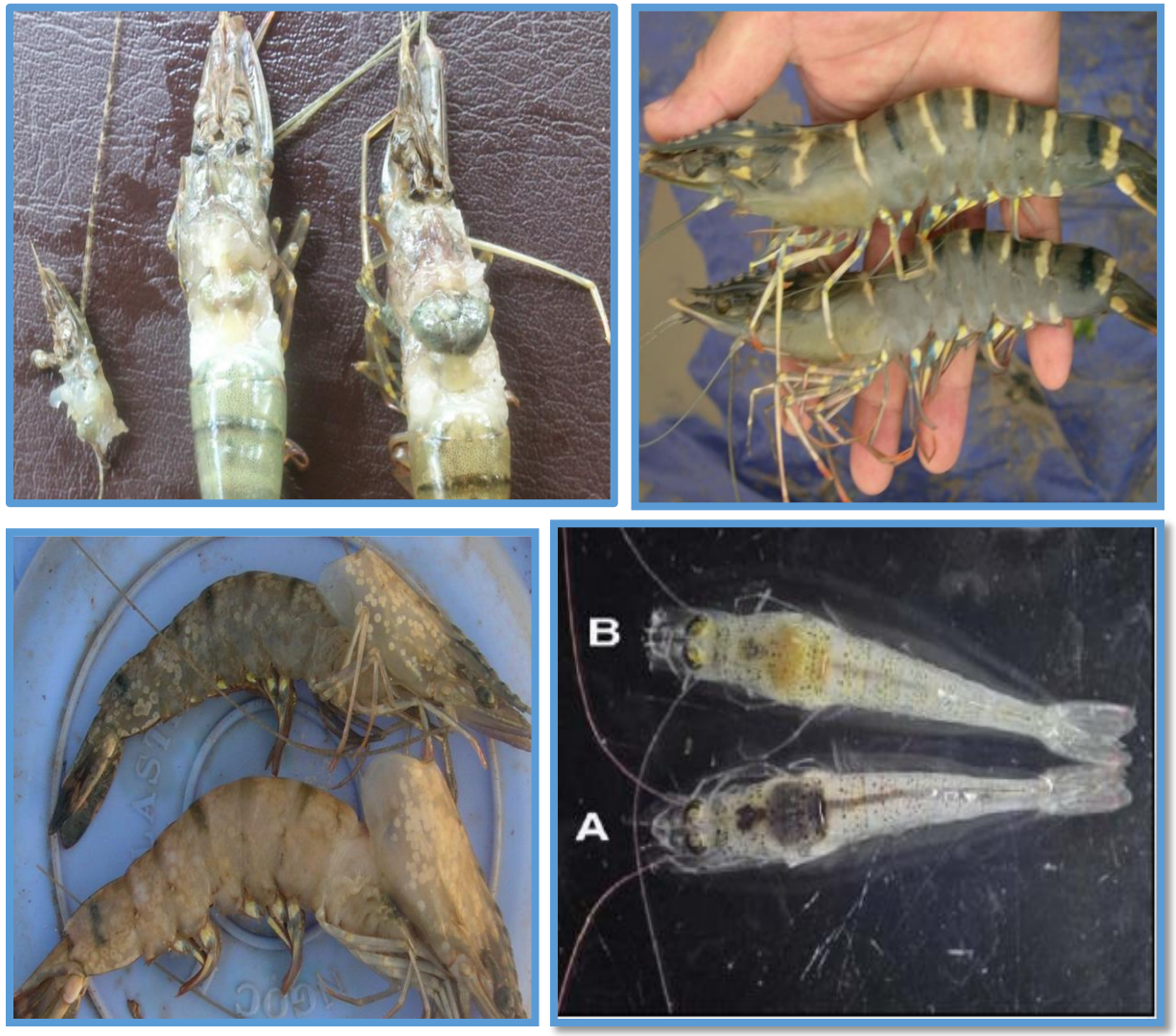

Emerging diseases of cultured shrimps in Southeast Asia

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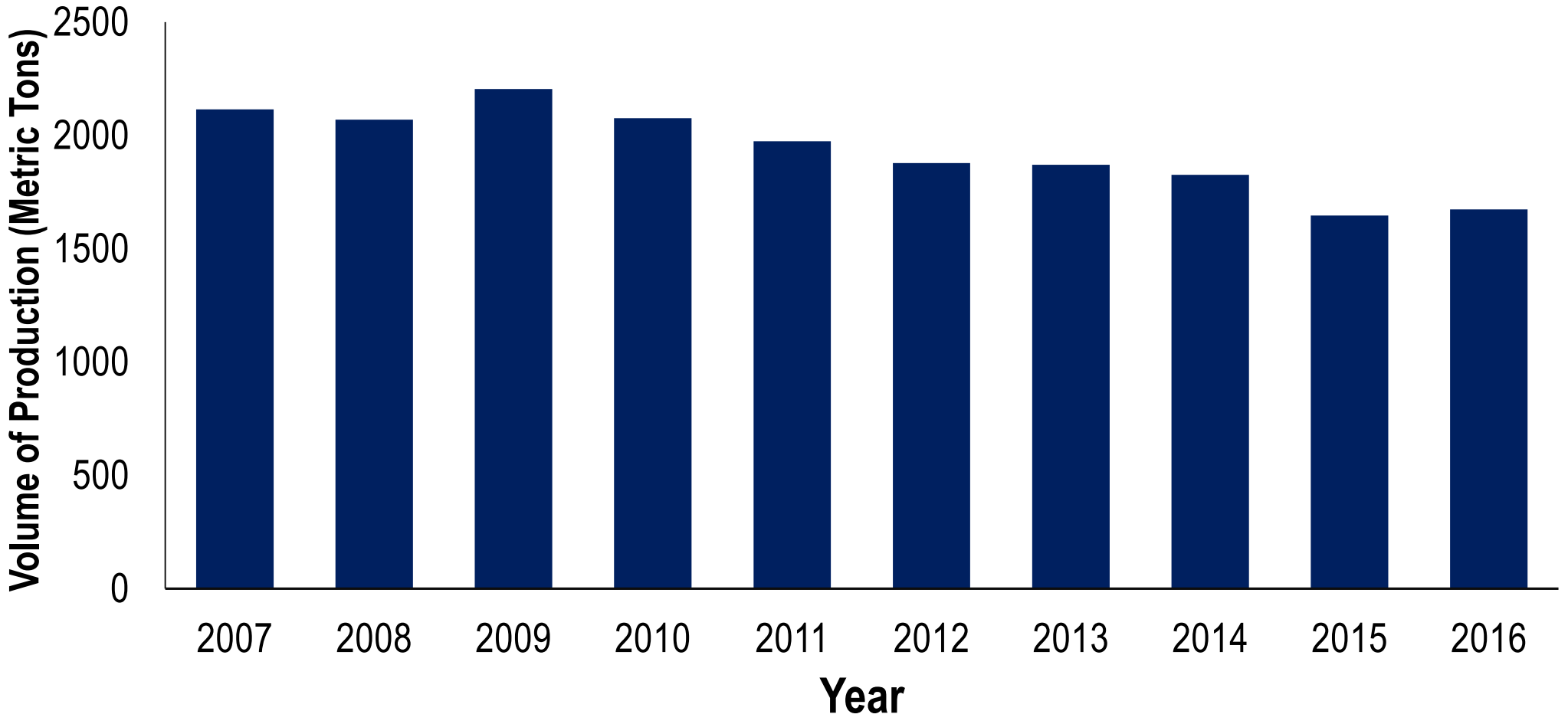
Topic Outline

