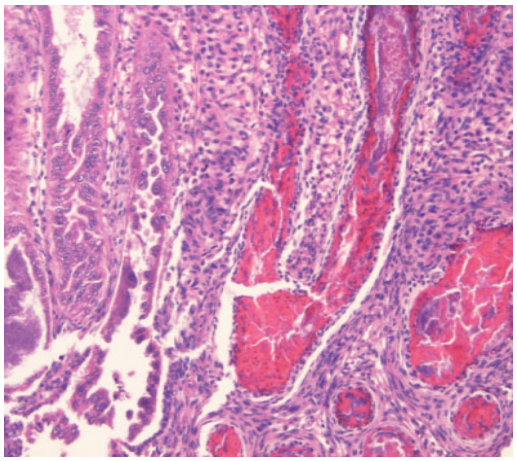
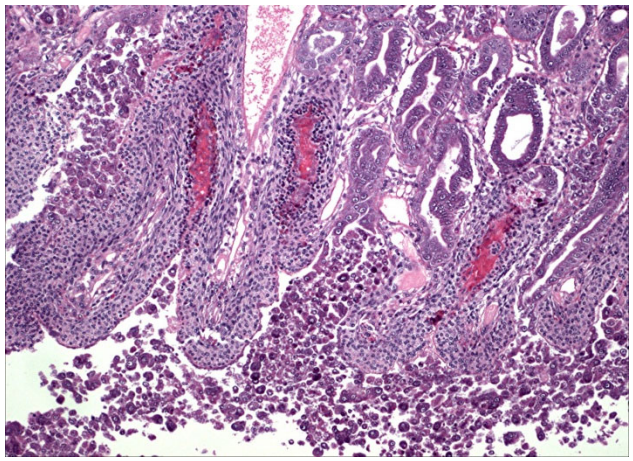
Australian Government
Department of Agriculture, Fisheries and Forestry

Final Report

Asia Pacific Emergency Regional Consultation on the Emerging Shrimp Disease: Early Mortality Syndrome (EMS) / Acute Hepatopancreatic Necrosis Syndrome (AHPNS)

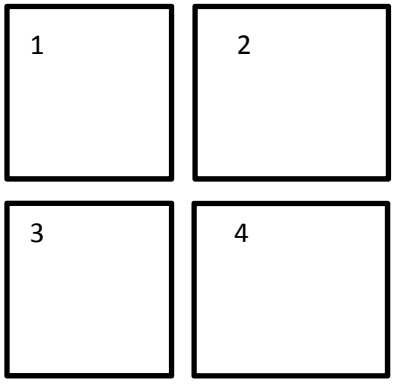


Network of Aquaculture Centres in Asia-Pacific

Bangkok, Thailand

9-10 August 2012



Cover Photo Credits:

1. Normal (left) and affected (right) white shrimp. HP shrunken in affected shrimp (E. Eknath).
2. Atrophied hepatopancrease indicative of AHPNS (D. Lightner).
3: Hepatopancreas showing pathology associated with terminal stages of the disease (T. Flegel).
4. Lesions in the hepatopancrease of affected shrimp (D. Lightner).

FINAL REPORT

ASIA PACIFIC EMERGENCY REGIONAL CONSULTATION ON THE EMERGING SHRIMP DISEASE: EARLY MORTALITY SYNDROME (EMS)/ACUTE HEPATOPANCREATIC NECROSIS SYNDROME (AHPNS)

Network of Aquaculture Centres in Asia-Pacific

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Our thanks

NACA wishes to sincerely thank the Australian Government Department of Agriculture, Fisheries and Forestry (DAFF, Australia) for funding the emergency consultation, and for their rapid and timely response to this issue, which has been of great assistance to the region. OIE support for the participation of OIE Crustacean disease experts is gratefully acknowledged. Finally NACA wishes to thank all the resource experts, national participants representing the respective national Competent Authorities (CA) and lead research institutions, regional and international organizations and private sector for their contribution to the regional consultation.

Abbreviations and Acronyms

AHPNS	Acute Hepatopancreatic Necrosis Syndrome
AG	Advisory Group
CA	Competent Authority
CMC	Crisis Management Center of FAO
DNA	Deoxyribonucleic Acid
DAFF	Australian Government Department of Agriculture, Fisheries and Forestry
EMS	Early Mortality Syndrome
ERAAD	Epidemiology and Risk Assessment of Aquatic Animal Diseases
FAO	Food and Agricultural Organization of the United Nations
GC	Governing Council of NACA
HP	Hepatopancreas
IGO	Intergovernmental Organization
IMN	Infectious myonecrosis
IMNV	Infectious myonecrosis virus
MPEDA	Marine Products Export Development Authority (India)
*Mr*NV	*Macrobrachium rosenbergii* nodavirus
NACA	Network of Aquaculture Centres in Asia-Pacific
OIE	World Organisation for Animal Health
PCR	Polymerase chain reaction
PL	Postlarvae
QAAD	Quarterly Aquatic Animal Disease
RNA	Ribonucleic Acid
RT-PCR	Reverse transcriptase PCR
SEAFDEC	Southeast Asian Fisheries Development Center
SPF	Specific pathogen free
TCP	Technical Cooperation Project
TEM	Transmission Electron Microscopy
TS	Taura syndrome
TSV	Taura syndrome virus
WFC	World Fish Centre
WSD	White spot disease
WSSV	White spot syndrome virus
YHV	Yellowhead virus

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Executive Summary

On 9-10 August 2012, an emergency regional consultation on Early Mortality Syndrome (EMS) of shrimp and associated pathology described as Acute Hepatopancreatic Necrosis Syndrome (AHPNS) was held in Bangkok, Thailand. The consultation brought together over 87 participants including international shrimp health experts, national governments in the Asia Pacific region and industry stakeholders to share information on this emerging disease, its occurrence, pathology and diagnosis, and to develop a coordinated regional response to the issue. The consultation was organised jointly by NACA and the Australian Government Department of Agriculture, Fisheries and Forestry (DAFF). The AHPNS news story and audio recordings of 19 technical presentations made at the regional Consultation meeting are available on NACA website at the following links.

http://www.enaca.org/modules/news/article.php?article_id=1952
http://www.enaca.org/modules/podcast/programme.php? programme_id=9

EMS or AHPNS?

The generic name EMS has been coined to describe unusually high mortality that can occur commonly within the first 30 days of shrimp grow-out due to a variety of pond management and pathogen related factors. In addition to pond management problems, various well studied pathogens like WSSV, YHV and vibriosis have been commonly linked to EMS. However, due to generic clustering of all potential causes of mortalities reported as EMS, this very broad and imprecise case definition provides little diagnostic value and has led to lot of confusion.

From 2009, however, a new distinctive pattern of mortalities has become evident in the early stages of grow-out of both *Penaeus vannamei* and *P. monodon*. The syndrome involves mass mortalities of up to 100% within 20-30 days after stocking. Affected shrimp consistently showed an abnormal hepatopancreas, which is usually shrunken and white and is accompanied by loose shells, pale overall colouration, slow growth, corkscrew swimming behavior and moribund shrimp sinking to die at the bottom of the pond. Examination of the histology of the hepatopancreas of affected shrimp revealed massive necrosis of the hepatopancreas. Given these specific signs, the name "acute hepatopancreatic necrosis syndrome" has been coined based on unique gross pathological lesions seen in the hepatopancreas and to qualify it amongst other potential causes of early mortalities. For clarity and to avoid confusion, the disease issue focus of the emergency regional consultation will be referred to throughout this document as AHPNS according to the detailed individual shrimp case definition described by Prof Don Lightner (see below).

Deaths consistent with AHPNS signs were first reported from China and Vietnam in 2010 followed by Malaysia in 2011 and Thailand early in 2012. The syndrome has caused substantial economic losses to shrimp farmers in the affected countries. The cause is not yet known.

A case definition for AHPNS

Reporting of AHPNS has been confounded by the lack of a clear case definition and by mortality events resulting from varied causes being reported broadly as EMS. To assist in accurate reporting of AHPNS amongst the background of potential causes of EMS, Prof Don Lightner has proposed the following animal-level case definition, which was agreed to in general by consultation participants:

Idiopathic

- No specific disease causing agent (infectious or toxic) has been identified so far.

Pathology

- Acute progressive degeneration of the hepatopancreas (HP) from medial to distal with dysfunction of B, F, R and E cells
- Prominent karyomegaly and necrosis and sloughing of HP tubule epithelial cells
- At the terminal stage, marked inter- and intra-tubular hemocytic inflammation and development of secondary bacterial infections become apparent in association with necrotic and sloughed HP tubule cells.

At the pond level, the following clinical signs could be used for presumptive diagnosis which can be further confirmed by histopathology observed at the animal level

- Often pale to white HP due to pigment loss in the connective tissue capsule
- Significant atrophy of the HP
- Often soft shells and guts with discontinuous contents or no contents.
- Black spots or streaks sometimes visible within the HP
- HP does not squash easily between the thumb and forefinger
- Onset of clinical signs and mortality starting as early as 10 days post-stocking
- Moribund shrimp sink to the pond bottom

Looking for the cause

While the apparent spread of AHPNS to various countries across Southeast Asia suggests that an infectious or at least biological agent might be involved, thus far, preliminary transmission trials using tissue filtrates of affected shrimp sent for laboratory analysis have failed to demonstrate that the disease is caused by a virus and no other infectious agent or toxin has been identified. AHPNS histopathology is suggestive of toxicity,

but testing of feeds from affected farms and two crustaceacides including cypermethrin have similarly failed to reproduce the disease. PCR testing has indicated that the disease is not caused by the known viral pathogens WSSV, YHV, IMNV or TSV. While the specific cause(s) of AHPNS remain unknown so far, the possibility of an infectious agent and/or toxin cannot be discounted. As such, immediate research investigations need to focus on resolving this knowledge void by exploring all possible causes of AHPNS with an open mind. It is very important to apply the case definition to all suspected AHPNS detections. It is strongly suggested this case definition be considered as essential for all future epidemiological studies, outbreak investigations and management, diagnosis and laboratory-based research to discover the cause of AHPNS. The need to determine if the cause of AHPNS is infectious is vital as this would have significant implications for biosecurity and response actions. A thorough epidemiological approach to outbreak investigation to improve knowledge about the disease, including to determine if the cause is infectious is urgently warranted. Implementation of precautionary measures to reduce the risk of a possible infectious agent spreading in the region (for example restricting movements from affected areas/countries to unaffected areas/countries) should be seriously considered.

Preparing for the future

As new diseases have emerged in aquaculture species with regularity, the consultation also discussed arrangements to improve response mechanisms to future disease emergencies. One constraint identified is the lack of funding for a rapid response capability in the region. At present, obtaining extra-budgetary funding to investigate and contain an emergency disease will often require lengthy approval processes that preclude funds being made available until the situation has become sufficiently 'hot' to persuade administrators to act.

As more options are available to contain a disease during early stages of it emerging, participants indicated a need to provide a mechanism for very early investigation and incident identification. Such a rapid response mechanism could provide information that could be used for any larger national or regional response (e.g. requests for activation of CMC of FAO, development of a TCP). One additional possibility proposed was to establish a 'regional emergency aquatic animal disease fund' and pre-agreed procedures for activating an investigation or response coordinated regionally by an independent agency such as NACA.

Government agencies were suggested as likely contributors to such a fund, industry representatives indicated they have also invested substantially to research the cause of AHPNS as well as other serious disease issues and they were open to the possibility of contributing to such a fund.

A. Background

A new/emerging disease of shrimp known as acute hepatopancreatic necrosis syndrome (AHPNS) has been reported to be the cause of significant financial losses at farms in China (2009), Vietnam (2010) and Malaysia (2011). Recently, it has been reported in shrimp being farmed in the eastern Gulf of Thailand (Flegel, 2012). AHPNS affects both *P. monodon* and *P. vannamei* and is characterized by mass mortalities (reaching up to 100% in some cases) during the first 20-30 days of culture (post-stocking in grow-out ponds). Considering the consistent characteristic pathology observed in the hepatopancreas of affected shrimp from each of the affected countries, a precise case definition has been provided by Lightner and his group. This case definition is being used with slight modifications to assist research being undertaken by other groups, notably Flegel and his coworkers, and is a major breakthrough in devising strategies to investigate the etiology of this new disease.

Anecdotal information suggests that AHPNS spread patterns may be consistent with an infectious agent. However, as yet no potential causative pathogen (if the disease is infectious) has been identified, and possible etiologies include toxins (biotic or abiotic), bacteria and viruses. Irrespective of the cause, the spread of the disease and its devastating impacts in the countries affected so far warrants increased disease awareness as well as preparedness and contingency planning by other countries in the region potentially at risk.

The NACA Asia Regional Advisory Group on Aquatic Animal Health recognized AHPNS as an emerging disease problem in its 10th AGM in 2011 and called for increased surveillance and reporting from the member governments in the region (http://www.enaca.org/modules/wfdownloads/singlefile.php?cid=132&lid=1053). Considering its potential severity and impact, as a first step, NACA circulated a Disease Advisory on AHPNS (Annex 1 EMS Disease Advisory) widely to Competent Authorities (CA) and concerned stakeholders in 18 member countries. NACA also took up the task of exploring various funding options for convening an emergency regional consultation and succeeded in getting support from the Department of Agriculture, Fisheries and Forestry (DAFF), Australia for convening a 2 day meeting "**Asia Pacific Emergency Regional Consultation on EMS/AHPNS**" in Bangkok on 9-10 Aug 2012.

NACA and DAFF convened this regional consultation in Bangkok involving global experts, national participants representing the Competent Authority and lead research institutions, regional and international organizations and private sector, with the purpose of knowledge sharing, information exchange and networking to help solve the AHPNS puzzle, prevent its further spread in the region and minimize its impact on shrimp farming industries (Annex 2 Prospectus).

B. Consultation objectives

The Primary Objectives of the Regional Consultation were to:

- Bring together global experts, national participants representing the CA and lead research institutions, regional and international organizations and the private sector
- Facilitate knowledge sharing, information exchange and networking for better understanding and dealing with AHPNS
- Document the current state of knowledge on AHPNS and lessons learned in dealing with disease emergencies at the national/regional levels
- Agree on a regional action plan for dealing with future aquatic disease emergencies in the region

The Specific Objectives included:

- Provide an overview of the current disease situation and its spread, with emphasis on the threats posed to shrimp industries in the region
- Situation analysis of outbreaks in China, Vietnam, Malaysia and Thailand
- Identify any similar occurrences in other countries in the region
- Develop guidance for future surveillance work by providing a field level disease card, case definition and outbreak investigation template
- Develop or plan collaborative research on AHPNS, intra-regionally and internationally, to identify the primary causative agent and risk factors and to develop management interventions including preventive measures
- Formulate a regional action plan to improve disease surveillance and reporting, and contingency measures to contain and prevent further spread of the disease

C. Participants

Over 87 people attended the 2 day event (Annex 3 List of Participants). This included 17 global experts (Australia, Brunei, Canada, China, Spain, Thailand, UK, USA, Vietnam), 40 national participants representing the Competent Authority and Lead research institutions (Australia, Bangladesh, Brunei, Cambodia, China, Indonesia, India, Myanmar, Malaysia, Nepal, Pakistan, Philippines, Thailand, Sri Lanka, Singapore, Vietnam), 10 private sector representatives (Alltech, Bayer, Cargil, CP, Pfizer, Inve, Novus, Pharmaq), 15 technical officers from regional and international organizations (OIE, FAO, SEAFDEC, MPEDA, WFC, NACA) and 7 post graduate research students (Thailand and Vietnam).

D. Process

The Regional Consultation was conducted as per the agenda (Annex 4 Agenda). Formal opening welcome remarks were provided by Dr Ambekar Eknath, DG of NACA and Dr Ingo Ernst, Director of Aquatic Animal Health of DAFF, Australia. Dr CV Mohan provided a brief presentation on workshop background, objectives, structure and expected outputs. The consultation was conducted in 3 parts. Technical presentations provided latest updates on AHPNS, country presentations shared experiences from the affected countries, four working groups had detailed discussions on different themes and reported back to the plenary session which developed recommendations and follow up actions.

Technical Presentations:

The following technical presentations were made at the consultation. PPTs provided as annex 5 (Annex 5 Technical Presentations)

- Characterization, Distribution, Impacts and Case Definition by Prof Don Lightner
- Research Progress on Bacterial and Viral Causes of AHPNS by Prof Tim Flegel
- Disease Emergence –Why and How? by Prof Peter Walker
- Novel Methods for "Hunting for Ghost Viruses" by Dr Jeff Cowley
- Epidemiology and Risk Factors-What We Know? by Dr Flavio Corsin
- Is EMS a Management Problem? by Dr Matt Briggs
- One Month Mortality Syndrome-Revisiting an old story by Dr Celia Pitogo
- Management of EMS-What Works and What Does Not? by Prof Chalor Limsuwan
- Disease Preparedness-Theory and Practice. What Have We Learnt? by Dr Ingo Ernst

Country Presentations:

The following presentations were made by the affected countries (Annex 6 Country Presentations)

- Experiences from China by National Team
- Experiences from Vietnam by National Team
- Experiences from Thailand by National Team
- Experiences from Malaysia by National Team

Audio recordings of all presentations are available on NACA website at the following links:

http://www.enaca.org/modules/news/article.php?article_id=1952

http://www.enaca.org/modules/podcast/programme.php

Group Discussions:

Four working group breakout sessions were organized as follows and specific tasks assigned to the groups to discuss and report back to the plenary session. Group findings were used to draw up recommendations and follow up actions.

Group 1: Current Knowledge, Knowledge Gaps and Research Priorities

- Team: Dr Don Lightner, Dr Tim Flegel, Dr Huang Jie, Dr Jason Weeks

Group 2: Detection, Reporting and Surveillance

- Team:, Dr Flavio Corsin, Dr Ian Gardner, Dr Jeff Cowley, Dr Celia Pitogo

Group 3: Biosecurity, Emergency Response and Disease Management

- Team: Dr Larry Hammell, Dr Matt Briggs, Dr Victoria Alday, Dr Ed Leano

Group 4: Regional Disease Response

- Team: Dr Ingo Ernst, Dr Brian Davy, Dr Peter Walker, Dr Supranee Chinabut

The summary and outcomes of the technical sessions and group discussions are captured separately in the sections below to enhance the quality and usefulness of the outcomes to stakeholders in member governments.

E. Technical Updates on AHPNS

Early Mortality Syndrome (EMS) is used generically to describe unusually high mortality that can occur commonly within the first 30 days of shrimp grow-out due to a variety of pond management and pathogen related factors. From 2009, however, a new distinctive pattern of mortalities has become evident in the early stages of grow-out of both *Penaeus vannamei* and *P. monodon*. The syndrome involves mass mortalities of up to 100% during the first 20-30 days after stocking. Affected shrimp consistently showed an abnormal hepatopancreas, which is usually shrunken and white and is accompanied by loose shells, pale overall coloration and moribund shrimp sinking to die at the bottom of the pond. Examination of the histology of the hepatopancreas of affected shrimp revealed massive necrosis of the hepatopancreas. Given these specific signs, the name "Acute Hepatopancreatic Necrosis Syndrome" has been proposed as a more appropriate term, to distinguish this condition from other causes of early mortalities.

Brief History and Spread in the Region

Beginning around 2009, EMS with disease characteristics indicative of AHPNS began to cause significant production losses in southern China. By 2010, the distribution of affected farms in China had expanded, and reports of EMS/AHPNS began to emerge from Vietnam. In 2011, the disease was reported to be in Malaysia and in early 2012, also in the eastern Gulf of Thailand.

China. The occurrence of EMS was recorded initially in Hainan in 2009 but was often confused with "covert disease" and thus ignored by most farmers. In 2011, however, disease occurrences became more serious, especially at farms with a history of culturing shrimp for >5 years and those closer to the sea using more saline water (salinity above 20 ppt) (Panakorn, 2012). Interestingly, reports also suggested that shrimp polycultured in freshwater ponds experienced lower mortality levels (however other confounding factors may have explained this finding). During the first half of 2011, about 80% losses in production were reported at shrimp farms in Hainan, Guangdong, Fujian and Guangxi.

Vietnam. EMS was first reported to be a serious problem in 2010, but widespread devastation in the Mekong Delta (South Vietnam) has occurred since March 2011. The main shrimp production areas affected are the provinces of Tien Gang, Ben Tre, Kien Giang, Soc Trang, Bac Lieu and Ca Mau and cover a total shrimp pond area of around 98,000 hectares. In June 2011, unprecedented losses in *P. monodon* were reported across 11,000 ha of shrimp farms in Bac Lieu, 6,200 ha in Tra Vinh (where it is estimated that in total, 330 million shrimp have died causing a loss of over VND12 billion), and 20,000 ha in Soc Trang (VND1.5 trillion in losses) (Mooney, 2012). As of the first quarter of 2012, the disease is still affecting the Mekong Delta area (Tien Gang: 28.5 ha; Tra Vinh: 1,642 ha; Soc Trang: 359 ha, Bac Liue: 98 ha; and Ca Mau: 4,007 ha) as well as the south central coast (Binh Dinh: 39 ha; Nin Thuan: 6.2 ha; Ba Ria-Vung Tau: 13 ha). The shrimp pond area affected in 2012 is estimated to be in the order of 39,000 ha (Vietnam Country presentation in this report).

Malaysia. EMS was first reported in late 2010 in the east coast of the peninsular state of Johor and subsequently in Pahang, Perak and Penang during 2011. Total production of cultured shrimp were 110,000mt in 2010, 75,000 mt in 2011 and 25,000 mt in 2012 (Jan-May) with 90% production contributed by P.vannamei (source: Malaysia Shrimp Industry Association). EMS resulted in a significant drop in *P. vannamei* production from 87,000 mt in 2010 to 67,000 mt in 2011 (source: annual Fisheries statistics). Production up to May in 2012 is only 25,000 mt (source: Malaysia shrimp industry association) and worse is expected due to EMS being reported in Kedah (May 2012) and Sabah (June 2012). Ongoing studies suggest links to water quality and possible predisposing factors such as paralytic shellfish poison, but these tentative findings require additional investigations to confirm their involvement in the syndrome.

Thailand. So far in 2012, 0.7% total shrimp ponds in Thailand have been affected by EMS, mostly in the coastal areas (Rayong, Chantaburi, Trat, Chacheongsao provinces) along the eastern Gulf of Thailand. To mitigate impacts, a variety of awareness/communication efforts involving close collaboration among government, researchers and the Thai shrimp farmers association at local and national levels are being made.

Species affected

AHPNS affects both *Penaeus monodon* and *P. vannamei* and there are reports that *P. chinensis* is also affected. It is characterized by mass and sudden mortalities (reaching up to 100% in some cases) during the first 20-30 days of culture (post-stocking in grow-out ponds).

Clinical Signs and Pathology (refer Presentation by Prof Don Lightner)

Clinical signs (field level) include a shrunken and white hepatopancreas, often accompanied by loose shells, pale overall body coloration, and moribund shrimp sinking to die at the bottom of the pond. The atrophied (shrunken) HP of affected shrimp are often pale to white because of pigment loss in the connective tissue of the HP sheath or capsule, and black spots or streaks are sometimes visible within the HP due to melanized tubules. The HP does not squash easily between the thumb and forefinger (i.e., it is more rigid, probably because of the large amount of fibrous connective tissue and hemocytes). Disease progression is as follows:

a) Idiopathic – no specific disease causing agent (infectious or toxic) has been associated with the lesion
b) Acute progressive degeneration of the hepatopancreas (HP) accompanied initially by a decrease of R, B and F-cells followed last by a marked reduction of mitotic activity in E-cells.
c) Progress of lesion development is proximal-to-distal with dysfunction of R, B, F, and lastly E-cells, with affected HP tubule mucosal cells presenting prominent karyomegaly (enlarged nuclei), and rounding and sloughing into the HP tubule lumens.
d) The sloughed HP cells provide a substrate for intense bacterial growth, resulting in massive secondary bacterial infection (putative *Vibrio* spp.) and complete destruction of HP at the terminal phase of the disease.
e) Accompanying the initial sloughing of HP tubule epithelial cells and the development of a secondary bacterial infection is intense intertubular hemocytic aggregation and hemocyte encapsulation of necrotic HP tubules and melanization of the more proximal portions of HP tubules in some shrimp.

