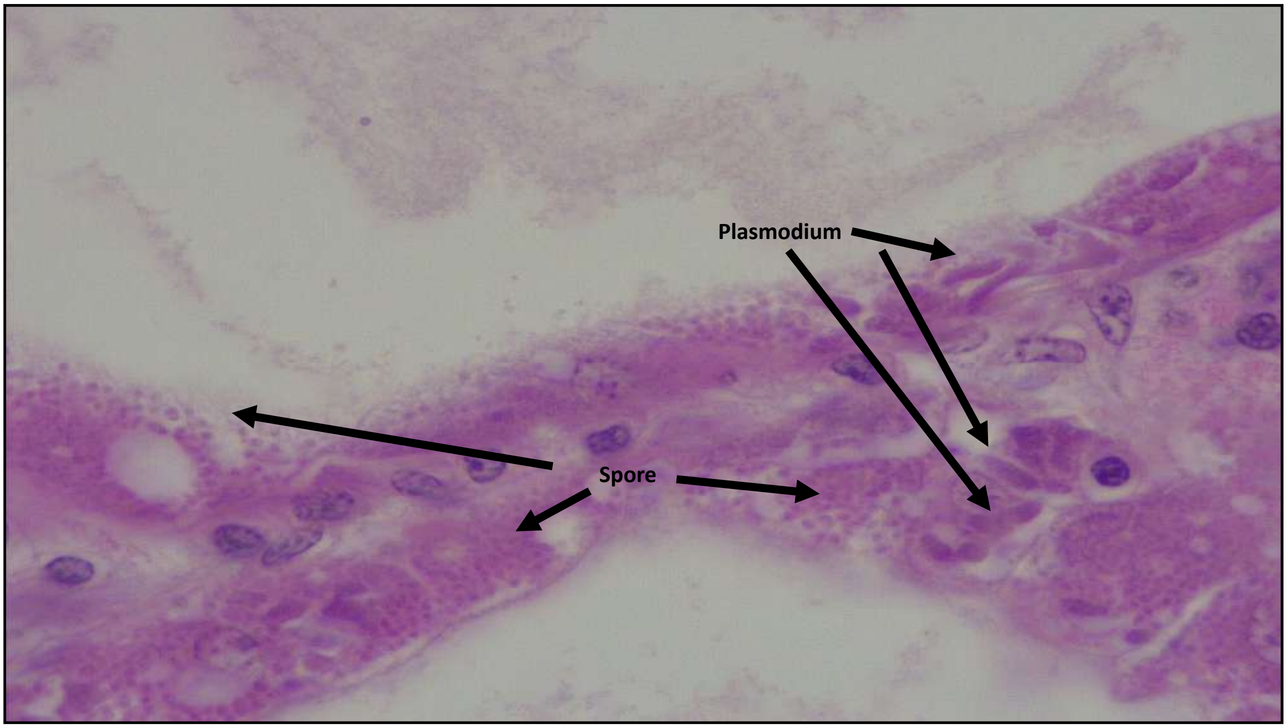


E. hepatopenaei spores in HP squashes
Courtesy of Natechanok Somboon, Thailand

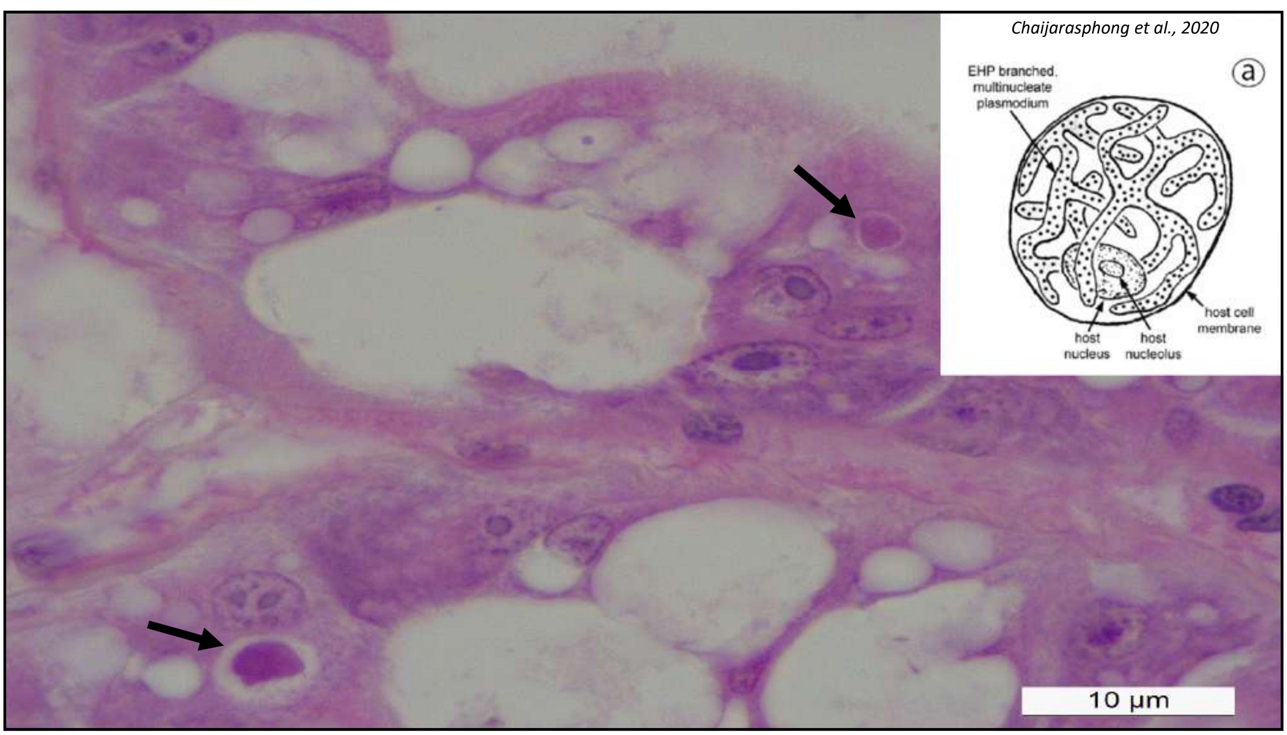
9



10


Plasmodium
Spore

11


Chaijarasphong et al., 2020
a
EHP branched, multinucleate plasmodium
host cell membrane
host nucleus
host nucleolus
10 µm

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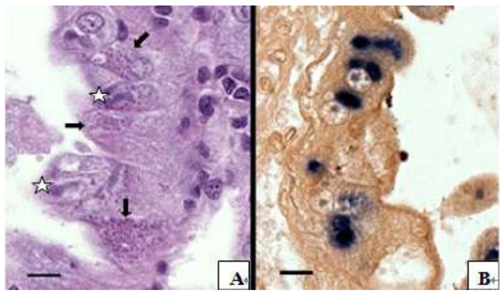
EHP - in situ hybridization *P. vannamei*



(A) H&E staining of hepatopancreas
(B) *in situ* hybridization of the consecutive section with a digoxigen-labeled EHP probe

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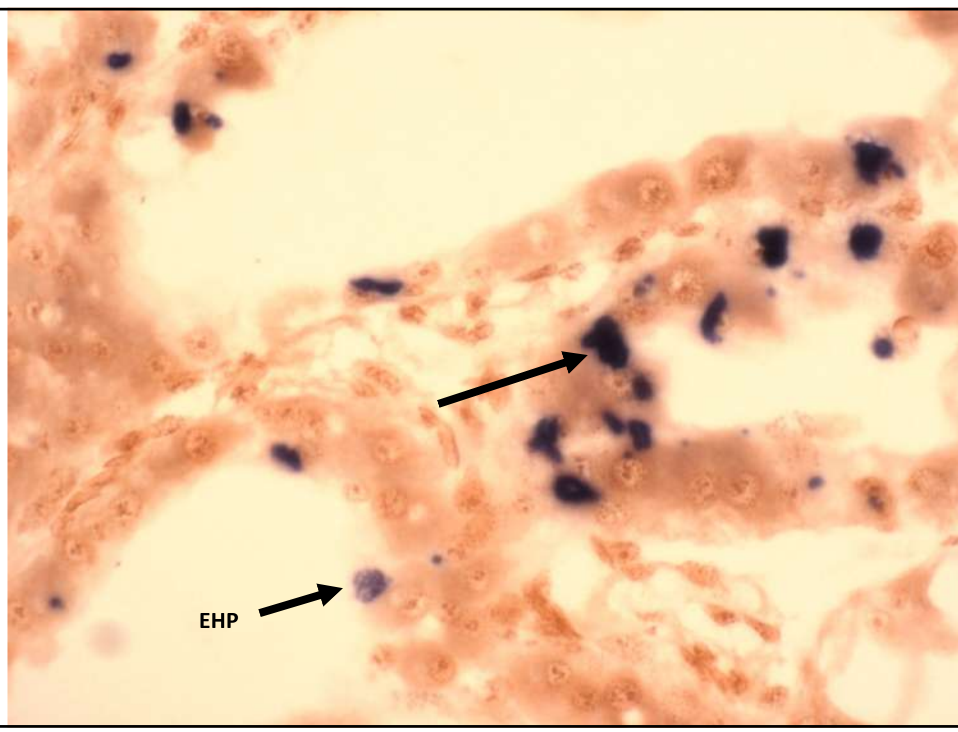

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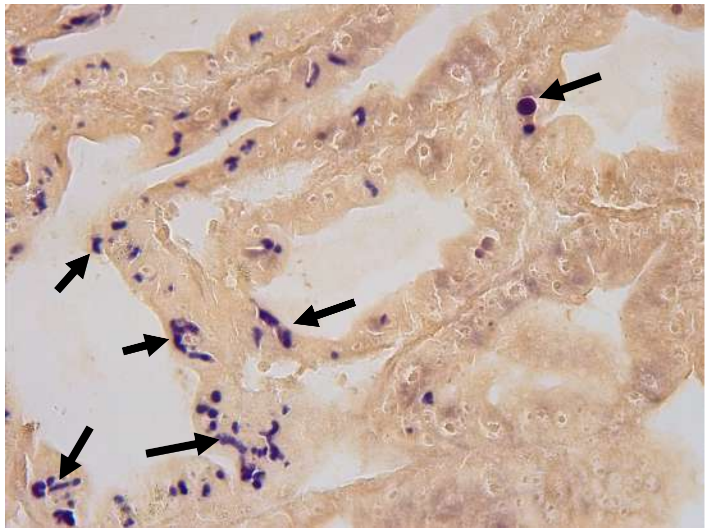
EHP *in-situ* hybridization *P. vannamei*



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Diagnostic PCR Methods for EHP

Primer	Target	Amplicon size	Sequence (5' to 3')	Reference
510F	18SrRNA	510bp	GCCTGAGAGATG GCTCCCACGT	Tang et al., (2015)
510R			GCGTACTATCCCCAGAGCCCGA	
F157	18S rRNA	157 bp	AGT AAA CTA TGC CGA CAA	Lui et al., (2018)
R157			AAT TAA GCA GCA CAA TCC	
Probe			FAM-TCC TGG TAG TGT CCT TCC GT-TAMRA	
SPW_1F	Spore wall	514 bp	TTGCAGAGTGTTGTTAAGGGTTT	Jaroenlak et al., (2016)
SPW_1R			CACGATGTGTCTTTGCAATTTTC	
SPW_2F	Spore wall	148 bp	TTGGCGGCACAATTCTCAAACA	
SPW_2R			GCTGTTTGTCTCCAACTGTATTTGA	

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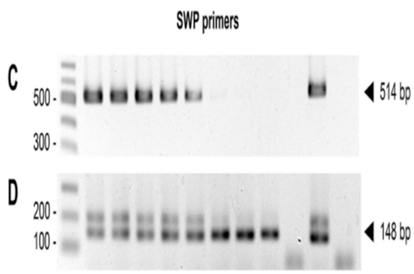
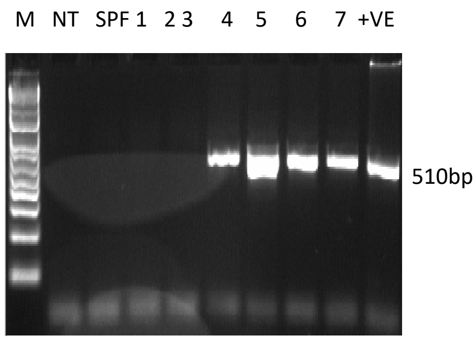
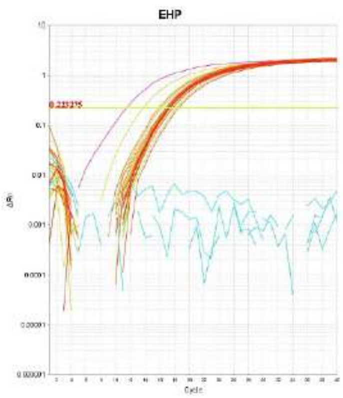


Jaroenlak et al., 2016.





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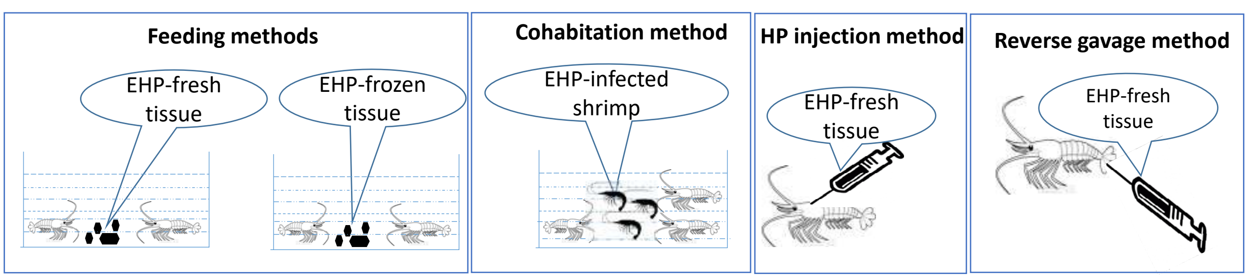
EHP Challenge Methods



- Samples were collected at 14 dpi, 30 dpi and 60 dpi
- PCR, Histology, and TEM were employed to detect EHP in collected samples

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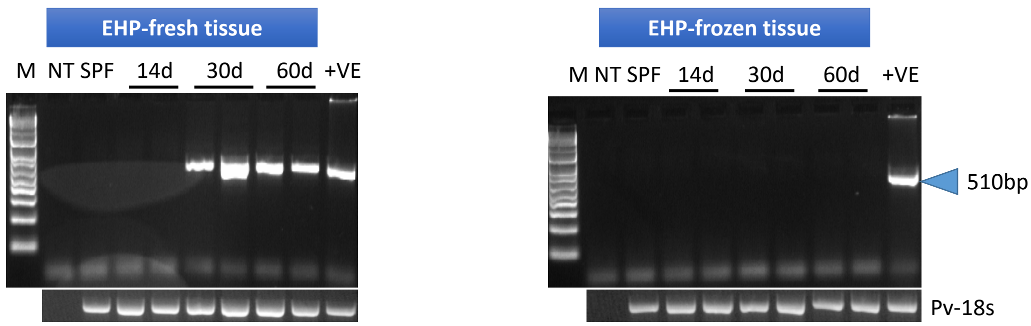
Feeding treatments



M=Marker; NT=non-template control; SPF=Specific Pathogen Free; 14d=14dpi; 30d=30dpi; 60d=60dpi; +VE=EHP positive control. 18S rRNA of *P. vannamei* was used as an internal control

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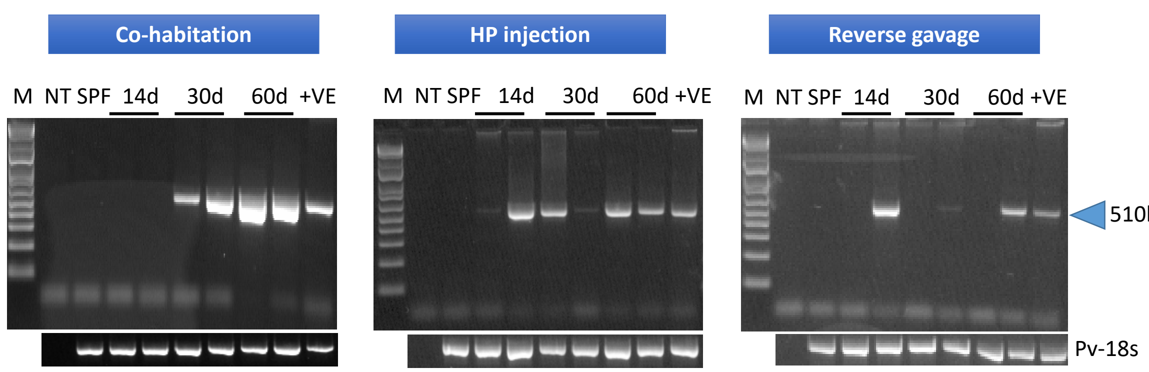
Co-habitation, HP Injection & Reverse Gavage Treatments



M=Marker; NT=non-template control; SPF=Specific Pathogen Free; 14d=14dpi; 30d=30dpi; 60d=60dpi; +VE=EHP positive control. 18S rRNA of *P. vannamei* was used as an internal control

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White Feces Syndrome (WFS) vs. EHP

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White Feces Syndrome (WFS) vs. EHP

The Israeli Journal of Aquaculture - Bamidgeh, IJA_67.2015.1212, 11 pages

***Vibrio cholerae*: a causal agent for the white feces syndrome in freshwater cultured whiteleg shrimp (*Penaeus vannamei*)**

Applied Microbiology and Biotechnology

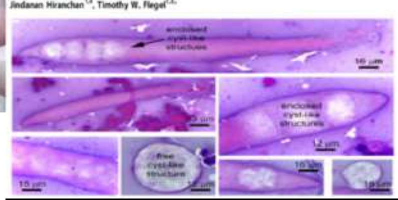
APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

Intestinal bacterial signatures of white feces syndrome in shrimp

Dongwei Hou[1] · Zhijian Huang[1] · Shenzheng Zeng[1] · Jian Liu[1] · Dongdong Wei[1] · Xisha Deng[1] · Shaoping Wang[1] · Qingyuan Yan[1] · Jianguo He[1,2]

White Feces Syndrome of Shrimp Arises from Transformation, Sloughing and Aggregation of Hepatopancreatic Microvilli into Vermiform Bodies Superficially Resembling Gregarines

Siripom Sriurairatana[1], Visanu Boonyawiwat[2], Warachin Gangnonngiw[3], Chaowanee Laosutthipong[1,4], Jindanan Hiranchan[1,4], Timothy W. Flegel[1,4,*]



Tangprasittipap *et al. BMC Veterinary Research* 2013, 9:139
http://www.biomedcentral.com/1746-6148/9/139