- Introduction
- Emerging Diseases
  - Acute hepatopancreatic necrosis disease (AHPND)
  - Hepatopancreatic microsporidiosis (HPM)
- Strategies for Disease Prevention & Control
- Future Directions

Top threats for both *P. vannamei* and *P. monodon*[1]

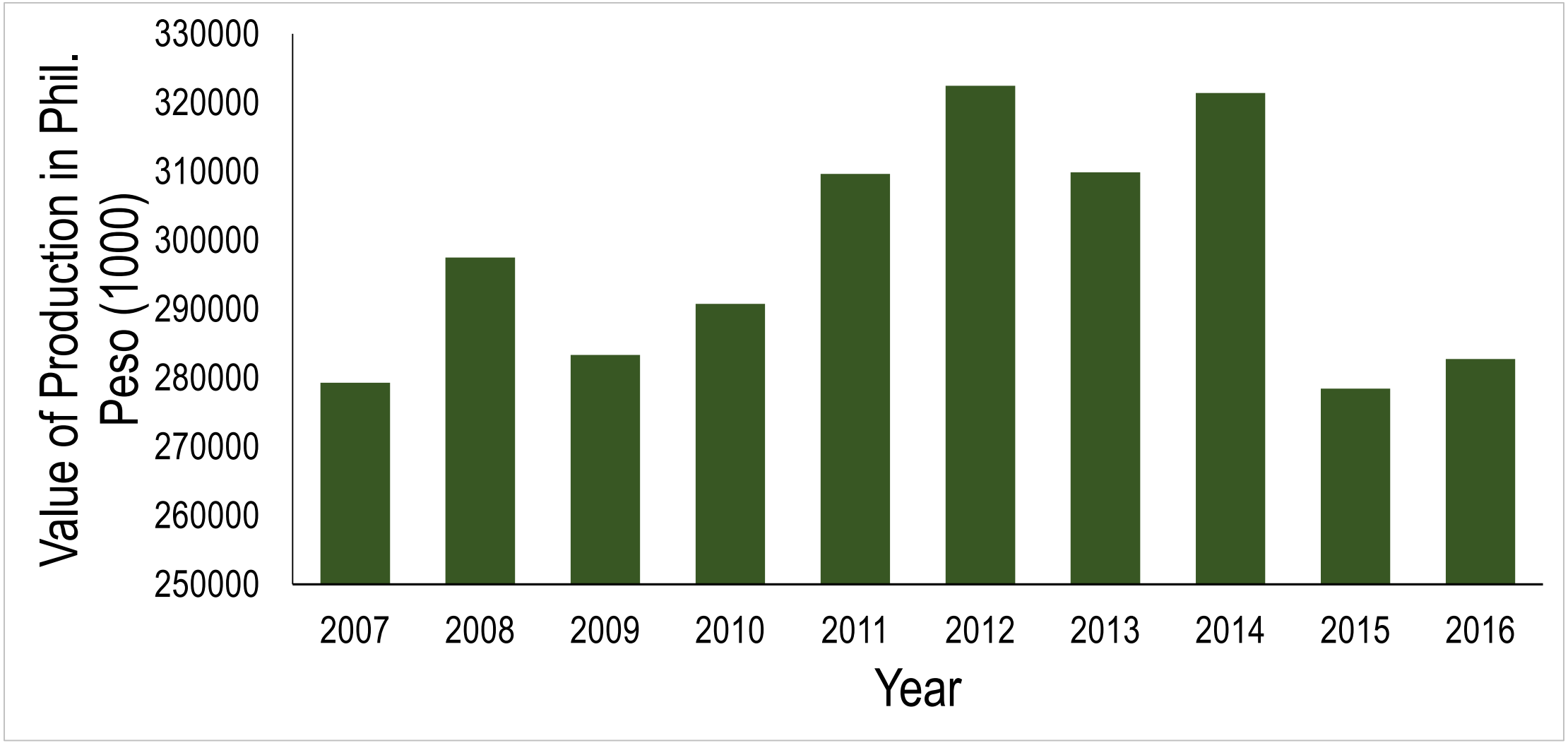
- Top viral threat for both species everywhere is still white spot disease (WSD)
- Next are YHV Types 1 & "8" with outbreaks only in Thailand & China, respectively
- Top bacterial threat is **Acute hepatopancreatic necrosis disease or AHPND caused by unique strain of *Vibrio parahaemolyticus* (VP_{AHPND})**
  – It is a component of early mortality syndrome (EMS)
- Next is **Hepatopancreatic microsporidiosis (HPM) caused by *Enterocytozoon hepatopenaei* (EHP)**
- WSSV and YHV will not be presented

[1] **Flegel, T (2016) 15th Meeting of the Asia regional Advisory Group on Aquatic Animal Health, 21-23 November 2016, Bangkok, Thailand**