In summary, the following pathological features have been observed consistently in the hepatopancreas of affected shrimp from all affected countries

1. Low activity of B, F and R cells
2. Low mitotic rate in E cells
3. Rounding-up and sloughing of HP tubule epithelial cells
4. Intertubular hemocytic congestion (inflammation)
5. Proximal- to-distal pattern of lesion spread
6. Distal end last to be affected
7. Enlarged nuclei (karyomegally) with prominent nucleoli

8. Bacterial infection during advanced/terminal stages of the disease
9. Bacterial phase appears to be secondary
10. Identical lesions found in *P. vannamei* and *P. monodon* tissue samples

Case Definition (refer Presentation by Prof Don Lightner)

Considering the consistent progressive pathology observed in the HP of juvenile shrimp that die soon after pond stocking, this newly emerged disease has been named Acute Hepatopancreatic Necrosis Syndrome (AHPNS). Dr Lightner has proposed the following animal level case definition for AHPNS to clearly distinguish it from other causes of EMS and as a base-line for future research on this specific condition.

- Idiopathic: No specific disease causing agent (infectious or toxic) has been identified.
- Pathology: Acute progressive degeneration of HP from medial to distal tubule regions with dysfunction of B, F, R and E cells; prominent karyomegaly and necrosis and sloughing of these tubule epithelial cells. In the terminal stage, marked inter- and intra-tubular hemocytic inflammation and development of secondary bacterial infections occur in association with necrotic and sloughed HP tubule cells.

At the pond level, the following clinical signs provide a presumptive diagnosis to be confirmed by animal level histopathology

- Often pale to white HP due to pigment loss in the connective tissue capsule.
- Significant atrophy (shrinkage) of the HP.
- Often soft shells and guts with discontinuous contents or no contents.
- Black spots or streaks sometimes visible within the HP.
- HP does not squash easily between the thumb & forefinger.
- Onset of clinical signs and mortality starting as early as 10 days post stocking
- Moribund shrimp sink to the pond bottom.

Primary Cause (refer Presentation by Prof Tim Flegel)

EMS is commonly used to describe unusually high mortality among shrimp within the first 30 days of culture. Such mortalities can be caused by various well known pathogens such as WSSV and YHV. This imprecise and very broad case definition for high mortality events is thus not particularly useful, and to avoid confusion, the newly emerged disease has been named AHPNS based on the specific animal-level case definition described by Dr Lightner. The precise case definition is critical to ensure research progress across various institutes and countries are focused on the same disease.

While the apparent spread of AHPNS to various countries across Southeast Asia suggests that an infectious or at least biological agent might be involved, thus far, preliminary transmission trials using tissue filtrates of affected shrimp sent for laboratory analysis have failed to demonstrate that the disease is caused by a virus and no other infectious agent or toxin has been identified. AHPNS histopathology is suggestive of toxicity, but testing of feeds from affected farms and two crustaceacides including cypermethrin have similarly failed to reproduce the disease. PCR testing has indicated that the disease is not caused by the known viral pathogens WSSV, YHV, IMNV or TSV. While the specific cause(s) of AHPNS remain unknown at yet, the possibility of an infectious agent and/or toxin cannot be discounted. As such, immediate research investigations need to focus on resolving this knowledge void by exploring all possible causes of AHPNS with an open line of investigation. Avenues to explore should include;

- biotic and abiotic toxins in:
    - Pond water & supply water, soils & sediments, etc.
    - Feed & feed ingredients, probiotics, etc.
    - Old and "new" agricultural pesticides, etc.
- possible new bacteria:
    - These might be revealed by shotgun sequencing of bacterial rDNA & *in situ*
    - There is also the possibility of a phage-bacterium partnership(s)
- possible unknown shrimp viruses that might be revealed by:
    - Challenge tests with filtered and unfiltered tissue extracts to see if a filterable agent is present
    - TEM examination of affected shrimp tissues for the presence of viral particles
    - Shotgun sequencing of "viral extracts" & *in situ*

Preliminary bacterial shotgun testing by PCR resulted in the identification of bacteria in the order Bukholderiales (genera *Ralstonia*, *Delftia* and *Pelomonas*) and Order Actinomycetales (genera *Leifsonia* and *Rhodococcus).* Next step is to make clones of these bacteria and use for *in situ* hybridization to test shrimps from test and control ponds. Probes specific to these bacteria now needed *in situ* hybridization confirmation of their involvement in causing AHPNS histopathology.

Disease Emergence and Spread (refer Presentation by Dr Peter Walker)

To assist guide approaches to identify the cause of AHPNS, it will useful to consider current knowledge and concepts on how new diseases emerge and spread. Disease emergence and subsequent spread often results from some disturbance in the ecology of an infectious agent. Potential pathogens are integral components of all ecosystems and their existence is perpetuated by them being able to be transmitted efficiently without necessarily causing disease. Many pathogens with potential to cause disease commonly infect healthy animals

with no pathology or mortality. A disturbance in ecology can upset the natural balance and result in a normally innocuous pathogen emerging as a new disease agent.

Aquaculture is an important contributor to socio-economic development in many countries, but intensive aquaculture practices often provide ideal environments for emergence and spread of disease because of the following reasons.

- Animals are often cultured in an unnatural environment
- Animals are often cultured at high stocking densities
- Animals are often stressed by culture conditions
- Unregulated trade in live animals occurs commonly

Only through early detection (rapid and accurate diagnosis, effective non-targeted surveillance), rapid response (national/international cooperation and information sharing, contingency planning, surge capabilities) and prediction and prevention **(**a more challenging option - but less costly socially, economically and environmentally; understanding biological and ecological drivers of pathogen emergence) can we reduce or limit the impact of emerging infectious diseases.

Molecular tools for discovering unknown pathogens (refer Presentation by Dr Jeff Cowley)

There are many methods now available for sequence-assisted and sequence-independent virus discovery that could be applied to help discover viruses or other pathogens if these are the cause of AHPNS. The selection of any particular approach can be guided by what clues become available on the etiological agent of AHPNS from epidemiological, histological and any other observational studies.

Molecular approaches to detect and characterize a pathogen rely mostly, but not exclusively, on some means of acquiring or enriching (i) the pathogen, from which DNA or RNA can then be extracted, or (ii) the pathogen nucleic acid itself. For example, virus particles can be acquired easily by microfiltration through 0.22 µm or 0.45 µm filters, or by high speed clarification of tissue homogenates followed by differential ultracentrifugation through, for example, a sucrose density cushion designed to exclude most organelles and other material of host cellular origin. Alternatively, some viruses have either double-stranded (ds)RNA genomes or synthesis dsRNA genomic intermediates during replication that can be distinguished from cellular RNA. For such viruses, dsRNA can be isolated, for example by gel purification following careful RNase A digestion of extracted total RNA, and used as source material for random cDNA synthesis, PCR amplification, cloning and sequence analysis. As another alternative, RNA from non-affected and AHPNS-affected shrimp could, for example, be randomly amplified by PCR (Differential Display) to identify DNA products unique to affected shrimp, and thus possibly derived from a pathogen.

Once suspected pathogen cDNA or DNA has been acquired by any method, even if in very low abundance, it can be amplified by random PCR methods for either direct sequence analysis or sequence analysis of clones containing amplified DNA fragments. Alternatively, very large amounts (>40 μg) of very long (>30 kb) DNA can be generated from <10 ng DNA by multiple displacement amplification (MDA) using phi29 DNA polymerase (REPLI-g, QIAGEN), which is ideal for amplifying representative DNA sequences of viruses with long genomes such as WSSV or herpes viruses.

Once amplified DNA is obtained, depending on its nature, options exist to sequence it using either the Sanger dideoxy-sequence terminator methods or any of the several NextGen multi-parallel sequencing platforms (eg. GL-Flex 454 pyrosequencing, Illumina, Ion Torrent). Indeed, based on the extraordinary capabilities of these NextGen platforms to generate massive amounts of sequence information, simple analysis of DNA or of cDNA prepared to total RNA of APHNS-affected shrimp should have the capability to identify pathogen genome/mRNA sequences, and with suitable coverage depth, allow *de novo* assembly of viral/pathogen genomes in the absence of available genome information or database searches to look for relationships to known pathogen sequence motifs.

Epidemiology and risk factors (refer Presentations by Dr Flavio Corsin and Dr Matthew Briggs)

Systematic robust epidemiological studies of AHPNS have not been conducted so far in any country, though considerable amounts of primary and secondary data have been gathered and epidemiological surveys conducted to identify potential risk factors. Working case definitions at the pond/farm level were also developed to complement the individual shrimp level case definition defined by Prof Don Lightner. Until epidemiological approaches are applied systematically to include hatchery, transport, pond, farm and location-specific data, it will be very difficult to pinpoint and prioritize risk factors for AHPNS. Based on circumstantial evidence, AHPNS appears to be associated with either an infectious agent or a (algal) toxin, although other "etiologies" cannot be ruled out. Hatchery and farm management processes might also play a key role.

Potential risk factors, which need to be reconfirmed with more robust quality data sets, have been deduced from observations that AHPNS outbreaks may be more likely to occur;

- in more intensive/high density systems
- with *P. monodon* compared to *P. vannamei*
- at locations closer to the sea with higher water salinity
- with seed sourced through some supply chains
- at farms not employing water reservoirs

Preliminary data also suggested that AHPNS severity may be greater;

- at older farms close to the sea
- at farms with poorly prepared ponds (no sludge removal) & poor management leading to excess nutrient pollution
- at locations with overcrowding of farms, sharing of water sources
- at farms/locations that overuse chemicals
- at farms using higher intensification
- when seed experiences stress during transportation
- when poor-quality (bacterially-infected) seed is used (although 54% of Malaysian farmers and many in Thailand report faster-growing SPF seed is affected more severely than slower-growing seed)
- when water salinity at stocking is high and with high and fluctuating temperatures,
- when seed are overstocked and overfed
- in ponds with inadequate aeration and evidence of toxic levels of H_2S

AHPNS severity appears to be lower at farms;

- using low salinity (<20 ppt) water
- inland and thus far from sea, using plastic-lined ponds, using biofloc systems (many, but not all, farms using biofloc or semi-biofloc report less problems)
- using high quality seed,
- using especially SPF *P. monodon*
- that strictly monitor and control early feeding rates
- using thorough pond and environment disinfection protocols (for both viruses and bacteria) prior to stocking, high quality probiotics and specific immune-stimulants

Future epidemiological studies should consider these factors to validate the observations and associations deduced from the rapid and thus preliminary survey. The need for systematic investigation of outbreaks was emphasized strongly in the consultation. All unusually high early mortalities of seed should be investigated thoroughly and only those that fit the pond-level and animal-level case definitions should be reported as AHPNS. Clinical signs (field level) should be observed carefully and be used only for presumptive diagnosis until confirmed by evidence of characteristic APHNS histopathology closely fitting the case definition.

Management (refer Presentations by Dr Matthew Briggs and Prof Chalor Limsuwan)

National Level: Assuming that AHPNS has an infectious etiology, the likelihood of AHPNS spreading to other countries in the region cannot be discounted. This could be mitigated especially by restrictions on movements of live seed and broodstock.

Measures suggested to contain AHPNS spread to non-affected countries include;

- only translocate live shrimp after conducting robust import risk analyses
- exercise considerable caution if seed and broodstock are introduced from affected countries
- increase surveillance efforts and study suspected AHPNS-like outbreaks thoroughly
- build capacity for early detection and rapid accurate diagnosis (especially histology), effective non-targeted surveillance of AHPNS
- developing contingency plans with agreed roles and responsibilities to mount a rapid response in the event of its occurrence
- enhance coordination and cooperation at an international level through constitution of a task force (e.g. Vietnam, Thailand) to deal with the disease

Farm/pond level: Since very little is known about AHPNS, including its primary etiology and whether it is infectious or not, it is very difficult as yet to recommend scientific management interventions. However, based on observations on AHPNS and past experience in dealing with various infectious diseases in shrimp, several options can be considered to manage or prevent it from occurring. Importantly, these management options remain good-practice recommendations, and their effectiveness has yet to be demonstrated fully.

Some of the generic management options suggested include;

- avoiding high risk practices (live feeds; co-cultivation)
- implementing pathogen exclusion practices (seed selection; pond environment considerations)
- employ stress reduction practices (good culture management systems)
- employ disease containment practices
- employ responsible trading practices

Some of the suggested shrimp health management practices that may be beneficial but have no proven or specific benefit for managing AHPNS include;

- Stocking with older seeds
- Use of only good and healthy post larvae at PL 10 or older
- Stocking with only healthy post larvae (e.g., check condition of the HP) from reliable hatcheries that use only approved probiotics
- Use of seeds from known sources since these present lower risk than those from nursery/middlemen
- Implementation of better management practices with a focus on pond preparation
- Use of approved quality immunostimulants
- Restriction of live shrimp movements
- Use of biofloc technology
- Disinfection of disease ponds as quickly as possible

- Implementation of surveillance, monitoring and proper reporting of all outbreaks
- Decrease in the stocking density (SD) to <100/m^2
- Increase in caution regarding the use of probiotics
- Use of appropriate water management to eliminate pathogens and their carriers
- Avoidance of probiotic over use during the first month post-stocking
- Maintaining pond water pH at 8.0$\pm$ 0.2
- Maintaining alkalinity at not lower than 100 mg/L (ppm)
- Maintaining DO at 4.0 mg/L at all times
- Maintaining consistent water color (phytoplankton)

There was considerable discussion and debate on the role of probiotics in AHPNS. It is widely believed that the use of probiotics is totally uncontrolled among shrimp farms. It was considered that the possibility of use of low quality, unapproved probiotics having a role in EMS/AHPNS could not be discounted. In view of this, caution was urged while using probiotics in shrimp ponds.

Preparedness and Response (refer to Presentation by Dr Ingo Ernst)

An aquatic animal disease incident would constitute an emergency if it could have significant impacts—either economic [production or trade], environmental or human health—and immediate response action might be needed to mitigate impacts and return industry to normal production and trade. Given the severe production impacts of AHPNS, its occurrence in a new country or region could be considered an emergency.

Should a disease emergency occur, the possible response actions would depend on the nature of the disease incident, for example: whether the disease occurred in closed or open systems, its distribution (e.g. restricted or widespread), existing knowledge on the disease (e.g. epidemiology), available tools (e.g. diagnostics), potential consequences, cost-benefit of response and technical feasibility. Each emergency response will differ, but basic response options include containment, eradication, and mitigation and management. Early responses provide more opportunities for effective response (e.g. eradication) and would usually deliver the highest return on investment in response activities. Activities that prevent a disease from entering a country or region are likely to provide the highest return on investment.

Where a disease becomes widespread in a country the opportunities for effective response become limited and mitigation and management of the disease impacts at an enterprise level may be the only way of managing the disease. Mitigation and management is likely to be the least cost-effective response option.

For countries where AHPNS occurs in a restricted distribution there may be opportunities to contain the disease (assuming it has an infectious etiology) to reduce its spread and limit impacts. For countries where AHPNS has not occurred the most cost-effective measures are likely to be those that prevent the entry of the disease—should it have an infectious etiology.

Emergency responses can be described by several response phases including disease freedom, alert, incident investigation, response and recovery. Each phase requires the activation of resources and processes to enable effective response actions. Some basic principles of an emergency response include:

- **Prevent** - program of risk reduction measures
- **Detect** - rapid detection and identification of the disease
- **Contain** - early implementation of control measures to prevent spread of the disease
- **Investigate** - rapid definition of the nature and extent of the outbreak
- **Decide** - decision on an appropriate response objective and plan
- **Respond** - marshal personnel and resources to implement the response plan
- **Recover** - undertake activities to return to production and trade

F. Group Discussion Findings

The issues identified by the groups and their recommendations were as follows:

1. Current Knowledge, Knowledge Gaps and Research Priorities

Group 1 sought to summarize the current state of knowledge on AHPNS, identify knowledge gaps and recommend research priorities, and to identify possible research networks and teams:

- Development of an information sheet summarising the gross signs of AHPNS was required as a priority to aid pond-side presumptive diagnosis. The gross signs of AHPNS were summarised as:
    - Often pale to white HP due to pigment loss in the connective tissue capsule.
    - Significant atrophy (shrinkage) of the HP.
    - Often soft shells and guts with discontinuous contents or no contents.
    - Black spots or streaks sometimes visible within the hepatopancreas.
    - Hepatopancreas does not squash easily between thumb and forefinger.
    - Onset of clinical signs and mortality starting as early as 10 days post stocking.
    - Moribund shrimp sink to the bottom.
- The case definition for AHPNS proposed by Prof Lightner was generally agreed on:
    - Idiopathic – no specific disease causing agent (infectious or toxic) has been identified.
    - Pathology: Acute progressive degeneration of the hepatopancreas from medial to distal region with dysfunction of B, F, R & E-cells, prominent karyomegaly and necrosis and sloughing of

these tubule epithelial cells. Terminal stage shows marked inter- and intra-tubular hemocytic inflammation and development of secondary bacterial infections that occur in association with necrotic and sloughed HP tubule cells.

- The immediate research priority was to identify the cause of AHPNS:
    - A robust challenge study is required to determine if AHPNS is transmissible and to fulfil Koch's-River's postulates (for example using non-frozen material by the oral route or by reverse gavage using filtered and unfiltered homogenates and by using water/sediment exposure.
    - Cohabitation challenge should be considered using affected shrimp cohabitated with non-affected SPF shrimp
    - Examination of cohabiting pond decapod species to determine if any other species are affected or can act as carriers.
    - If evidence of transmission is found and the pathogen remains elusive, consider using molecular tools such as pyrosequencing, subtractive hybridisation libraries and computational subtraction to search for genome sequences of cryptic pathogens.
- A thorough and robust epidemiological study is a priority that needs to consider a range of parameters including abiotic, edaphic and climatic, etc.
    - Fit available data retrospectively and make a predictive model based on data to date? Farm infectivity rates etc.
    - Develop a risk model based on existing information?
- The toxico-pathology should be investigated (biotic and abiotic toxins should be considered)
- As a lower priority, investigate moulting frequency and possible factors disrupting moulting due to damage to the hepatopancreas and hence soft and loose shells.
- Investigate the possibility of immune-deficiencies using genetic markers.
- Overall, a more forensic approach to the investigation of AHPNS is required, using a chain-of-evidence approach to separate facts from fiction.
- Countries could consider combining available resources for investigating AHPNS to avoid duplication of effort. A joint program would help create consensus on the best scientific approaches, develop the best team drawing on experts from different countries, and facilitate coordination of remedial or regulatory action, inspections etc across jurisdictions. Such a program could be coordinated by NACA or a similar regional mechanism, and overseen by an international steering committee.
- NACA is well placed to act as a clearing house for information, communication of R&D outcomes or establishing a community dialogue hub for researchers to share experience on AHPNS, for example through email listserve, wikis, social media, the web etc.
- Recommend to investigate the possibility of convening a biannual meeting for researchers to share experiences and research outputs on AHPNS.

- Recommend that NACA lobby to establish a joint/community funding pool shared between countries to address common objectives and facilitate exchange of histology slides and other materials between researchers.
- Important to begin to responding to AHPNS now based on the limited information that is available, rather than to wait for more information to come to light.

2. Detection, reporting and surveillance

Group 2 considered issues relating to the detection, reporting and surveillance for AHPNS:

- Surveillance needs to consider the capacity, desire (may be related to the availability of compensation) and resources available in a country, as well as the perceived level of threat.
- Surveillance needs to consider the capacity, desire (may be related to the availability of compensation) and resources available in a country, as well as the perceived level of threat.
- Criteria for identifying a suspected AHPNS outbreak at the pond / farm level were proposed:
    - Known affected country/area (highly specific criteria are desirable)
        - >10 dead shrimp/day (or expressed as a percentage of shrimp in the pond)
        - <30days (but later can change)
        - Gross signs (hepatopancreas white/small and resists compression)
    - Country/ area not known to be affected (highly sensitive criteria are desirable).
        - >2 dead shrimp/day
        - <40days (but later can change)
        - Gross signs (hepatopancreas white/small and difficult to squeeze)
    - Pond (confirmed): at least one positive shrimp meeting the case definition proposed by Dr Lightner
    - Farm (confirmed): at least one confirmed positive pond
- When collecting data about an affected pond or farm, data should also be collected from unaffected 'control' ponds or farms without "unusual" mortalities where possible for comparison.
- Recommend developing a sampling kit for the benefit of government officers, including guidance/photographs on selection of ponds and collection of samples. The kit should include standardized data collection sheets to ensure that required information is collected, including data on likely risk factors. The kit should include access to fixatives such as Davidson's (ideally) or formalin.
- Recommend developing an information kit (flyer etc) for farmers including guidance on gross signs and control measures.
- Develop a national/international list of reference laboratories that have the capacity to diagnose AHPNS, and web sharing mechanism for pathology and reference slides.

- Encourage affected countries to provide reports and epidemiological information to existing regional/international reporting mechanism (eg. OIE or NACA QAAD program) including information on changes in the distribution or behavior of the disease.

3. Biosecurity, emergency response and disease management

Group 3 considered issues related to biosecurity, emergency response and disease management, including:

- Necessary measures to reduce the risk of AHPNS spreading (if considered an infectious aetiology).
- Guidance on response actions in the event of new detections of AHPNS affected (including outbreak investigation).
- Measures to manage the impact of AHPNS in endemic areas.

Measures to reduce the risk of AHPNS spreading:

- Control transboundary movement of live shrimp particularly from affected areas to unaffected areas.
- Zoning of affected countries and restriction of movement from affected areas to unaffected areas, creation of a buffer zone to be monitored, tracking of live shrimp moved from affected areas within a specified time (1-2 months?) and followed up by surveillance.
- There is a concern on the importation of commodity shrimp for reprocessing from affected areas into free areas
- Treatment of outbreak ponds before the release of the water.
- Implementation of adequate biosecurity at harvest and post-harvest in affected countries/areas including harvest, effluent and solid waste from processing plants.
- Capacity building of national reference laboratories for AHPNS diagnosis (also service labs if available).

While information is required to make some of these decisions, waiting for 'enough' information may be too late. Better to start reacting now. Specific areas of research required to support control measures are:

- Transmission trials at pond level.
- Study of possible pond-related trigger factors (toxins?).
- Identification of possible sources: Broodstock and postlarvae, possible carriers including wild aquatic animals, plants and phytoplankton etc.
- Risk factors (eg. soil versus lined ponds, fresh water versus sea water etc).
- Economic impact of the disease, to help raise awareness of the need to address AHPNS and invest in research and control measures by all stakeholders.

Development of an AHPNS awareness program was suggested to:

- Disseminate findings of scientifically reliable research studies undertaken by institutes/private companies.
- Involve the private sector including farmer associations and corporations.
- Disseminate concern on probiotic use (quality and quantity) and forestall a possible jump into the alternative of antibiotic use, which is unlikely to provide a solution.
- Disseminate information on the economic impact of the disease.

Establishment of an international task force to coordinate and direct efforts was suggested to consolidate information on AHPNS across countries (globally FAO, regionally NACA). Confidentiality would have to be assured for the task force to function, and the group should also seek to gather information from local producers, as the main source of primary data.

4. National / regional disease response

Group 4 addressed issues relating to a coordinated national and regional/international response to AHPNS. The group sought to:

- Document lessons learned in dealing with emergencies at the national and regional level, including lessons learned in:
    - Moving the decision making scale from local to national responses.
    - Governance and decision making around high profile economic / trade issues.
- Recommend priorities for strengthening national and regional responses (e.g. access to expertise, resources, emergency funds).
- Discuss existing mechanisms and the role of regional and international organisations and past experiences.

Overall it was felt that capabilities and arrangements for responding to disease emergencies across the region had improved considerably in recent years. The apparent containment of IMNV to Indonesia suggests that national biosecurity arrangements are stronger than they once were.

The key lessons learned were as follows:

- Greater emphasis should be placed on improving preventative measures, as this is the most cost-effective point to deal with AHPNS
- Vietnamese experience with AHPNS suggests that it is important to activate a national response group as early as possible. In the Vietnamese example, a task force is responsible for:
    - Facilitating communication and cooperation across ministries and provinces.
    - Preparing situation reports.
    - Coordinating research across institutes.
    - Determining research priorities and budgetary requirements.