The microsporidian *Enterocytozoon hepatopenaei* is not the cause of white feces syndrome in whiteleg shrimp *Penaeus (Litopenaeus) vannamei*

Amornrat Tangprasittipap[1,2†], Jiraporn Srisala[2†], Saisunee Chouwdee[1,2], Montagan Somboon[4], Niti Chuchird[4], Chalor Limsuwan[4], Thinnarat Srisuvan[6], Timothy W Flegel[1,3,5] and Kallaya Sritunyalucksana[1,2,5*]

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WFS Geographical Distribution In the World

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WHITE FECES SYNDROME (WFS) Indonesia, East Java 2016, 2017 & 2018 OIE Twinning Project

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Indonesia 2016, WFS Endemic Region

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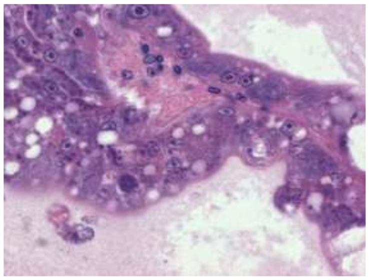
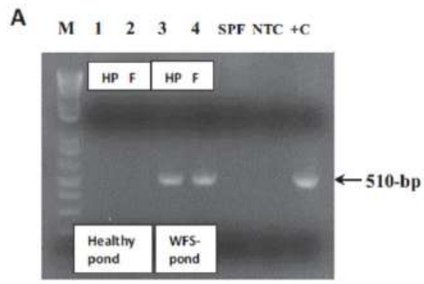
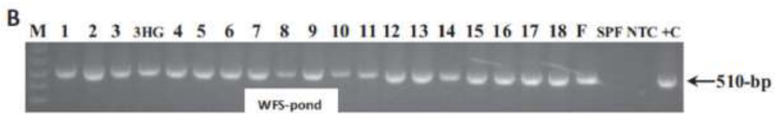
Indonesia 2016, WFS Endemic Region





Shrimp displaying WFS=EHP Positive 18/18



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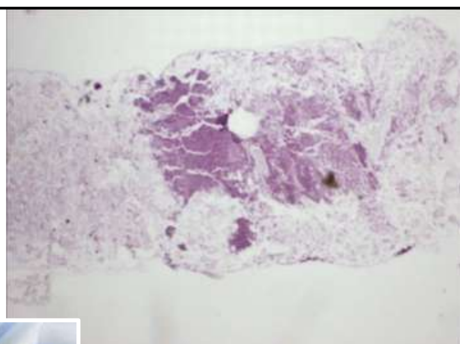
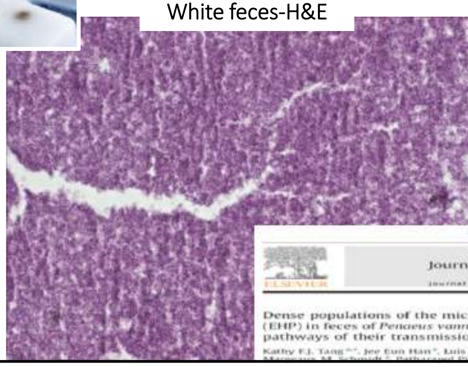
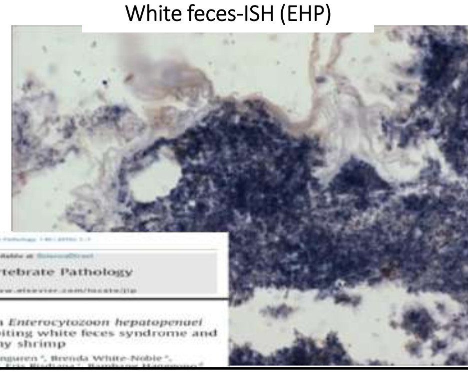


White feces-H&E

White feces-ISH (EHP)





Journal of Invertebrate Pathology

Dense populations of the microsporidian *Enterocytozoon hepatopenaei* (EHP) in feces of *Penaeus vannamei* exhibiting white feces syndrome and pathways of their transmission to healthy shrimp

Kathy F.J. Tang, Jee Eun Han, Luis Fernando Aranguren, Brenda White-Noble,

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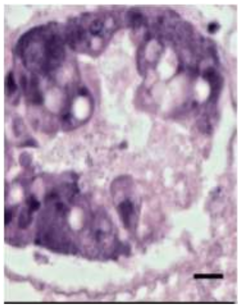
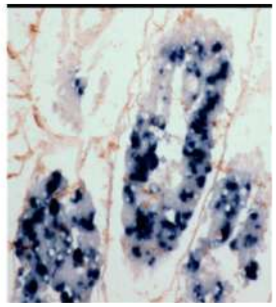
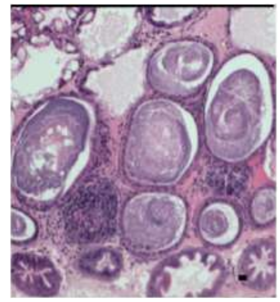
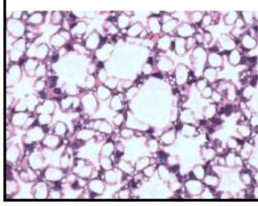
Shrimp Displaying WFS-Present-EHP and Septic hepatopancreatic necrosis (SHPN)

Aquaculture

Enterocytozoon hepatopenaei (EHP) is a risk factor for acute hepatopancreatic necrosis disease (AHPND) and septic hepatopancreatic necrosis (SHPN) in the Pacific white shrimp *Penaeus vannamei*

Luis Fernando Aranguren *, Jee Eun Han, Kathy F.J. Tang

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Grow out pond displaying white feces

Grow out pond without white feces


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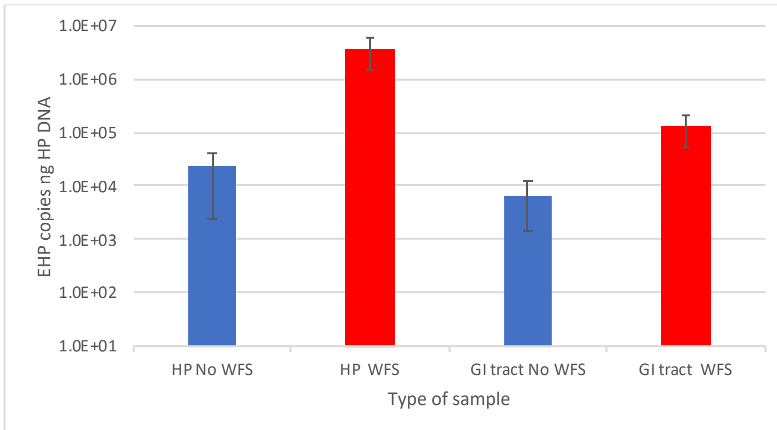
EHP Quantification in Grow-out Pond with WFS vs. no WFS



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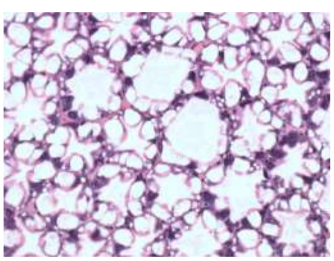
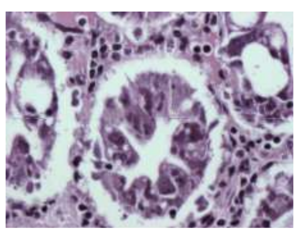
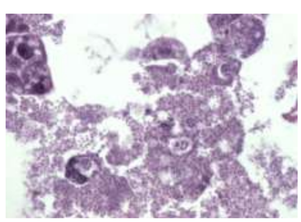
Histological findings:

Healthy

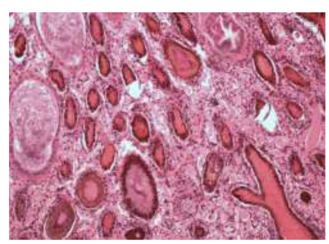


WFS: EHP





WFS: SHPN



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Healthy grow-out pond

Grow-out pond displaying white feces

Grow-out pond with history of white feces

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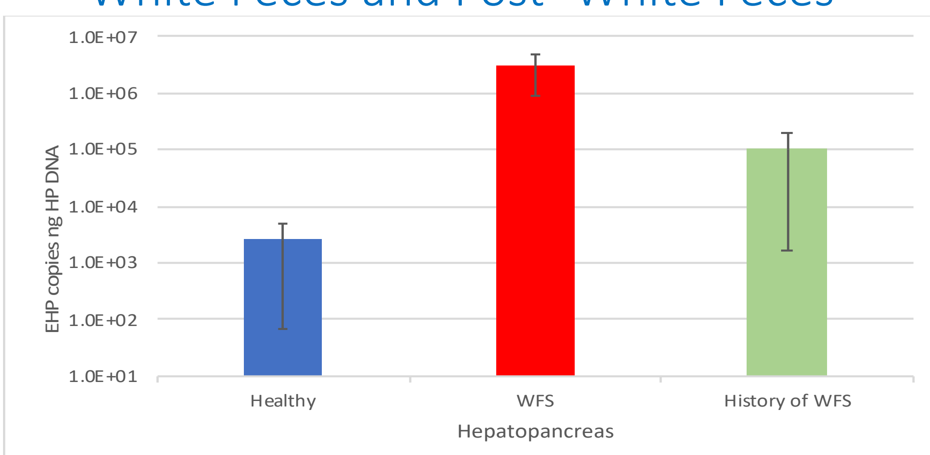
EHP Quantification in Grow-out Pond Healthy, White Feces and Post -White Feces



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India, WFS endemic region

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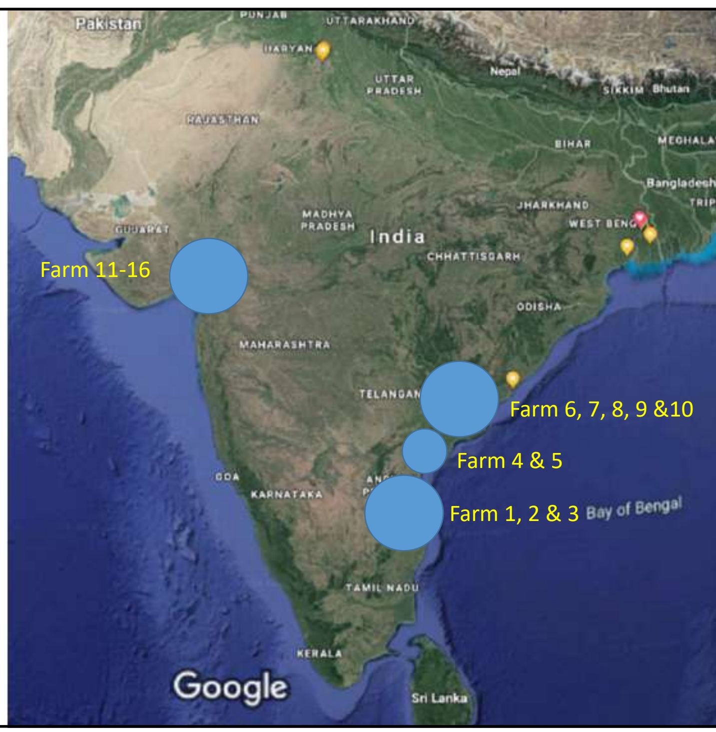

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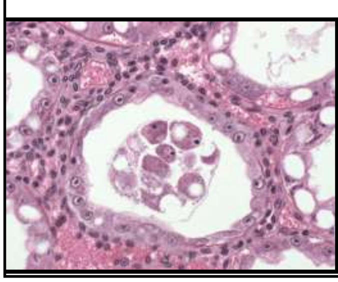

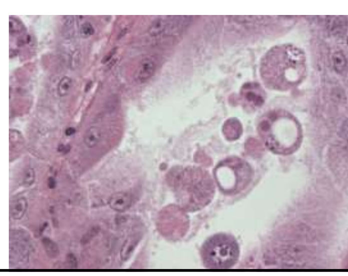

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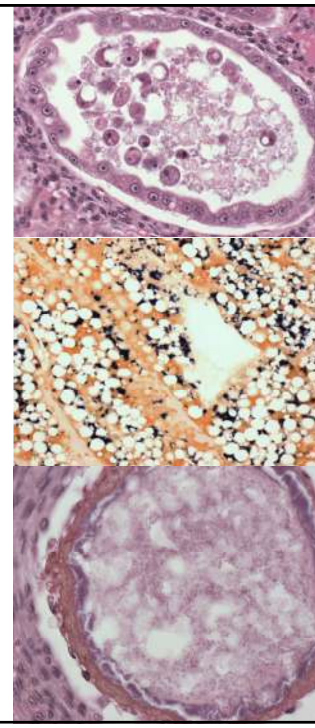
Results

Farm	DOC	ABW (g)	Salinity (ppt)	Stocking density (s/m2)	EHP
1	65	8	55	25	POS
2	45	7	35	10	POS
3	49	5.5	25	25	POS
4	20	5.6	35	30	POS
5	65	14	15	40	POS
6	53	6.32	10	25	POS
7	110	32	10	10	POS
8	65	20	4	30	POS
9	45	6	6	30	POS
10	41	6	25	25	POS
11	141	26.5	25	30	POS
13	105	15	18	80	POS
14	105	14	22	80	POS
15	69	10	30	24-25	POS
16	150	28	35	35	POS



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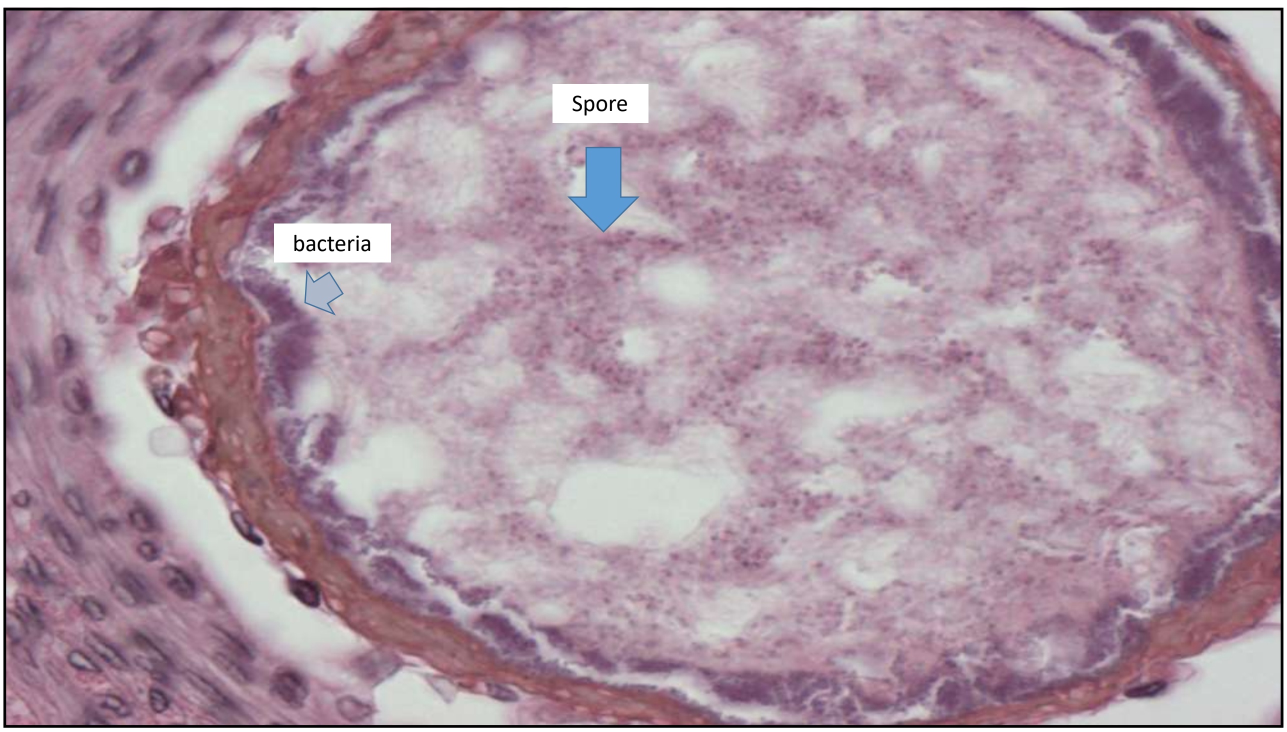

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EHP Geographical distribution in the Americas

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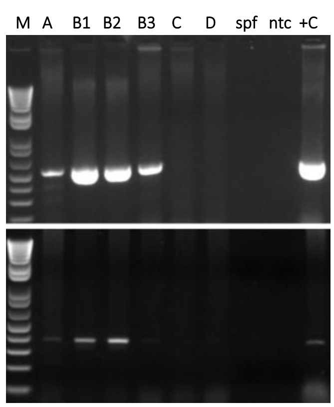
EHP of Shrimp Cultured in Venezuela

- **Slow growth**
- **Size disparity**
- **Chronic mortalities**
- **High FCR**
- **Soft shell**
- **CV% >25%**



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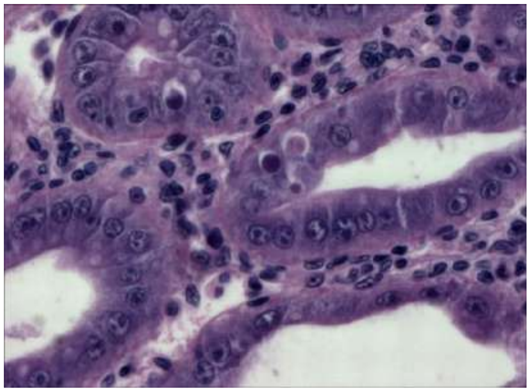

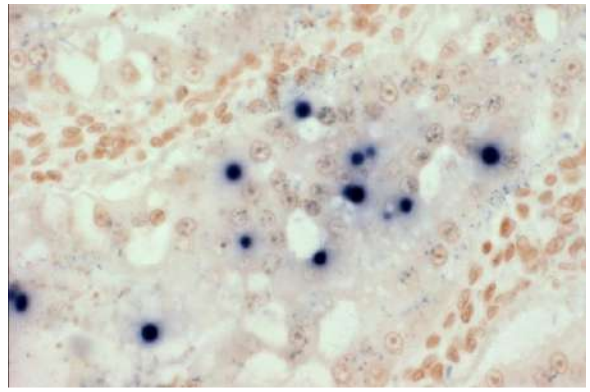

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EHP VS. WFS in the Americas

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Detection of the microsporidian *Enterocytozoon hepatopenaei* (EHP) and Taura syndrome virus in *Penaeus vannamei* cultured in Venezuela