Penaeus vannamei: Volume of Production in the Philippines

Source: Philippine Statistics Authority, retrieved 6 April 2017

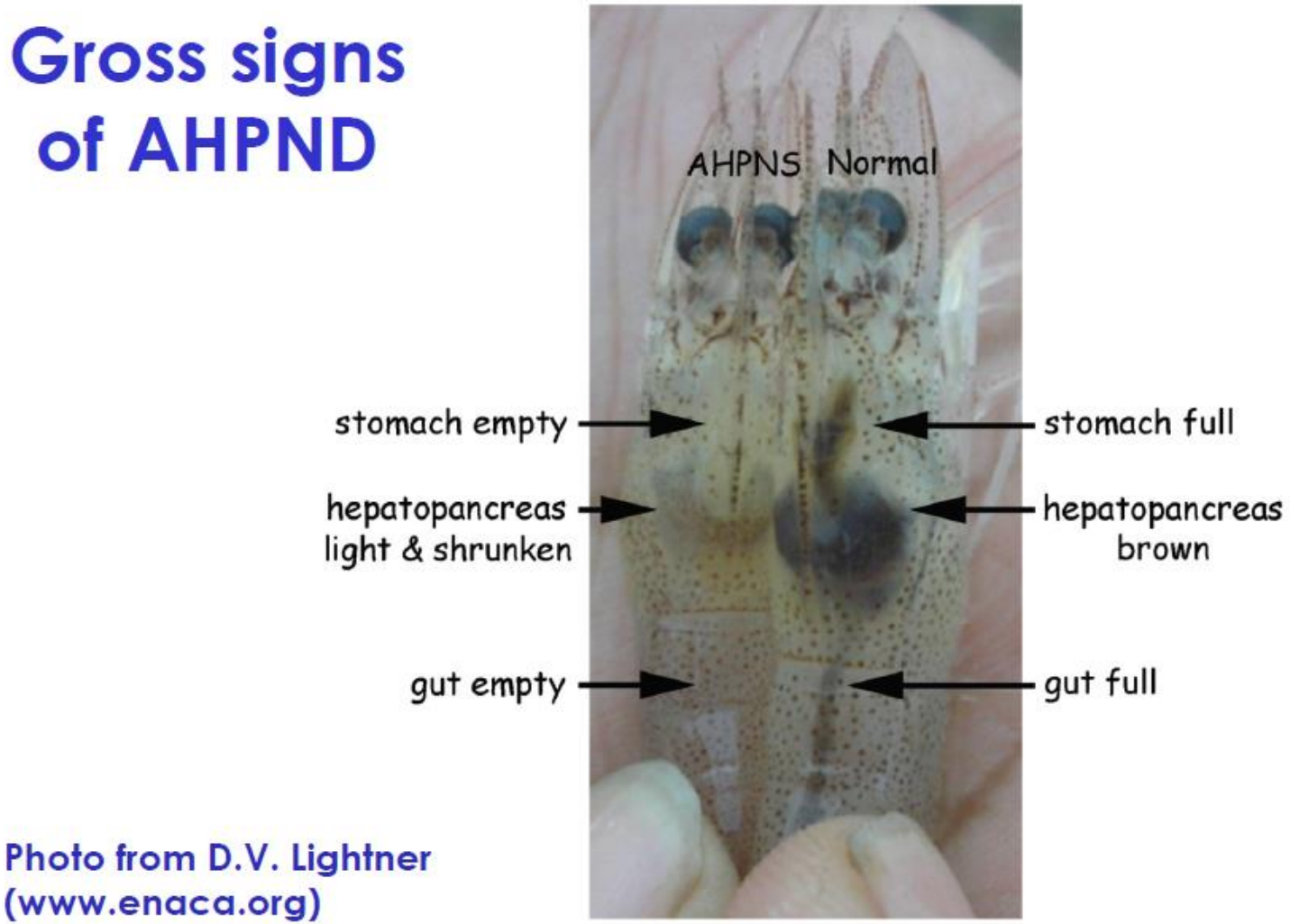


Penaeus vannamei: Value of Production in Philippine pesos

Source: Philippine Statistics Authority, retrieved 6 April 2017

Acute Hepatopancreatic Necrosis Disease (AHPND)

- Earlier known as early mortality syndrome (EMS) because mortality occurs in the early stages of culture (30-35 days after stocking shrimp post larvae in ponds)
- AHPND is caused by unique strain of *Vibrio parahaemolyticus* that possesses a unique plasmid which contains an operon coding for homologs of *Photorhabdus* insect-related (Pir) binary toxin, Pir A and Pir B (Tinwongger et al., 2014)



Host and Geographical Distribution

- mainly implicated in mass mortalities of the whiteleg shrimp (***P. vannamei***) but has also been reported in tiger prawn (*P. m*onodon) and fleshy prawn (*P. chinensis*)
- **AHPND outbreaks**: China in 2009; Viet Nam (2010); Malaysia (2011); Thailand (2012); Mexico (2013), and most recently in the Philippines (2015)

Now officially listed by the OIE

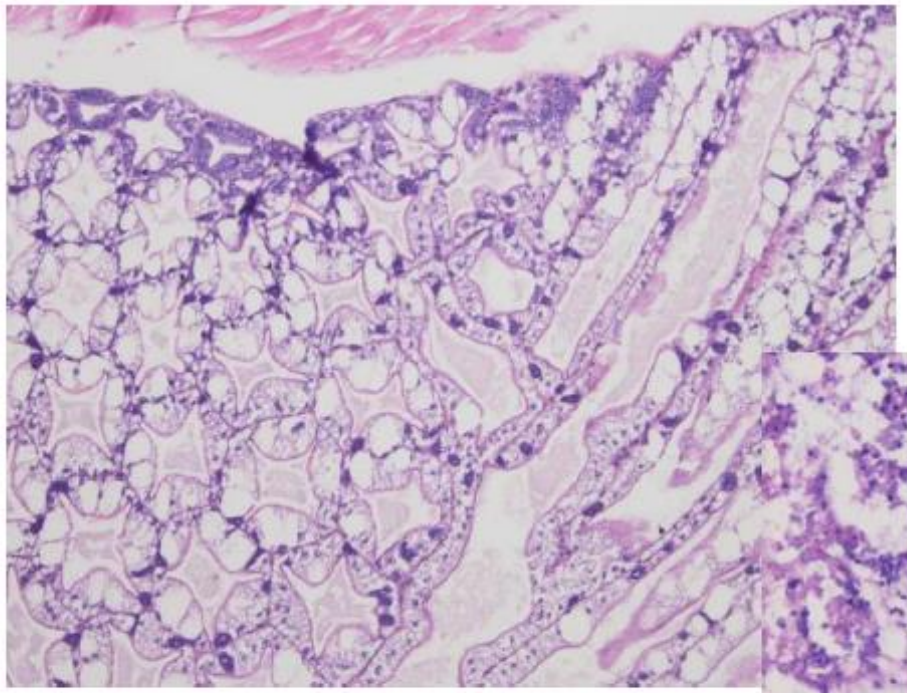
- First appeared on the NACA list in 2012
- A disease card is available at NACA website
- A chapter for the OIE Manual of Diagnostic Tests for Aquatic Animals is available on line