- While there has been investment of resources by governments and IGOs, the reaction has been too slow and some actions, such as outbreak investigation, might not have been undertaken in the best way.
- The development of a pond or farm level case definition is key to surveillance and monitoring programs. However, the case definition needs to be based on a sufficient number of cases.
- Thorough investigation of outbreaks is important and requires an appropriate approach supported by appropriate skill sets such as epidemiology. Skills requirements may change over time as the investigation evolves.
    - The development of documents providing guidance on investigation procedures would be useful, particularly where non-specialists are involved.
- Rapid access to resources is required to support timely deployment of investigation or response teams.
    - OIE and FAO already provide resources in some circumstances.
    - FAO can assist rapidly, but requires a formal approach to be made by national governments. NACA could approach FAO on behalf of member governments with their approval.
- Development of a regional response fund could encourage regional communication and cooperation at a senior level, and perhaps provide motivation to report disease issues earlier.
    - There is a need to support countries that do not have resources necessary to address a disease emergency.
    - A fund would need adequate governance mechanisms without restricting the ability to allocate resources quickly.
- There are some issues with coordination within countries, and it is important to engage with the correct contact points and ensure effective communication with IGOs.
- Availability of expertise in aquatic animal health is a concern, particularly with regards to histopathology and epidemiology. There is a need for succession planning as many available experts are close to retirement, and a need to provide training on an ongoing basis to maintain capacity.
- Reporting of disease through the current QAAD system is working and participation is improving. However there is sometimes a reluctance at the level of industry and government to share information, which can result in significant delays in responding to new diseases and slow recognition of disease severity.
    - There seems to be a gap between on-farm events and national/regional reporting.
- Accelerating the speed of information sharing would be beneficial, as information flow and recognition of disease severity takes time.
- The preparation of disease advisory cards as per IMNV and AHPNS examples is beneficial, but cards need to be circulated faster and more widely.
- NACA may be well placed to solicit information (e.g. pro forma situation reports) to support investigation of disease emergencies.

G. Workshop conclusions, recommendations and way forward

Case Definition: Development of a shrimp-level case definition for AHPNS by Prof Lightner is a breakthrough that will help direct and progress future research on this distinctive disease as opposed to other varied causes of early mortalities often seen in poorly managed farms. It is strongly suggested this case definition be considered as essential for all future epidemiological studies, outbreak investigations and management, diagnosis and laboratory-based research to discover the cause of AHPNS.

Presumptive Diagnosis: Gross clinical signs of AHPNS have been more or less consistent and it is suggested that they be used at the farm/pond level for presumptive diagnosis for confirmation of animal-level case definition histopathology. It was recommended that NACA develop a disease card with case definitions and appropriate pictures and disseminate this widely across member countries to increase awareness and support surveillance efforts.

Harmonization: Rigorous use of the animal-level and pond-level case definitions would enable direct comparison of data across AHPNS-affected countries, and also help to better describe new suspect cases in AHPNS-affected countries and unaffected countries potentially at risk.

Diagnosis: AHPNS pathology, in particular the progression of lesions from proximal to distal regions of the hepatopancreas in the absence of pathogens is suggestive of a toxic etiology, while the nature of its spread is suggestive of infectious etiology. At this stage, the primary cause is unknown, and the possibility of an infectious agent and/or toxin cannot be discounted. In view of this, research efforts should focus on all possible causes of AHPNS and on confirmation through robust challenge studies.

Epidemiology and Risk Factors: The consultation recognized that robust epidemiological studies have not been conducted so far in any country, even though a considerable amount of primary and secondary data have been gathered and preliminary epidemiological methods applied to identify potential risk factors. The consultation strongly recommended that a regionally-coordinated (e.g. by NACA, OIE Collaborating Centre ERAAD) epidemiological study be undertaken to better understand risk factors and disease spread so as to develop predictive models.

Capacity Building: The consultation recognized that the availability of all-round expertise (e.g. Prof Don Lightner and Prof Tim Flegel) to deal with emerging disease situations in the region will decrease as senior people retire. The need for succession planning and developing necessary skill sets and expertise to respond to disease emergencies should be taken up on high priority and NACA has agreed to continue a process of consultation with experts as part of a wider updating review of present and future capacities to provide appropriate responses as suggested by the Consultation. At the national and regional levels, expertise in

shrimp disease outbreak investigation, epidemiology and histopathology should be further developed and strengthened.

Local, National and Regional Response mechanisms and increased effort in capturing lessons learned: e.g. Better Organized and Coordinated Research: It was agreed in the workshop discussions based on the approaches taken by different AHPNS affected countries that it is becoming clear that each country and often separate responsible organizations in each country are developing responses and spending separate budgets on identifying key issues and there is limited information exchange and coordination both within and amongst affected countries. More effective pooling, particularly of human and financial resources within countries and regionally through mechanisms for both national and regional coordination can be improved and making more effective use of regional bodies such as NACA could result in more cost effective outputs and outcomes. Such approaches could also create wider consensus based thinking incorporating the best scientific approach to follow and the more effective team delivery mechanisms. For example, oversight could be provided by regional/international experts via more broadly based steering Committee approaches. Overall deliverables could be monitored and knowledge generated more effectively and shared amongst all members.

Knowledge Sharing and Communication: Communication and information sharing was recognized as very important constraint in terms of more effective responses to new/emerging diseases. It was suggested that NACA orchestrate a community dialogue hub for AHPNS for all researchers to share experiences (web-based, List-serve, Wiki, Facebook etc.), seek to host biannual meetings for researchers and other key stakeholders to share experiences and research outputs, as well as providing a platform for simple steps such as promoting the wider exchange of key research materials and exchange of histopathology slides.

Regional Emergency Fund: Considering the importance of ready access to funds to rapidly respond to disease emergencies, it was suggested that NACA and its partners seek to develop a community emergency fund mechanism that can be accessed by all member countries in the face of an aquatic animal disease emergency. Such funds could be used for fielding a rapid emergency mission to affected countries, commission a systematic outbreak investigation and for developing project/funding proposals to address the emergency. NACA Asia Regional Advisory Group on aquatic animal health and the NACA Governing Council could provide the overseeing and monitoring role for operation of such an emergency fund.

Annex 1: Disease Advisory

Asia Regional Aquatic Animal Health Programme

DISEASE ADVISORY

Early Mortality Syndrome (EMS)/Acute Hepatopancreatic Necrosis Syndrome (AHPNS): An emerging threat in the Asian shrimp industry

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The Asia-Pacific region, being the top producer of aquaculture products in the world, is continuously beset by emerging aquatic animal disease problems causing high mortalities and economic losses among small farmers as well as commercial producers. Over the last couple of decades, several diseases (e.g. luminous vibriosis, white spot syndrome, yellowhead disease, Taura syndrome) have caused significant devastation in the shrimp aquaculture of the region, causing the collapse of some industries (e.g. *Penaeus monodon*). Recently, a new/emerging disease known as early mortality syndrome (EMS) in shrimp (also termed acute hepatopancreatic necrosis syndrome or AHPNS) has been reported to cause significant losses among shrimp farmers in China (2009), Vietnam (2010) and Malaysia (2011). It was also reported to affect shrimp in the eastern Gulf of Thailand (Flegel, 2012).

The disease affects both *P. monodon* and *P. vannamei* and is characterized by mass mortalities (reaching up to 100% in some cases) during the first 20-30 days of culture (post-stocking in grow-out ponds). Clinical signs observed include slow growth, corkscrew swimming, loose shells, as well as pale coloration. Affected shrimp also consistently show an abnormal hepatopancreas (shrunken, small, swollen or discouloured). The primary pathogen (considering the disease is infectious) has not been identified, while the presence of some microbes including *Vibrio*, microsporidians and nematode has been observed in some samples. Lightner et al. (2012) described the pathological and etiological details of this disease. Histological examination showed that the effects of EMS in both *P. monodon* and *P. vannamei* appear to be limited to the hepatopancreas (HP) and show the following pathology:

1) Lack of mitotic activity in generative E cells of the HP;
2) Dysfunction of central hepatopancreatic B, F and R cells;
3) Prominent karyomegaly and massive sloughing of central HP tubule epithelial cells;
4) Terminal stages including massive intertubular hemocytic aggregation followed by secondary bacterial infections.

Similar histopathological results were obtained by Prachumwat et al. (2012) on Thai samples of *P. vannamei* collected from Chantaburi and Rayong provinces in late 2011 and early 2012 (Figure 1). The progressive dysfunction of the HP results from lesions that reflect degeneration and dysfunction of the tubule epithelial cells that progress from proximal to distal ends of HP tubules. This degenerative pathology of HP is highly suggestive of a toxic etiology, but anecdotal information suggests that disease spread patterns may be consistent with an infectious agent.

In China, the occurrence of EMS in 2009 was initially ignored by most farmers. But in 2011, outbreaks became more serious especially in farms with culture history of more than 5 years and those closer to the sea using very saline water of 20 (Panakorn, 2012). Shrimp farming in Hainan, Guangdong, Fujian and Guangxi suffered during the first half of 2011 with almost 80% losses

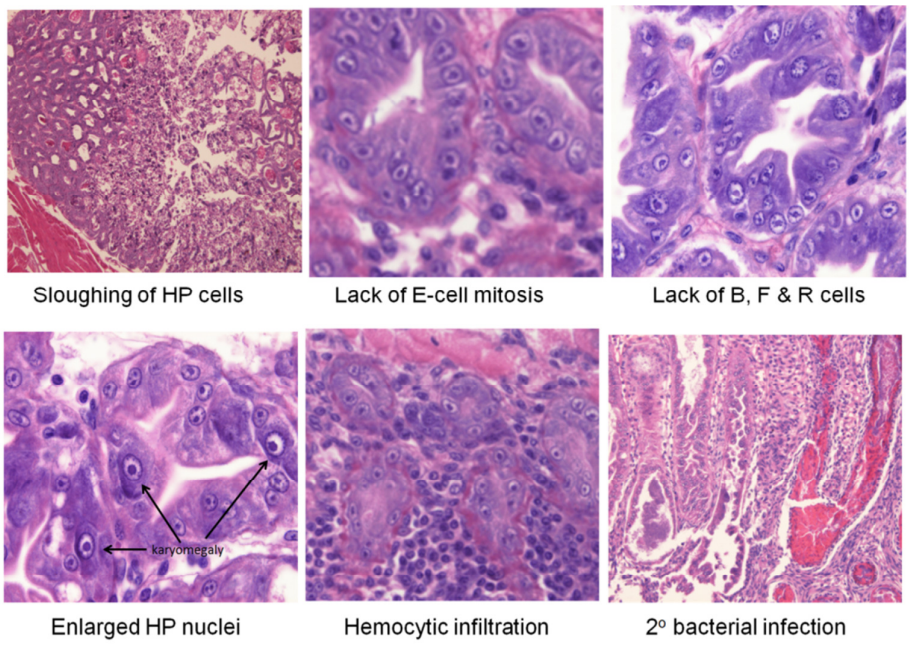

Figure 1. Histopathology of *Penaeus vannamei* hepatopancreas from Thailand affected by EMS/AHPNS. Photos courtesy of T.W. Flegel.

In Vietnam, the disease has been observed since 2010 but the most widespread devastation due to EMS has only been reported since March 2011 in the Mekong Delta (South Vietnam). It affects the main shrimp production areas of Tien Gang, Ben Tre, Kien Giang, Soc Trang, Bac Lieu and Ca Mau provinces with a total shrimp pond area of around 98,000 hectares. In June 2011, unprecedented losses in *P. monodon* were reported in 11,000 ha of shrimp farms in Bac Lieu, 6,200 ha in Tra Vinh (total of 330 million shrimp have died causing a loss of over VND12 billion), and 20,000 ha in Soc Trang (causing VND1.5 trillion in losses) (Mooney, 2012).

In Malaysia, EMS was first reported in mid-2010 in the east coast of peninsular states of Pahang and Johor. The outbreaks of EMS resulted in the significant drop in *P. vannamei* production, from 70,000 mt in 2010 to 40,000 mt in 2011. Production for 2012 (up to May) is only 30,000 mt and worse is expected to come as unconfirmed reports on EMS outbreaks in the states of Sabah and Sarawak came in April 2012.

So far no potential causative pathogen has been found and possible etiologies include toxins (biotic or abiotic), bacteria and viruses (NACA-FAO 2011). Nonetheless, the spread of the disease and its devastating effect in the shrimp industry of the countries affected so far, will require proper contingency planning in other countries in the region, especially in *P. vannamei* culture which is commonly cultivated at present in many Southeast Asian countries. Added to this is the standing threat of infections myonecrosis (IMN) on *P. vannamei* culture, which is now somehow contained within Indonesia. Rumors of disease outbreaks caused by IMNV from other countries in Asia have so far been false (Senapin et al., 2011). With Vietnam suffering the greatest loss due to EMS outbreak, the Food and Agriculture Organization of the United Nations (FAO) undertook an emergency mission in 2011 to assess the disease situation in the country, in collaboration with national as well as international shrimp health experts. As a follow-up on this emergency mission, FAO also developed a national TCP on emergency assistance to control the spread of this shrimp disease. Implementation of the national TCP in Vietnam has commenced in April 2012.

Identifying the primary cause of the disease is necessary, but while this information is still not yet available, increased disease awareness and preparedness should be implemented by every shrimp-producing country in the region. Considering the great economic loss that EMS will cause in the region's shrimp industry, ways of preventing the spread and/or occurrence of this disease should be formulated by

concerned experts, officials and other regulatory bodies. Farmers, on the other hand, should also properly cooperate with the concerned agencies by promptly reporting any suspected mortalities among cultured shrimp that appear to be similar to the clinical description of EMS/AHPNS. It is important that histological examination be carried out to confirm that suspected occurrences fit the AHPNS case definition devised by Dr. Lightner.

The purpose of this short communication is to inform all NACA members of the emerging threat and request respective Competent Authorities (CA) and concerned stakeholders to increase surveillance and reporting efforts. Only through surveillance, early response, contingency planning and disease preparedness, can countries minimize the impact of the impending threat. NACA Secretariat will approach the CA of the four member governments currently affected by EMS to put up a multi-disciplinary team of experts to understand more about the disease and develop contingency measures to prevent its further spread in the region.

NACA will greatly appreciate receiving any relevant information pertaining to EMS/AHPNS from all member countries in the region. Information can be sent by e-mail to the authors at eduardo@enaca.org and mohan@enaca.org.

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Annex 2: Prospectus

Australian Government
Department of Agriculture, Fisheries and Forestry

ASIA PACIFIC EMERGENCY REGIONAL CONSULTATION ON SHRIMP EARLY MORTALITY SYNDROME (EMS)/ ACUTE HEPATOPANCREATIC NECROSIS SYNDROME (AHPNS)

9-10 August 2012
Bangkok, Thailand

Prospectus

Rationale

The Asia-Pacific region, being the top producer of aquaculture products in the world, is continuously beset by emerging aquatic animal disease problems causing high mortalities and economic losses among small farmers as well as commercial producers. Recently, a new/emerging disease known as early mortality syndrome (EMS) in shrimp (also termed acute hepatopancreatic necrosis syndrome or AHPNS) has been reported to cause significant losses among shrimp farmers in China (2009), Vietnam (2010) and Malaysia (2011). It was also reported to affect shrimp in the eastern Gulf of Thailand (Flegel, 2012)[1]. Outbreaks in Vietnam and Malaysia have caused severe economic losses and significantly lowered annual shrimp production.

The disease affects both *P. monodon* and *P. vannamei* and is characterized by mass mortalities (reaching up to 100% in some cases) during the first 20-30 days of culture (post-stocking in grow-out ponds). Clinical signs observed include slow growth, corkscrew swimming, loose shells, as well as pale coloration. Affected shrimp also consistently show an abnormal hepatopancreas (shrunken, small, swollen or discolored). The primary pathogen (considering the disease is infectious) has not been identified, while the presence of some microbes including *Vibrio*, microsporidians and nematode has been observed in some samples. Lightner et al. (2012)[2] described the pathological and etiological details of this disease. Histological examination showed that the effects of EMS in both *P. monodon* and *P. vannamei* appear to be limited to the hepatopancreas (HP) and show the following pathology:

1) Lack of mitotic activity in generative E cells of the HP;
2) Dysfunction of central hepatopancreatic B, F and R cells;

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[1] Flegel, T.W. 2012. Historic emergence, impact and current status of shrimp pathogens in Asia. Journal of Invertebrate Pathology 110:166-173.

[2] Lightner, DV, Redman, RM, Pantoja, CR, Noble, BI, Tran, L. 2012. Early mortality syndrome affects shrimp in Asia. Global Aquaculture Advocate, January/February 2012:40.

3) Prominent karyomegaly and massive sloughing of central HP tubule epithelial cells;
4) Terminal stages including massive intertubular hemocytic aggregation followed by secondary bacterial infections.

So far no potential causative pathogen has been found and possible etiologies include toxins (biotic or abiotic), bacteria and viruses (NACA-FAO 2011)[3]. Nonetheless, the spread of the disease and its devastating effect in the shrimp industry of the countries affected so far, will require proper contingency planning in other countries in the region, especially in *P. vannamei* culture which is commonly cultivated at present in many Southeast Asian countries. Added to this is the standing threat of infections myonecrosis (IMN) on *P. vannamei* culture, which is now somehow contained within Indonesia. Rumors of disease outbreaks caused by IMNV from other countries in Asia have so far been false (Senapin et al., 2011)[4].

Identifying the primary cause of the disease is necessary, but while this information is not yet available, increased disease awareness and preparedness should be implemented by every shrimp-producing country in the region. Considering the great economic loss that EMS will cause in the region's shrimp industry, ways of preventing the spread and/or occurrence of this disease should be formulated by concerned experts, officials and other regulatory bodies. Farmers, on the other hand, should also properly cooperate with the concerned agencies by promptly reporting any suspected mortalities among cultured shrimp that appear to be similar to the clinical description of EMS/AHPNS. It is important that histological examination be carried out to confirm that suspected occurrences fit the AHPNS case definition devised by Dr. Lightner.

Considering the seriousness of this emerging shrimp disease, NACA and DAFF are convening this regional consultation involving global experts, national participants representing the Competent Authority and lead research institutions, regional and international organizations and private sector with the following objectives.

Objectives

This regional consultation will:

a) Provide an overview of the current disease and its spread, with emphasis on the threat that it poses in the shrimp industry of the region;
b) Assess the economic effects of the disease: outbreaks in China, Vietnam, Malaysia and Thailand;
c) Identify any similar occurrences in other countries in the region;
d) Develop a field level disease card and case definition as easy reference in monitoring the occurrence of the disease;
e) Formulate a regional action plan – improved disease surveillance and reporting, and contingency measures to contain and prevent further spread of the disease;
f) Develop or plan collaborative research on EMS/AHPNS, inter-regionally and internationally, to identify the primary causative agent, develop preventive measures, etc.; and,
g) Formulate other regulatory measures for overall management of the disease.

[3] NACA-FAO 2011. Quarterly Aquatic Animal Disease report (Asia and Pacific Region), 2011/2, April-June 2011. NACA, Bangkok, Thailand.

[4] Senapin, S., Phiwsaiya, K., Gangnonngiw, W., Flegel, T.W. 2011. False rumours of disease outbreaks caused by infectious myonecrosis virus (IMNV) in the whiteleg shrimp in Asia. Journal of Negative Results in BioMedicine. 10: 10

Participants

Participants will be representatives from shrimp producing countries of the region including:

- NACA member countries
- ASEAN member countries
- Private sector

International and regional experts, including OIE experts, will be invited to make presentations and facilitate series of discussions pertaining to the disease.

Process

The workshop will include detailed lecture on the description of the new disease including gross signs, histopathological characteristics, production losses, suspected pathogens/causative agent, etc. An open discussion will follow the lecture so that the participants will have more insights on the importance of the disease. This will be followed by presentations on disease outbreak cases in China, Vietnam, Malaysia and Thailand. Similar cases observed in other countries (if any) will then be tackled in the discussion/forum.

Group discussions on various key issues will be facilitated by experts to develop recommendations and follow up actions. A disease card will be developed for wider dissemination as first-hand reference for the disease. Formulation of important regulations to contain and prevent the spread of the disease will be of high importance. Finally, collaborative research will be planned to pin point the primary causative agent of the disease, which is necessary for the development of prevention and control measures.

Expected Outputs

At the end of the two-day workshop, the following outputs are envisaged:

- Increased awareness on EMS/AHPNS;
- Field level Disease Card and case definition for EMS/AHPNS developed for publication and dissemination to shrimp-producing sectors in the region;
- Development of a template for outbreak investigation
- Regional action plan on emergency response and contingency planning developed;
- Surveillance, monitoring and reporting of EMS/AHPNS outbreaks improved;
- Collaborative research to identify the primary causative agent and development of preventive and control measures planned/developed.