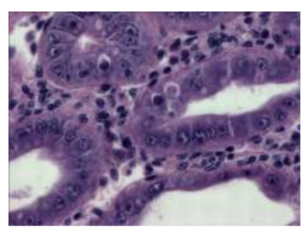
Kathy F.J. Tang[a,*], Luis Fernando Aranguren[b], Patharapol Piamsomboon[c], Jee Eun Han[d], Irina Y. Maskaykina[b], Margeaux M. Schmidt[b]

Vol. 141: 71–78, 2020 | DISEASES OF AQUATIC ORGANISMS Dis Aquat Org | Published online September 17

Evidences supporting *Enterocytozoon hepatopenaei* association with white feces syndrome in farmed *Penaeus vannamei* in Venezuela and Indonesia

Luis Fernando Aranguren Caro[1,*], Hung N. Mai[1], Orlando Pichardo[2], Roberto Cruz-Flores[1], Bambang Hanggono[3], Arun K. Dhar[1]

EHP in the Americas 2016



White Feces in the Americas 2018

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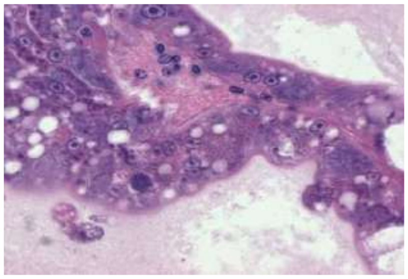
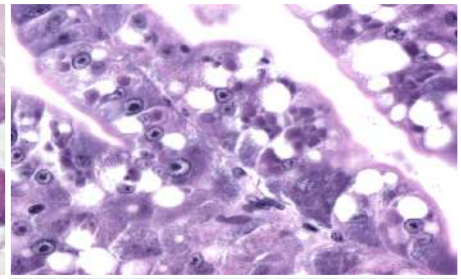
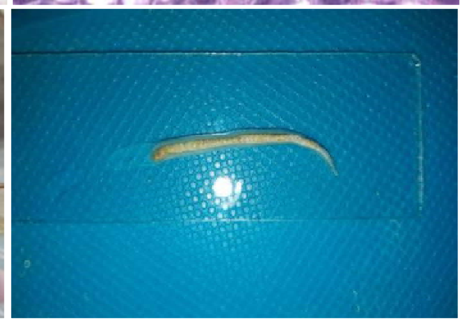
White Feces Syndrome in the Americas







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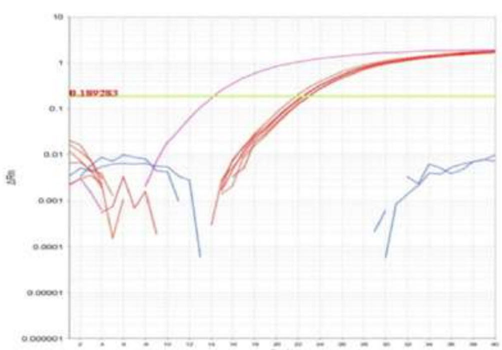
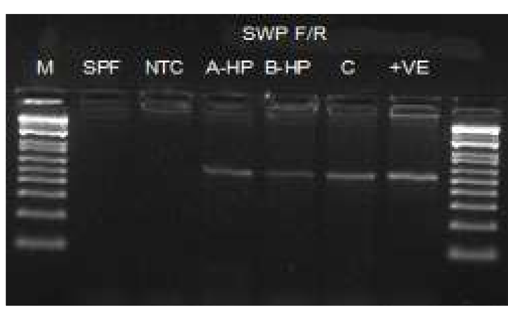
Detection of EHP using qPCR Taqman Probe (SSU) (Liu et al., 2018.)



EHP Spore wall protein gene (SWP-PCR)



514 bp

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Aranguren Caro *et al. BMC Veterinary Research* (2021) 17:65
https://doi.org/10.1186/s12917-021-02778-0

BMC Veterinary Research

RESEARCH ARTICLE Open Access

The effect of salinity on *Enterocytozoon hepatopenaei* infection in *Penaeus vannamei* under experimental conditions

Check for updates

L. F. Aranguren Caro[1*], F. Alghamdi[2], K. De Belder[1], J. Lin[1], H. N. Mai[1], J. Millabas[1], Y. Alrehaili[2], A. Alazwari[2], S. Algetham[2] and A. K. Dhar[1]

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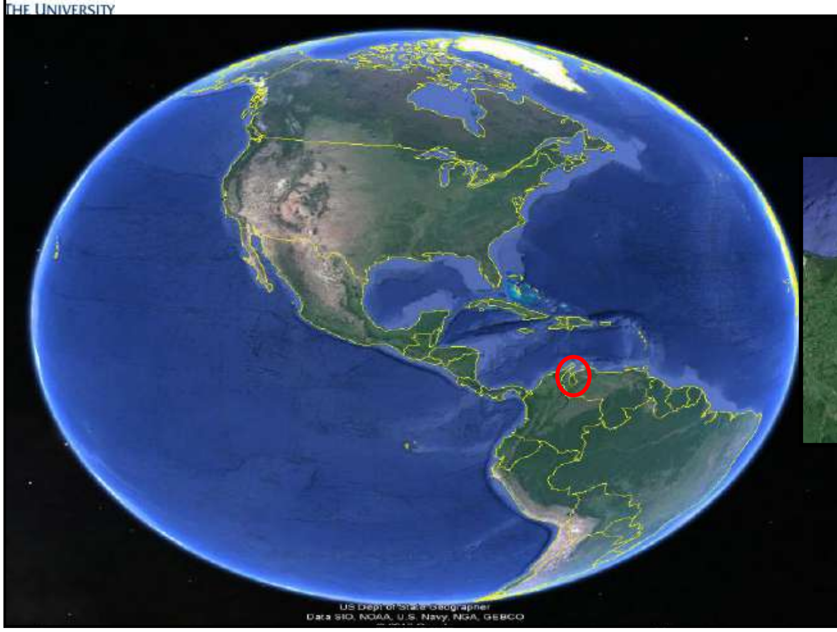

High salinity

Low salinity

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High salinity

Low salinity

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Fecal Strings Used as Source of Inoculum for EHP Challenge

Experimental Challenge	Duration	Final survival (%)					
		2 ppt EHP treatment	2 ppt control	15 ppt EHP treatment	15 ppt control	30 ppt EHP treatment	30 ppt control
1	20 days	95 ± 7	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
2	26 days	90 ± 0	100 ± 0	100 ± 0	100 ± 0	90 ± 7	100 ± 0

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Prevalence and Severity of EHP Based on H&E

2 ppt treatment		15 ppt treatment		30 ppt treatment	
Severity	Prevalence (%)	Severity	Prevalence (%)	Severity	Prevalence (%)
G4 (10.0%) G3 (10.0%) G2 (0.0%) G1 (10.0%) G0 (70.0%)	30%	G4 (0.0%) G3 (0.0%) G2 (0.0%) G1 (31.2%) G0 (68.8%)	31.2%	G4 (25.0%) G3 (8.3%) G2 (16.7%) G1 (16.7%) G0 (33.3%)	66.6%

Grade scale based on Lightner (1996)

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Experimental reproduction of White Feces Syndrome in whiteleg shrimp, *Penaeus vannamei*

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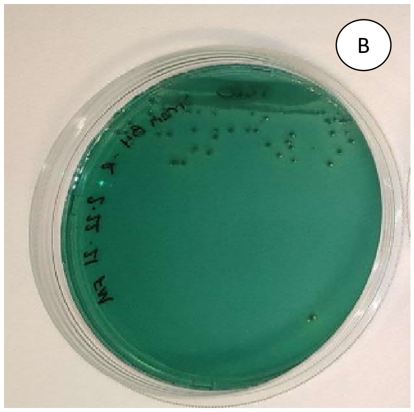
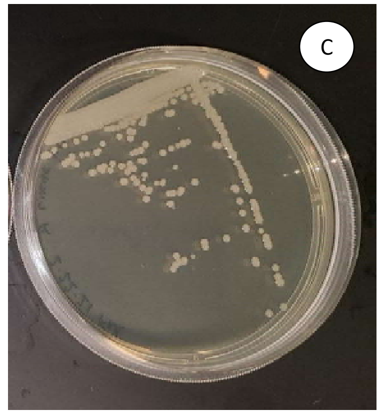
Experimental Design: Bacterial Strain





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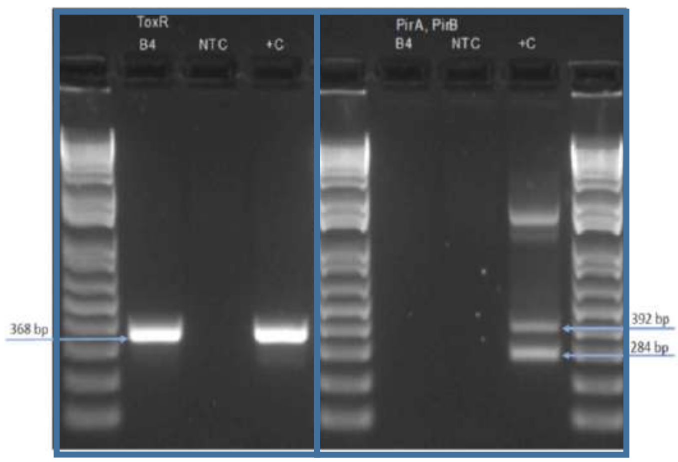
PCR Amplification of *ToxR* and *pirA/B genes*

ToxR: Kim *et al*., (1999)
AHPND: Han *et al*., (2015)



Sequencing of the
16S rRNA region,
MW526256

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Vibrio parahaemolyticus strain India 2019 16S ribosomal RNA gene, partial sequence	Vibrio parahaemolyticus	2687	2687	100%	0.0	100.00%	1455	MW526256.1
☑	Vibrio parahaemolyticus strain MC32 16S ribosomal RNA gene, partial sequence	Vibrio parahaemolyticus	2676	2676	100%	0.0	99.86%	1487	MT534026.1
☑	Vibrio parahaemolyticus strain VC005 16S ribosomal RNA gene, partial sequence	Vibrio parahaemolyticus	2676	2676	100%	0.0	99.86%	1487	MT534020.1
☑	Vibrio parahaemolyticus strain VC004 16S ribosomal RNA gene, partial sequence	Vibrio parahaemolyticus	2676	2676	100%	0.0	99.86%	1487	MT534019.1
☑	Vibrio parahaemolyticus strain VC002 16S ribosomal RNA gene, partial sequence	Vibrio parahaemolyticus	2676	2676	100%	0.0	99.86%	1487	MT534018.1
☑	Vibrio parahaemolyticus strain VC001 16S ribosomal RNA gene, partial sequence	Vibrio parahaemolyticus	2676	2676	100%	0.0	99.86%	1487	MT534017.1
☑	Vibrio parahaemolyticus strain 2012V-1165 chromosome 1	Vibrio parahaemolyticus	2676	39509	100%	0.0	99.86%	3411422	CP051111.1

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Experimental design: Set up

Trial	Tank	Group	Number of animals (initial)	Number of animals (final)	Final survival	CV%
1 23 days	1	SPF Negative control	7	6	85.7%	21.4
	2	SPF +V. parahaemolyticus	7	6	85.7%	27.6
	3	EHP positive control	8	8	100.0%	41.6
	4	EHP + V. parahaemolyticus	7	4	57.1%	40.0
2 30 days	1	SPF Negative control	10	9	90.0%	12.0
	2	SPF + V. parahaemolyticus	10	8	80.0%	10.1
	3	EHP positive control	9	4	44.4%	30.8
	4	EHP + V. parahaemolyticus	8	2	25.0%	40.7

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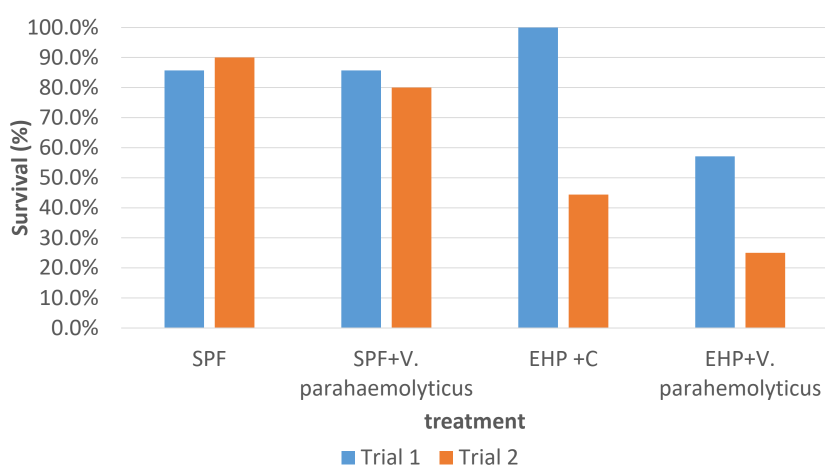
Final survival



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Results

Trial	Treatments	Clinical signs	EHP & SHPN detection by H&E histology EHP (%)	SHPN (%)	EHP detection by real time-PCR
1	SPF Negative control	No WFS	0	0	0%
	SPF+V. parahaemolyticus.	No WFS	0	0	0%
	EHP positive control	No WFS	100	37.5%	100%
	EHP+ V. parahaemolyticus.	WFS	100	83.3%	100%
2	SPF Negative control	No WFS	0	0%	0%
	SPF+ V. parahaemolyticus	No WFS	0	0%	0%
	EHP positive control	No WFS	100	60%	100%
	EHP+ V. parahaemolyticus.	WFS	100	60%	100%

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Shrimp displaying White fecal strings

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Shrimp displaying White fecal strings

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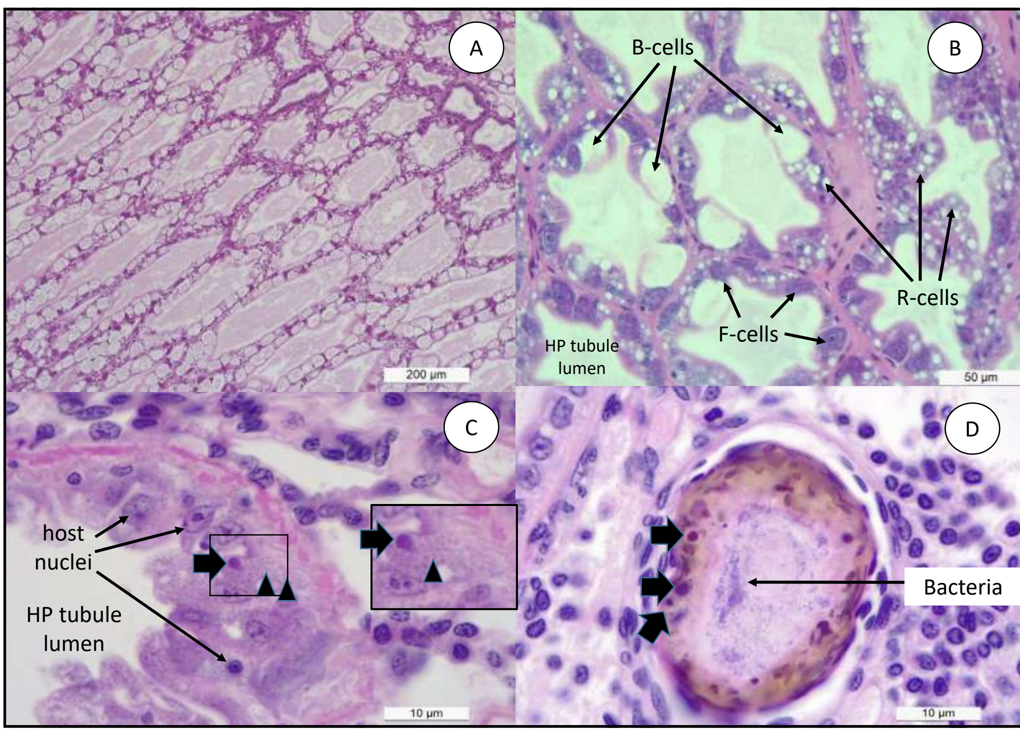

Fig 4. (A,B,C & D

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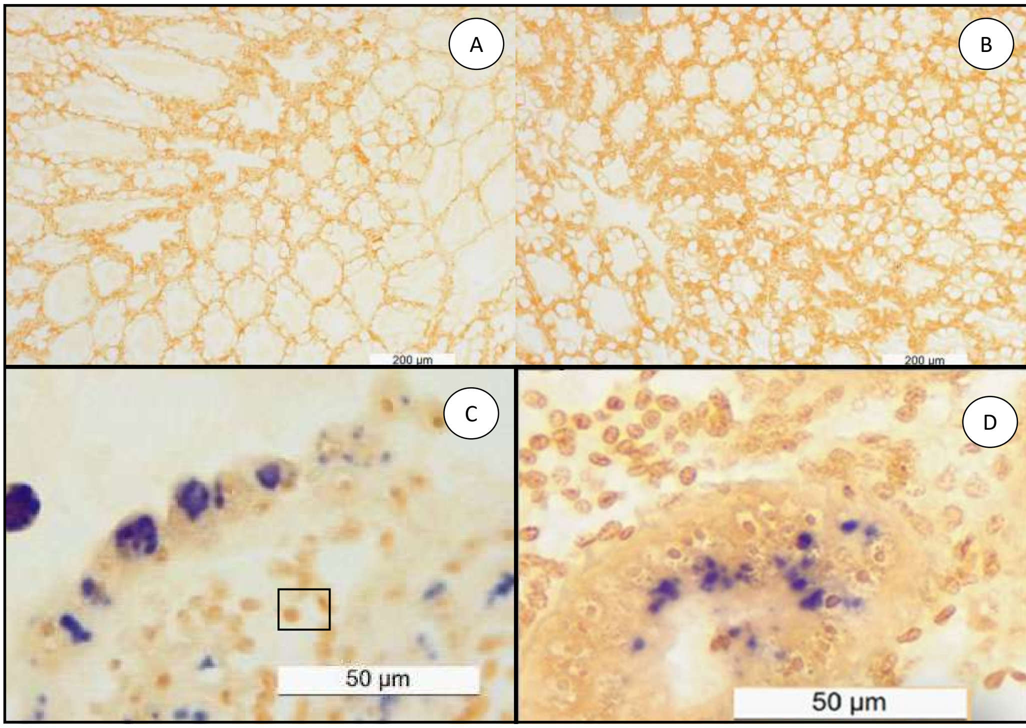