Tests and Diagnostic Methods

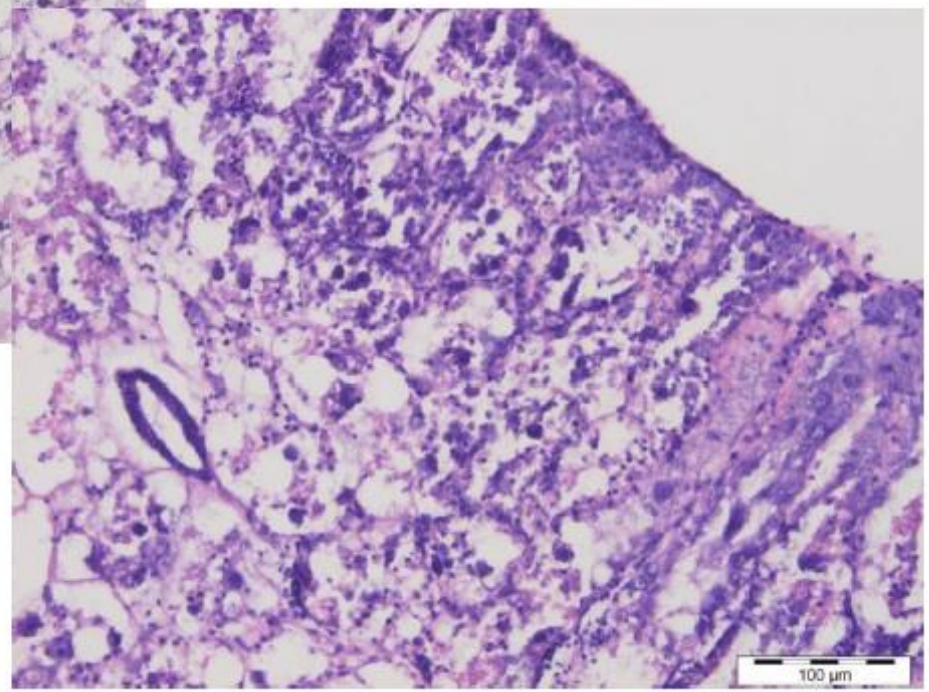
- **Histopathology** (**sloughing of the hepatopancreatic cells**)
- PCR detection methods include AP1, AP2, AP3, and AP4
- Methods AP3 and AP4 target the AHPND toxin genes pirA and pirB (Sirikharin et al. 2015. PLoS ONE. 10, e0126987; Dangtip et al. 2015. 2: 158-162)
- AP3: exhibits excellent sensitivity and specificity; require enrichment
- AP4 nested PCR: suitable for the archived DNA extracts and for tissues or environmental samples preserved in lysis buffer or alcohol
- Similar methods in other publications (Tinwongger et al. 2014. Fish Pathol. 49: 159-164; Lee et al. 2015. PNAS 112: 10798-10803; Han et al. 2015. Dis Aquat Org. 113: 33-40)
- Loop-mediated isothermal amplification (LAMP) method

Medial sloughing of HP cells

The key diagnostic feature needed for confirmation



Normal
hepatopancreas



AHPNS
hepatopancreas
(pathognomic lesion)

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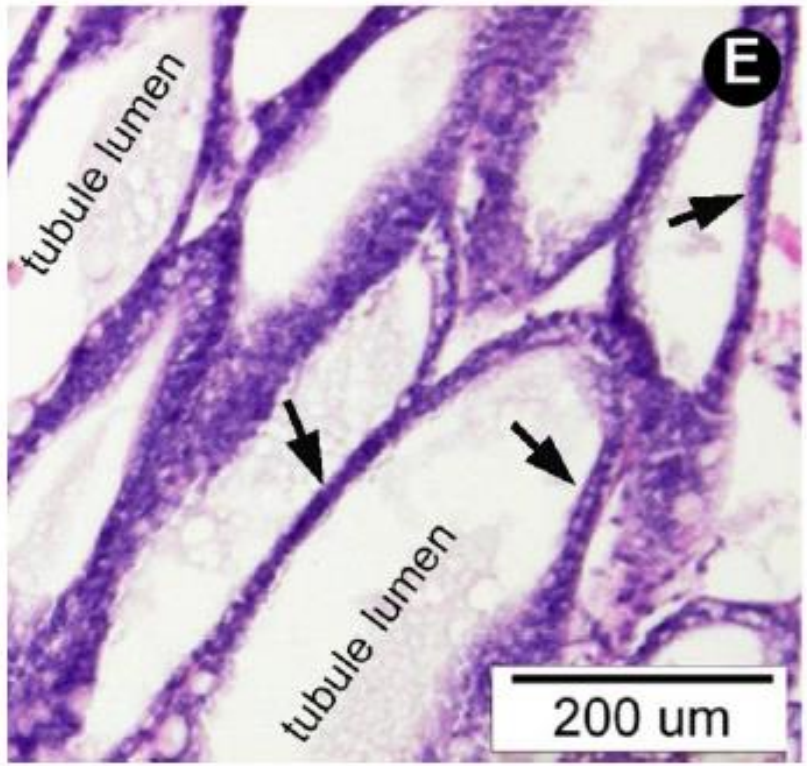
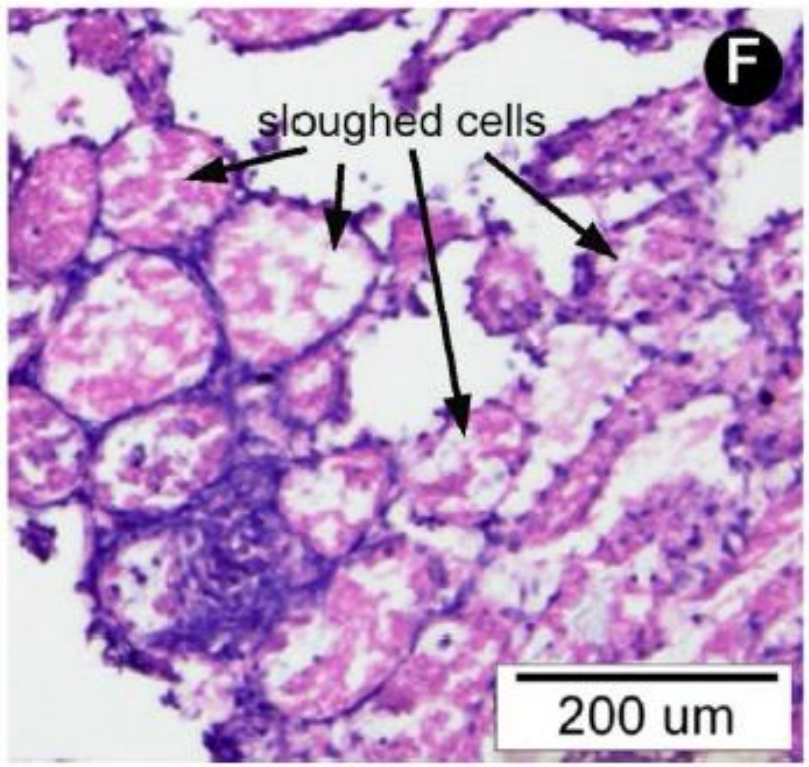
Collapsed epithelia with dilute Pir toxins

- Both Pir toxins are needed to cause AHPND
- Diluted, expressed Pir toxins also cause collapsed HP tubule epithelia (Sirikharin et al. 2015. PLoS ONE. 10, e0126987)