Information Dissemination

Workshop outputs will be circulated to national stakeholders, regional and international organizations and made available on NACA website for free download

Annex 3: List of participants

Emergency Regional Consultation on EMS/AHPNS
9-10 Aug 2012, Bangkok, Thailand

List of Participants

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Annex 4: Provisional Agenda

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ASIA PACIFIC EMERGENCY REGIONAL CONSULTATION ON SHRIMP EARLY MORTALITY SYNDROME (EMS)/ ACUTE HEPATOPANCREATIC NECROSIS SYNDROME (AHPNS)

9-10 August 2012
Bangkok, Thailand

Provisional Agenda

8 August 2012 - Arrival of Participants		
Day 1: Thursday, 9 August, 2012		
0830-0900h	Registration	
0900-0915h	Formal Opening Session • Opening Welcome Remarks by Dr Ambekar Eknath, DG, NACA • Opening Welcome Remarks by Dr Ingo Ernst, DAFF, Australia	
0915-1000h	Background, Objectives, Structure and Expected Outputs by Dr CV Mohan (NACA) and Dr Ingo Ernst (DAFF) Introduction of Participants Announcements and Local Logistics Group Photo	
1000-1030h: Coffee Break		
	Moderators: Dr Ingo Ernst and Dr CV Mohan	
1030-1050h	Characterization, Distribution, Impacts and Case Definition By Prof Don Lightner	
1050-1110h	Research Progress on Bacterial and Viral Causes of AHPNS By Prof Tim Flegel	
1110-1130h	Q&A Session	
1130-1150h	Disease Emergence –Why and How? Prof Peter Walker	
1150-1210h	Novel Methods for "Hunting for Ghost Viruses" by Dr Jeff Cowley	
1210-1230h	Q&A Session	
1230-1330h: Lunch Break		
1330-1350h	Epidemiology and Risk Factors-What We Know? By Dr Flavio Corsin	
1350-1410h	Is EMS a Management Problem? By Dr Matt Briggs	
1410-1430	Q&A Session	
1430-1450h	Experiences from Vietnam by National Team	

1450-1510h	Experiences from China by National Team	
1510-1530h	Q&A Session	
1530-1600h: Coffee Break		
1600-1620h	Experiences from Thailand by National Team	
1620-1640h	Experiences from Malaysia by National Team	
1640-1700h	Q&A Session	
1700-1730h	Experiences from Other Countries (if any)	
1730-1800h	General Discussions and Wrap Up for the day	
1830-2000h	WORKSHOP DINNER	
Day 2: 10 August, 2012		
	Moderators: Dr Jeff Cowley and Dr Ed Leano	
0900-0920h	One Month Mortality Syndrome-Revisiting an old story by Dr Celia Pitogo	
0920-0940h	Management of EMS-What Works and What Does Not? By Prof Chalor Limsuwan	
0940-1000h	Disease Preparedness-Theory and Practice. What Have We Learnt? By Dr Ingo Ernst	
1000-1020h	Q&A Session	
1020-1030h	Group Discussion Themes and Expected Outputs By Dr Ingo Ernst and Dr CV Mohan	
1030-1045h: Coffee Break		
1045-1300h	Group 1: Current Knowledge, Knowledge Gaps and Research Priorities Team: Dr Don Lightner, Dr Tim Flegel, Dr Huang Jie, Dr Jason Weeks Group 2: Detection, Reporting and Surveillance Team:, Dr Flavio Corsin, Dr Ian Gardner, Dr Jeff Cowley, Dr Celia Pitogo Group 3: Biosecurity, Emergency Response and Disease Management Team: Dr Larry Hammell, Dr Matt Briggs, Dr Victoria Alday, Dr Ed Leano Group 4: Regional Disease Response Team: Dr Ingo Ernst, Dr Brian Davy, Dr Peter Walker, Dr Supranee Chinabut	
1300-1400h: Lunch Break		
	Moderator: Dr Supranee Chinabut and Dr Peter Walker	
1400-1530h	Presentations of Group Findings and Discussions	
1530-1600h: Coffee Break		
	Moderators: Dr Ambekar Eknath, Dr Ingo Ernst, Dr Tim Flegel, Dr Don Lightner	
1600-1700h	Plenary Discussions, Recommendations and Follow up Actions	
1700-1730h	Closing Formalities • Closing Remarks by Dr Ingo Ernst, DAFF, Australia • Closing Remarks by Dr Ambekar Eknath, DG, NACA	
11 August, 2012 – Departure of Participants		

Annex 5: AHPNS Technical Presentations

Australian Government
Department of Agriculture, Fisheries and Forestry

Asia Pacific Emergency Regional Consultation on EMS/AHPNS

Background, Objectives, Structure and Expected Outputs

CV Mohan (NACA) and Ingo Ernst (DAFF)

Background

- New/emerging disease
  - Early mortality syndrome (EMS) in shrimp
  - Also termed acute hepatopancreatic necrosis syndrome or AHPNS
- Reported to cause significant losses
- China (2009), Vietnam (2010), Malaysia (2011) and eastern Gulf of Thailand (2012)
- Affects both *P. monodon* and *P. vannamei*
- Characterized by significant mortalities during the first 20-30 days of culture (post-stocking in grow-out ponds)

Background

- Considerable research work done in the region and outside the region
- BUT, so far potential causative pathogen has NOT been described
- From the nature of spread, EMS appears to be infectious??
- NACA Asia Regional Advisory Group on aquatic animal health recognized EMS as an emerging problem in its 10th AGM in 2011and called for increased surveillance and reporting from member governments in the region

Rationale

- Considering the importance of this emerging problem to EMS affected countries and countries that are potentially at risk
  - EMS disease advisory was sent to Competent Authorities and lead shrimp researchers of all member countries and widely disseminated
  - In addition, EMS circular sent to all governments, funding agencies and regional/international organizations seeking support to convene a regional consultation
- Considering the seriousness of the problem and its potential impact, DAFF Australia came forward to support this 2 day consultation
- OIE is supporting participation of two OIE Experts

Primary Objectives

- Bringing together global experts, national participants representing the CA and lead research institutions, regional and international organizations and private sector
- Facilitating networking and information sharing for better understanding and dealing with EMS
- Documenting the current state of knowledge on EMS and lessons learned in dealing with disease emergencies at the national/regional levels
- Agreeing on a regional action plan for dealing with future aquatic disease emergencies in the region

Specific Objectives

- Provide an overview of the current disease situation and its spread, with emphasis on the threat that it poses to the shrimp industry of the region
- Situation analysis of outbreaks in China, Vietnam, Malaysia and Thailand
- Identify any similar occurrences in other countries in the region
- Develop guidance for future surveillance work by providing a field level disease card, case definition and outbreak investigation template
- Develop or plan collaborative research on EMS/AHPNS, intra-regionally and internationally, to identify the primary causative agent, risk factors and develop management interventions including preventive measures
- Formulate a regional action plan to improve disease surveillance and reporting, and contingency measures to contain and prevent further spread of the disease;

Participation

- 16 Resource experts
- About 40 national participants representing the CA and lead research institutions from NACA member countries
- 10 leading private sector representatives
- 8 regional/international organizations
- 8 Post graduate researchers

Consultation Structure

- Presentations by resource experts
- Sharing of country experiences
- Working group discussions
- Working group presentations
- Plenary session to develop recommendations and follow up actions

Working Groups

- Group 1: Current Knowledge, Knowledge Gaps and Research Priorities
  - Team: Dr Don Lightner, Dr Tim Flegel, Dr Huang Jie, Dr Jason Weeks
- Group 2: Detection, Reporting and Surveillance
  - Team:, Dr Flavio Corsin, Dr Ian Gardner, Dr Jeff Cowley, Dr Celia Pitogo
- Group 3: Biosecurity, Emergency Response and Disease Management
  - Team: Dr Larry Hammell, Dr Matt Briggs, Dr Victoria Alday, Dr Ed Leano
- Group 4: Regional Disease Response
  - Team: Dr Ingo Ernst, Dr Brian Davy, Dr Peter Walker, Dr Supranee Chinabut
- Each group to have a maximum of 22 members
- Kindly fill in your names for the groups you are interested

Scope of the consultation

- This is not a unique circumstance. Serious aquatic animal diseases will continue to emerge in aquaculture
- While this workshop is about EMS, we will discuss broader issues about how response to serious aquatic animal diseases are managed regionally
- We won't be able to address all of those broader issues but we should try to capture the major points and possible solutions.

Expected Outputs

- Increased awareness on EMS/AHPNS
- Current state of knowledge documented
- Field level Disease Card, case definition and outbreak investigation template produced
- Collaborative research to identify the primary causative agent and development of preventive and control measures planned/developed
- Surveillance, monitoring and reporting of EMS/AHPNS outbreaks improved
- Lessons learned in dealing with disease emergencies at the national/regional level documented
- Regional action plan on emergency response and contingency planning developed
- Report of the Regional Consultation as a NACA/DAFF 2012 document for wider dissemination (draft outline provided)

Thank you....

Visit us at www.enaca.org for more details

Documentation of a "New" Disease (Early Mortality Syndrome) in South China in 2010 & Vietnam in 2011 & 2012

D.V. Lightner, R.M. Redman, C.R. Pantoja,
B.L. Noble, L.M. Nunan and Loc Tran
OIE Reference Laboratory for Shrimp Diseases
Department of Veterinary Science & Microbiology
The University of Arizona, Tucson, AZ, USA

THE UNIVERSITY OF ARIZONA

OIE

1

Early Mortality Syndrome

A more descriptive name is:

AHPNS

for Acute Hepatopancreatic Necrosis Syndrome

Topics in this presentation:

- Samples & observations from South China, August/September 2010.
- Samples from Me Kong Delta & South Vietnam, July 2011, December 2011 & July-August 2012.
- Brief results of studies on:
  - Feed from affected farms.
  - Toxicity studies with crustacides.
  - Infectivity studies using frozen samples from affected farms.

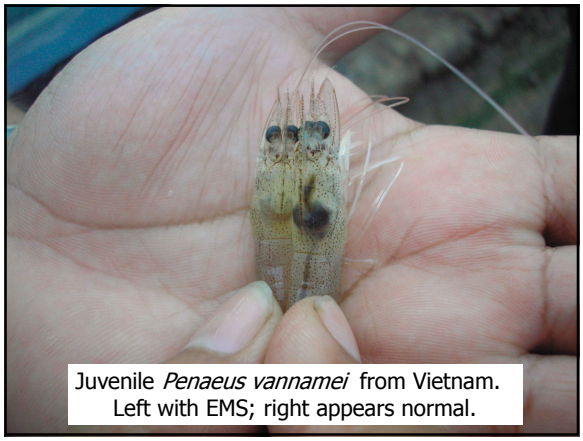
3

Gross Signs of EMS/AHPNS

- Significant atrophy of HP.
- Often pale to white within HP connective tissue capsule.
- Black spots or streaks sometimes visible.
- HP does not squash easily between thumb & finger.

4

Juvenile *Penaeus monodon* from Vietnam with EMS. The HP is pale & atrophied; the midgut is empty except for sloughed HP cells.



Juvenile *Penaeus vannamei* from Vietnam. Left with EMS; right appears normal.

Juvenile *Penaeus vannamei* from Vietnam.
Both with atrophied HP indicative of EMS.

Juvenile *Penaeus vannamei* from Vietnam.
Both show pale or atrophied HP indicative of EMS.

Juvenile *Penaeus monodon* from Vietnam.
Left shrimp appears normal (except for black gills), while right 2 show dark, atrophied HPs typical of EMS.

9

Histopathology shows two lesions of the HP

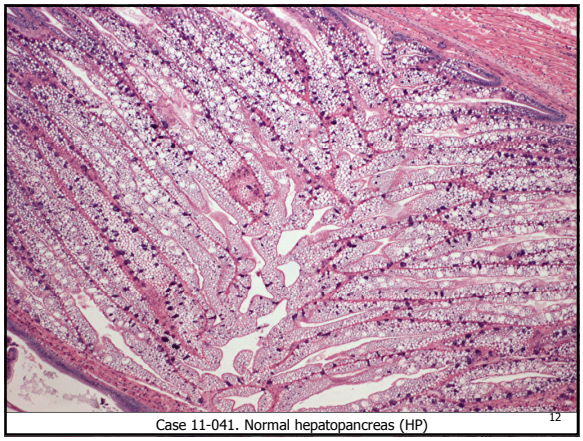
- Dysfunction of the HP:
  - Putative infectious agent, or toxicant, results in loss of HP tubule structure & cell morphology.
  - Infectious agent(s) suspected, but not yet demonstrated.
  - Toxic (environmental, feed, etc.?)
- A massive secondary bacterial (due to a putative *Vibrio* spp.) infection of the HP.

10

Samples from South China August/September 2010 & Vietnam July & December 2011

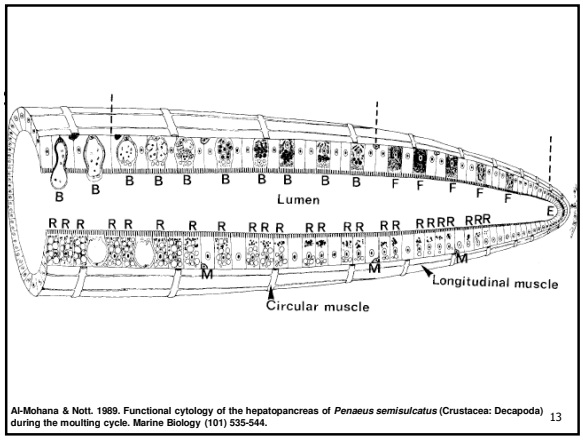
Lesions showing hepatopancreas dysfunction

11

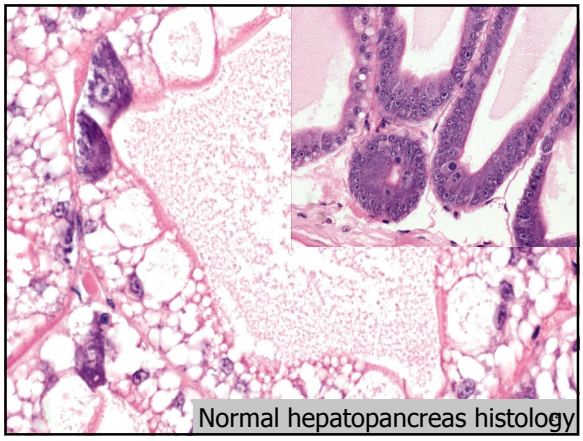


Case 11-041. Normal hepatopancreas (HP)

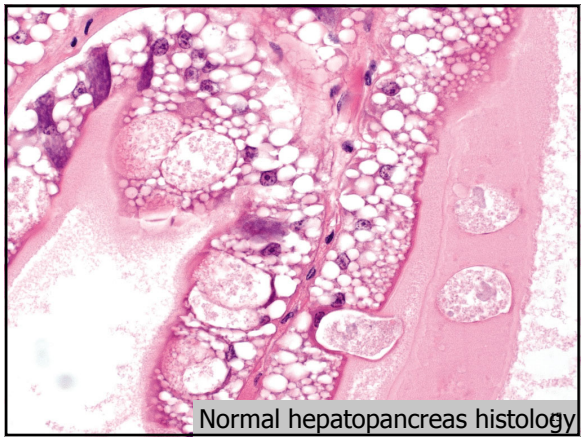
12



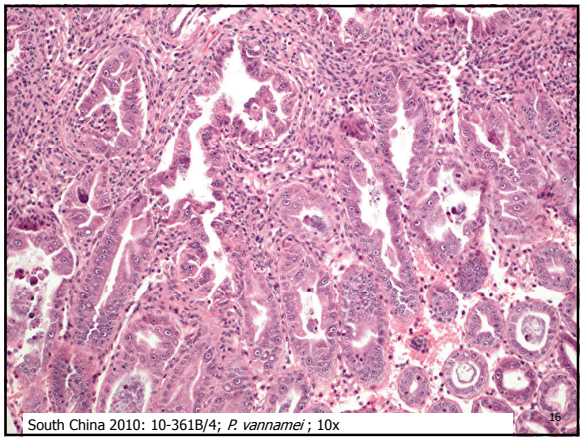
Al-Mohana & Nott. 1989. Functional cytology of the hepatopancreas of *Penaeus semisulcatus* (Crustacea: Decapoda) during the moulting cycle. Marine Biology (101) 535-544.



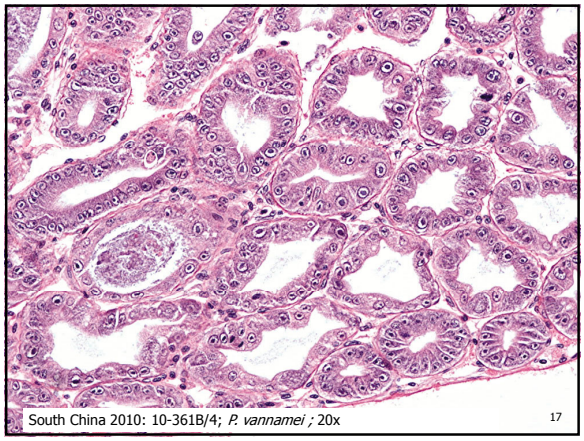
Normal hepatopancreas histology



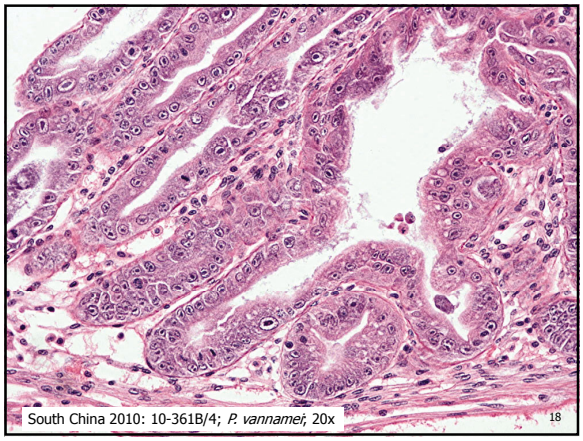
Normal hepatopancreas histology



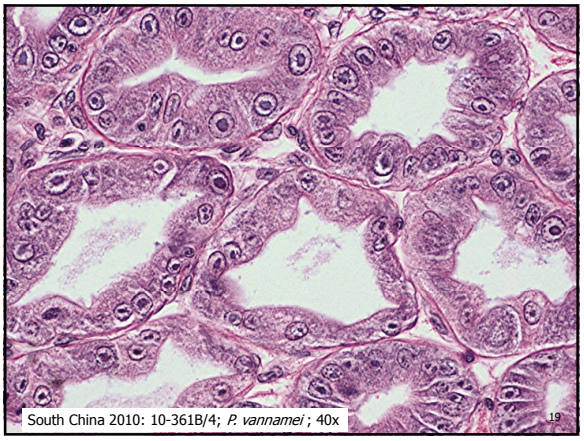
South China 2010: 10-361B/4; *P. vannamei* ; 10x



South China 2010: 10-361B/4; *P. vannamei* ; 20x

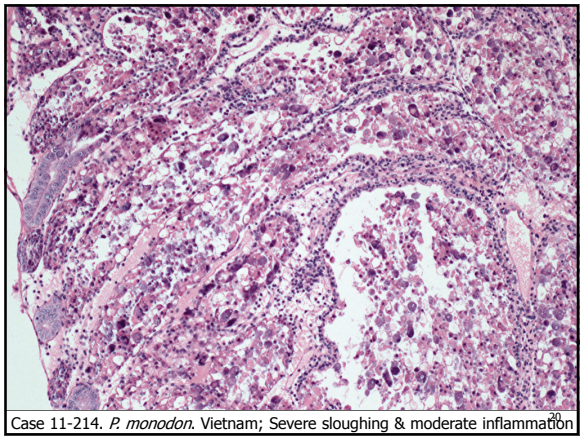


South China 2010: 10-361B/4; *P. vannamei*; 20x



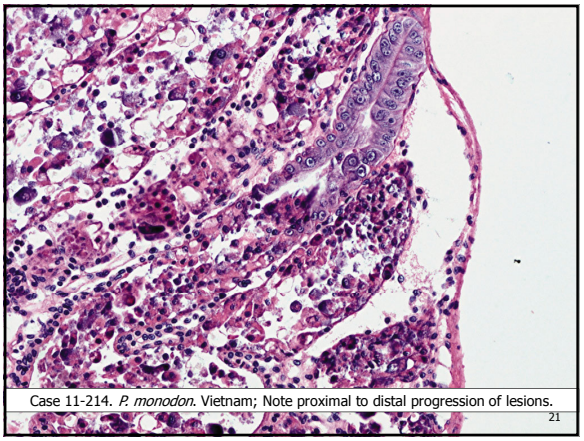
South China 2010: 10-361B/4; *P. vannamei* ; 40x

19



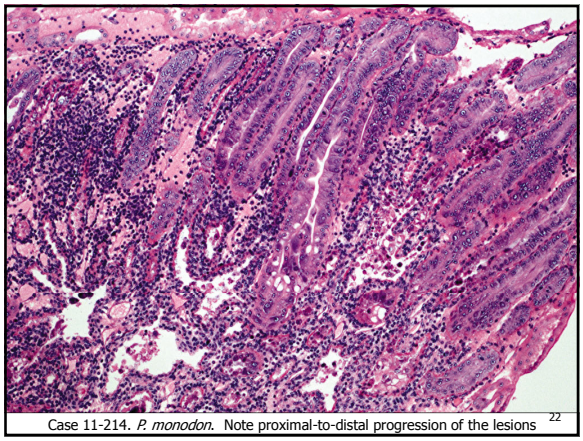
Case 11-214. *P. monodon*. Vietnam; Severe sloughing & moderate inflammation

20



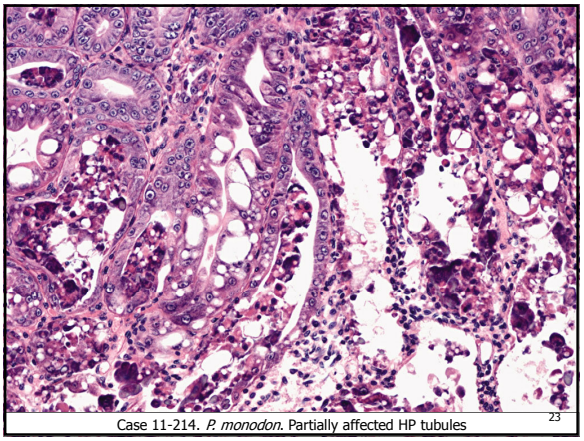
Case 11-214. *P. monodon*. Vietnam; Note proximal to distal progression of lesions.

21



Case 11-214. *P. monodon*. Note proximal-to-distal progression of the lesions

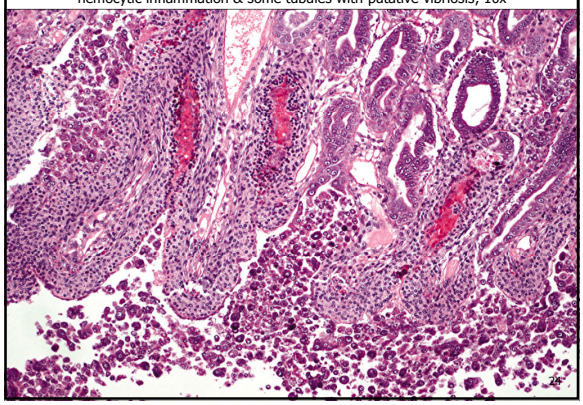
22



Case 11-214. *P. monodon*. Partially affected HP tubules

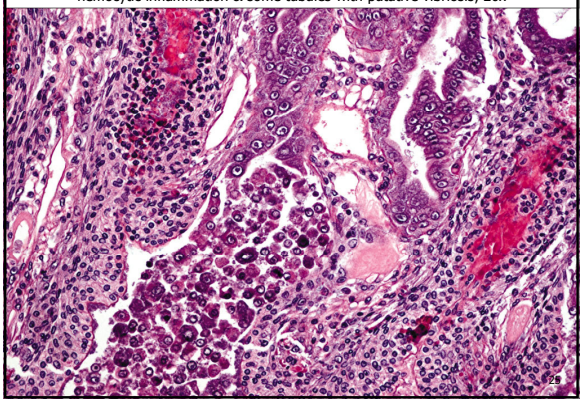
23

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



24

Case 11-254. *P. vannamei*. Vietnam; HP tubule epithelium sloughing, significant proximal hemocytic inflammation & some tubules with putative vibriosis; 20x



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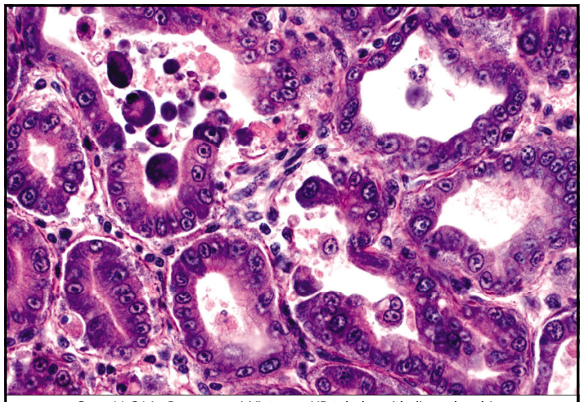
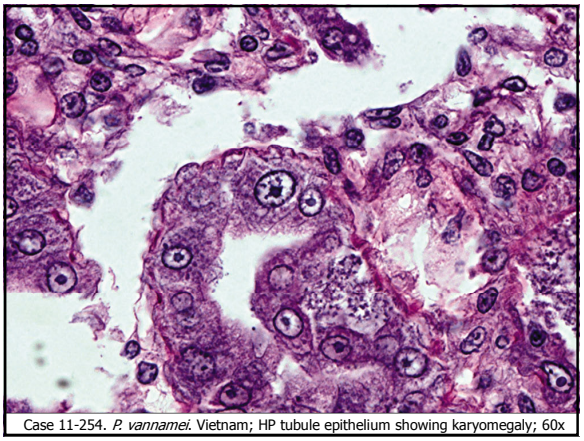


Case 11-214. *P. vannamei*. Vietnam; HP tubule epithelium sloughing & with slight hemocytic inflammation

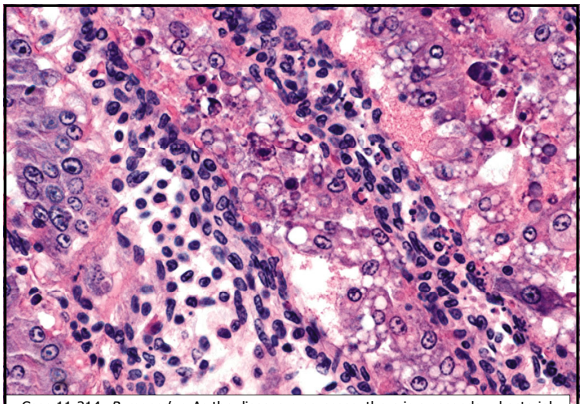
26



Case 11-254. *P. vannamei*. Vietnam; HP tubule epithelium showing karyomegaly; 60x

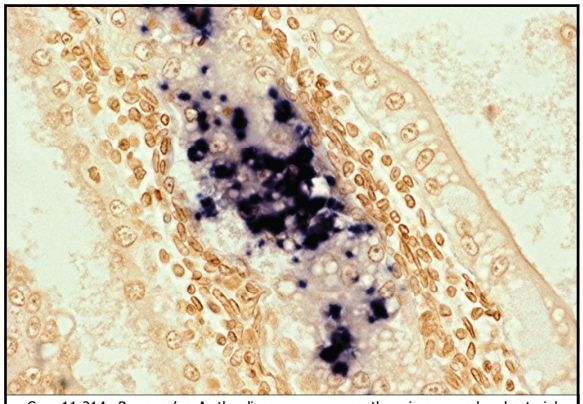
HP lesions showing HP destruction due to Vibriosis

28



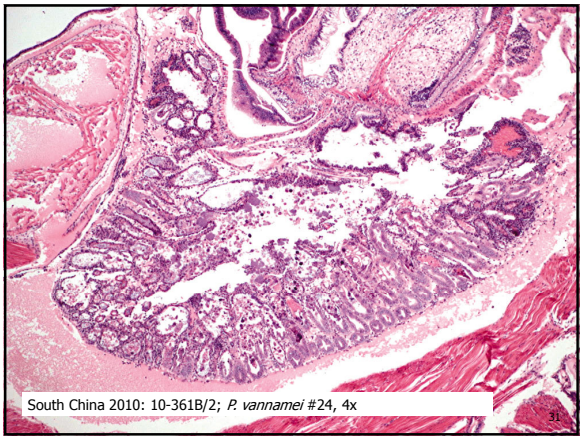
Case 11-214. *P. monodon*. As the disease progresses, there is a secondary bacterial infection of the HP (probably by opportunistic *Vibrio* spp.)