Fig 5. (A,B,C & D

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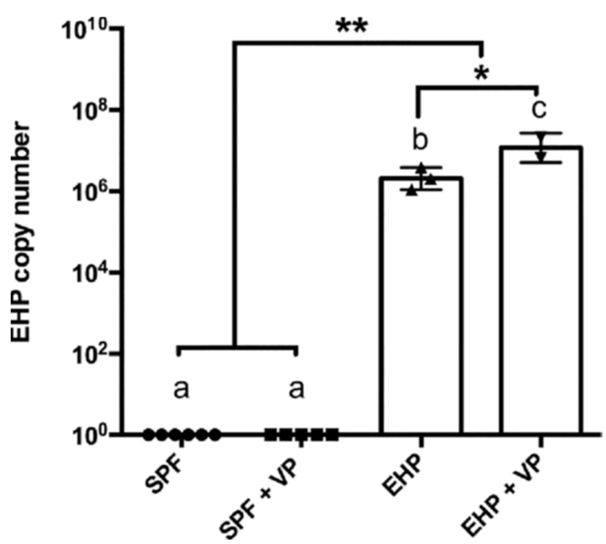
EHP copy number



1.4×10^{7} copies/ng of DNA

2.3×10^{6} copies /ng of DNA

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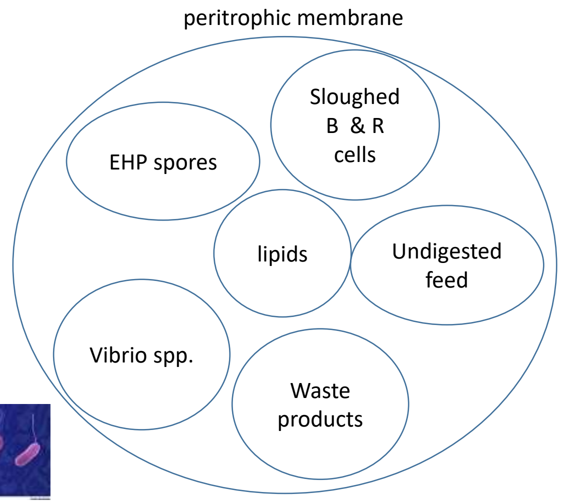
White Fecal strings

Environmental change e.g pH

digestive enzymes; proteases, including specific collagenases; lipolytic enzymes; chitinase; cellulase; laminarinase; alP-glucosidase and/or a-amylase



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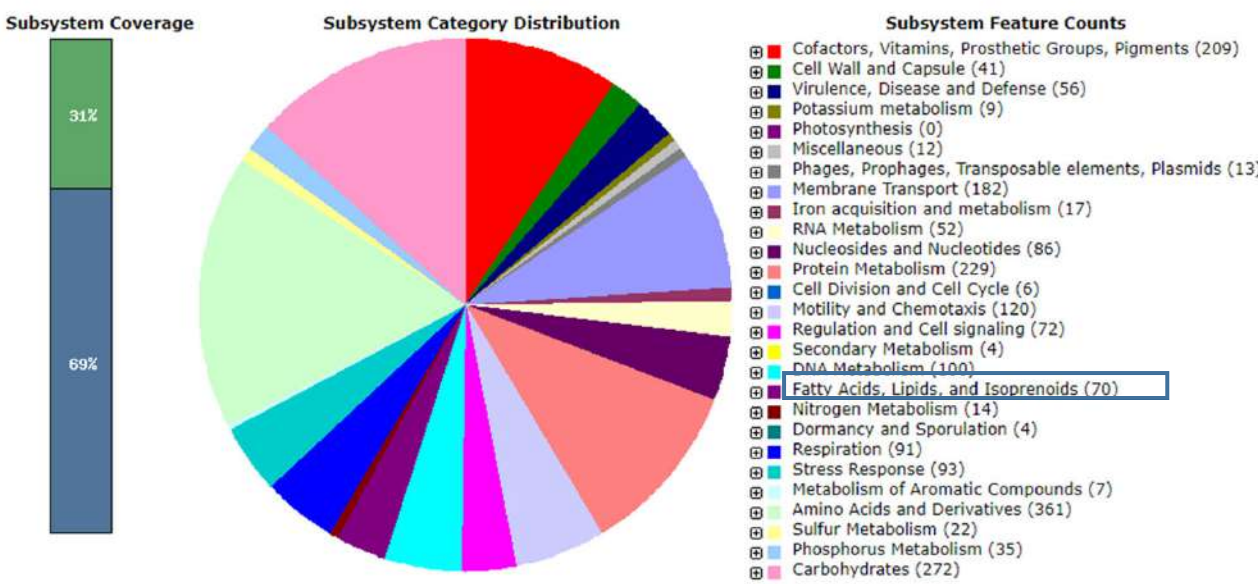
Vibrio parahaemolyticus NGS analysis



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Summary and Future Studies

- We describe for the first time the experimental reproduction of WFS in shrimp *P. vannamei* pre-infected with EHP and challenged with a particular strain of *Vibrio parahaemolyticus*.
- We consider EHP as the primary pathogen and the strain of *Vibrio parahaemolyticus as the* secondary pathogen.
- Not only an association among EHP vs. WFS vs. *Vibrio parahaemolyticus* was found, but also causality was proved. EHP in combination of *V. parahaemolyticus* can cause WFS under laboratory conditions.
- The mechanism by which WFS is produced is unknown
- EHP is not the only causative agent involved in the reproduction of WFS

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Summary and Future Studies

- Analyze the NGS data in the strain of *V. parahaemolyticus* (B4) to determine the possible proteases / lipases that enhance the sloughing of HP epithelial cells
- Establish the composition of the Fecal strings

2003-2005 in Latin America

66

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PLOS ONE

RESEARCH ARTICLE

Experimental reproduction of White Feces Syndrome in whiteleg shrimp, *Penaeus vannamei*

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Check for updates

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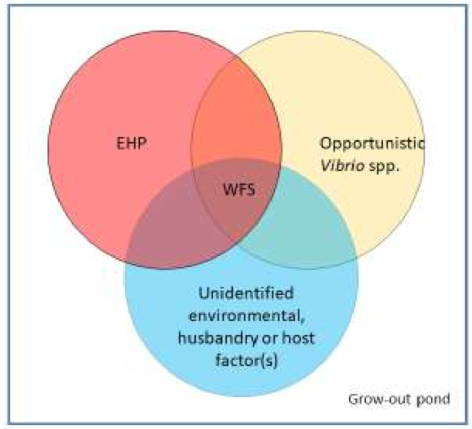
Pathogenesis of WFS in EHP-endemic Areas at Farm Level

Normal pond
↓
Floating fecal strings
↓
Size disparity
↓
Increase FCR
↓
Shrimp stop eating
↓
Chronic mortalities

68

Final Remarks

- There is a strong association between White feces syndrome and EHP
- In EHP endemic regions, EHP can explain the clinical manifestation of white feces syndrome
- Not all cases of WFS are associated to EHP



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EHP Management

Avoid live feed

- Never use live animals (e.g., live artemia, polychaetes, clams, oysters, etc.) as feeds for broodstock
- At a minimum, live feed should be frozen or dried before use

Avoidance by exclusion

- Early detection by molecular methods (fast & sensitive diagnosis)

70

Thank you!
Questions?

Wet mounts/Microscopy
Direct microscopy in tissues

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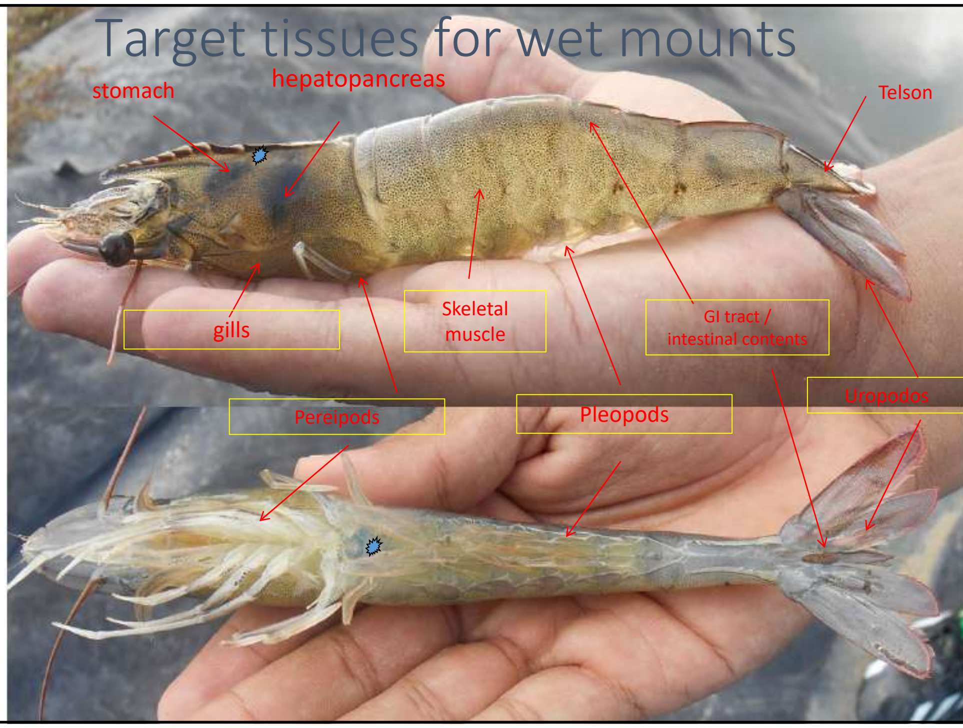
1

Purpose of wet mounts

- To have a presumptive diagnosis of presence of a pathogen in a given tissue
- To determine what is the tissue/organ affected:
  - **Systemic**
  - **Enteric**

2

Target tissues for wet mounts



3

Disection

4

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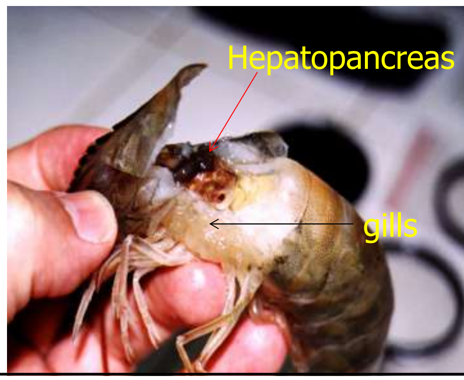
Disection



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Hepatopancreas

6

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EMS

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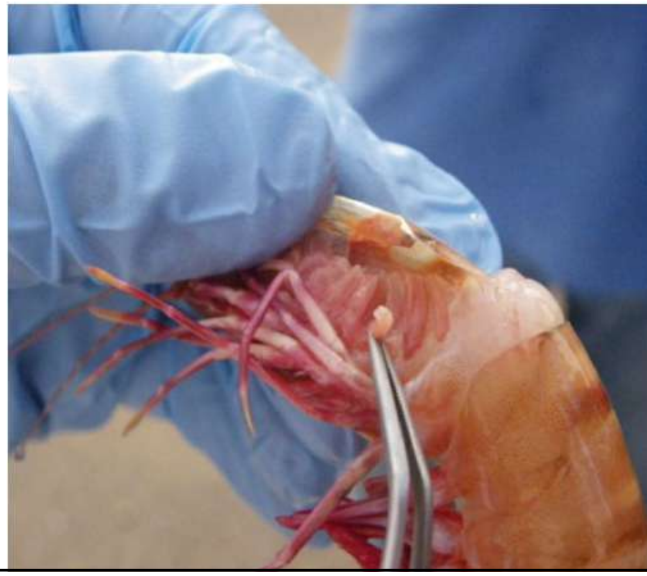
Gill sampling



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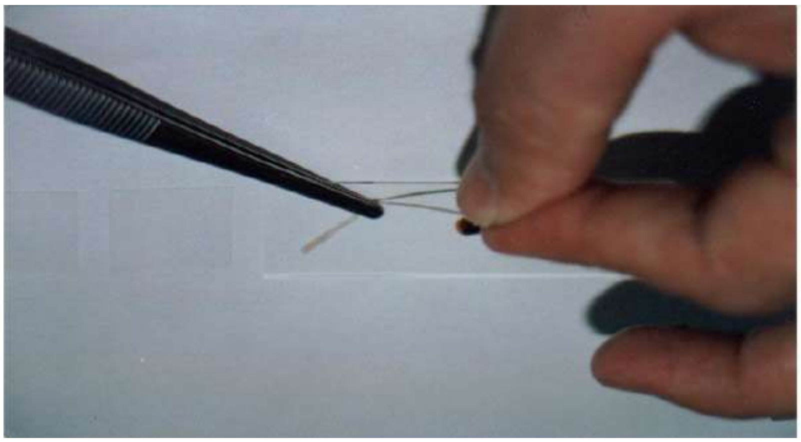
hemolymph

10

Sampling GI tract



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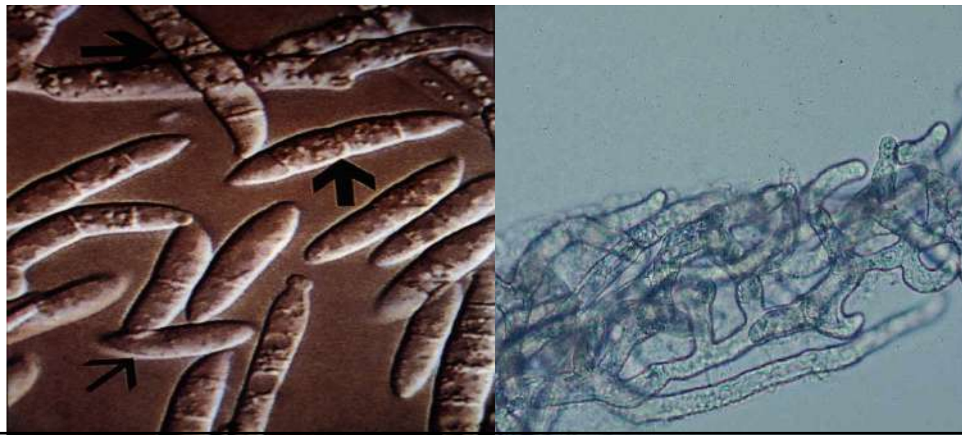
Disection

12

Hyphaes/spores in skeletal muscle



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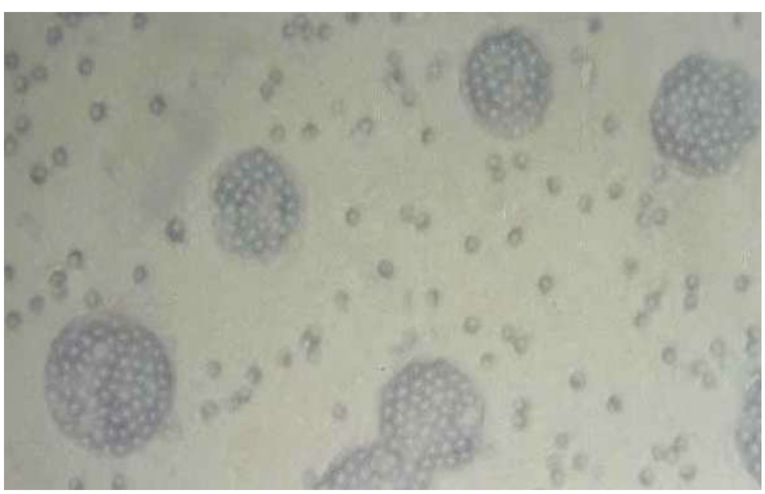
Microsporidia in skeletal muscle



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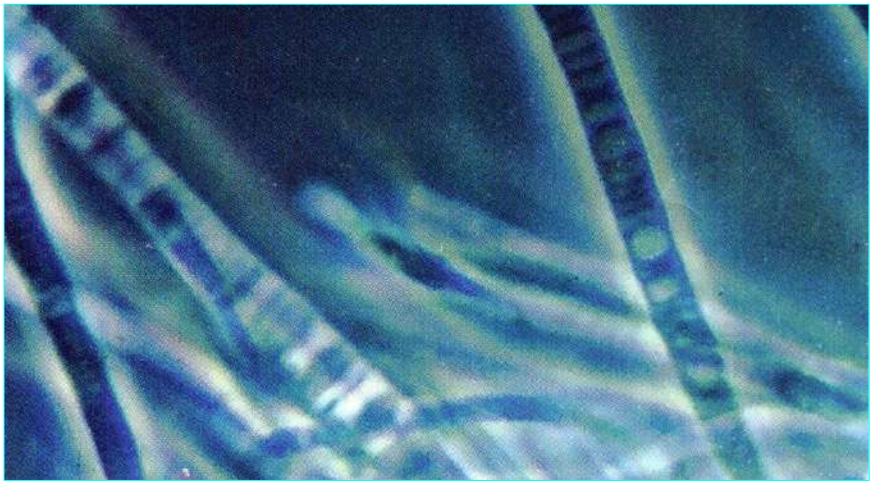
Leucothrix sp. In gill filaments



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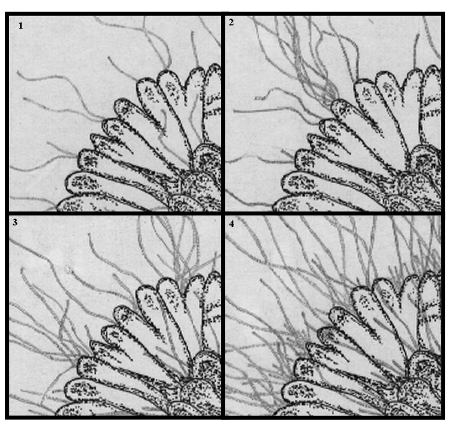
Grados de severidad



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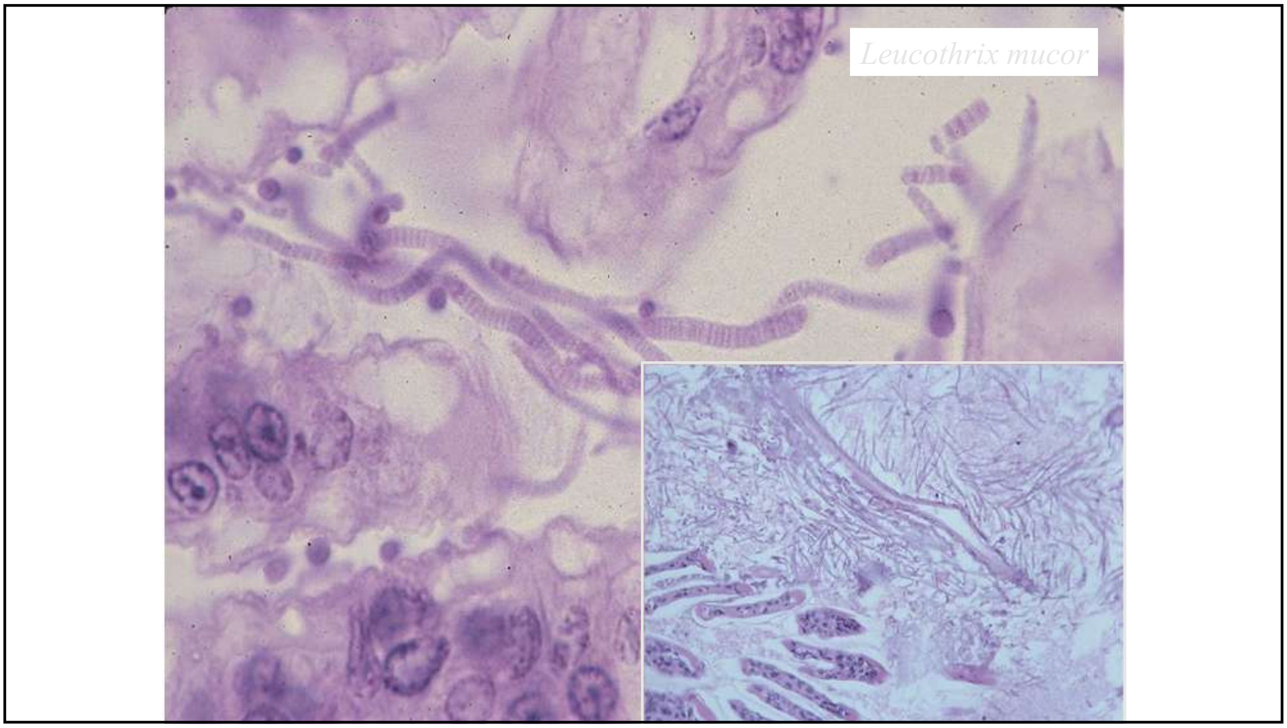