5 µg each
collapsed epithelia



10 µg each
AHPND sloughing

AHPND potentiating factors (Flegel, 2016)

- The expressed proteins caused AHPND at 10 µg per g shrimp each (total 20 µg)
- But, $(NH_4)_2SO_4$ precipitate from culture broth required only 1 µg/g shrimp (20x less)
- Thus, other broth components must potentiate ToxA and ToxB virulence
- Two additional proteins in the $(NH_4)_2SO_4$ precipitate are being examined
- They occur only in VP_{AHPND} isolates and not in a non-AHPND VP isolate
- It is important to identify such potentiators and determine if they play any role in AHPND

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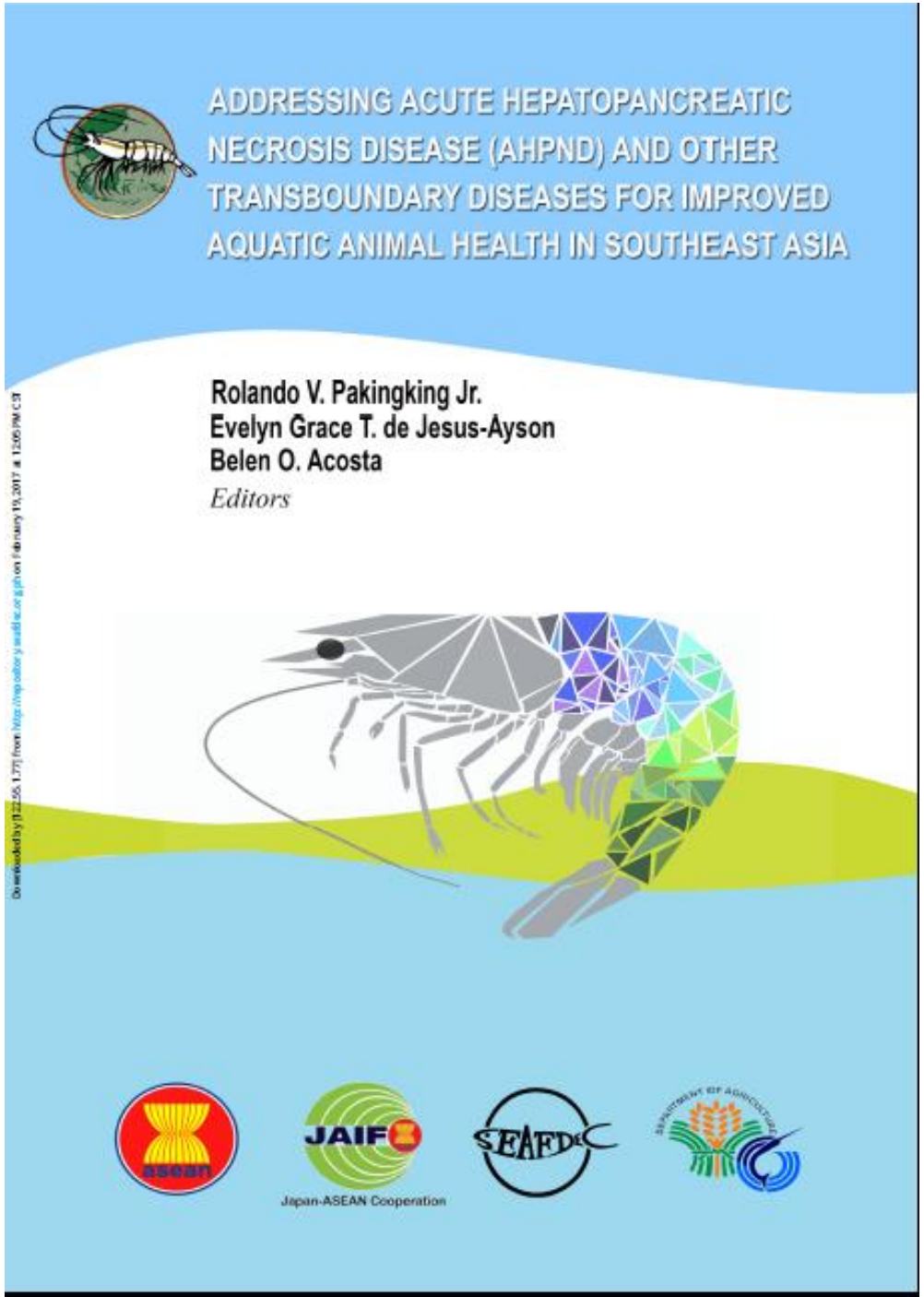

- ASEAN Regional Technical Consultation (RTC) on EMS/AHPND and Other Transboundary Diseases for Improved Aquatic Animal Health Management in Southeast Asia, 22-24 February 2016, Makati, Philippines

Strategies for disease prevention, control, and biosecurity*

- Compliance with good aquaculture practices to maintain optimal environmental conditions during the culture period
- Establish effective prevention system for EMS/AHPND and other diseases based on recent R&D results
  - ✓ Marker-assisted selective breeding
  - ✓ Development of vaccine against AHPND
  - ✓ IgY as feed additive (based on experiments, high concentration of IgY in egg yolk can reduce the mortality of AHPND-infected shrimp)
  - ✓ Nano-bubble technology: ozone nano-bubble can prevent AHPND
  - ✓ Pond bottom management: use of central drain system
  - ✓ Phage therapy

* **Pakingking, R. V., Jr.,** de Jesus-Ayson, E. G. T., & Acosta, B. O. (Eds.). (2016). *Addressing acute hepatopancreatic necrosis disease (AHPND) and other transboundary diseases for improved aquatic animal health in Southeast Asia: Proceedings of the ASEAN Regional Technical Consultation on EMS/AHPND and Other Transboundary Diseases for Improved Aquatic Animal Health in Southeast Asia, 22-24 February 2016, Makati City, Philippines*. Tigbauan, Iloilo, Philippines: Aquaculture Dept., Southeast Asian Fisheries Development Center. 109 p.

- ✓ Use of AHPND-free live feeds for broodstock (specifically polychaetes)
- ✓ Use of greenwater technology
- ✓ Use of indigenous probiotics

- Develop and implement the Guidelines on Health Management and Good Practices to Prevent EMS/AHPND and other trans-boundary diseases
- Strictly implement the reporting system to relevant authorities and/or Competent Authority at country, regional and international levels (Early warning system, Monitoring system, Information for the regular report, annual report)
- Emergency preparedness and contingency plan (should be the responsibility of Competent Authority)

* **Pakingking, R. V., Jr.,** de Jesus-Ayson, E. G. T., & Acosta, B. O. (Eds.). (2016). *Addressing acute hepatopancreatic necrosis disease (AHPND) and other transboundary diseases for improved aquatic animal health in Southeast Asia: Proceedings of the ASEAN Regional Technical Consultation on EMS/AHPND and Other Transboundary Diseases for Improved Aquatic Animal Health in Southeast Asia, 22-24 February 2016, Makati City, Philippines*. Tigbauan, Iloilo, Philippines: Aquaculture Dept., Southeast Asian Fisheries Development Center. 109 p.