29



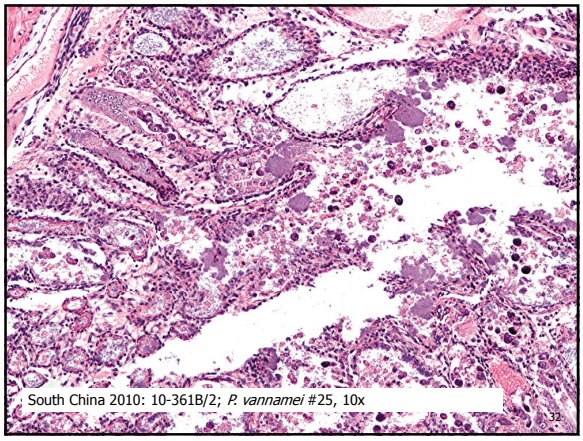
Case 11-214. *P. monodon*. As the disease progresses, there is a secondary bacterial infection of the HP (probably by opportunistic *Vibrio* spp.). Positive ISH results.

30



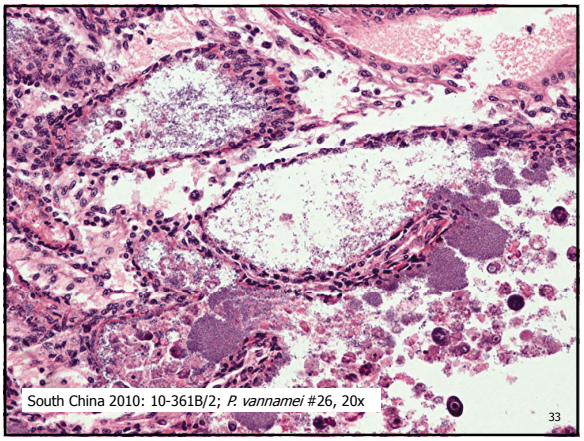
South China 2010: 10-361B/2; *P. vannamei* #24, 4x

31



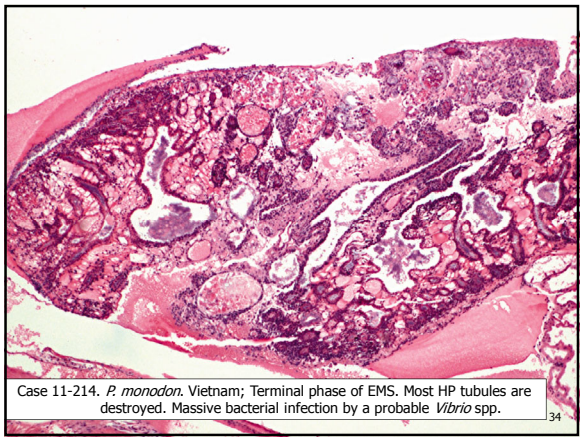
South China 2010: 10-361B/2; *P. vannamei* #25, 10x

32



South China 2010: 10-361B/2; *P. vannamei* #26, 20x

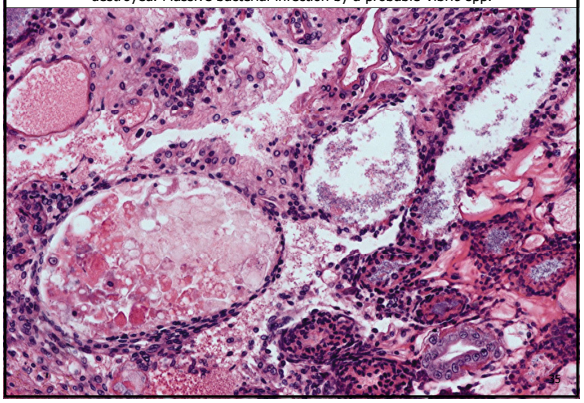
33



Case 11-214. *P. monodon*. Vietnam; Terminal phase of EMS. Most HP tubules are destroyed. Massive bacterial infection by a probable *Vibrio* spp.

34

Case 11-214. *P. monodon*. Vietnam; Terminal phase of EMS. Most HP tubules are destroyed. Massive bacterial infection by a probable *Vibrio* spp.



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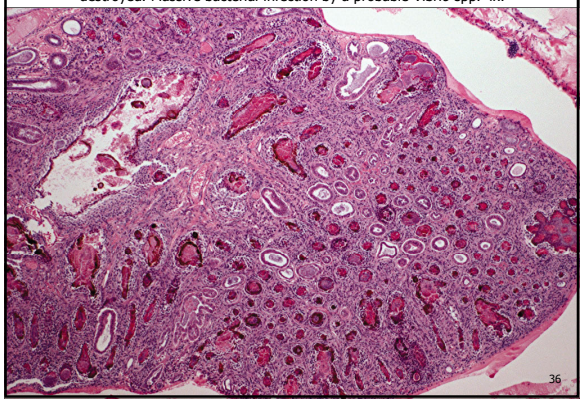
Case 11-254. *P. vannamei*. Vietnam; Terminal phase of EMS. Most HP tubules are destroyed. Massive bacterial infection by a probable *Vibrio* spp. 4x.



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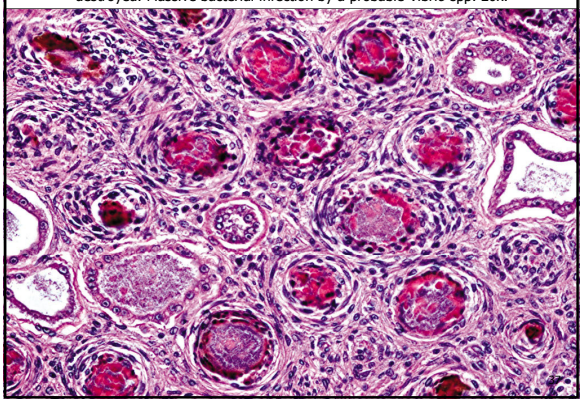
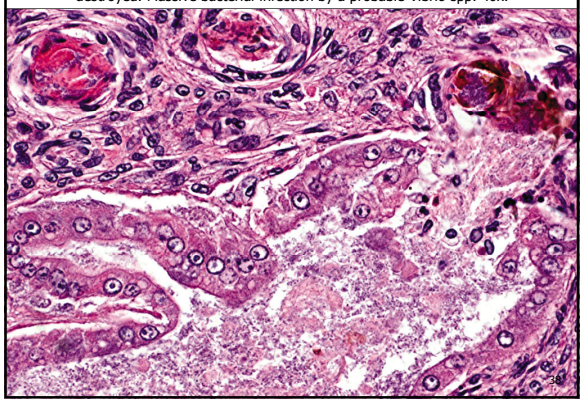
Case 11-254. *P. vannamei*. Vietnam; Terminal phase of EMS. Most HP tubules are destroyed. Massive bacterial infection by a probable *Vibrio* spp. 20x.



37

Case 11-254. *P. vannamei*. Vietnam; Terminal phase of EMS. Most HP tubules are destroyed. Massive bacterial infection by a probable *Vibrio* spp. 40x.



38

Summary

Lesions found in the juvenile hepatopancreata samples

- Low activity of "B" cells and "R" cells.
- Low mitotic rate in "E" cells.
- Rounding-up & sloughing of HP tubule epithelial cells.
- Intertubular hemocytic congestion (inflammation).
- Proximal-to-distal pattern of lesions.
- Distal end of tubules are the last to be affected.
- Enlarged nuclei (karyomegaly), with prominent nucleoli.
- Additionally…

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Summary

- Bacterial infection (probably by a *Vibrio* spp.) during advanced/terminal stages of the disease.
- Bacterial phase appears to be secondary.
- Identical lesions found in *P. vannamei* and *P. monodon* samples.

40

Proposed name for the disease

Acute hepatopancreatic necrosis syndrome

= AHPNS

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Proposed Case Definition for 30-Day Mortality Syndrome (= AHPNS)

- Idiopathic – no specific disease causing agent (infectious or toxic) has been identified.
- Pathology:
  - acute progressive degeneration of hepatopancreas (HP) from medial to distal with dysfunction of B, F, R & E-cells, prominent karyomegaly & necrosis & sloughing of these tubule epithelial cells.
  - terminal stage shows marked inter- & intra-tubular hemocytic inflammation & development of secondary bacterial infections that occur in association with necrotic & sloughed HP tubule cells.

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Possible Etiological Agents

- Infectious agent:
  - 37-day per os & injection infectivity study gave negative results.
- Toxicant(s):
  - 37 day study using feeds from affected farms gave negative results.
  - Two commercially available & commonly used crustacides tested gave negative results.
  - Algal or other environmental toxin(s)?

Possible Etiological Agents

- Infectious agent – bacteria (*Vibrio* sp.), virus, parasite?
- Unlikely:
  - Bacterial phase of disease occurs after HP begins to degenerate.
  - **37-day per os & injection infectivity study gave negative results.**

Design of Infectivity Study

- ~0.5 g *P. monodon* from affected farms transported frozen to UAZ.
- Some used in per os infectivity study with similar size SPF *P. monodon*.
- A second batch was:
  - homogenized, diluted 1:20 in 2% sterile saline.
  - filtered through a 0.45 micron filter to remove bacteria.
  - 100 ul injected in the 3rd abdominal segment.
- Histology for AHPNS at termination (day 36).

Summary of Infectivity Study

Tank	Treatment	Number	No. Day 36	% Survival
1	Negative control	10	10	100%
2	Per os*	10	10	100%
3	Injection	10	10	100%

* MBV was passed to the SPF *P. monodon* in the per os group.

Possible Etiological Agents

- Toxicant
  - From feed (new ingredient(s)?)
  - Remember the melamine contamination of "wheat gluten" of 2-3 years ago.
  - **37 day study using feeds from affected farms gave negative results.**

Design of Feed Toxicity Study

- 3 starter feeds (2 Uni-President & 1 CP) were collected at farms with ongoing EMS.
- At UAZ each feed was provided to 30 ~1g SPF *P. monodon* for 37 days.
- Fed at 5% body weight in 2 equal feedings.
- Histology of samples at termination (day 37) examined for AHPNS.

Feed Toxicity Study Results

Tank Number	Treatment	No. Stocked	Day 37; No. Collected	% Survival
1	Rangen	30	30	100%
2	Uni-Pres #1	30	29	97%
3	Uni-Pres#2	30	30	100%
4	CP feed	30	29	97%

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Possible Etiological Agents

- Toxic agent is possible:
  - Algae (bluegreen, dinoflagellate) in ponds?
  - Crustacides used in pond preparation prior to stocking?
  - Other?
- Hepatotoxic effect of 2 brands of crustacides?
  - Two commercially available & commonly used crustacides were tested & gave negative results.

50

Toxicity Trials with Cypermethrin

- Assumption: Cypermethrin binds to suspended sediments & is available in pond bottom detritus when PLs are stocked.
- Commercial grade cypermethrin was purchased in Vietnam.
- Pesticide was mixed with soil to give 0 ppb, 50 ppb, 200 ppb & 400 ppb.
- A plastic grate with 1 cm^3 cells was added to each experimental tank with soil to reduce turbidity.
- 40 *P. vannamei* & 40 *P. monodon* were used in replicates per dose level of cypermethrin.
- Samples for histology were taken at 20 & 40 days post stocking & examined for signs of EMS.

51

Toxicity Trials with Cypermethrin

Concentration of Cypermethrin	20 day histological findings*	40 day histological findings*	Final Adjusted Survival (%)**
0 ppb	AHPNS N/D	AHPNS N/D	100%
50 ppb	AHPNS N/D	AHPNS N/D	100%
200 ppb	AHPNS N/D	AHPNS N/D	100%
400 ppb	AHPNS N/D	AHPNS N/D	100%

* AHPNS = acute hepatopancreatic necrosis syndrome.
N/D = not detected.
** Survival adjusted for histological samples.

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Possible Etiological Agents – What We Know to Date:

- Severe HP dysfunction followed by a terminal *Vibrio* infection of the HP.
- *Vibrio* sp. may not be the agent of AHPNS as terminal phase of EMS appears to be opportunistic.
- Feeds (e.g. a new ingredient) tested do not cause AHPNS.
- Cypermethrin in static renewal bioassays or when added to soil does not cause AHPNS in lab trials.
- Tests for an infectious agent (viral, parasitic, bacterial) have been negative to date.

Acknowledgements

- OIE (World Organization for Animal Health) for travel to Vietnam & Thailand.
- Department of Animal Health, MARD, Vietnam for local arrangements in Vietnam.
- Uni-President feed company in Vietnam for funding toxicity & infectivity studies.
- CP Foods, Thailand for funding recent work on EMS.
- World Bank & Global Aquaculture Alliance for travel.

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THE UNIVERSITY OF ARIZONA

AQUACULTURE PATHOLOGY
Tucson, Arizona

Thank you for your attention!

oie

NACA AHPNS meeting August 9-10, 2012

Centex Shrimp *Center of Excellence for Shrimp Molecular Biology and Biotechnology*

Research progress on bacterial and viral causes of AHPNS

Anuparp Prachumwat, Siripong Thitamadee, Siriporn Sriurairatana, Niti Chuchird, Chalor Limsuwan, Wassan Jantratit, Sage Chaiyapechara, Kallaya Sritunyalucksana, Timothy W. Flegel

Centex Shrimp, Mahidol University, Faculty of Fisheries, Kasetsart University and National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand

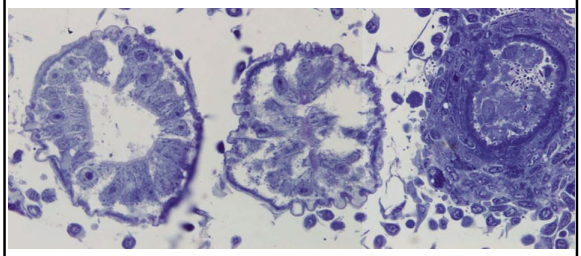
National Center for Genetic Engineering and Biotechnology BIOTEC a member of NSTDA

Acute hepatopancreatic necrosis syndrome (AHPNS) a sub-category of early mortality syndrome (EMS)

Cause of problems in Vietnam & China

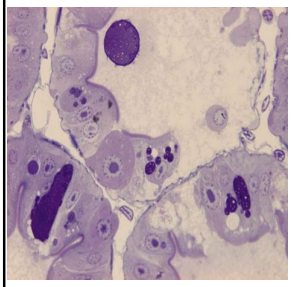
- Definitely NOT caused by IMNV
- Definitely NOT caused by the microsporidian *Enterocytozoon hepatopenaei*
- Dr. Lightner has given a case definition of a new disease that may be the major cause
- To the case definition, we propose to add blebbing of the HP tubule epithelium

Semi-thin sections Vietnam

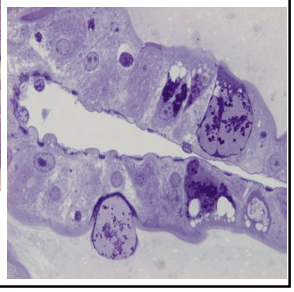


Altered HP tubule sheath and blebbing of basement membrane We have tentatively added this to the case definition

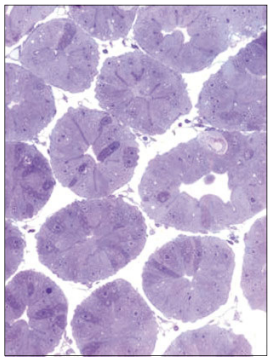
Semi-thin sections Thailand

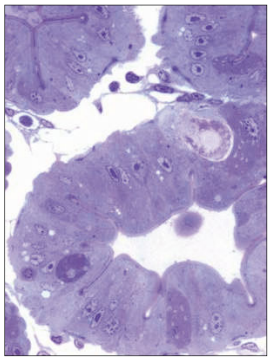


Same crenulations with and without bacteria present



Semi-thin sections from Malaysia





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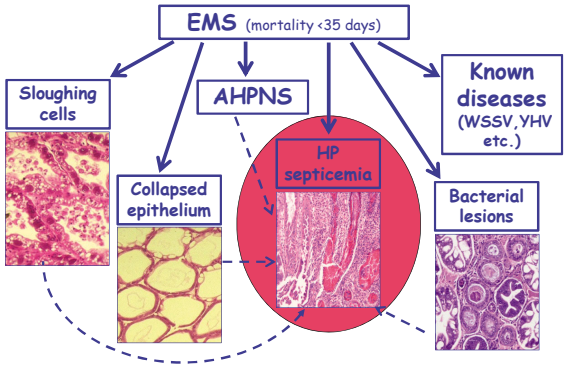
Early mortality syndrome (EMS)

- Name for unusually heavy shrimp mortality approx. within the first 35 days of culture
- This very imprecise case definition that has led to confusion in diagnosis
- Many possible causes including diseases of well-known pathogens like WSSV & YHV
- We are examining only ponds that fit Dr. Lightner's new case definition
- This precise case definition is a major breakthrough for further study
- It is important because research progress depends on analysis of identical cases

EMS versus AHPNS

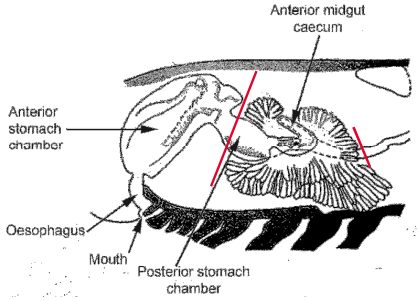


Cannot use septicemia alone to diagnose AHPNS

Possible causes of AHPNS

- Should check for biotic and abiotic toxins in:
  - Pond water & water supply, soils & sediments, etc.
  - Feed & feed ingredients, probiotics, etc.
  - Old and "new" agricultural pesticides, etc.
- Should investigate possible new bacteria:
  - Do shotgun sequencing of bacterial rDNA & *in situ*
  - Check for a possible phage-bacterium partnership
- Should investigate possible viral etiology:
  - Do challenge tests with filtered and unfiltered tissue extracts to see if a filterable agent is present
  - Check with TEM for viral particles
  - Do shotgun sequencing of "viral extracts" & *in situ*

Importance of sample collection



Adapted from D'Abramo LR, Conklin DE, Akiyama DM (1997) Crustacean nutrition, World Aquaculture Society, Boca Raton

Bacterial shotgun testing

Shrimp samples from 1 normal and 6 AHPNS ponds (1N+2AHPNS Vietnam and 4 AHPNS Thailand)

Extract DNA from outer part of HP of individual shrimp, avoiding contamination from posterior stomach chamber and midgut

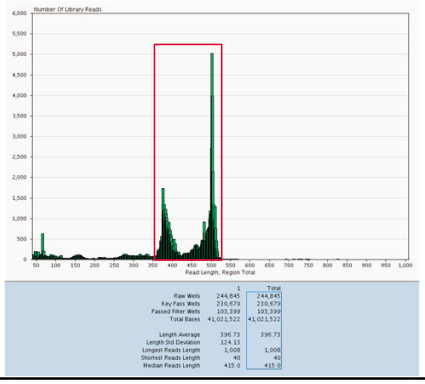
Pool the DNA extracts from each pond (7 pooled samples total)

Individual PCR with universal PCR primers for 400-500 bp amplicons

Add individual 9 bp identity tags to amplicons of each sample

Pool and sequence all amplicons by Roche 454

Overall sequencing result



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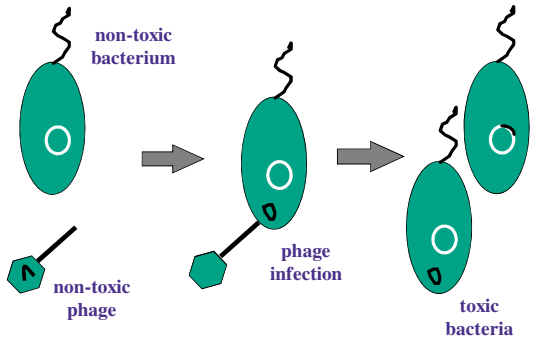
Summary of initial results

- Total number of unique sequences with more than one read = 1205 (singletons = 7123)
- Highest difference between test and control ponds:
  - Burkholderiales:
    - *Burkholderiaceae* genus ***Ralstonia***
    - *Comamonadaceae* genus ***Delftia*** (*acidovorans* & *tsuruhatensis*); ***Pelomonas*** (aquatica); (all former *Pseudomonas* species)
  - Actinomycetales:
    - *Microbacteriaceae* genus ***Leifsonia***
    - *Nocardiaceae* genus ***Rhodococcus*** (*globerulus, erythropolis, boritolerans*)
- Next step - make clones of these, and examine shrimp from test and control ponds by *in situ* hybridization

Possible phage involvement?