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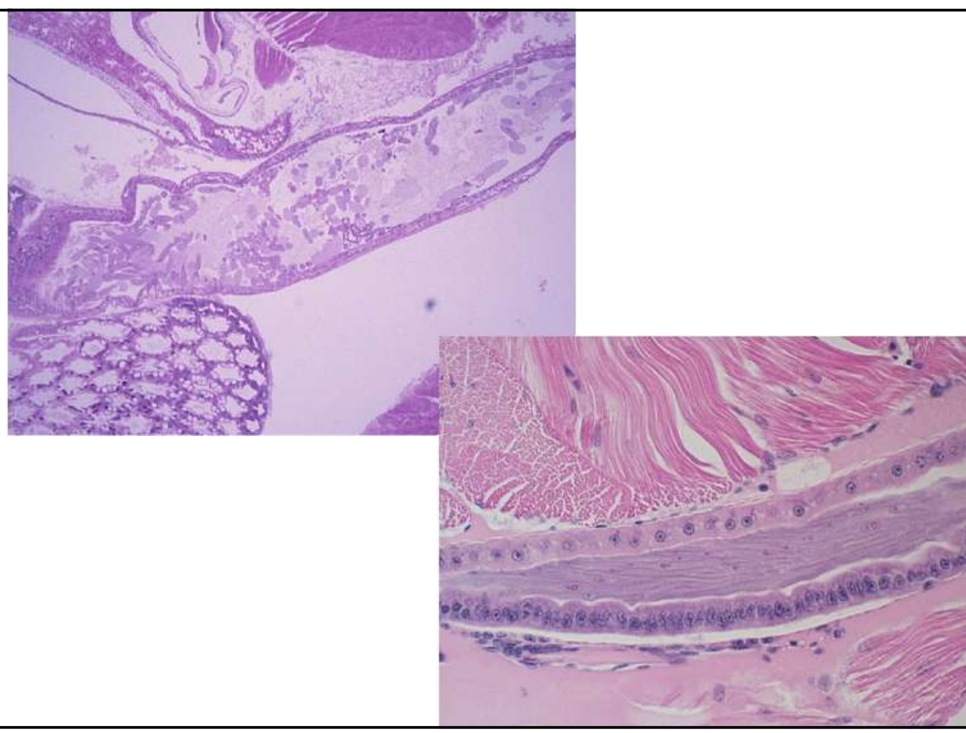
Contenido intestinal

19



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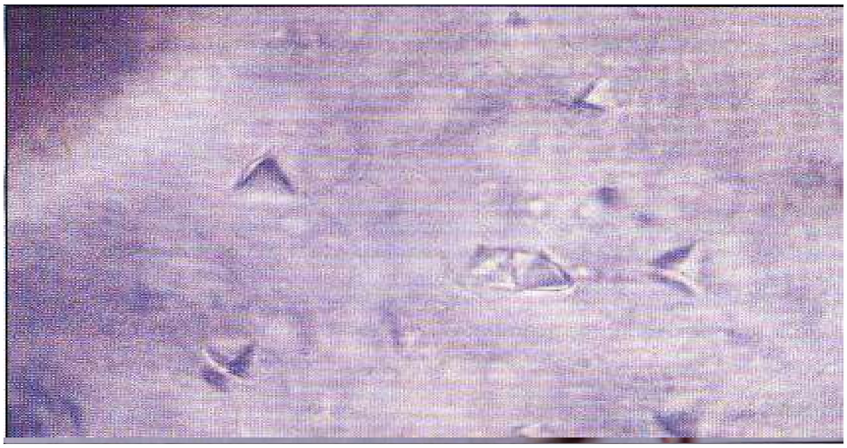
Intestinal content



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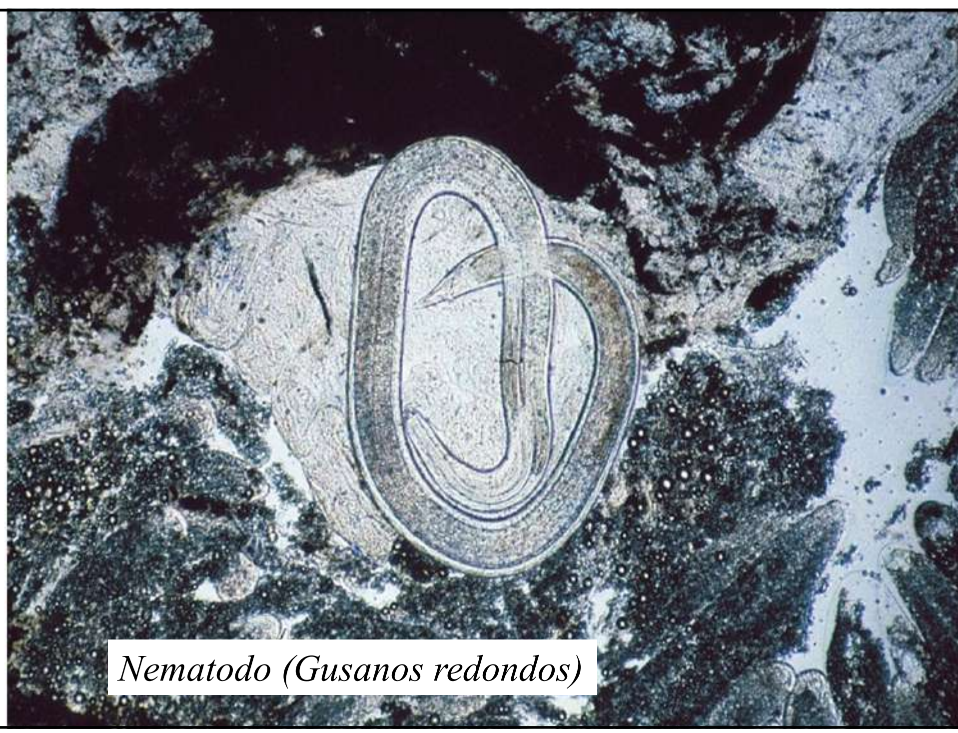

Nematodo (Gusanos redondos)

22

Lab practice

pathogen	Stain	Target Organ/tissue
WSSV	Hematoxylin	Gills
AHPND	None	Hepatopancreas

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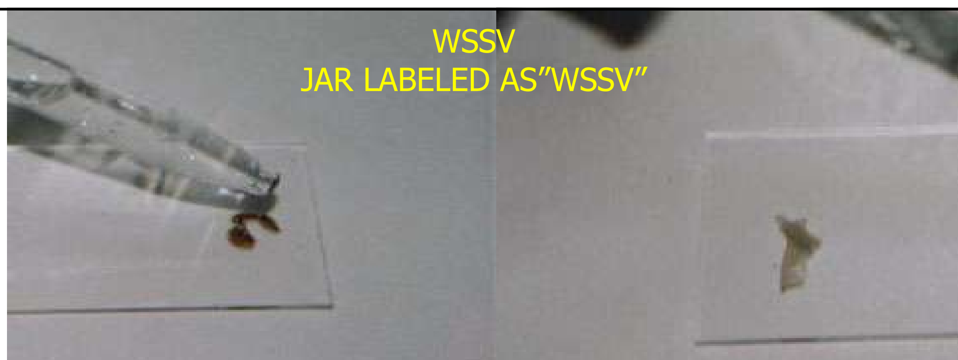
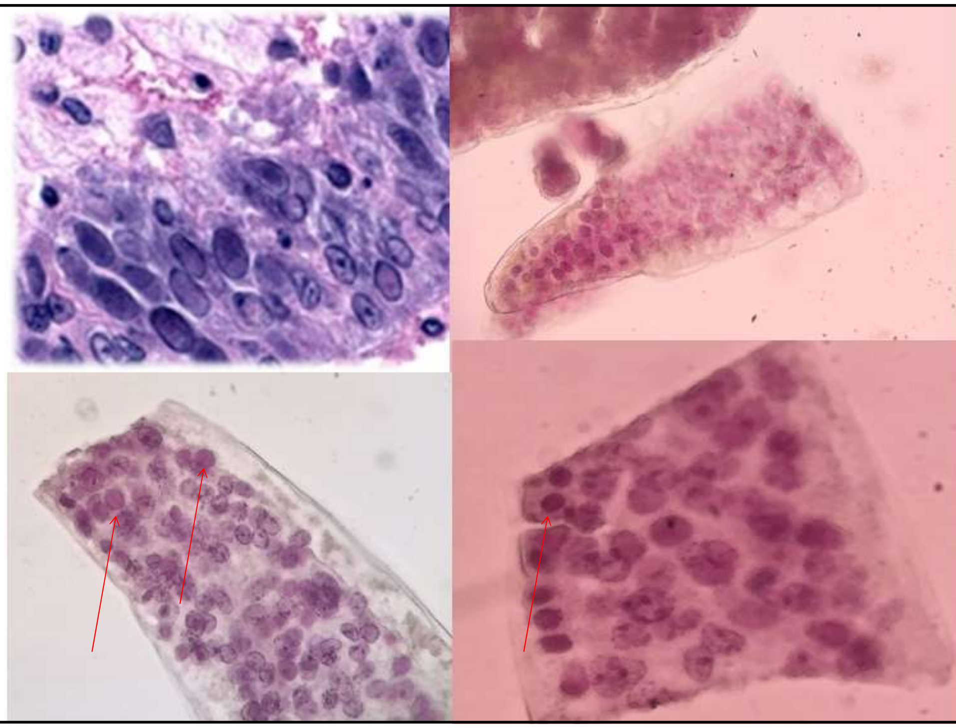


Cut the gills filaments in small sections with a blade
Add 3-5 drops of hematoxylin
Lets stand for 1 min
Put coverslip and press it down

24



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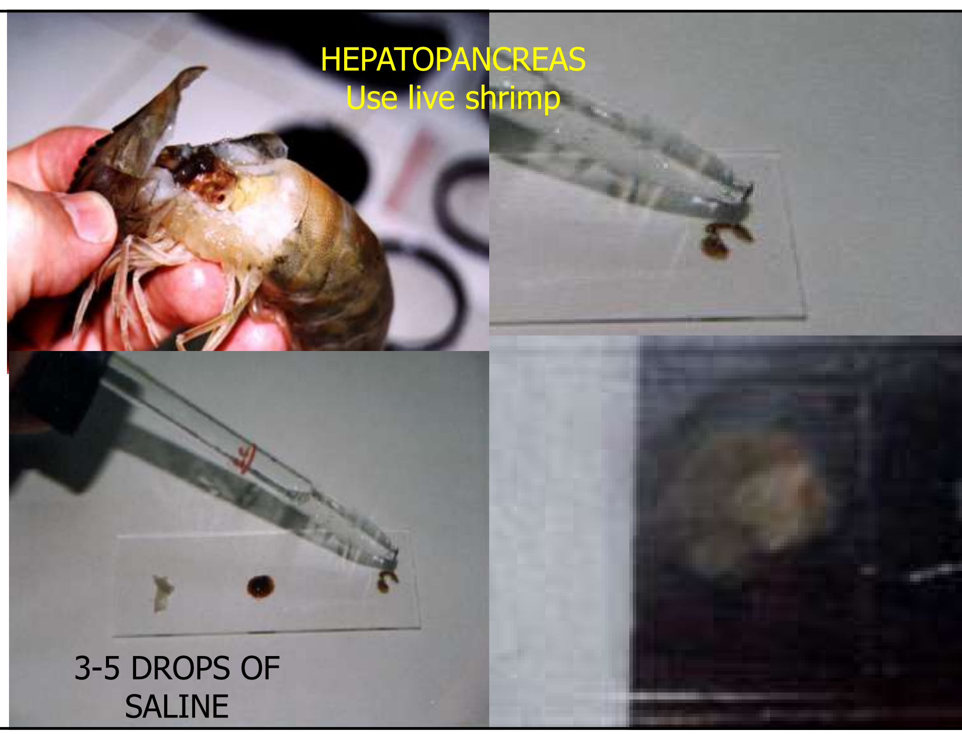

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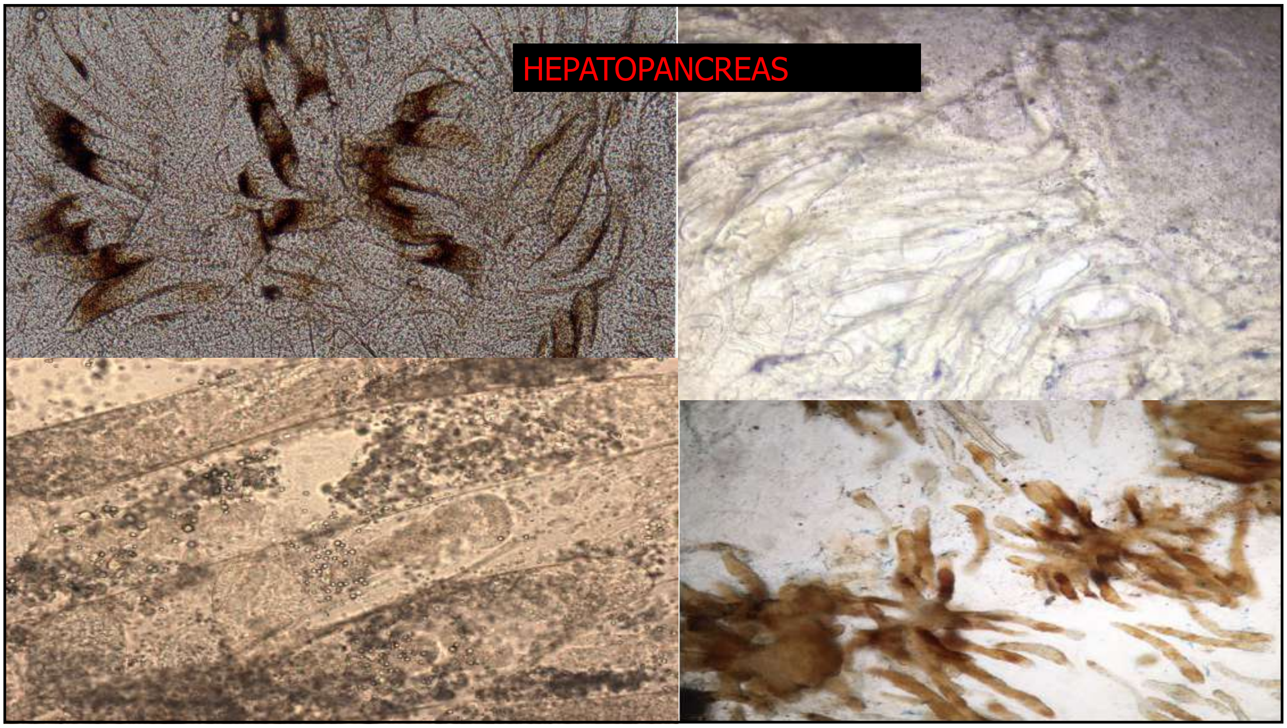

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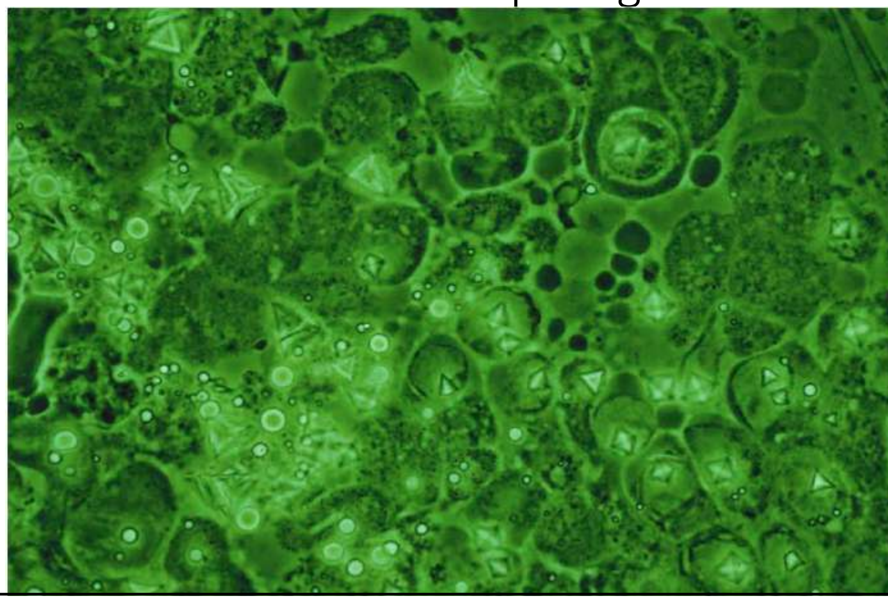
Hepatopancreas

28

BP occlusion bodies stained with malaquite green



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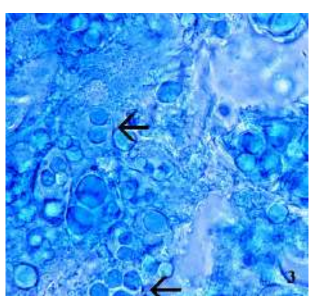
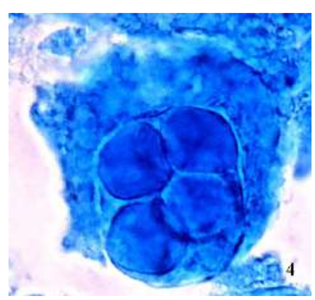
Cuerpos de occlusion (MBV) tenidos con azul de metileno