Hepatopancreatic microsporidiosis (HPM) caused by *Enterocytozoon hepatopenaei* (EHP)

Hepatopancreatic Microsporidiosis

- First seen in 2001 in the HP of *P. monodon* (Chayaburakul et al. 2004. Dis Aquat Org 60:89-96)
- Considered to be of no impact at the time
- Causative agent: *Enterocytozoon hepatopenaei* (EHP) (Tourtip et al.2009. J. Invertebr. Pathol. 102, 21-29)
- Now reported from China, Vietnam, Thailand,India, Indonesia, **Philippines** (probably Malaysia & Australia) (Flegel, 2016)
- Probably endemic in Australasia but not yet reported from some countries (Flegel, 2016)

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Asia-Pacific QAAD Reporting

❖ Newly listed diseases

Hepatopancreatic microsporidiosis caused by *Enterocytozoon hepatopenaei* (HPM-EHP)

Country	1Q2016	2Q2016
India	+	+
Philippines	0000	+
Thailand	+?( )	+?( )

+?( ) Confirmed infection/infestation limited to one or more zones of the country, but no clinical disease

- EHP may severely retard shrimp growth
- Degree of retardation is proportional to severity of infection (Liu et al. 2016. Prog Fish Sci. 37, 119-126)
- Infection up to 10^3 copies/µg total DNA by PCR seems to have no negative effect (Flegel, 2016)
- Copy numbers above 10^7 per µg DNA may give slow cumulative mortality (Flegel, 2016)
- HPM may predispose shrimp to bacterial pathogens
- EHP can be transmitted by feeding infected HP tissue to naïve shrimp
- It can also be transmitted by co-habitation of naïve shrimp separated from infected shrimp

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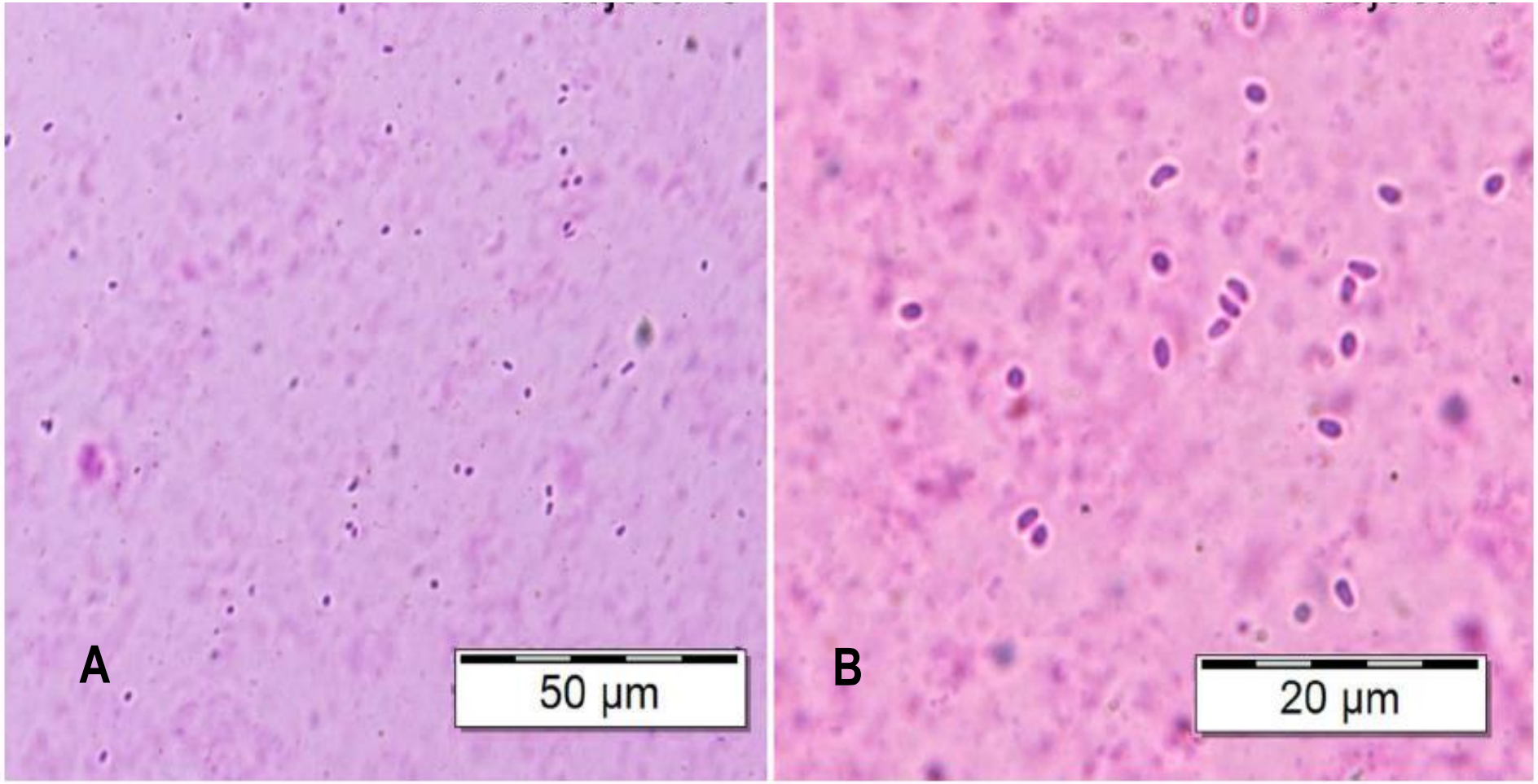
Diagnosis of HPM

- No clear gross signs for diagnosis
- It may be suspected as a cause in shrimp showing severe growth retardation
- To confirm diagnosis, histological analysis or molecular methods are needed
- Histological diagnosis is dependent on finding the presence of EHP spores
    - Difficult by light microscopy because
        - Spores are very small and a 100x lens is required
        - Sometimes spores are present in low numbers
- New wet mount method using **2% aqueous solution of Phloxine B stain**
    - Slightly egg-shaped spores of about 1.1 micron in length indicate the presence of EHP