- It is well-known that bacteriophages can transfer lethal toxin genes to bacteria:
  - *Corynebacterium diphtheriae* produces diphtheria toxin when lysogenized by beta phage.
  - *Vibrio cholerae* produces cholera toxin when lysogenized by CTX phage
  - *Vibrio harveyi* produces a lethal shrimp toxins when lysogenized by VHS1 phage and VHML phage
- Sometimes the lysogenized bacteria lose phages upon serial subculture (they become "cured")
- We believe that this possibility should also be examined

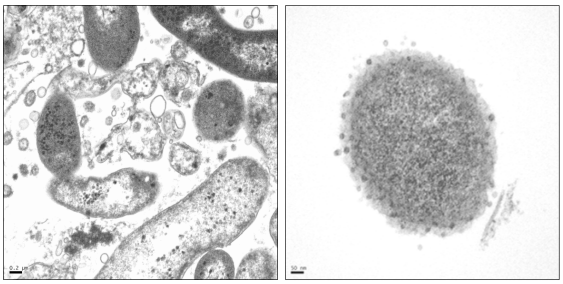
Working hypothesis on *V. harveyi*-phage interaction



Testing for phage involvement

- Can be studied by looking for clear plaques in bacterial colonies on isolation plates
- Other methodology:
  - Isolate bacteria from HP of affected shrimp (several types by streaking on both TSA andTCBS)
  - Avoid stomach and midgut contamination
  - Homogenize HP tissue from same shrimp
  - Centrifuge at 3000 xg and filter the clear supernatant solution with 0.22μ filter and dilute 100x
  - Test filtrate for no bacteria and inject into SPF shrimp: if no disease then no direct viral pathogen present
  - Test individual bacterial isolates injected alone and injected together with the filtrate: higher mortality with filtrate indicates a possible phage
  - Also test the filtrate on bacterial lawns for plaques

TEM phage evidence from Vietnam



Phage from Rayong samples

-The phage is being amplified

-TEM and sequencing will be possible

-Will need bioassays with bacteria and phage alone and mixed

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Testing for unknown shrimp viruses

- This refers to viruses other than phages
- Remove HP tissue of AHPNS shrimp (remember to avoid stomach and midgut contamination)
- Homogenize in buffer and centrifuge at 3,000 to 5,000 g to precipitate debris
- Remove the clear supernatant solution, filter through 0.22 μ filter and check bacterial free
- Dilute 100x and inject into SPF shrimp with buffer control
- If no AHPNS mortality, there should be no viral cause
- Unpublished tests like this have so far been negative

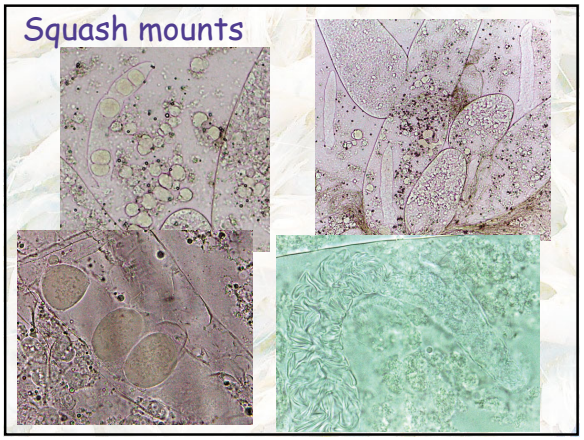
Shotgun cloning for viruses

- We are doing this but have no results so far
- Prepare samples as in the previous slide up to the 0.22μ filtered HP supernatant solution
- Transfer the tube to an ultracentrifuge to precipitate any viruses that might be present
- Extract the total nucleic acid from the pellet (both RNA and DNA)
- Use the extract as template for random prime RT to produce cDNA from any RNA present
- Use the mixed cDNA and any original DNA as template for random prime PCR
- Sequence the whole PCR product mixture

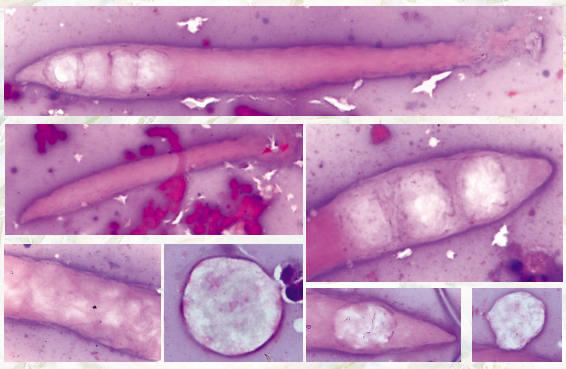
New "gregarine" from WFS shrimp

- Unlike any gregarine previously reported from shrimp; found together with *E. hepatopenaei*
- Un-segmented with an apparent meront stage in the shrimp hemolymph
- Trophozoites in HP tubule lumens and easily degraded by Davidson's fixative
- Trophozoites can be seen in squash mounts with careful examination
- Fibrous lattices formed in the interstitial spaces of the HP, surrounding tubules
- Appears to be a meront amplification stage

Squash mounts



Stained smears



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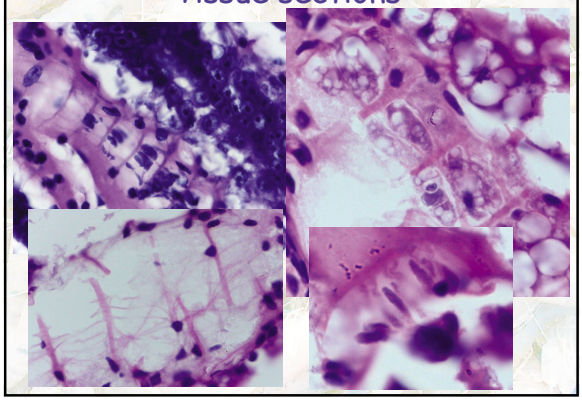
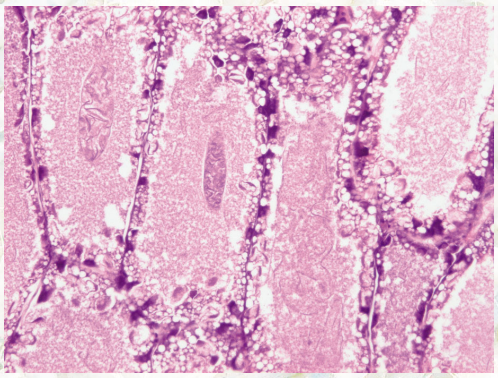
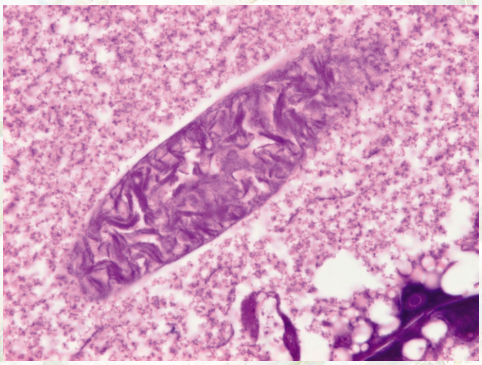
Tissue sections



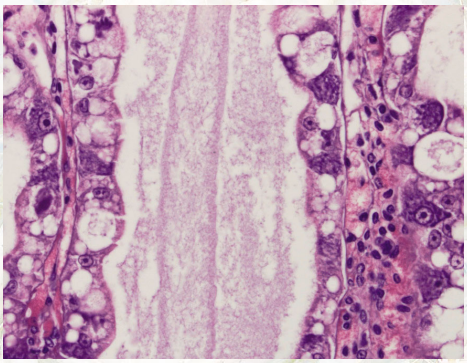
Degraded by Davidson's 1



HigDegraded by Davidson's 2



Degraded by Davidson's 3



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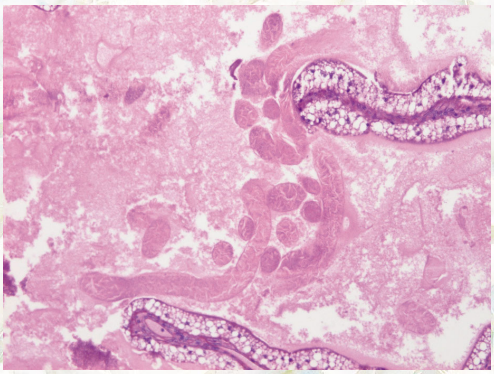
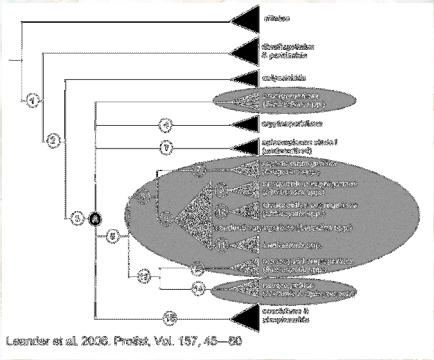


Appears not to fit in phylogenetic tree



Disease emergence – why and how?

Peter Walker

9 August 2012

AUSTRALIAN ANIMAL HEALTH LABORATORY
www.csiro.au

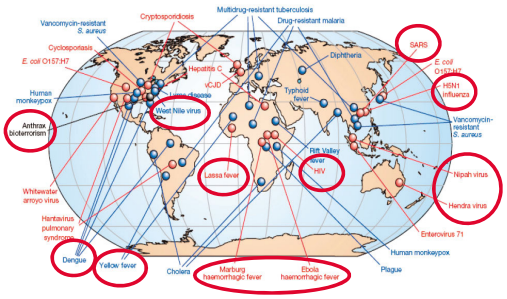
Emerging Infectious Diseases (EIDs)

EIDs are infectious diseases that have first appeared or whose incidence has increased in the past 20 years

Term is usually applied to human diseases:

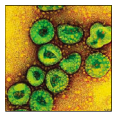
- EIDs account for at least 12% of all human pathogens
- 54.3% are caused by bacteria or rickettsia (mostly drug-resistant strains)
- 25.4% are caused by viruses
- 60.3% of EIDs are zoonotic (human diseases of animal origin)
- 71.8% of zoonotic EIDs originate in wildlife
- During the past decade, 28.8% of EIDs have been vector-borne (transmitted by insects)

Disease emergence is a global problem



Case study – SARS virus

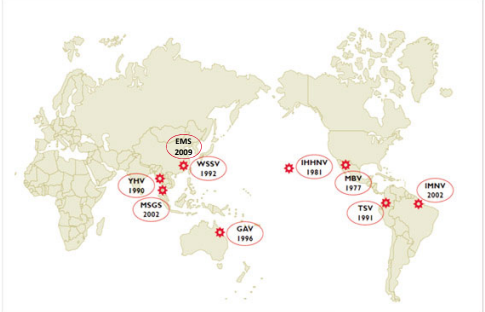
- Emerged in Guangdong Province, China in November 2002
- Spread to 37 countries in 6 months
- Estimated economic impact $20 billion
- Emerged from wildlife, probably bats to infect palm civets
- Humans exposed to civets in markets
- 8422 cases, 916 deaths
- Containment and eradication
  - Rapid response
  - International cooperation (WHO coordinated)
  - Rapid identification of the pathogen
  - Rapid diagnostic test development
  - Travel restrictions
  - Face masks
  - Isolation of infected patients



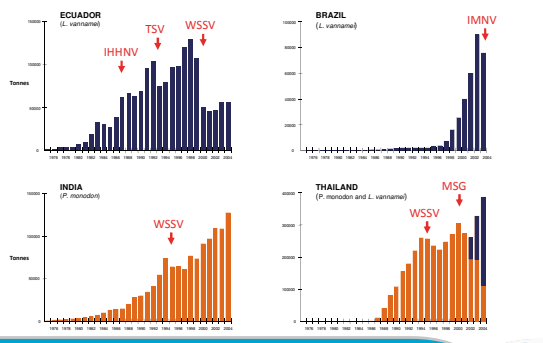
Case study – Chikungunya virus

- Viral disease transmitted by urban *Aedes aegypti* mosquitoes
- First reported in East Africa in 1952
- Major epidemic emerged on Reunion Island in the Indian Ocean in 2005
- 244,000 inhabitants infected, 204 deaths
- Moved to India in 2006, more than 1 million cases
- Now epidemic throughout Africa, SE Asia and into Italy
- Single point mutation in the E1 glycoprotein (A226V) allowed adaptation to a new mosquito species (*Aedes albopictus*)
  - Higher virus replication rate (more efficient transmission)
  - *Aedes albopictus* has much wider geographic distribution
  - Distribution expanding globally due to climate change and other factors

Global emergence of shrimp diseases



Disease impacts both major shrimp species

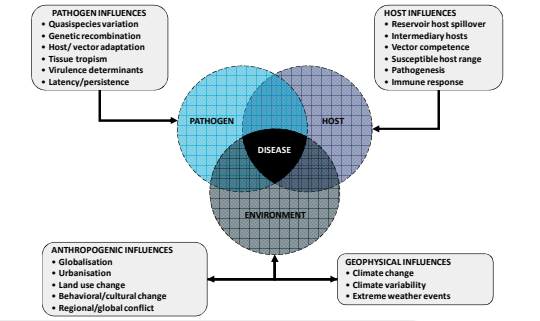


Why do new diseases emerge?

Disease emergence and spread results from a disturbance the ecology of infection

- Pathogens are an integral part of the ecosystem
- Infection and efficient transmission do not necessarily require the appearance of disease
- Many pathogens commonly infect healthy animals with no pathology or mortality
- A disturbance in the ecology of the pathogen can upset the natural balance, resulting in the emergence and spread of disease

Complex dynamics of disease emergence



Ecological changes on a massive scale

Changes in land-use

- Over 2 million Ha of shrimp ponds

Changes in the ecology of aquatic animals

- More than 220 farmed species recorded
- Terrestrial ponds, sea cages
- Artificial or alternative feeds

Changes in the patterns of trade

- Est. 28,000 *P. vannamei* broodstock/ month to Asia
- Over 1.5 M tonnes of American prawns farmed in Asia
- Over 4.5 M tonnes of shrimp product traded globally

Aquaculture and disease emergence

Aquaculture is an important contributor to socio-economic development in many countries

BUT

Aquaculture practices provide an ideal environment for emergence and spread of disease

- Animals are often cultured in an unnatural environment
- Animals are often in high stocking densities
- Animals are often stressed by culture conditions
- Unregulated trade in live animals commonly occurs

Categories of EID

- **Diseases that emerge in the natural host**
  - e.g., YHV, MBV
- **Diseases that emerge by spill-over of infection to a new host**
  - e.g., TSV, WSSV, IMNV
- **Diseases that are increasing in geographic distribution**
  - e.g., MrNV

Diseases that emerge in the natural host

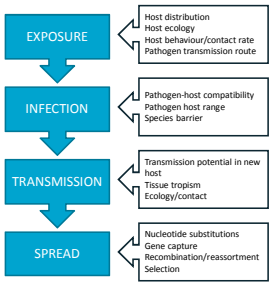
- Infections are non-pathogenic in the natural host
  - Long period of adaptation
  - Efficient replication and transmission are not dependent on the development of disease
- Disease occurs as a result of:
  - Change in the pathogen (mutation, recombination, acquired virulence factor)
  - Change in the environment (stress, climate or weather)
  - Change in the host (genetic selection, immunodeficiency, concurrent infection)

Diseases that emerge by spill-over to a new host

- Infections are non-pathogenic in the natural host (reservoir)
- Exposure and spill-over may be as a result of:
  - Increased proximity to a reservoir host
  - Increased frequency of exposure to a reservoir host
  - Genetic change in the pathogen permitting infection of a new host
  - Unusual amplification of pathogen levels (or increases shedding) in the reservoir host
    - physiological stress
    - change in ambient temperature
- Spill-over occurs more commonly between related host organisms

Spill-over to a new host

- a change in environmental conditions or social behaviour to create the opportunity for exposure of a new host to a potential pathogen
- conditions that permit infection and on-going replication of the pathogen in a new host
- on-going transmission of the adapted pathogen in the new host population
- genetic adaptation of the pathogen to replication in the new host population



Parrish et al Microbiol Mol Biol Rev, 2008

Diseases that are increasing in geographic distribution

- Changes in human behaviour (anthropogenic influences)
  - Increased travel and trade
  - Increased urbanisation
  - Changes in social practices
  - Expansion of new industries
  - Human conflict
- Changes in environmental conditions
  - Climate change
  - Climate variability
  - Extreme weather

How do we reduce or limit the impact?

- Early detection
  - rapid and accurate diagnosis, effective non-targeted surveillance
- Rapid response
  - national/international cooperation and information sharing, contingency planning, surge capabilities
- **Prediction and prevention**
  - a more challenging option - but less costly socially, economically and environmentally
  - understanding biological and ecological drivers of pathogen emergence

How can we predict disease emergence?

- Case studies of known EIDs (e.g., SARS, Hendra virus, etc)
  - e.g., the role of rodents and bats as sources of disease emergence
- Identification and characterisation of new or poorly described pathogens
- Experimental studies of the host-pathogen interface and the evolution pathogen-host adaptation
  - e.g., understanding influenza virus adaptation to human-human transmission
- Landscape-level modelling of host and pathogen population dynamics and the influence of environment variables
  - application of new high-throughput genomic technologies
  - requires fusion of genomics, epidemiology, ecology, geography, meteorology, social sciences, etc
- Correlative studies to identify high risk scenarios and 'hot spots' of disease emergence
  - exposure avoidance, changes in policies, practices and social behaviour

Implications for shrimp health management

- Avoid high risk practices
  - Live feeds
  - Co-cultivation (especially more closely related species)
- Pathogen exclusion practices
  - Manage seed selection and the pond environment stock to ensure stock are free of pathogenic viruses
- Stress reduction practices
  - Manage stock during grow-out to ensure that, if stock are inadvertently infected with pathogenic viruses, they do not develop disease
- Disease containment practices
  - Manage disease outbreaks to prevent spread of pathogens to the environment and other farms
  - Report new or unusual diseases
- Responsible trading practices
  - Manage the international movement of infected shrimp or shrimp products to prevent the global spread of disease

The ongoing problem of disease emergence

- **New diseases continue to emerge**
- **Diseases continue to spread to new areas**
- **Losses due to disease continue to impact significantly on production**
- **Disease losses are driving environmental impacts**
- **Industry seeks technological solutions**
- **The real solution may lie in attitudes and behavior**

Thank you

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Novel Methods for "Hunting for Ghost Viruses"

Jeff Cowley

CSIRO ANIMAL, FOOD & HEALTH SCIENCES, QBP, BRISBANE
www.csiro.au

Molecular options for investigating the cause of AHPNS

If clues indicative of a specific known virus type

- gross disease signs
- histopathology
- virion or nucleocapsid morphology, etc

Educated guess

Sequence-assisted genome amplification methods

- PCR using degenerate primers targeted to functional motifs of proteins highly conserved across related viruses
- PCR using primers with 3'-terminal sequences targeted to conserved inverted terminal repeat sequences at the end of virus genome segments

Molecular options for investigating the cause of AHPNS

Suspect a viral pathogen but few or no obvious clues

Sequence-independent genome amplification methods

- Differential display ☺
- Subtractive hybridisation
- Random PCR ☺
- Whole genome amplification ☺
- Crude shotgun sequencing
- Several others

Approach selection based on available starting material

InterScience
Reviews in Medical Virology

Rev. Med. Virol. 2006; **16**: 365–383.
Published online 23 August 2006 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/rmv.515

REVIEW

Virus discovery by sequence-independent genome amplification

Helen E. Ambrose and Jonathan P. Clewley*
Virus Reference Department, Centre for Infections, Health Protection Agency, London NW9 5EQ, UK

SUMMARY

Genome sequences from several blood borne and respiratory viruses have recently been recovered directly from clinical specimens by variants of a technique known as sequence-independent single primer amplification. This and related methods are increasingly being used to search for the causes of diseases of presumed infectious aetiology, but for which no agent has yet been found. Other methods that do not require prior knowledge of the genome sequence of any virus that may be present in the patient specimen include whole genome amplification, random PCR and subtractive hybridisation and differential display. This review considers the development and application of these techniques. Copyright © 2006 John Wiley & Sons, Ltd.

Received: 31 May 2006; Revised: 2 June 2006; Accepted: 5 June 2006

Differential display (DD) RT-PCR method

Identify mRNA in Sample A NOT PRESENT in Sample B

Synthesise cDNA from mRNA 3'-polyA tail using an oligo-dT primer

1. 5'-AAGCTTTTTTTTTTC-3'
2. 5'-AAGCTTTTTTTTTTG-3' ☺
3. 5'-AAGCTTTTTTTTTTA-3'

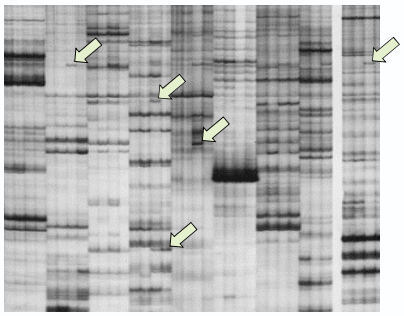
PCR amplify cDNA using short unique primers and α[^{35}S]-dATP

1. 5'-AAGCTTACAACGAGG-3'
2. 5'-AAGCTTGGATTGGTC-3'
3. 5'-AAGCTTCTTTCTACCC-3' ☺

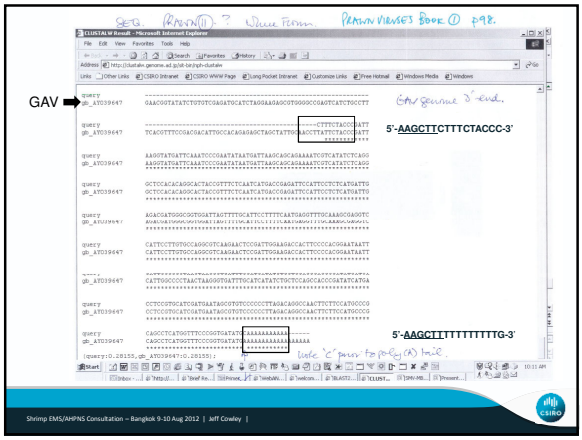
Identify unique bands in gels, isolate, re-amplify by PCR, clone and sequence

DD-RT-PCR – gel exposed to X-ray film

long



short



Random PCR method

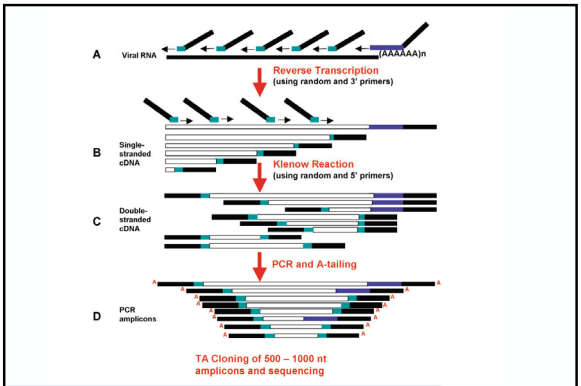
Uses special primers to synthesise cDNA from RNA and second-strand DNA from this cDNA → dsDNA

Uni-P-N_6 5'-GCCGGAGCTCTGCAGAATTCNNNNNG-3'
5'-GCCGGAGCTCTGCAGAATTCNNNNNA-3'
5'-GCCGGAGCTCTGCAGAATTCNNNNNT-3'
5'-GCCGGAGCTCTGCAGAATTCNNNNNC-3'

Uni-P-T_{14}A 5'-GCCGGAGCTCTGCAGAATTCTTTTTTTTTTTTTTA-3'
Uni-P-T_{14}G 5'-GCCGGAGCTCTGCAGAATTCTTTTTTTTTTTTTTG-3'
Uni-P-T_{14}C 5'-GCCGGAGCTCTGCAGAATTCTTTTTTTTTTTTTTC-3'

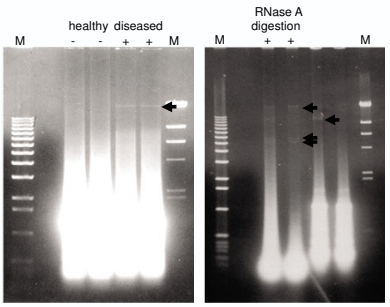
Amplify random-sized PCR products using a single Universal primer

Uni-P 5'-GCCGGAGCTCTGCAGAATTC-3'

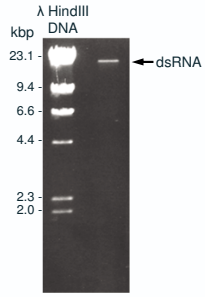


What starting material ?