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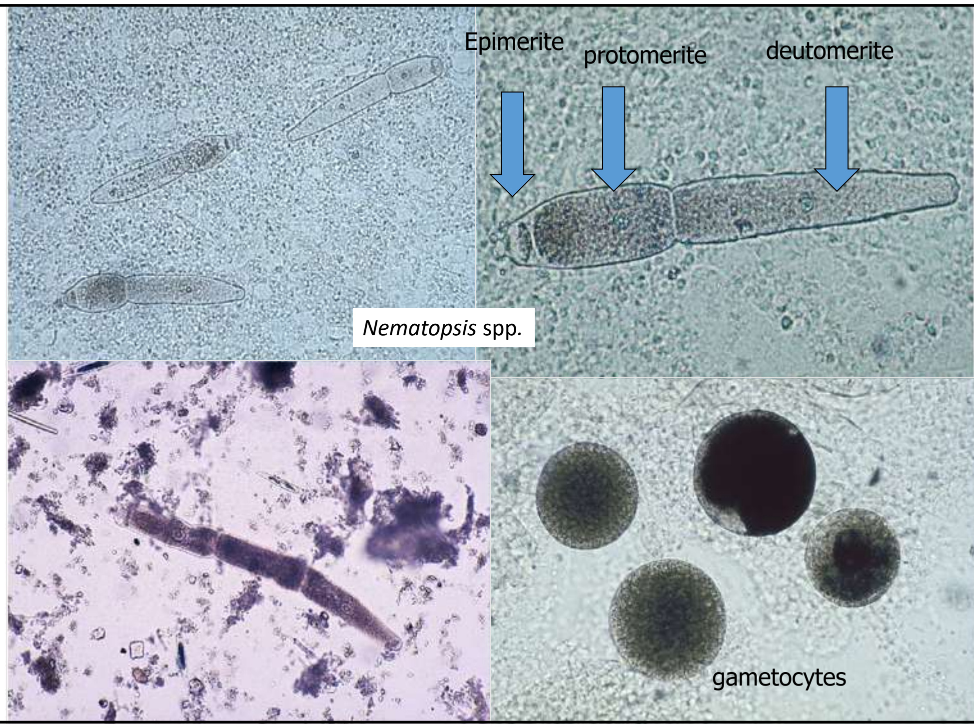

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Hands on

1. Take a shrimp:
- Healthy
- AHPND infected shrimp

2. Take gills sample and placed in the slide
3 Take a HP (2-4 mm) and placed in the slide
Take a Gastrointestinal content and placed in the slide
Add saline to each sample
Put a coverslip and squash down the sample:
Observe in the microscope

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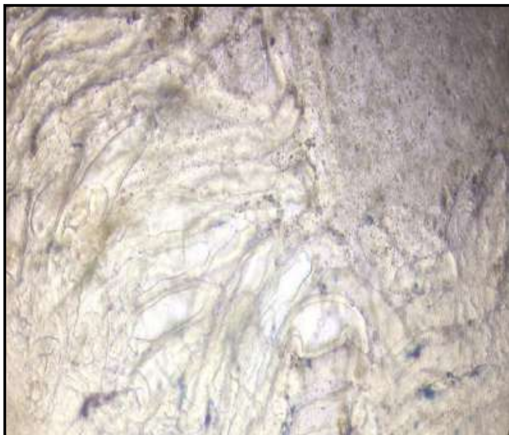
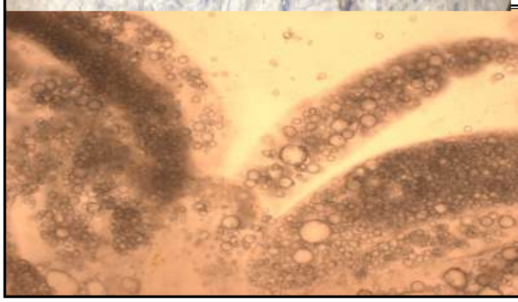
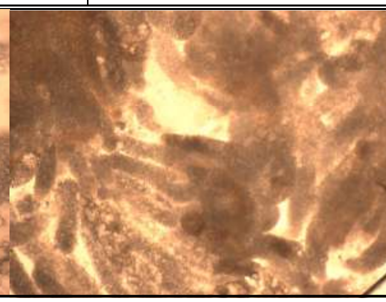
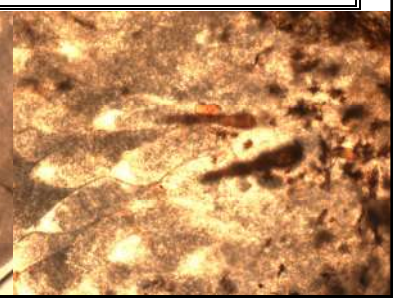


Severity Grade	Clinical Findings
0	> No signs of infection/infestation by pathogen, parasite, or epicommensal present.
trace	> Signs of infection/infestation by pathogen, parasite or epicommensal are present at just above diagnostic procedure minimum detection limits.
1	> Signs of infection/infestation by pathogen, parasite or epicommensal are present, but at levels that may be below those needed for clinical disease. > Agent detected may be in early stages of infection and represent preclinical disease.
2	> Moderate signs of infection/infestation as shown by low to moderate numbers of parasite or epicommensal, or by number and severity of pathogen caused lesions. > Prognosis is for possible production losses and/or slight increases in mortality if no treatment (if treatable) or management change is applied.
3	> Moderate to high signs of disease apparent as shown by relatively higher numbers of parasite or epicommensal, or by number and severity of pathogen caused lesions. > Potentially lethal prognosis if no treatment (if treatable) or management change is applied.
4	> High numbers of parasite or epicommensal present, or for pathogen caused infections the presence of severe lesions and advanced tissue destruction. > Lethal prognosis, especially under conditions conducive to disease development (i.e. with low oxygen, ecdysis, changes in salinity or temperature, etc.).

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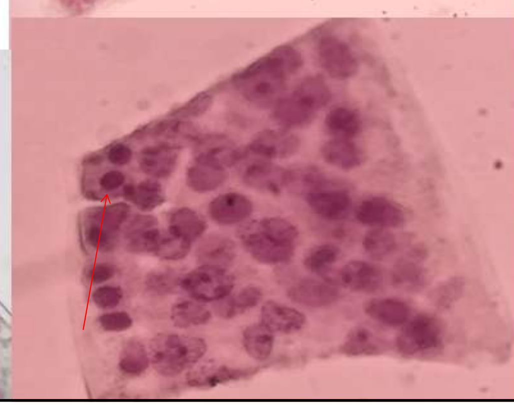
Cut the gills filaments in small sections with a blade
Add 3-5 drops of hematoxylin
Lets stand for 1 min
Put coverslip and press it down





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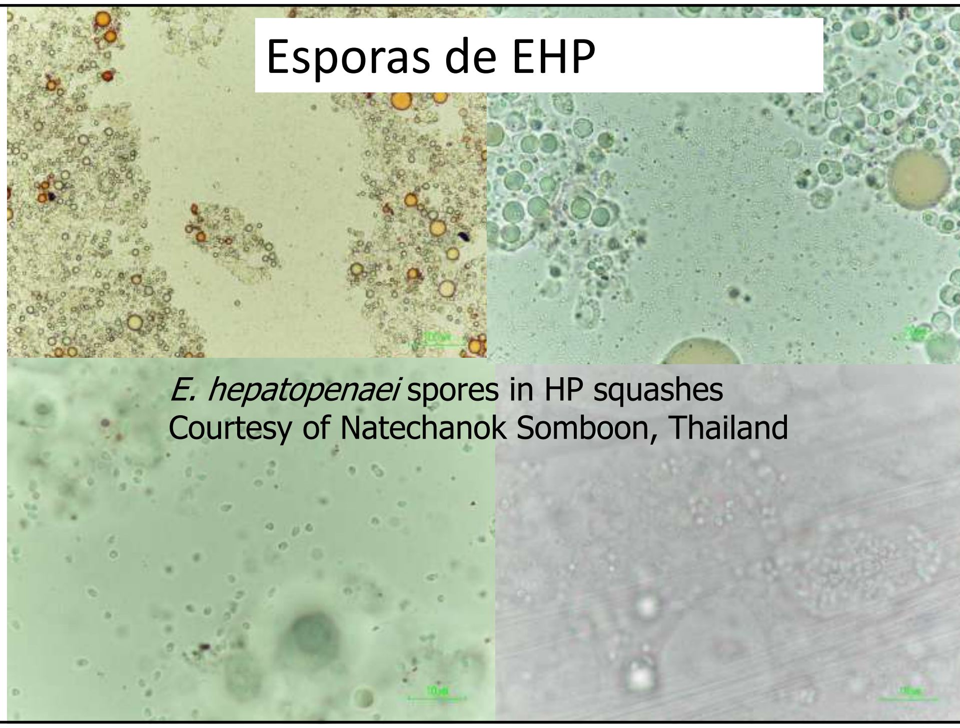
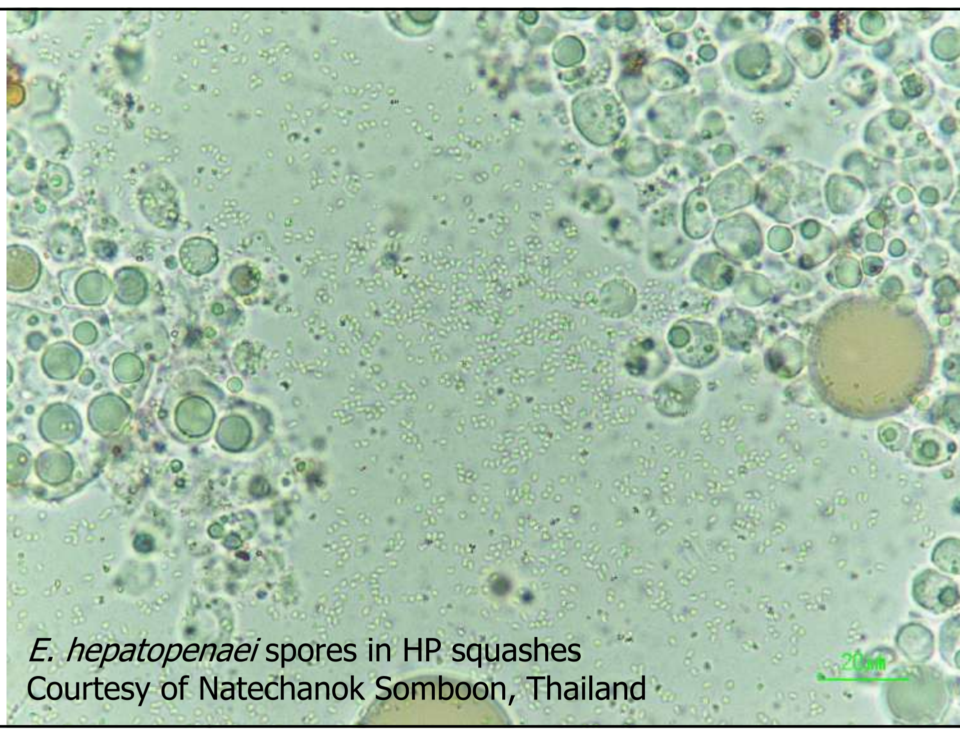
Esporas de EHP



E. hepatopenaei spores in HP squashes
Courtesy of Natechanok Somboon, Thailand

35



E. hepatopenaei spores in HP squashes
Courtesy of Natechanok Somboon, Thailand

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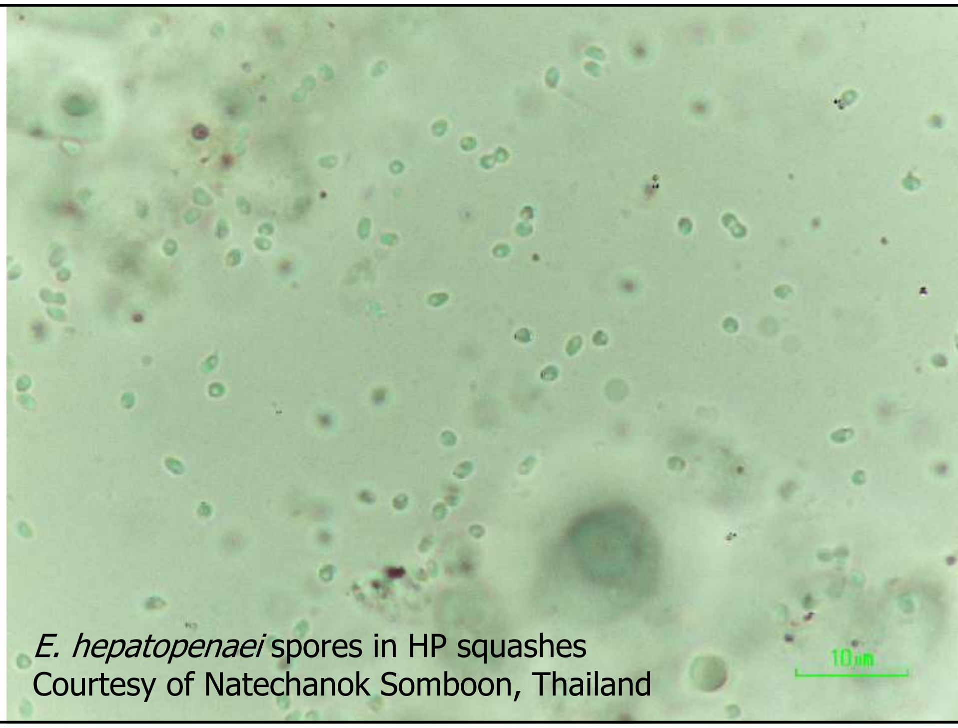
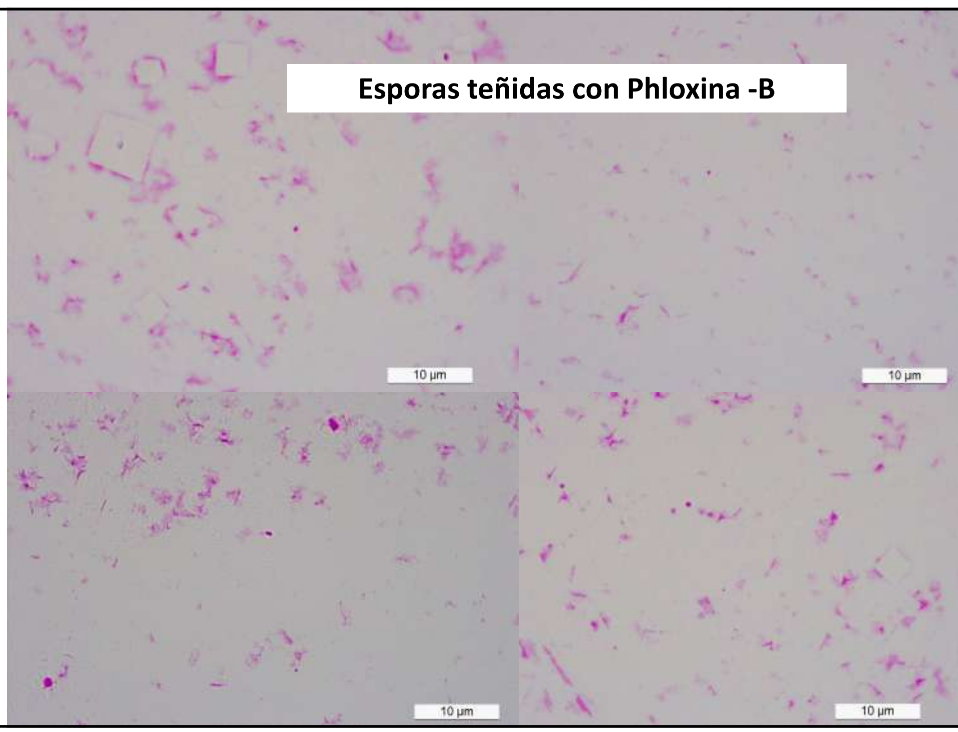


E. hepatopenaei spores in HP squashes
Courtesy of Natechanok Somboon, Thailand

37

Esporas teñidas con Phloxina -B



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H&E slides

Slot	pathogen	Slot	pathogen
1	EHP	26	HPV
2	EHP	27	WSSV
3	EHP	28	WSSV
4	EHP	29	WSSV
5	EHP	30	WSSV
6	EHP	31	TSV
7	EHP	32	TSV
8	EHP	33	YHV
9	HPM	34	IMNV
10	HPM	35	PVNV
11	HPM	36	HP MICROSPORIDIUM
12	HPM	37	MUSCLE MICROSPORIDIUM
13	AHPND/EMS	38	GREGARINES
14	AHPND/EMS	39	NEMATODS
15	AHPND/EMS	40	FUSARIUM
16	AHPND/EMS	41	AHPND/EMS
17	AHPND/EMS	42	EHP IN SITU
18	BP	43	EHP IN SITU
19	BP	44	EHP IN SITU
20	MBV	45	SPF
21	MBV	46	SPF
22	NHP	47	SPF
23	NHP	48	SPF
24	NHP IN SITU	49	SPF
25	HPV	50	SPF

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♦ Questions?

Thank you

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Biosecurity in shrimp farming

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1

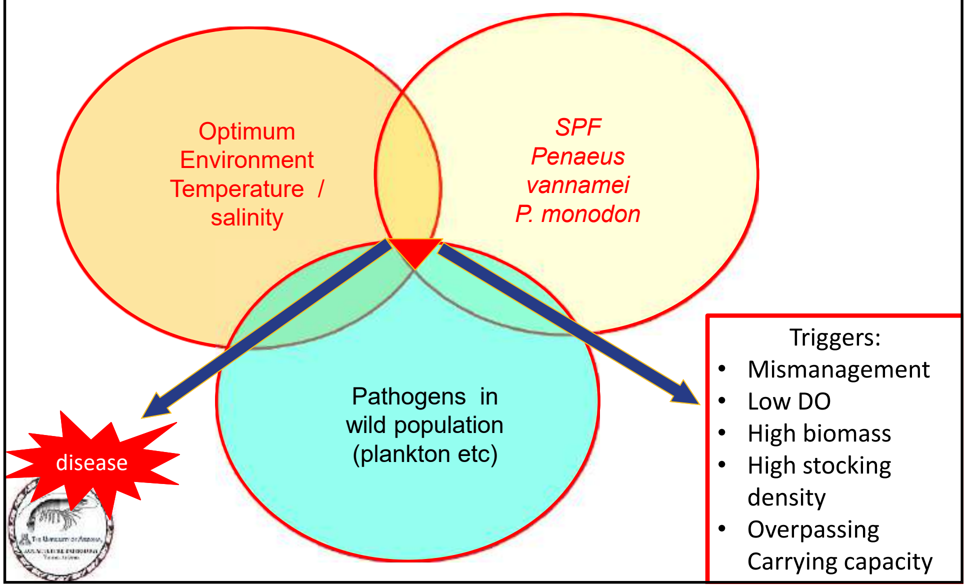
BIOSECURTIY – Definition for Shrimp Aquaculture

Biosecurity - The practice of exclusion of specific pathogens from cultured stocks at broodstock facilities, hatcheries and farms, or from entire regions or countries to prevent the occurrence of economically important diseases.