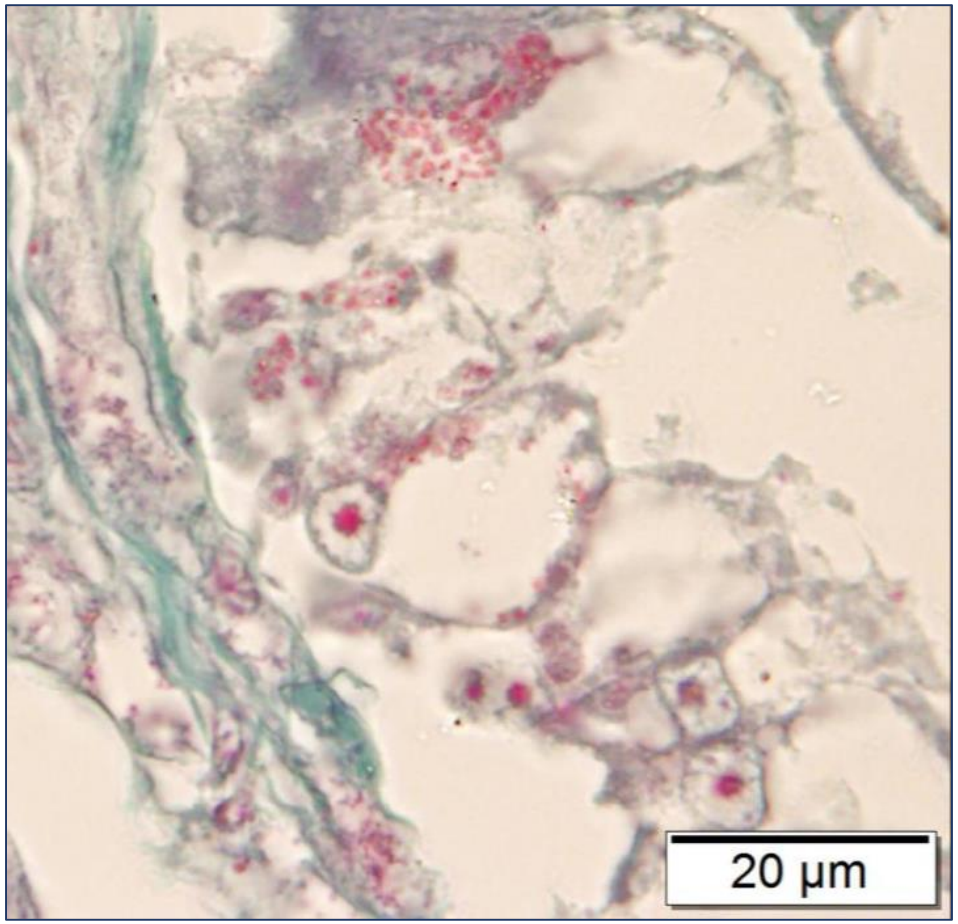


Spores of *Enterocytozoon hepatopenaei* stained with Phloxine B solution (2% w/v) examined by light microscopy at 40X (A) and 100X (B) magnification (Source: Sritunyalucksana, K. Biotec Thailand)

- Chromotrope stain for spore detection
  - First used for EHP by Tourtip et al. (2009. *J. Invertebr. Pathol. 102, 21-29)*
  - Obtained from Weber et al. (1992. N Engl J Med. 326, 161-166)
  - A more rapid “hot” method was developed later (Moura et al. 1997. Arch Pathol Lab Med. 121, 888- 893)
  - These methods reveal spores better but still do not detect infected cells without spores