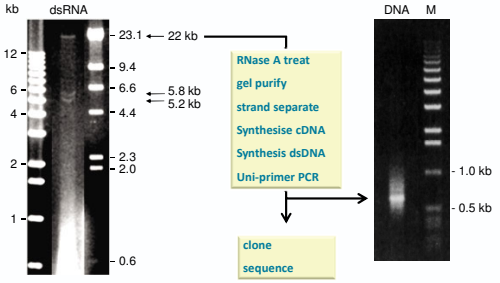
dsRNA in total RNA - lymphoid organ GAV-infected shrimp



dsRNA gel purified from *P. monodon* LO total RNA

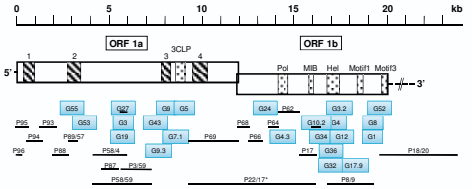


cDNA synthesis - random PCR amplification of ~22 kb dsRNA

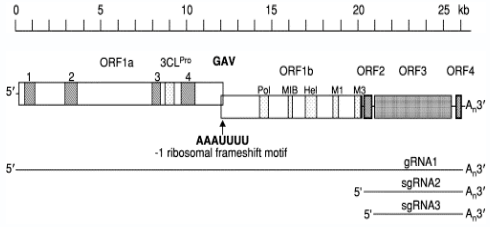


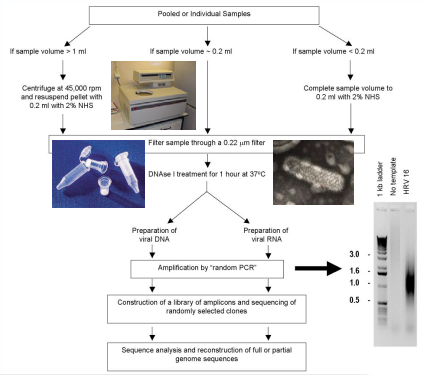
Clones amplified randomly across GAV ORF1a-1b gene

(gaps filled by PCR or semi-random PCR approaches)



Complete GAV genome sequence

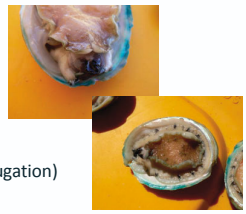




Whole genome amplification

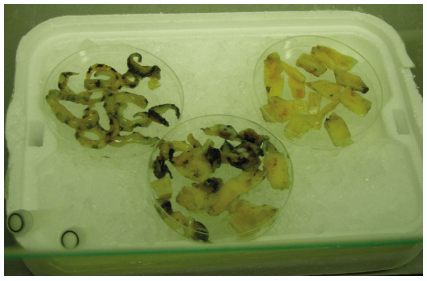
Abalone herpesvirus (AbHV)

Abalone viral ganglioneuritis (AVG)

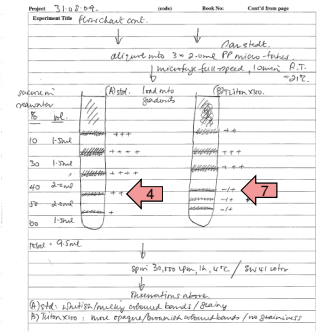


1. Extract nerve tissue
2. Homogenise, sonicate, clarify
3. Semi-purify virions (ultracentrifugation)
4. Extract DNA
5. **Amplify DNA (REPLI-g)**
6. NextGen sequencing (454, Illumina)
7. Bioinformatics – genome assembly

AbHV-infected abalone muscle pieces cut to access nerves easily

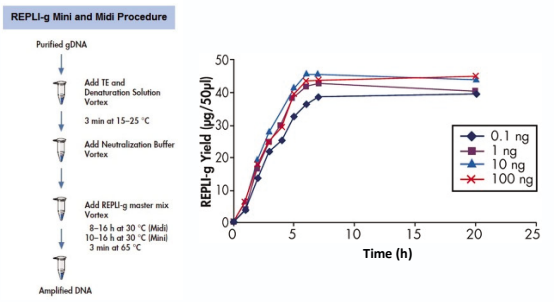


AbHV purification – sucrose density gradient



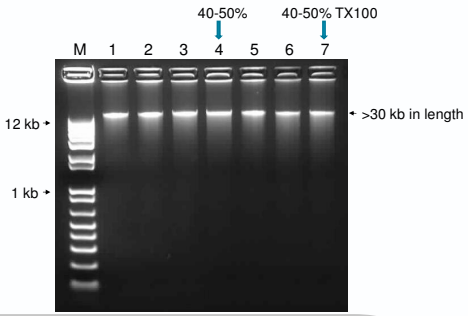
phi29 DNA polymerase MDA

REPLI-g Midi Kit (QIAGEN)



REPLI-g Multiple Displacement Amplification

(phi29 DNA polymerase + random primers)



REPLI-g able to enrich AbHV DNA

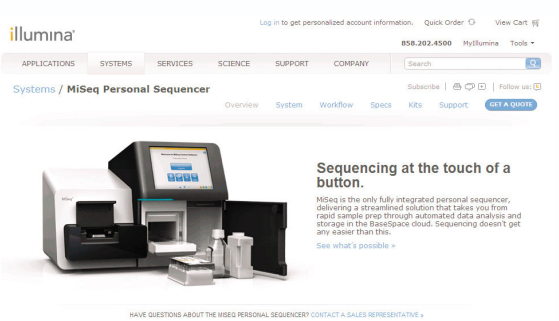
TaqMan **real-time PCR analyses – Ct values**

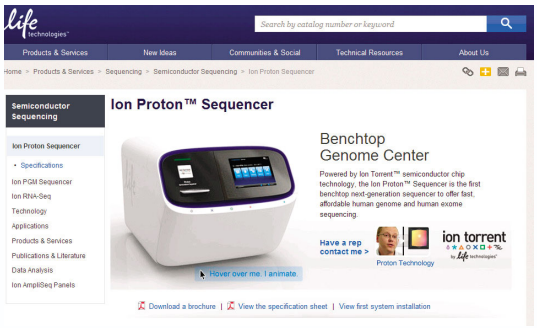
	DNA			DNA after REPLI-g		
No.	AbHV	18S rDNA	Difference	AbHV	18S rDNA	Difference
1	20.4	18.7	-1.7	16.0	22.6	6.6
2	24.1	21.7	-2.4	16.4	25.7	9.3
3	23.8	21.9	-1.9	16.5	27.7	11.2
4*	23.0	21.2	-1.8	16.1	28.0	11.9
5	20.4	18.7	-1.7	16.2	23.0	6.9
6	24.1	21.7	-2	17.6	25.4	8
7*	23.0	21.2	-1.8	16.7	30.1	13.4

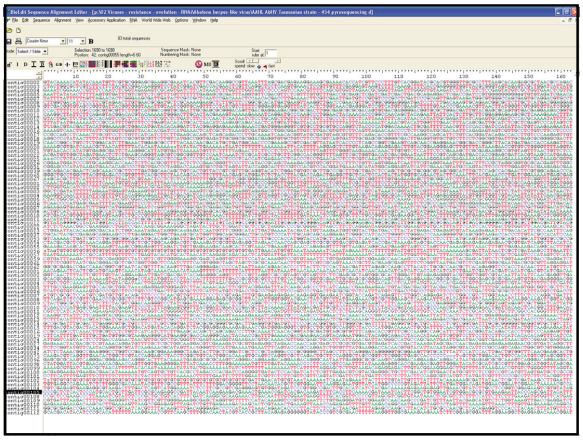
AbHV DNA enriched 50,000 to 100,000-fold / abalone DNA

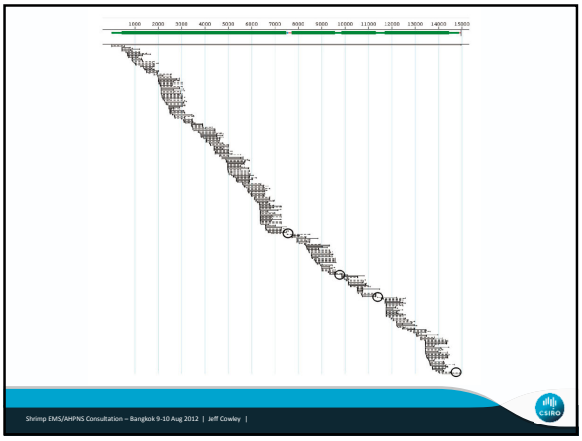
GS FLX+ System

Sanger-like read lengths - the power of next-gen throughput

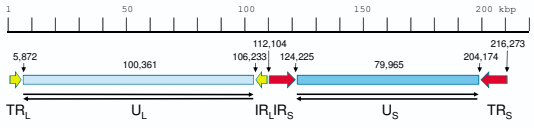








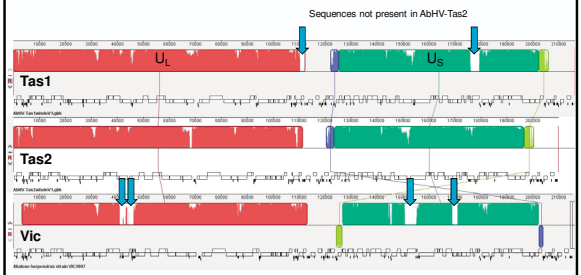
Complete genome sequences 3 x AbHV strains



Region	AbHV-Tas1	AbHV-Tas2	AbHV-Vic	OsHV-1
TR_L / IR_L	5,871	5,968	5,873	7,584
TR_S / IR_S	12,103	11,333	11,876	9,774
U_L	100,361	99,204	102,249	167,843
U_S	79,965	76,084	78,650	3,370
IR_L-X-IR_S	-	-	-	1,510
Total =	**216,273**	**209,897**	**215,337**	**207,439**

AbHV genome differences - Tas1 vs Tas2 vs Vic strains

(progressive genome alignment - Mauve 2.3.1)



Molecular options for investigating involvement of a viral/other pathogens in EMS/AHPNS ?

Approaches guided by whatever solid clues surface on the possible cause

Shotgun Sequencing' using NetGen sequencing platforms - easy using crude material – attempts made already at CENTEX

Other sequence-independent approaches will need

Pathogen enrichment/purification strategies

Pathogen nucleic acid enrichment/purification/amplification strategies

Which in turn will direct sequencing strategy

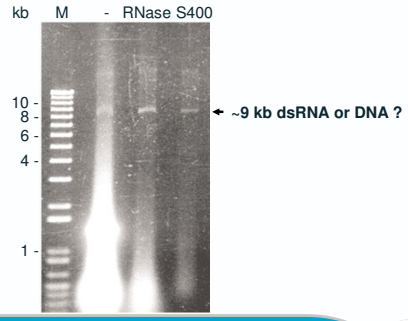
Acknowledge

NACA	**Ambekar E Eknath** **CV Mohan**
Aust Govt DAFF	**Ingo Ernst**

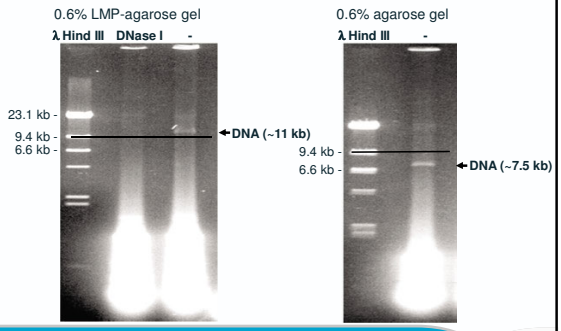
Thank you

Gill total RNA of diseased *P. monodon* affected by MCMS

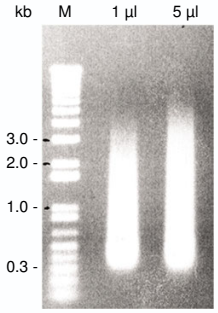
(GAV, MoV and others)



DNase 1 digested gill total RNA – band confirmed as DNA



Gel-purified ~9 kb DNA amplified randomly by PCR



Amplified DNA cloned and sequenced

Clones possessed retrovirus/transposon-like sequences (ORF errors)

1. *gag*-like protein with Zn-finger domain
2. *pol*-like protein with N-/C-terminal *integrase*-like components
3. *reverse-transcriptase*-like protein
4. Env-like protein sequences not detected

BLAST searches – invertebrate transposon sequences most related (eg. Silk worm, fruit fly, freshwater snail, mosquito, fungus etc)

Mobile DNA element (transposon or retrotransposon) possibly activated in response to stress ?

5'-RACE used to determine the 5'-terminal sequence of the MoV M mRNA

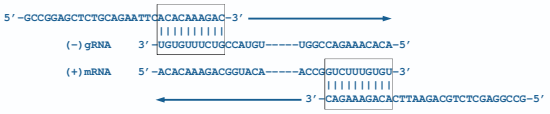
Absolute conservation of the terminal 10 nucleotides of MoV M RNA with the Uukuniemi virus ITR consensus sequence

```
MoV (+)mRNA   5'-ACACAAAGAC..
   (-)gRNA    3'-UGUGUUUCUG..
                 ||||||||||
Phlebovirus   3'-UGUGUUUCUG..
Tenuivirus    3'-UGUGUUUCAG..

Nairovirus    3'-AGAGUUUCUG..
Bunyavirus    3'-UCAUCACAU...
Hantavirus    3'-AUCAUCAUCUG.
Tospovirus    3'-UCUCGUUAG...
```

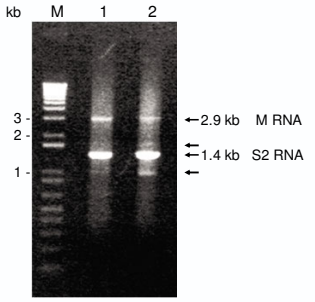
Single-primer RT-PCR to amplify full-length genomic RNA segments of MoV

A primer containing a 3' sequence with 10 nt complementary to the 3'-terminus of the MoV M gRNA was used to amplify the expected L, M and S RNA segments

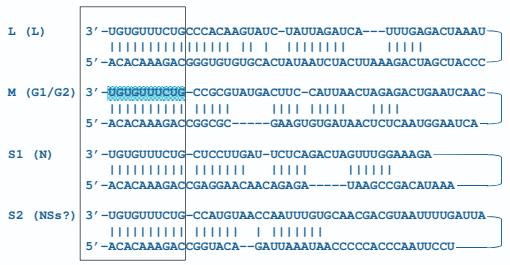


This primer used in conjunction with L RNA segment primers to amplify regions extending to the 3'- and 5'-termini of the L RNA

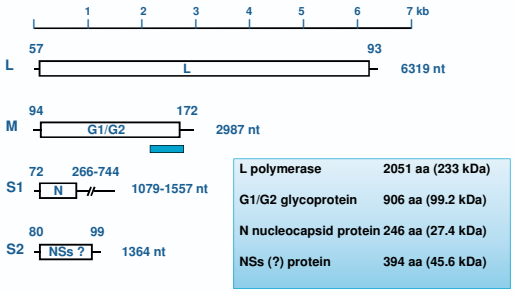
Single-primer RT-PCR products - MoV RNA segments



Inverted terminal repeat (ITR) sequences in the 4 (-) gRNA segments of MoV



Complete sequences of the 4 RNA genome segments of MoV



L polymerase	2051 aa (233 kDa)
G1/G2 glycoprotein	906 aa (99.2 kDa)
N nucleocapsid protein	246 aa (27.4 kDa)
NSs (?) protein	394 aa (45.6 kDa)

EMS Epidemiology & Risk Factors: what do we know?

Flavio Corsin
Vietnam Manager &
Asia-Pacific Aquaculture Manager

the sustainable trade initiative

Why is IDH involved?

- The Sustainable Trade Initiative (IDH)
  - convenes and co-invests with the private sector to achieve sustainable production
  - Funded by the Dutch (and Danish) governments
  - We can spend only if private sector spends
  - Work on 12-16 sectors including aquaculture (≈8M €)
  - Aquaculture: focus on pangasius, shrimp and tilapia
  - Shrimp disease major challenge to sustainability
    - EMS recognised as a major challenge!

Source of information

- FAO/OIE CMC-AH mission: 11-19 July 2011 (VN)
  - Questionnaires/interviews (17 farmers)
    - case-control approach
  - Participatory epidemiology (other 17 farmers)
- FAO supported analysis of DAH survey (VN)
  - Questionnaire/interviews (20,584 farmers; 29 key shrimp farming provinces)
  - Descriptive analysis
  - Statistical analysis
- Mixed sources (Regional)
- **Note: not "proper" epi investigations**

EMS: characteristics

- Mass & sudden mortality (>10/day)
  - About 1 month after stocking
- Abnormal HP (shrunken, white, swollen)
- *Apparently* spreading
- Outbreaks 4-5 days apart (direct transmission?)

Case definition

- Don Lightner: animal-level case definition
- Pond-level case definition
  - Mortality (mass, eg >10/day)
  - Abnormal HP
  - Time after stocking?

Risk factors?

General
- Intensive systems ↑ risk
- Larger farms ↓ risk (of course)
- Inland districts & lower salinity ↓ risk
- Having a reservoir ↓ risk
- Not all farms affected in 2010 experienced EMS in 2011

Pond preparation
- Saponin ↓ risk
- Longer drying time ↑ risk
- Ploughing ↑ risk
- Use more lime ↑ risk

Risk factors?

Stocking
- Monodon more susceptible. Vannamei also affected
- Stocking later & older seed ↓ risk
- Source of seed may play a role. Seed from "known sources" as opposed to nursery/middlemen ↓ risk

No association with insecticide use

Other "non-biological" associations
- Testing water, using "certified" seed, sharing equipment, using vitamins/antibiotics… ↑ risk

Careful about bias!

If it is a pathogen, where from?

- Sudden appearance in 2010
- Conditions changed?
  - No major/unusual weather event
  - No recorded/detectable change in farming practice
- Possibly associated with an introduction

Conclusions & recommendations

- Likely infectious
- Can be controlled
- Seed appear to be important (pathogen introduction?)
- Need a properly designed epi investigation
  - NACA to coordinate?
  - ERAAAD to support?

the sustainable trade initiative

www.idhsustainabletrade.com

corsin@idhsustainabletrade.com

Is EMS A Management Problem ?

Dr. Matthew Briggs

Director

Vannamei 101

What is EMS ?

- EMS = Early Mortality Syndrome
- Early mortality syndrome can be caused by a number of different pathogenic, environmental and management issues. Some, but not all of this appears to be caused by a new and previously unknown syndrome called Acute Hepatopancreatic Necrosis Syndrome (AHPNS)
- EMS is associated with mass shrimp mortality (40-60 % typically, but sometimes 80-100%) in shrimp farms of both *monodon* and *vannamei* occurring within 10-30 days of stocking and within a week of first symptoms
- A Vietnamese study found *monodon* were twice as likely to be infected than *vannamei,* although there have recently been signs that domesticated *monodon* are less affected. Other species –unclear !

EMS History

- EMS first noticed in in intensive *vannamei* ponds in S. China in 2009 (ignored) and then in early 2010 all over S. China and in *monodon* ponds in S. Vietnam and from mid 2010 in West Malaysia (Pahang and Johor States)
- Came back stronger in March 2011 in up to 100,000 ha in Mekong delta and Central Vietnam, plus S. China and most of W. Malaysia much worse & again in early 2012
- From September 2011 spread to Chantaburi/Rayong/ Chachoengsao in Thailand (becoming more serious in 2012) and in April 2012 Sabah & Sarawak, E. Malaysia
- Unsure if same problem or not, but histo-pathological symptoms characteristic of AHPNS can sometimes be seen, but not in all cases of EMS

EMS/AHPNS Gross Signs

- Lethargy, slow growth, corkscrew swimming, loose shells, initially swollen but then progressively shrunken, pale & degenerating hepatopancreas with less lipid droplets (consistent with toxic agent) & massive bacterial infection. Also - soft, dark, mottled carapace, white patches on body and sometimes white faeces and fouled gills. Dead shrimp on pond bottom (not sides) usually during/immediately after moulting process
- Haemolymph clotting time extended >1.5 minutes indicating stress

Early Mortality

- EMS itself is not a new issue, only the name
- Shrimp of many species in many areas have long had a difficult period around one month of culture, resulting in early mortality (one month syndrome)
- However, recent problems around Asia often appear much more serious than previously encountered
- Many of the reports of this EMS which have been occurring throughout Asia since 2009-2010 could rightly be termed EMS, but may be due to the same (often undiagnosed) diseases and poor management that have been experienced for some time, whilst others may be due to something new

Early Mortality

- Previous experience has shown that often, with newly stocked ponds, levels of potentially toxic bacterial species (especially vibrios) increase quite rapidly after about one month of culture as feed levels start to rise, algal blooms become unstable and inadequately treated pond soils begin to release toxic nutrients such as ammonia and nitrite
- Such increases in pathogenic bacteria cause stress and, if further stresses due to the presence of other pathogens (including viruses), rapid environmental changes or poor management practices occur, this can lead to mortality

EMS and AHPNS

- So is EMS merely a worse expression of existing problems – or is there something different or new out there which we have not experienced before ?
- What appears likely now - due to the severity, sudden onset and rapid spread of this new syndrome, is that there is one particular type of EMS, which has been termed AHPNS by Dr. Lightner, with new, specific and characteristic symptoms, which does indeed appear to be caused by a new pathological or toxic agent
- But that many other cases may be EMS from known causes, provoked by bad management
- However, whether "normal" EMS or the "new" AHPNS is the cause, it is to be expected that implementation of better management practices should still be able to reduce losses

Clinical Signs of AHPNS

- Dr. Donald Lightner says possible idiopathic condition with a clear case definition:
- Stage 1: Acute progressive degeneration from proximal to distal end of the tubules of the HP. Medial to distal destruction of B, R & F cells, Decreased cytokine function, No cellular differentiation, and Lack of mitotic spindles in E-cells, Prominent karyomegaly and sloughing of tubular epithelial E-cells in HP
- Stage 2: Intertubular aggregation of haemocytes
- Stage 3: 2º bacterial invasion leading to multiple *Vibrio Spp.* (& other) infections especially in the HP
- Also, Flegal recently found abnormal blebbing of the HP tubule margins in the absence of bacteria (happens with toxins or cell apoptosis)
- These histological signs must be present in AHPNS

Disease Identification

- When farmers experience early mortality, the tendency in many areas of Asia is not to conduct a thorough analysis of the actual causes of the mortality, but to point the finger at the latest new disease and blame that (i.e. the mis-diagnosis of IMNV in Asian countries outside of its known range of Indonesia)
- Without thorough and rapid analysis, correct diagnosis is often elusive
- In the case of AHPNS this means histopathology by a trained vet, together with other analysis of potential viruses, bacteria and other pathogens using histopathology, PCR and/or bacteriology
- Thus, we must all be careful when we try to ascribe a cause to a particular episode of EMS in our ponds

Our Questions ?

- Is EMS (Early Mortality Syndrome) caused by poor management or specific disease(s)?
- Should it be renamed BMS (Bad Management Syndrome) ?
- In many cases, the answer to this question is probably yes, but how much of EMS is caused by BMS ?
- And is BMS the cause in the sub-set of cases where EMS is caused by AHPNS ?
- What are the causes (and potential solutions) for EMS and AHPNS ? and
- What can we do to improve management to prevent EMS and AHPNS ?

EMS - causes ?

- Main potential causes of EMS could include: Pathogens such as viruses, bacteria & microsporidians; Toxins from either algae or applied pesticides; and Poor pond management practices
- Viruses (especially WSSV). WSSV has long known to be cause its major effects on juvenile shrimp (often 20-30 days after stocking), especially in combination with increasing vibrio loads and especially when stocking coincides with either cold or fluctuating temperatures
- Some Chinese farmers are suggesting that outbreaks of WSSV, TSV, YHV and other viral pathogens are responsible for much of the losses due to EMS, but that they are not correctly diagnosed

EMS - causes ?

- Bacteria (especially vibrios). Bacterial concentrations in both the water and the shrimp tend to increase during the first month, leading to increasingly stressed shrimp and thus mortality, and in many cases heavy bacterial infection of the hepatopancreas is characteristic of shrimp dying during outbreaks of EMS
- Early reports from Vietnam implicated microsporidian infections, and it is implicated in white faeces disease, but not in mass mortalities and is not common in EMS cases
- Algal toxins – typically originating from unwanted blooms of blue-green or dinoflagellate species – always a possibility, but no proof of this yet
- Overexposure to pesticides (from crustacides like cypermethrin) – often blamed in Vietnam and may cause EMS. But seems not AHPNS as cannot exactly replicate symptoms in shrimp exposed to these chemicals

EMS - causes ?

- Poor management practices – It is clear that in many cases, lack of proper management has lead to early mortality
- Examples of poor management practices include: poor pond preparation, lack of drying and removal of sediments, stocking in cold weather, lack of testing of PL for major pathogens, lack of control over algal blooms, lack of screening of inlet water, poor biosecurity leading to disease and disease vectors entering ponds, insufficient aeration for the biomass under culture, use of poor quality diets, use of contaminated water supplies, overuse of potentially harmful chemicals, lack of monitoring and control over bacterial concentrations in the ponds etc etc.

Risk Factors of EMS/AHPNS

- Older farms close to the sea, poor pond preparation (no sludge removal) & management leading to excess nutrient pollution, overcrowding of farms, sharing water sources, overuse of chemicals (pesticides/ABs/Chlorine/lime), intensification, seed transportation stress, poor (bacterially infected) seed quality (although 54% of Malaysian farmers and many in Thailand report faster growing SPF seeds worse affected than slower-growing seeds – perhaps due to increased moulting rate ?), high salinity at stocking, high and fluctuating temperature, overstocking & overfeeding (especially blind at start of cycle), inadequate aeration and H_2S toxicity

Risk Factors of EMS/AHPNS

Farms less affected:

- Low salinity (<20 ppt), far from sea, plastic liners, biofloc systems, (many biofloc or semi-biofloc farms report less problems – but not all), use of high quality seed, especially SPF *monodon* stocks, strict control of early feeding rates, thorough pond and environment disinfection protocols (for both virus and bacteria) prior to stocking, use of high quality probiotics and specific immunostimulants
- Risk to wild shrimp fisheries (UNKNOWN ?)