2

2

Pathogen + Host + Environment + Trigger = disease



3

Biosecurity :

- Identifying risk(s)
  - hazard identification
  - risk assessment
  - risk management
  - risk communication

- Putting in place methods to reduce risk(s)
  - Facility design
  - Standard operating/management procedures (SOP)
  - Use of "disease-free" (SPF) or resistant stocks
  - Routine surveillance
  - Contingency plans ready when necessary.

4

Hazard identification: Diagnosis methods

- **Histopathology**
  - H&E special stains
  - In situ hibridisation immunohistochemistry
- **Microbiology**
  - Bacteria identification, antibiogram, MIC
- **Molecular biology**
  - PCR RT-PCR
  - sequencing gene expression
- **Pond history**
  - Mortality feed consumption
  - Physico-chemical parameters
- **Fresh mount**
  - Organ affected
- **bioassays**
  - Challenge test. Determine the infectious nature of the risk

5

Hazard identification: Routine surveillance

6

Routine surveillance: targeted surveillance

7

Farms: Degree of Risk

Risk	Source
High 1000X	Untested live shrimp from + zones.
	Tested live shrimp from + zones.
	Frozen shrimp for reprocessing.
Moderate 100X	Use of untreated/filtered source water.
	Fresh or frozen feed stuffs for larval rearing or broodstock from + zones.
Low 10X	Fresh or frozen feeds from neg. zones.
	SPF shrimp.
Very Low 1X	Cooked/dried crustacean products.
	Processed feeds with shrimp meal.

8

8

➢Risk assessment:

Disease	Category
1. White spot disease	C1
2. Taura syndrome	C1
3. Yellow head disease – Yellow head virus	C1
4. Infectious hypodermal and hematopoietic necrosis	C3
5. Infectious myonecrosis	C1, C2
6. Necrotising hepatopancreatitis	C2
7. Monodon Baculovirus (MBV)	C2, C3
8. Muscle Microsporidia	C2, C3
9. Gill-associated virus	C1, C2
10. Monodon slow growth syndrome	
11 Baculovirus penaei (BP)	C3
11. Acute hepatopancreatic necrosis disease AHPND /(EMS)	C1
12. Hepatopancreatic microsporidia	C1

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Risk assessment
What should be on the disease & pathogen exclusion list?

- ➢ Disease must be caused by a pathogen whose biology is well known.
- ➢ Agent must have a limited geographic or environmental distribution.
- ➢ Agent might cause significant production losses.
- ➢ Cost-effective exclusion methods are available.
- ➢ Accurate methods available for pathogen detection.

10

10

➢Risk Management.

Broodstock stocks

11

➢Risk Management.

12

➢Risk Communication/reporting.

	WSSV			TSV			YHV			IMNV			NHP			BP			IHHNV			MBV			PvNV		
Laboratory	N°	N° Pos (+)	% Pos (+)	N°	N° Pos (+)	% Pos (+)	N°	N° Pos (+)	% Pos (+)	N°	N° Pos (+)	% Pos (+)	N°	N° Pos (+)	% Pos (+)	N°	N° Pos (+)	% Pos (+)	N°	N° Pos (+)	% Pos (+)	N°	N° Pos (+)	% Pos (+)	N°	N° Pos (+)	% Pos (+)
Lab 1																											
Lab 2																											
Lab n																											
Total																											

2. NUMBER OF SAMPLES ANALYZED BY SHRIMP CULTURE DIVISIONS

	WSSV			TSV			YHV			IMNV			NHP			BP			IHHNV			MBV			PvNV		
Sector	N°	N° Pos (+)	% Pos (+)	N°	N° Pos (+)	% Pos (+)	N°	N° Pos (+)	% Pos (+)	N°	N° Pos (+)	% Pos (+)	N°	N° Pos (+)	% Pos (+)	N°	N° Pos (+)	% Pos (+)	N°	N° Pos (+)	% Pos (+)	N°	N° Pos (+)	% Pos (+)	N°	N° Pos (+)	% Pos (+)
Hatchery																											
Farm																											
Maturation																											
fresh food																											
Wild samples																											
Total																											

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Biosecurity :

- ➢ Identifying risk(s)
  - ➢ hazard identification
  - ➢ risk assessment
  - ➢ risk management
  - ➢ risk communication
- ➢ Putting in place methods to reduce risk(s)
  - ➢ Exclusion strategy
  - ➢ Facility design
  - ➢ Standard operating/management procedures
  - ➢ Use of “disease-free” (SPF) or resistant stocks
  - ➢ Routine surveillance
  - ➢ Contingency plans ready when necessary

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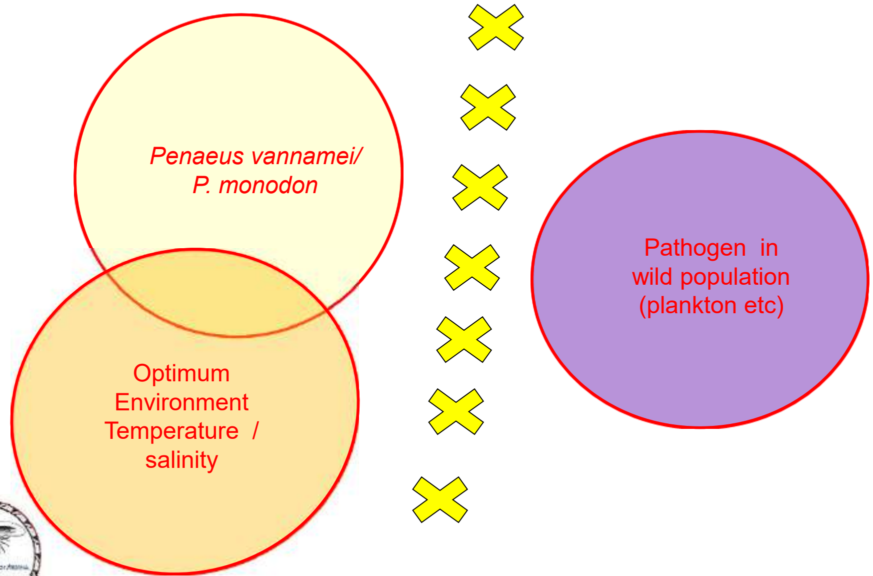
Pathogen exclusion= no disease



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➢Putting in place methods to reduce risk(s)

A. Livestock
B. Water
C. Bottom of the pond
D. Fresh feed
E. Materials/equipment
F. People
G. Vehicle
H. Processing plants

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A. livestock

- Introduction of animals (broodstock, PL, nauplii) increase the risk of diseases introduction
- allow no imports of live/frozen shrimp.
- if importation is allowed, require SPF certification.
- use of quarantine & re-test confirmation of SPF status.

17

17

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SPF / SPR - Definitions

- Specific Pathogen Free (SPF) - a domesticated line or stock of shrimp subject to a routine surveillance program carried out under the supervision of an approved diagnostic lab for ~$\geq$ 2 years & demonstrated to be SPF for the OIE listed pathogens (OIE 2018).
- Specific Pathogen Resistant (SPR) - a domesticated shrimp line that resists one or more of the diseases or agents from the OIE or USMSFC lists.
  - TSV-Resistant line
  - IHHNV resistant line
  - WSSV Tolerant/resistant
  - AHPND Tolerant/resistant

18

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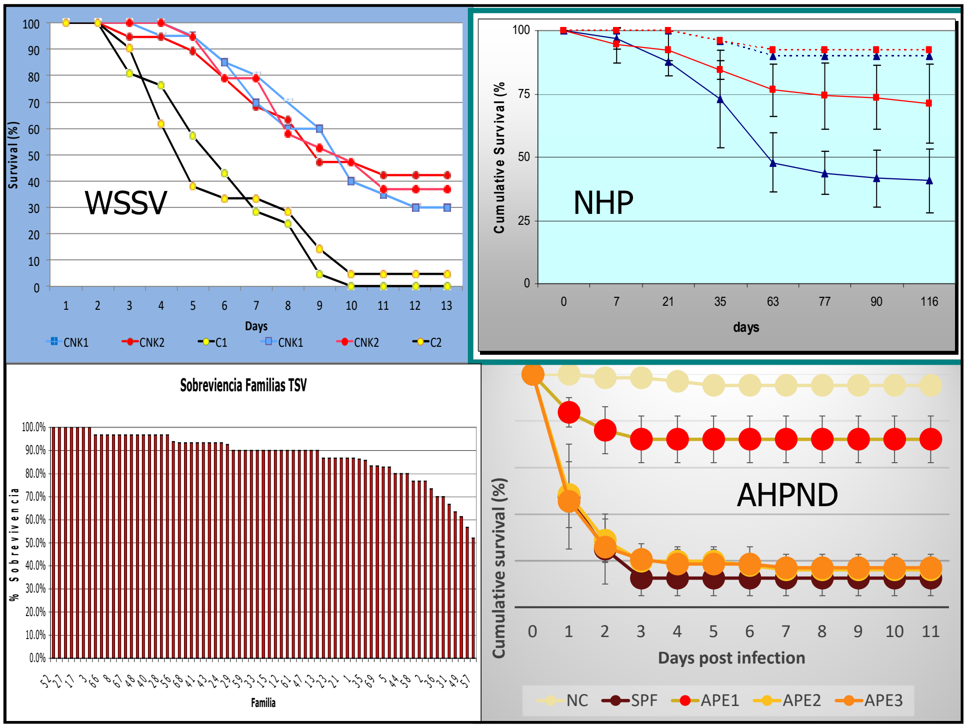

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Strategies for the Development of SPF/SPR Stocks

- Develop new SPF/SPR stock from a founder population in quarantine.
  - founder population from wild stocks.
  - from culture (survivors, high performers, etc.)

20

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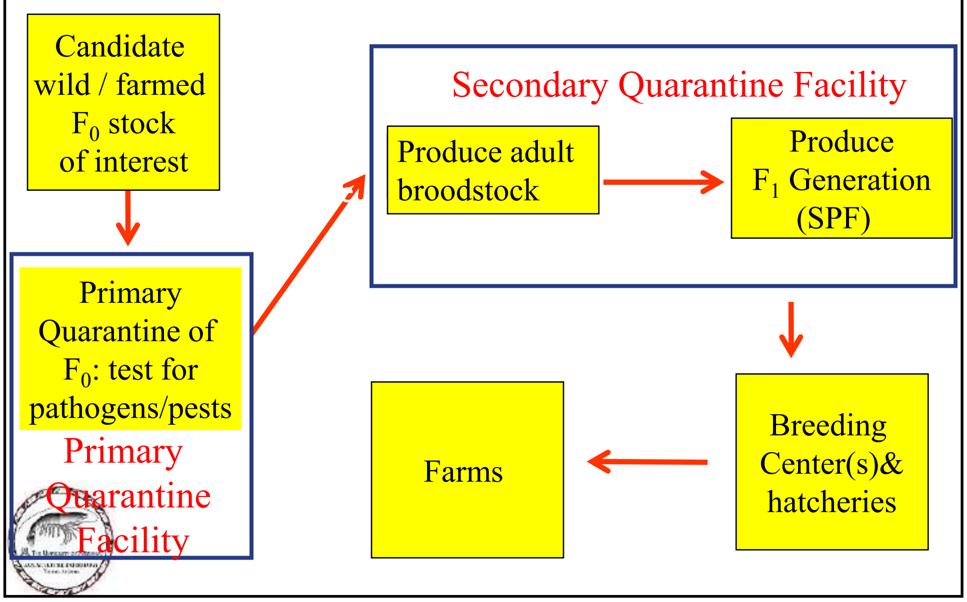
Steps to SPF Stock Development



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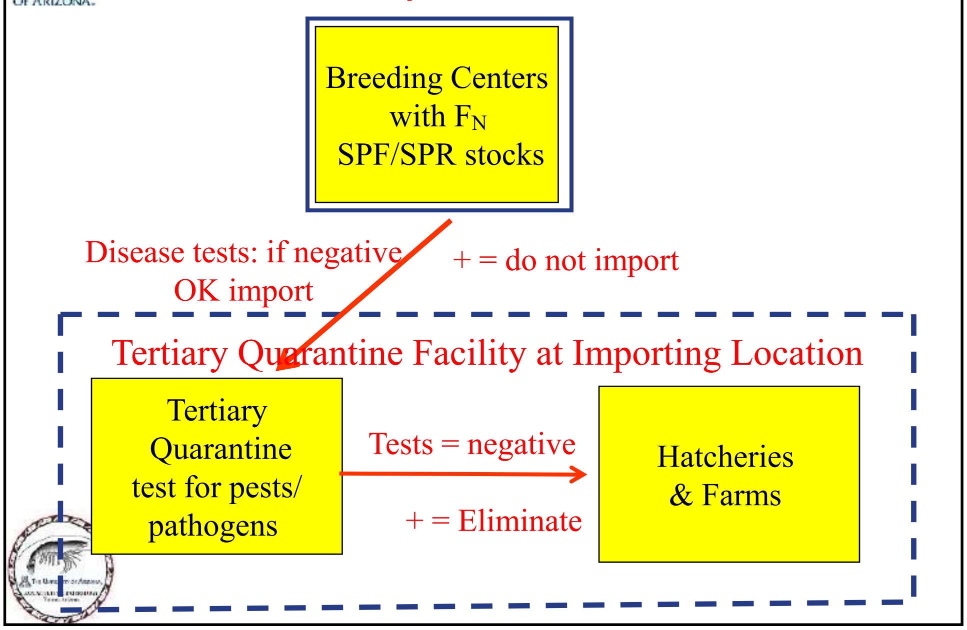
Tertiary Quarantine



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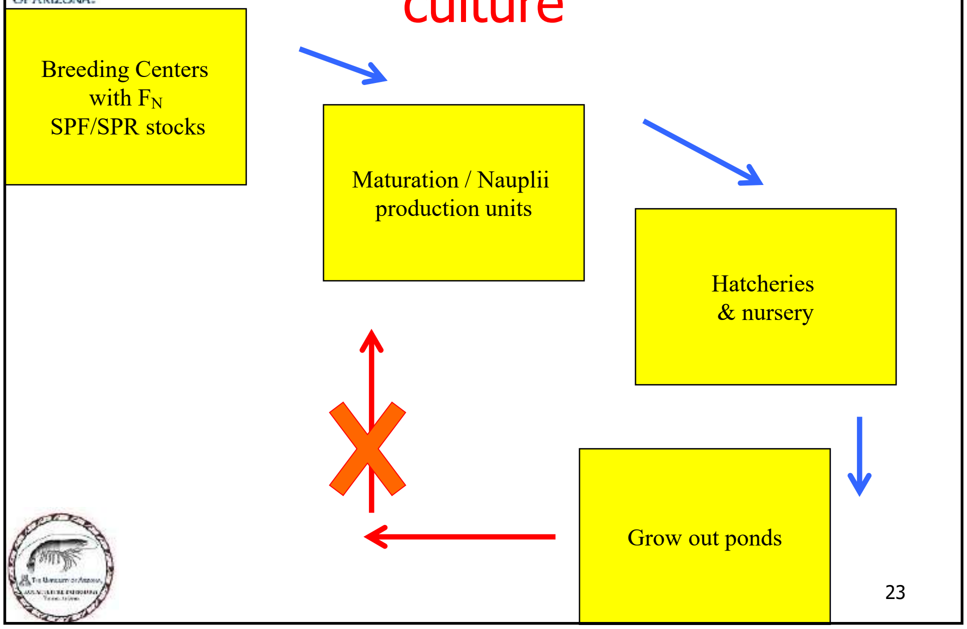
SE Asia shrimp culture



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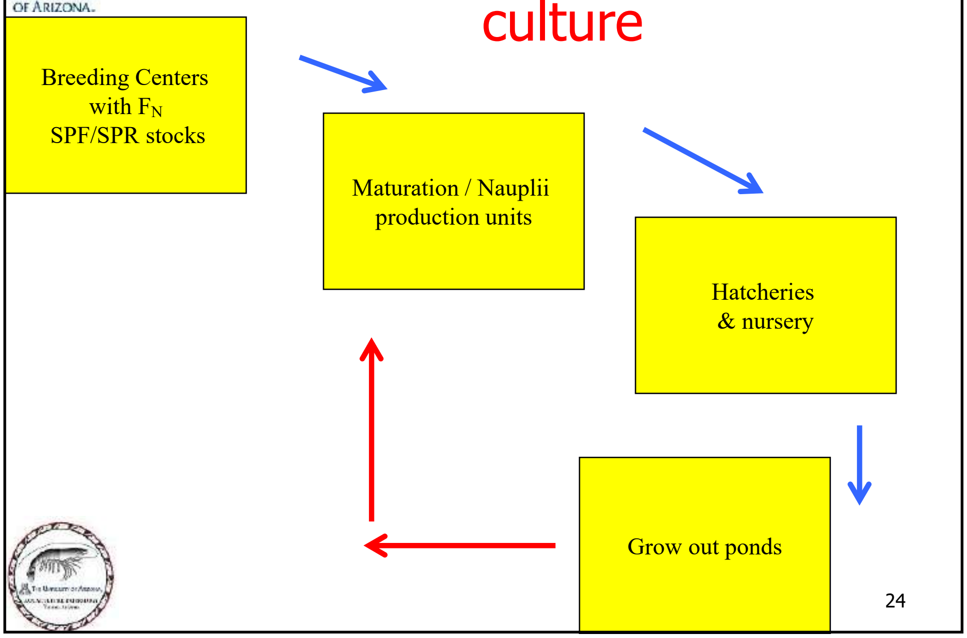
Latin-American shrimp culture