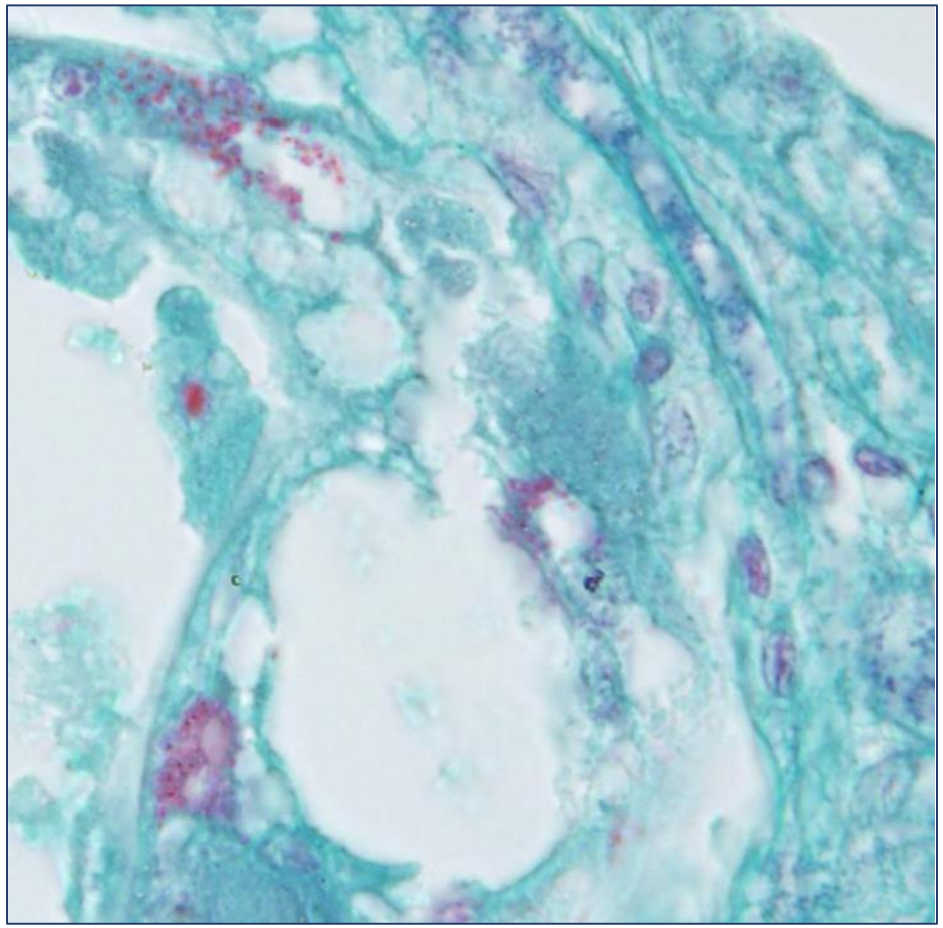
Chromotrope staining

Normal method



Weber et al., 1992. N Engl J Med. 326, 161-166

Hot method



Moura et al. 1997. Arch Pathol Lab Med. 121, 888-893

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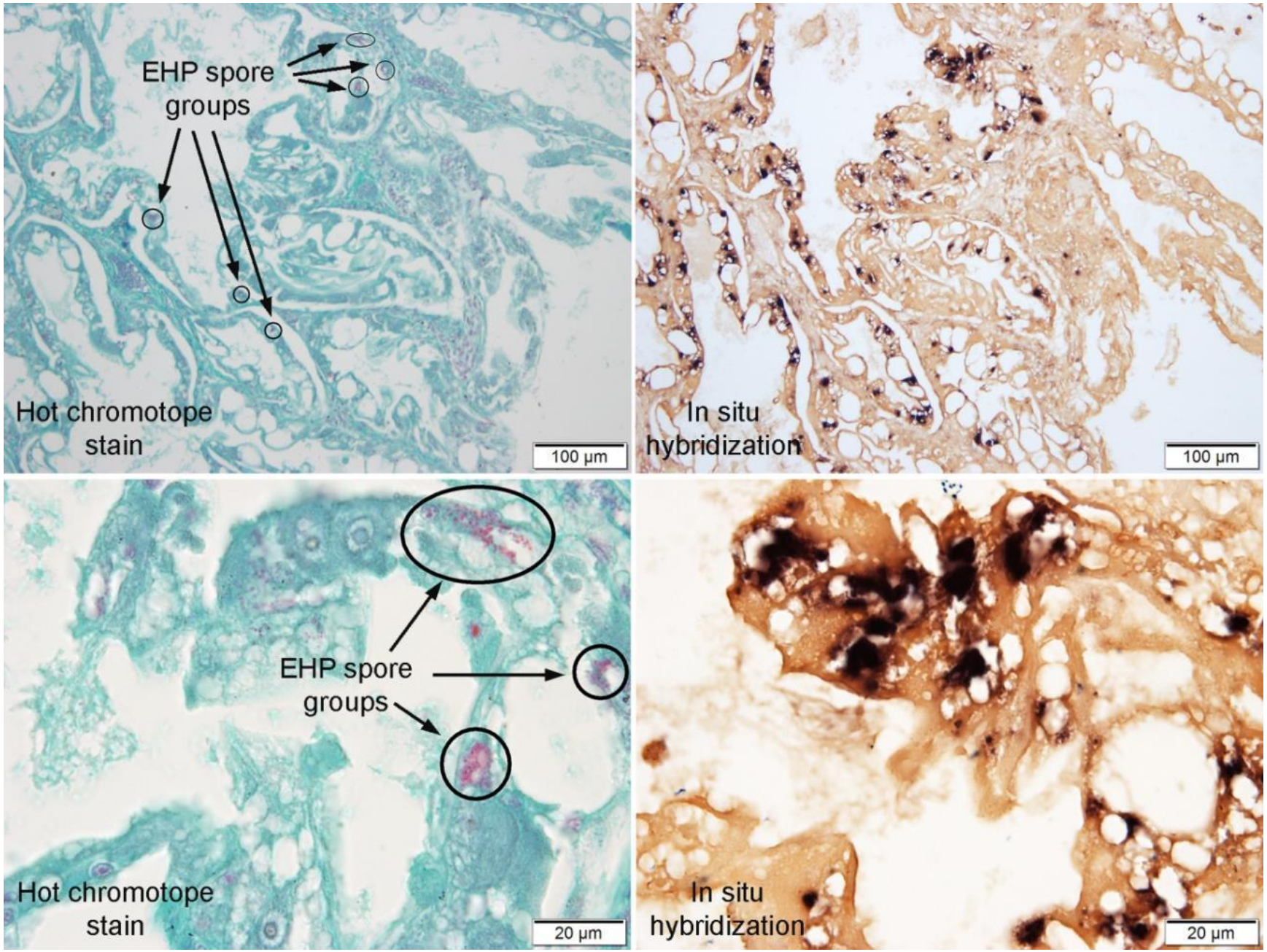
Spores poorly indicate HPM severity



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qPCR is better than microscopy (Flegel, 2016)

- qPCR is the best way to quantify EHP infections
- qPCR is needed in grow-out ponds to determine whether EHP exceeds ~1000 copies per µg DNA
- The whole HP must be homogenized and sub-sampled for qPCR because lesions are unevenly distributed

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Prevention and control of HPM (Flegel, 2016)

- Broodstock and PL should be monitored by PCR to ensure freedom from EHP
- Broodstock should not be fed with live, non-SPF polychaetes, mollusks, etc.
- Reservoir species need to be identified and eliminated from the culture system
- Increased water exchange may reduce spore density and reduce transmission
- Treatment at -20ºC for 48 h before use is recommended (Leiro et al. 2012. Int J Food Microbiol. 156: 152-160)

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Future Directions

- To date, strategies pertinent to prevention and control of AHPND and HPM are the 2 major concerns for shrimp farmers
- Detection of AHPND has improved the supply of clean broodstock and PL
- Unwarranted outbreaks of AHPND have been improved by management changes, based on recommendations
- For HPM, work is needed on:
    - Identification of infected carriers (reservoirs)
    - Modes of transmission and interventions
    - Possibility of therapeutics
- Attention and guidance should be provided to farmers who are into small-scale shrimp culture as they represent a weak link in the system posing high risk for diseases

- Detection methods should be adherent to gold standards such as those indicated in the OIE guidelines, and whenever possible should be harmonized in the region
- Development of production systems based on cultivation of SPF/SPT/ SPR stocks in a biosecure environment should be pursued to ensure constant supply of SPF/ SPT/ SPR stocks
- Government and private sectors at national and regional levels should work together to generate sufficient funds and resources for the conduct of R & D programs
- To prevent illegal transboundary movement of living aquatic animals including shrimp broodstocks or their offspring for cultivation as well as polychaetes used as broodstock feeds, countries in the region should work in concert to harmonize national legislations and regulations related to aquatic animal health management

Back Row (From Left to Right)

Prof. Mohamed Shariff (AAHSC, OIE; Malaysia), **Dr. Andy Shinn** (FishVet Group, Thailand), **Dr. Hirofumi Kugita** (OIE-RRAP, Japan), **Prof. Timothy Flegel** (Centex Shrimp, Thailand), **Mr. Simon Wilkinson** (NACA), **Dr. Eduardo Leaño** (NACA), **Dr. Chien Tu** (Observer, AHRI, Chinese Taipei), **Dr. Kjersti Gravningen** (Aquafuture, Norway), **Dr. Derun Yuan** (NACA).

Front Row (From Left to Right)

Dr. Diana Chee (AVA, Singapore), **Dr. Thitiporn Laoprasert** (AAHRDD, Bangkok, Thailand), **Dr. Janejit K** (AAHRDD, Bangkok, Thailand), **Dr. Supranee Chinabut** (Thailand), **Dr. Melba Reantaso** (FAO, Rome, Italy), **Dr. Cherdsak Virapat** (NACA), **Dr. Fania Dwi** (OIE-RRAP, Tokyo, Japan), **Dr. Hong Liu** (AQSIQ, P.R. China), **Dr. Rolando Pakingking, Jr.** (SEAFDEC AQD, Philippines), **Dr. Carolyn Benigno** (FAO-RAP, Thailand)