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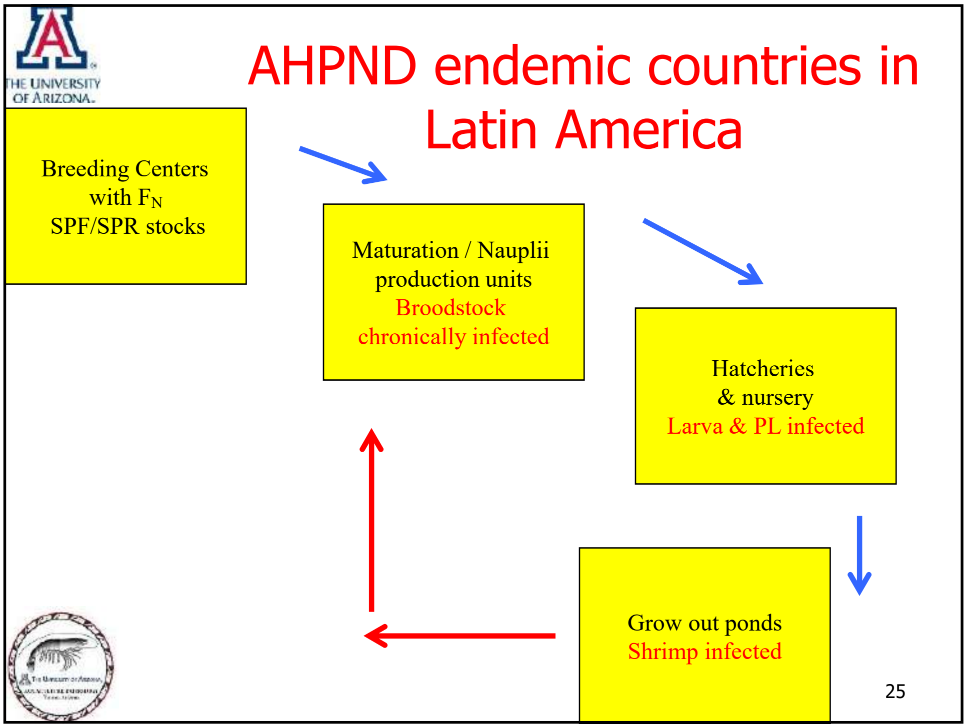
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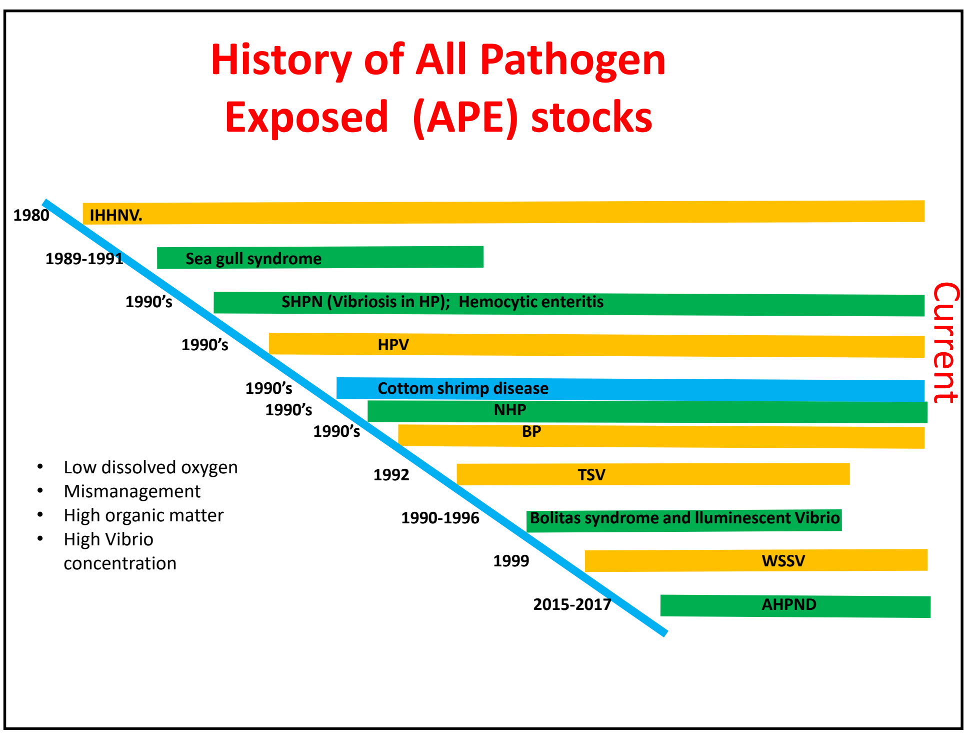
AHPND endemic countries in Latin America
Breeding Centers with F_N SPF/SPR stocks
Maturation / Nauplii production units
Broodstock chronically infected
Hatcheries & nursery
Larva & PL infected
Grow out ponds
Shrimp infected
25

25


History of All Pathogen Exposed (APE) stocks
1980
IHHNV.
1989-1991
Sea gull syndrome
1990's
SHPN (Vibriosis in HP); Hemocytic enteritis
1990's
HPV
1990's
Cottom shrimp disease
1990's
NHP
1990's
BP
1992
TSV
1990-1996
Bolitas syndrome and Iluminescent Vibrio
1999
WSSV
2015-2017
AHPND
Current
• Low dissolved oxygen
• Mismanagement
• High organic matter
• High Vibrio concentration

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SPF vs. APE

- SPF areas where major shrimp pathogens are not present
- Areas where the exclusion strategy can be achieved easily

- Areas where major shrimp pathogens are present
- Areas where the exclusion strategy can NOT be achieved

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➢Putting in place methods to reduce risk(s)

A Livestock…

- ➢ All in all out in the Batch culture
- ➢ Disinfection.
- ➢ Disinfection of eggs,
- ➢ Disinfection of nauplii
- ➢ Disinfection of artemia nauplii.

Chemical / disinfection method	Active ingredients	Dosage of active ingredient	Contact time	Scope	Application	Elimination of residues
Formalin	Formaldehyde	100 ppm	30 sec	eggs, nauplii, PLs	Baths	Natural breakdown
Povidine	Iodine	25 ppm	30 sec	eggs disinfection	Baths	Natural breakdown
		50 ppm	30 sec	nauplii disinfection	Baths	Natural breakdown

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➢Putting in place methods to reduce risk(s)

A. Livestock
B. Water
C. Environment
D. Fresh feed
E. Materials/equipment
F. People
G. Vehicle
H. Processing plants

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B. Water

- ➢ Some pathogens can be present in the water as a particle
- ➢ Several shrimp pathogens can have carriers:
  - ➢ arthropod:
  - ➢ Fish
  - ➢ Wild shrimp
  - ➢ others

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BIOFLOC & RAS

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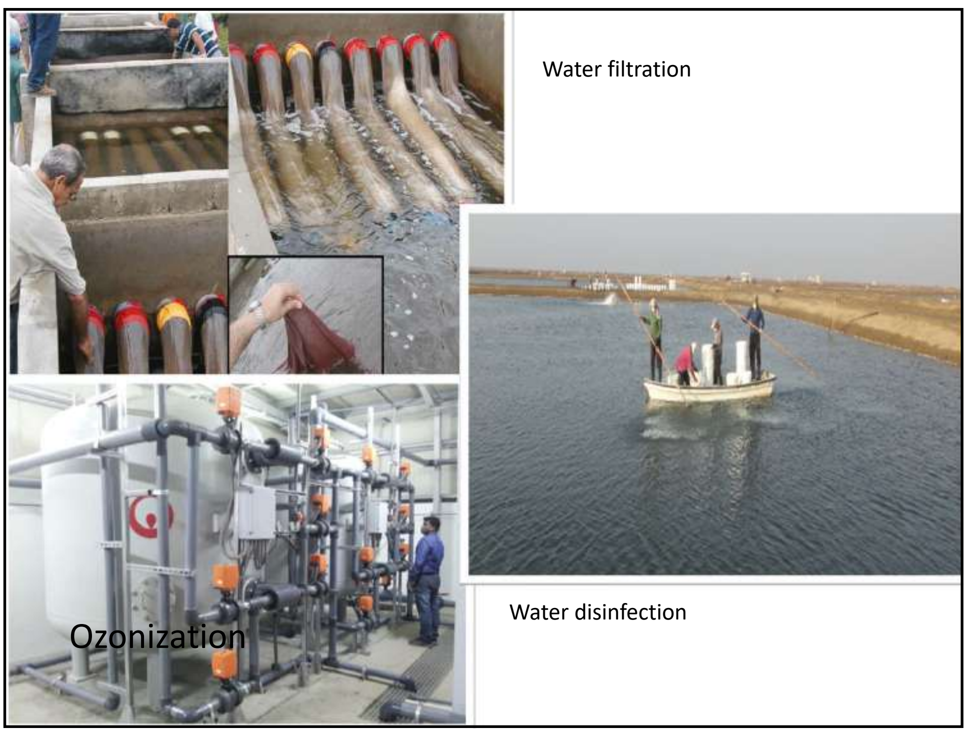
Water filtration
Water disinfection
Ozonization

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Seawater intake, reservoir
& chlorine gas - Malaysia

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Filter Bags Farm Supply
Canal/Reservoir (KSA)
Biosecurity strategy
Main feeder canal (1000 um)
Sub feeder canal (500 um)
Well point
Grow-out ponds (250 um)

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Filter Bags Farm Supply Canal/Reservoir (Texas)

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➢Putting in place methods to reduce risk(s)

A. Livestock
B. Water
C. Environment
D. Fresh feed
E. Materials/equipment
F. People
G. Vehicle
H. Processing plants

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Lined pond cleaning
& drying - Malaysia
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Crab Fence
(Ecuador)
40

40


Covered & lined ponds - Peru
41

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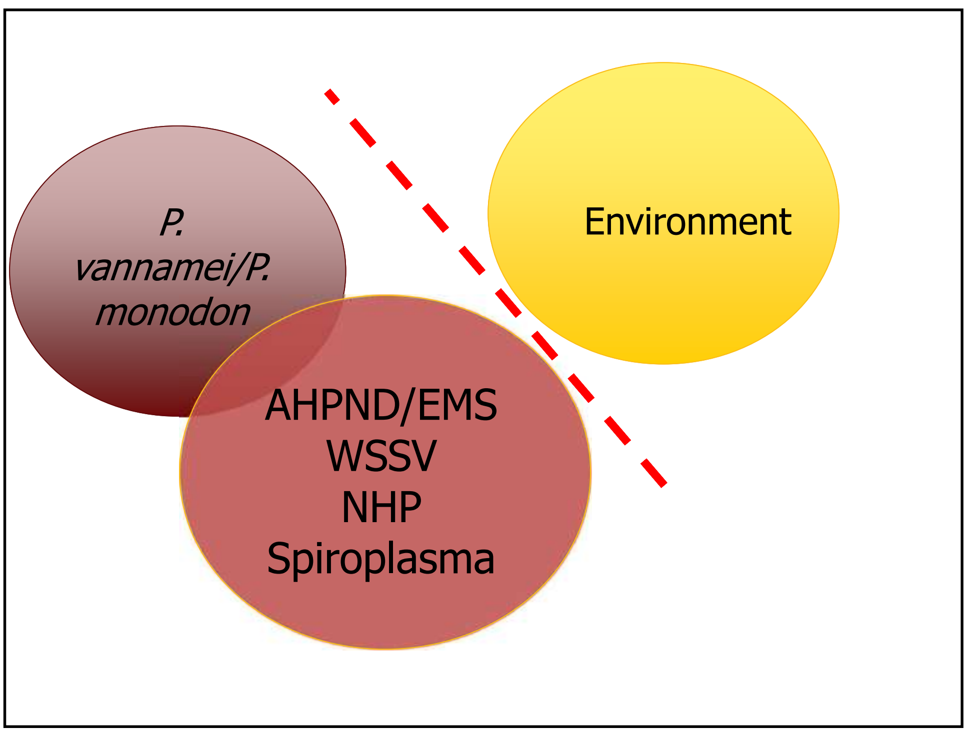
P. vannamei/P. monodon
Environment
AHPND/EMS
WSSV
NHP
Spiroplasma

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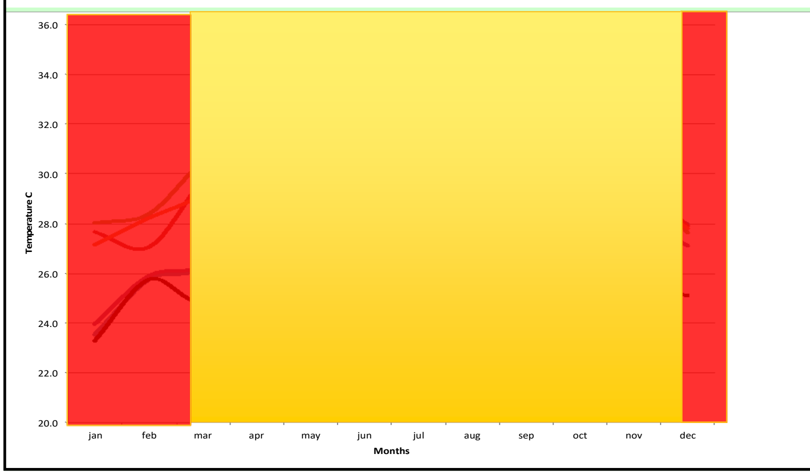
WSSV outbreak in low temperatures:
Seasonal stocking / harvesting



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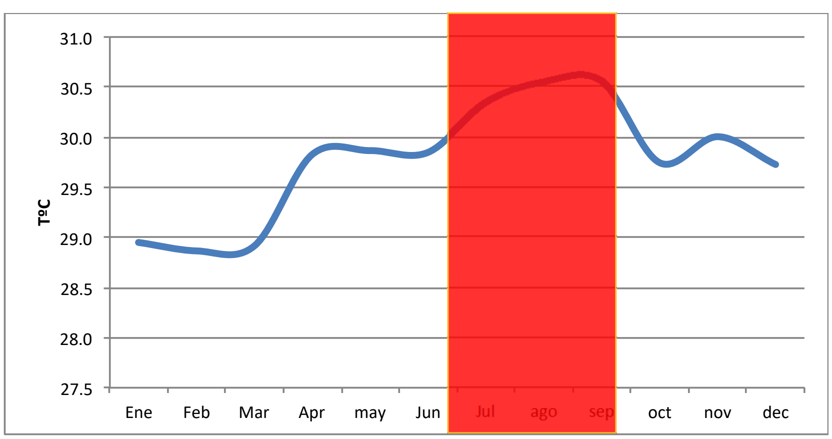
C. Environment

NHP is favored by high temperatures and high salinities



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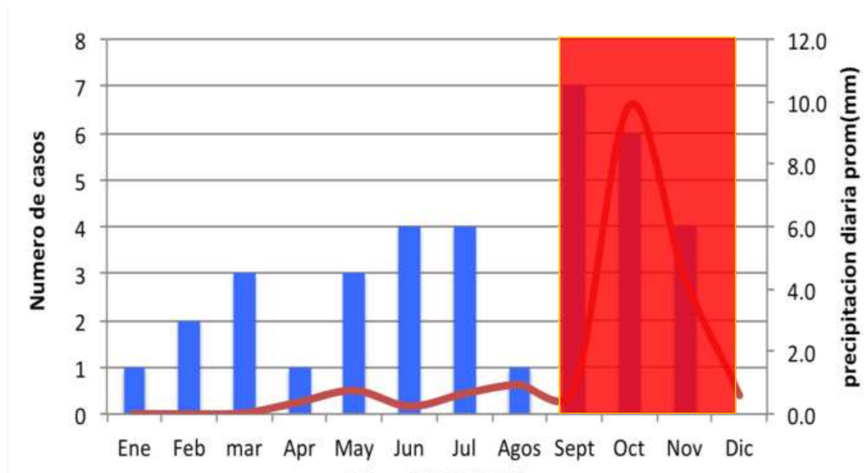
C. Environment

Spiroplasma penaei is favored by the low water salinity (<10 ppt)



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➢Putting in place methods to reduce risk(s)

A. Livestock
B. Water
C. Environment
D. Feed: Pellet feed and fresh feed
E. Materials/equipment
F. People
G. Vehicle
H. Processing plants

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[redacted]
LiquaLife
~~C042.100.020347~~
~~7685~~
29116NF

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LOS CABOS
Shrimp
Fresh frozen
Superba Krill
TOPSY
BAITS
LIVE
SEA ANGLING BAIT
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Fresh feed use is very common in maturation labs

- Origin of fresh feed must be from areas where non-OIE diseases have been reported
- Areas where shrimp is not under culture: E.J Maine Polychaeta
- Still SPF status must be proven

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➢Putting in place methods to reduce risk(s)

A. Livestock
B. Water
C. Environment
D. Fresh feed
E. Processing plants
F. Materials/equipment
G. People
H. Vehicle

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E. Processing plants

- Several shrimp farmers from different geographical areas might take the harvested product at the same time to the same processing plant
- Bin can be cross-contaminated
- Some times processing product from different countries
- Wastewater and solids must be properly treated before releasing to the environment. E.g WSSV Introduction in th Western hemisphere

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Putting in place methods to reduce risk(s)

A. Livestock
B. Water
C. Environment
D. Fresh feed
E. Processing plants
F. Materials/equipment
G. People
H. Vehicle

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Equipment /material

- No sharing equipment/material with neighboring farms
- Material must be disinfected when returned from processing plant before entering to the farm. The same processing plant might be receiving product from other shrimp farms. Cross-contamination
- Cast net /boat should be specific for each pond/zone
- Material/equipment must be restricted to each division: Broodstock, hatchery, grow-out ponds

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Visitors

- Restrict the number of visitors
- Conduct a risk assessment to visitors e.g visit to other shrimp facilities the same day. Introduction of shrimp
- A guided tour should start from most biosecurity location to the least biosecurity areas
- Maturation, hatchery, larviculture, farm
- Use of gumboots / clothing

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Movement of staff within zones
Shrimp hatchery

Algae → larvae → Postlarvae → Artemia

Cleanest → dirtiest

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Movement of staff between zones
Shrimp farm

New population/new pond → Old pond/old population

Unaffected pond → Affected pond

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Movement of staff between zones Shrimp farming

Maturation/NPU Hatchery → Nursery → Grow out → Processing plant/ Accomodation

Cleanest → dirtiest

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Biosecurity requirements in the different phases of shrimp farming

		Biosecurity levels						
		High level				Medium level		Low level
requirement	Area	Quarantine	Broodstock Breeding Center	Maturation/Nauplii production unit	Larval culture	Nursery	Grow-out ponds	Processing plant
Pathogen free status		M	M	M	M	M	N/A	N/A
Intensive surveillance		M	R*	R*	N/A*	N/A*	N/A*	N/A
Inlet water Disinfected		M	M	M	M	R	N/A	N/A
RAS		M	R	R	N/A	R	N/A	N/A
Indoors		M	M	M	M	R	N/A	N/A
Effluent treatment		M	R	R	R	N/A*	N/A*	M
Solid wastes treatment		M	M	M	M	M	M	M
Restricted access		M	M	M	M	R*	R*	R

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Fallowing for disease control

Fallowing - *is a procedure used to restore the 'pre-disease' environment of an aquaculture establishment.*

- starts immediately after depopulation.
- necessary cleaning, disinfection & dry-out have been completed.
- facility remains unstock for a defined period.

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How are pathogens eliminated once they are present?

Facility (i.e. hatchery, farm):

- depopulate affected tanks, ponds
- partial disinfection (lime, chlorine, drying)
- fallow entire farm
- re-stock with SPF shrimp stock
- partial disinfection (lime, chlorine, drying)
- fallow entire facility

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Thanks

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