EMS - Management

- EMS can be caused by many different factors, which largely can be avoided, prevented or minimized through proper management, even though real causes may be unknown
- It has long been known that effective control over the 3 main elements of management-namely the shrimp, the pathogens and the environment can reduce risks and increase the likelihood of producing successful harvests

EMS - Management

- Experience has shown that proper management techniques can result in good production even when multiple pathogens are present within the culture environment, provided that pathogen loads are reduced and shrimp stress levels are minimized – this should also hold true for management in the presence of EMS/AHPNS
- There are already some recommendations which have been published suggesting methods of management that can either prevent or eliminate these problems due to EMS

Management methods to reduce impacts of EMS/AHPNS

- Good preparation of the pond environment : Remove sludge &/or completely dry the pond bottom to control organic nutrient loading
- Thoroughly disinfect (of virus and bacteria) water in RV and ponds prior to stocking
- Consider employing biofloc management technology or control algal blooms to limit b/g or d/f blooms
- Take care with mineral balance with low salinity ponds
- Stock only healthy post larvae (preferably > PL10) from reliable hatcheries (check condition of the HP). Ensure use of only approved probiotics

Management methods to reduce impacts of EMS/AHPNS

- Stock based on pond carrying capacity (max for *vannamei* 13-15 mt/ha and *monodon* 6-8 mt/ha)
- When CC reached (and feeding/growth slows and/or DO falls) conduct partial or full harvests
- Control feeding to about 12 kg per 100,000 pcs/day of shrimp at 30 d with average body weight at 2.0-2.5 g. The accumulated feed at 30 d should not be <250 kg. Reduce feeding when water temperatures <26 °C. Maximum daily feed increment should only be 500g/100,000 shrimp.
- Add good probiotics and/or immune stimulants to diets to improve resistance

Management methods to reduce impacts of EMS/AHPNS

- Maintain DO >3-4.0 ppm near the edge of sludge. Provide 1 HP/400 kg of biomass. When adequate oxygen is provided, problems with toxic hydrogen sulphide, ammonia and nitrite can be avoided
- Try to avoid stocking in cold weather or during periods of fluctuating environmental parameters (temperature/salinity)
- Use of domesticated, selected *monodon* stocks where available ?

Use of Immunostimulants to Control EMS ?

- Some work done by GS Biotech this year in East Malaysia with their general immunostimulant product Beta defence feeding at 3% vol/wt in diet from day 1 throughout 78 day cycle with *vannamei*
- Product contains LPS, Beta-Glucans, Amino Acids and Nucleotides developed using a special fermentation technique
- Previously found effective at preventing WSSV in *monodon* farming in Malaysia and the Philippines

Results against EMS:

- Stocked 40-50 PL/m^2 – 6 treated ponds got average 80-90% SR at FCR 1.34 and 16 g at 6.1 mt/ha after 78 days, whilst 6 control ponds were all harvested by 50 days when they were at just 11 g with <50% SR

Second trials using Beta-defense in West Malaysia - ongoing

- Where product was used from day 1 – up do date (day 35-37) only 1 out of 4 ponds were hit with EMS
- However, where treatment was started late (day 9-12), mortality due to EMS occurred in all 4 ponds by day 23-29
- Whilst in the 2 control ponds, both ponds were hit by EMS within 28-38 days
- **Note:** Correct dose rate was not administered until day 21, with only 20% of recommended dose rate fed before that
- Nonetheless, if given from day 1, indications were that it was effective at preventing EMS (even at low dose rates) hence appears that EMS could be preventable !

Cause of AHPNS ?

- Testing conducted so far on animals suffering from the subset of EMS termed AHPNS showed that it appears to be caused by an unknown agent, but that it does appear (from epidemiological analysis of its appearance and spread) to be an infectious agent, or a toxic agent of unknown origin
- Thus still unknown, but some clues as to what it isn't :
- injection of algal or pesticide toxins into SPF shrimp are not able to reproduce AHPNS symptoms, so it does not look likely to be caused by these
- Antibiotic treatments are not successful, meaning that it is unlikely to be a bacteria unless it is species which are resistant to antibiotics

Cause of EMS/AHPNS ?

- Challenge tests with homogenates show that the causative agent is not filterable - so not virus (also no virus seen by TEM) and PCR negative for IMNV, MrNV & all other viruses in EMS-infected shrimp
- Frozen shrimp infected with AHPNS have not been able to induce lesions consistent with this syndrome when injected or fed to healthy shrimp
- No evidence for a rickettsial agent (like NHP-B)
- Not from diet as feeding commercial feeds to healthy experimental shrimp in tanks – no signs of AHPNS
- Some microsporidian spore infection in affected shrimp by I-SH or microscopy, but not likely the main cause

Recent Analysis by Flegal/Chalor, Bangkok, Thailand

- To test for the possible involvement of bacteria in causing AHPNS, conducted shotgun sequencing of bacterial ssrDNA fragments amplified from HP of shrimp from 6 AHPNS infected (in Vietnam and Thailand) and 1 control pond. From analysis of approximately 100,000 fragments, a total of 8,327 unique sequences (TU) of approximately 400 bp were obtained. A comparison of the read frequency for these yielded 5 TU with the highest difference between test and control ponds. These consisted of the following genera; *Ralstonia*, *Delftia* and *Pelomonas* (both formerly *Pseudomonas spp.), Leifsonia* and *Rhodococcus spp.* The sequences from these bacterial genera will now be used to design specific probes for *in situ* hybridization assays with HP tissues from AHPNS-infected shrimp (but no results yet)

Recent Analysis by Flegal/Chalor, Bangkok, Thailand

- According to Dr Chalor, these are not naturally occurring bacteria in the ponds and should not be in the shrimp HP, and are present at higher numbers than could be considered a coincidence
- A common trait amongst these bacteria is the ability to survive and grow well at low pH, hence Chalor suspects that these bacteria were selected for these particular traits and used as probiotics so that the hatchery tanks can be maintained at low pH to reduce *Vibrio* infections
- Thus the hatcheries may have unknowingly infected all the PL with these pathogenic bacteria
- Subsequently, infected shrimp then start to succumb from HP necrosis due to stress on transport/acclimation from hatchery to ponds

Recent Analysis by Flegal/Chalor, Bangkok, Thailand

- Thus, elimination of these types of probiotics in the hatcheries should prevent such problems
- The presence of APHNS in hatchery-reared PLs is supported by the fact that classic AHPNS histopathology has been found in PL from pond - reared vannamei broodstock in Vietnamese hatcheries In addition, certain hatcheries seem to produce PL more susceptible to this syndrome and it may also explain the spread of this problem –i.e. from China to Vietnam with imported PLs and from West to East Malaysia (using PLs imported from W. Malaysia)
- Although this does not explain how this problem started in Thailand (which does not import PLs.) But: Thai hatcheries do also use a lot of probiotics !

Recent Analysis by Malaysian National Fish Centre

- Tests done on infected shrimp from Perak and Pahang States in Malaysia found colonization of haemolymph of infected individuals with high concentrations of bacteria including *Vibrio sp.* and *Photobacterium damseliae* (formerly *Pastuerella piscicida* and part of Vibrionacea family)
- Pasteurelosis has been known to cause mortality in various cultured finfish species and is common in wild fish populations. Also known to have caused black gill, HP necrosis and mortality in *monodon* in India and has been associated with disease in juvenile and adult *vannamei* in Ecuador

Recent Analysis by Malaysian National Fish Centre

- Fish can be long term carriers, and it can also be transmitted through water
- This bacteria is known to be difficult to eliminate with antibiotic treatments, with stressed fish and shrimp immuno-compromised and easily re-infected, which could explain the lack of success in treating EMS with antibiotics so far
- Already exist PCR and bacteriology tests for this bacteria, which should now be tested for more extensively in affected shrimp to see if this is a component of the syndrome

Conclusions

- A large (but undefined) portion of EMS is likely to result from poor management, although in specific cases of AHPNS, a new and as yet unverified pathogenic/toxic agent is implicated – but still may be preventable through better management
- More thorough analysis of the real causes of EMS/AHPNS should be conducted to determine the real spread of AHPNS and develop management tools and strategies to reduce its impact
- The possible role of new potentially pathogenic bacterial species and/or possible role of bad probiotics should be further investigated
- Meanwhile, use of probiotics containing suspect species in both hatchery and farm should be discontinued and only "good" probiotics used

Conclusions

- Management methods already in use to reduce stress and risks of shrimp farming in environments infected with pathogens can and should be employed to try to minimize losses due to EMS/AHPNS
- The ability of high quality probiotics and immunostimulants to prevent outbreaks should be further investigated
- Training of hatchery and farm staff in better management practices and avoidance of known risk factors is required
- PLs from all hatcheries should be screened for AHPNS symptoms prior to transfer to farms
- Movements of live shrimp stocks should be restricted until protocols for screening for EMS/AHPNS can be developed to ensure that syndrome is not transferred to new areas/countries

One-month Mortality Syndrome – Revisiting an old story

Celia R. Lavilla-Pitogo
Integrated Aquaculture International LLC
Brunei Darussalam

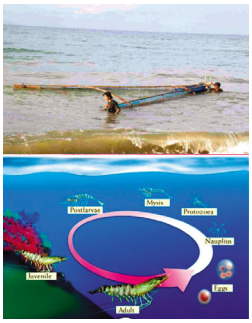
Once upon a time …

Aquaculture in the Philippines

- Ranks high in world production mainly due to seaweeds
- In polyculture systems, milkfish is traditionally the main crop in brackishwater ponds and shrimps are their minor crops .
- The major cultured shrimp is *Penaeus monodon*.
- Shrimp export averaged 30,000 (!!!) metric tons per year.

Shrimp culture in the Philippines

- Traditional ponds used wild-caught penaeid postlarvae
- In the 1980s, intensive pond technology was introduced leading to increased demand for postlarvae



Luminescent vibriosis in hatcheries

- Occurrence was reported when the hatchery system shifted from community culture to Galveston method and its modifications
- In 1990, we reported the causative agent as luminescent *Vibrio harveyi*
- At present, *V. harveyi* infection is reported in cultured aquatic animals over a wide geographical area
- Species affected not only include shrimps, but also various marine fish, lobster, and seahorse

Penaeus monodon pond culture

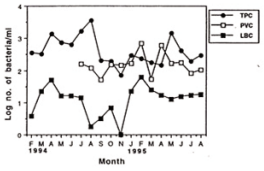
- Based on stocking density, shrimp farms are classified as:
  - Traditional = < 1 shrimp/m^2
  - Extensive = 1-3 shrimp/m^2
  - Semi-intensive = 3-10 shrimp/m^2
  - Intensive = 10-30 shrimp/m^2 or more
- Ponds are mostly earthen, but a few have concrete dikes & water canals, or are plastic lined.

Effect of luminescent vibriosis on the performance of grow-out ponds

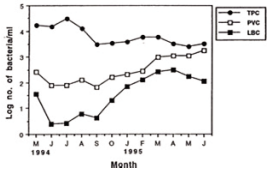
Year	Culture Period (Days)	Stocking Density (shrimp/m^2)	Survival (%)	Average Weight at Harvest (g)	Aborted Runs (%)
1992	174	20.0	86.6	28.3	0
1993	220	28.0	63.0	33.6	0
1994	174	40.3	29.3	25.6	65

Average Bacterial Load of Seawater

Nearshore



River

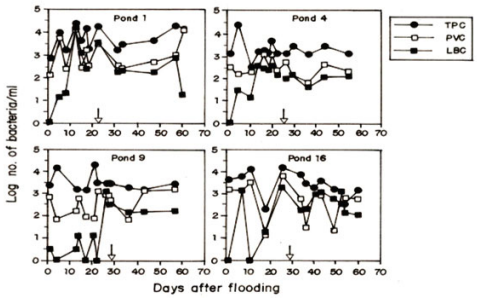


Bacterial load of soil samples from earthen ponds during pond preparation

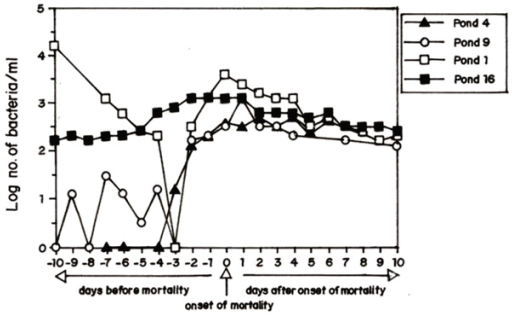
Sample	Total bacteria (cfu/g)	Presumptive vibrios (cfu/g)
Dry soil		
A	6.2×10^2	0
B	5.3×10^2	0
C	7.0×10^3	0
D	4.7×10^3	0
Moist soil		
A	3.7×10^5	9.5×10^3
B	1.1×10^5	5.0×10^2
C	2.0×10^5	2.5×10^3
D	4.3×10^4	5.0×10^2

Drying is an effective way of eliminating vibrios in the soil.

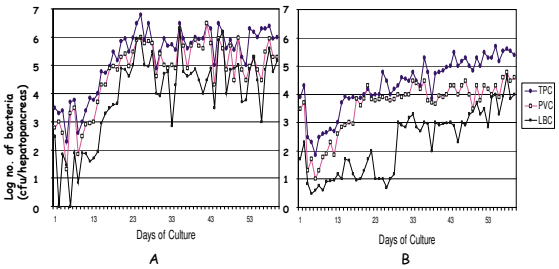
Bacterial profile of shrimp ponds from first day of flooding to 60 days



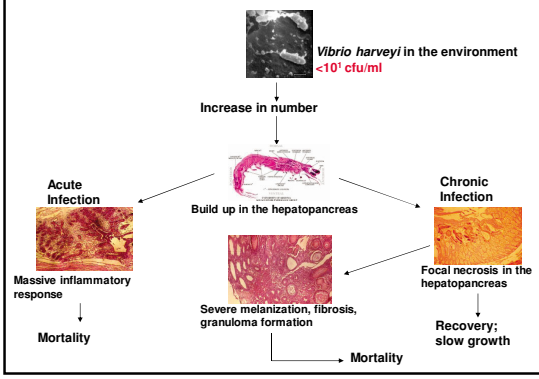
Comparison of luminescent bacterial load of 4 ponds



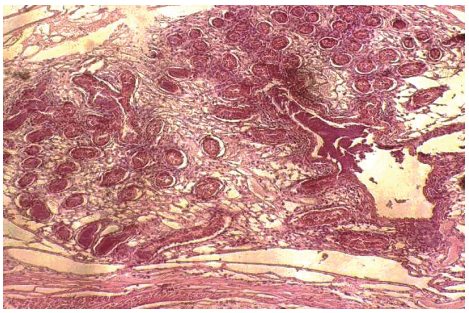
Bacterial load of the hepatopancreas of pond-reared *P. monodon* juveniles with (A) and without (B) luminescent vibriosis



Fate of *Penaeus monodon* juveniles to sub-adults after infection with *Vibrio harveyi*



Massive inflammatory response in the hepatopancreas of infected shrimp
(Not NHP after tests by Dr. Paul Frelier)



Summary

- Microbial diversity in the rearing water lost 2 - 3 weeks after flooding
- Shrimp mortality occurred within a few days of exposure to levels of luminous bacteria (LB) above 10^2 cfu/ml
- Shrimp mortality occurred when LB exceeded 50% of the total *Vibrio* load of the digestive tract
- Younger stages were more vulnerable to infection than older shrimps

Main Problems:

- Development of a niche for opportunistic/pathogenic bacteria
- Loss of microbial diversity in the rearing system

Solutions:

- Sustain microbial diversity
- Add niche-filling but benign bacteria, which can either exclude pathogens or compete for nutrients
- These bacteria may be indigenous in the system, such as those associated with phytoplankton and fish, or introduced into the system from commercial sources

Did hatcheries play a role?

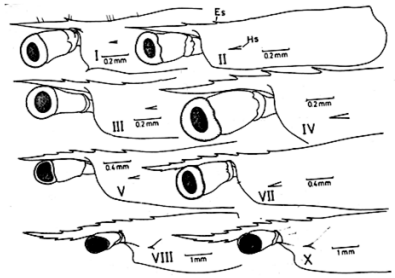
Country	Estimated number of hatcheries	Estimated number of farms
Thailand	1,000	25,000
China	1,500	8,000
Indonesia	400	60,000
India	200	100,000
Bangladesh	45	32,000
Vietnam	900	8,000
Taiwan	200	2,500
Philippines	90	2,000
Malaysia	60	800
Australia	12	35
Sri Lanka	40	800
Japan	100	135
Total	4,547	239,270

1:53 ratio

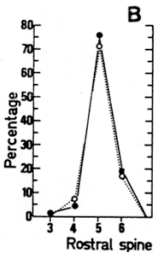
Before there were hatcheries, seeds were caught from the wild.

- Seasonal availability (March - September)
- Motoh (1981) determined the stage of wild-caught postlarvae based on rostral spine count and body measurements

How old are your postlarvae?

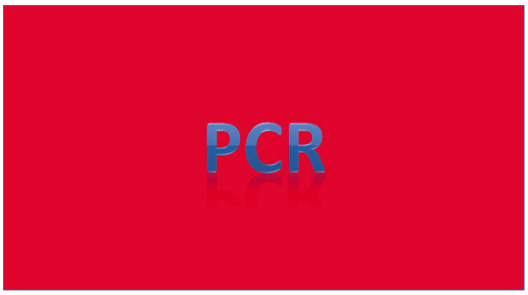


Development of rostral spines in shrimps



Number of rostral spines of wild-caught *P. monodon* PLs

Determinants of hatchery-reared shrimp postlarval quality:



Comparing PLs from Wild and Hatchery

Wild-caught

Rostral spines = 4 - 6 dorsal; 1-3 ventral

MBV infection = 0 out of 40 batches

Luminous bacterial load
- 40% are positive
- counts range from 5.0 95 cfu/postlarva

Hatchery-reared

Rostral spines = depends on age

MBV infection = 23 % positive out of 40 batches

Luminous bacterial load
- 79% are positive
- counts range from 10 - 99,000 cfu/postlarva

Range of bacterial populations associated with hatchery-reared and wild-caught postlarvae.

Stage	Total Plate Count	Presumptive *Vibrio* Count
Hatchery-reared postlarvae		
PL 12	1.4×10^1 - 8.8×10^4	1.0×10^0 - 1.7×10^4
PL 13	3.4×10^3 - 9.0×10^4	3.5×10^1 - 8.9×10^4
PL 14	5.0×10^2 - 7.5×10^4	4.7×10^2 - 2.5×10^4
PL 15	2.7×10^2 - 8.7×10^4	1.0×10^2 - 1.7×10^4
PL 16	3.5×10^1 - 9.9×10^4	5.0×10^0 - 9.9×10^4
PL 17	1.1×10^3 - 7.4×10^4	2.9×10^2 - 4.6×10^4
PL 18	6.7×10^2 - 1.3×10^5	2.0×10^2 - 2.7×10^4
Wild-caught postlarvae		
Stage V-VI	3.8×10^3 - 3.0×10^5	1.6×10^2 - 1.4×10^5

Comparison of luminescent bacterial load of hatchery-reared and wild-caught postlarvae

Stage	Number of Batches Examined	Batches Negative for Luminescent Bacteria (%)	Range of Associated Luminescent Bacteria
Hatchery-reared postlarvae = 272 batches			
PL 12	97	59 (60.8)	5.0×10^0 - 1.3×10^5
PL 13	36	12 (33.3)	2.5×10^0 - 8.9×10^4
PL 14	25	11 (44)	5.0×10^0 - 2.5×10^4
PL 15	37	11 (29.7)	7.0×10^1 - 1.7×10^4
PL 16	18	6 (33.3)	5.0×10^0 - 9.9×10^4
PL 17	28	9 (32)	5.0×10^0 - 3.0×10^5
PL 18	31	7 (22.6)	2.0×10^2 - 4.0×10^4
Wild-caught PLs	31	18 (58)	5.0×10^0 - 3.5×10^2

Summary of antibiotic reactions of bacteria from wild-caught and hatchery-reared postlarvae to antibiotics

Source of Bacteria/ Antibiotics	Resistant	Intermediate	Sensitive
Wild-caught postlarvae = 32 isolates			
TE-30	2 (6.25)	17 (53.13)	13 (40.63)
SXT	2 (6.25)	4 (12.5)	26 (81.25)
F/M 300	9 (28.13)	13 (40.63)	10 (31.25)
S-10	17 (53.13)	9 (28.13)	6 (18.75)
NOR-10	0 (0)	3 (9.38)	29 (90.63)
E-15	15 (46.88)	17 (53.13)	0 (0)
AM-10	14 (43.75)	6 (18.75)	12 (37.50)
RA-5	18 (56.25)	7 (21.88)	7 (21.88)
P-10	19 (59.38)	0 (0)	13 (40.63)
C-30	1 (3.13)	3 (9.38)	28 (87.50)
Hatchery-reared postlarvae = 48 isolates			
TE-30	21 (43.75)	14 (29.17)	13 (27.08)
SXT	24 (50)	4 (8.33)	20 (41.67)
F/M 300	6 (12.5)	25 (52.08)	17 (35.42)
S-10	36 (75)	8 (16.67)	4 (8.33)
NOR-10	0 (0)	9 (18.75)	39 (81.25)
E-15	25 (52.08)	22 (45.83)	1 (2.08)
AM-10	37 (77.08)	0 (0)	11 (22.92)
RA-5	18 (37.5)	19 (39.58)	11 (22.92)
P-10	38 (79.17)	0 (0)	10 (20.83)
C-30	16 (33.33)	6 (12.5)	26 (54.17)

Summary of Findings

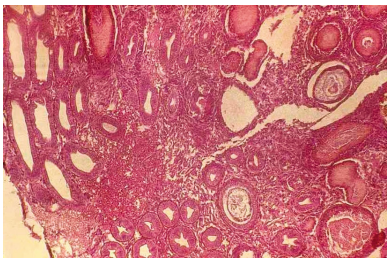
- The total bacterial populations associated with hatchery-reared and wild-caught *Penaeus monodon* postlarvae increased in proportion to their age.
- Although all postlarvae have associated vibrios, not all of them harbor luminescent bacteria and lower populations of the latter are associated with wild-caught postlarvae.

Summary of Findings

- Relatively higher percentage of antibiotic resistance is exhibited by bacteria associated with hatchery-reared than wild-caught PLs in more kinds of antibiotics.
- There is an urgent need to find and implement alternative methods of disease control to replace antibiotics.

Thank you very much for your attention

Histological section of shrimp hepatopancreas showing severe melanization, fibrosis, and hemocytic infiltration in the tubules and surrounding spaces



Management of EMS

What works and What does not?

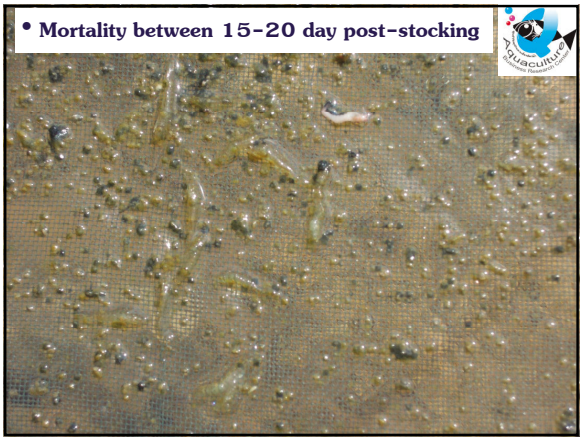
Dr. Chalor Limsuwan
Faculty of Fisheries, Kasetsart University
Thailand

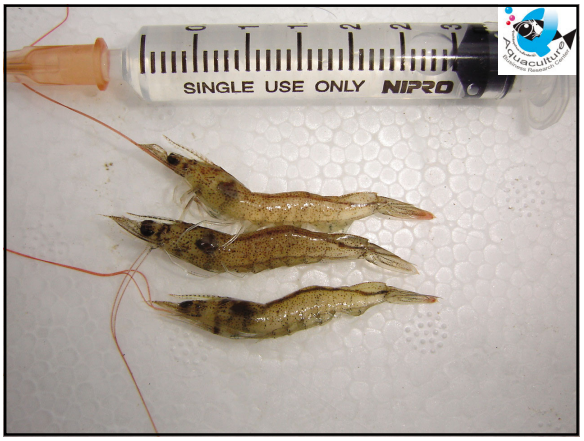
Early Mortality Syndromes: EMS

Acute Hepatopancreatic Necrosis Syndrome : AHNS

- Mortalities occur in the first 15-50 days post-stocking
- Not related to WSSV, YHV, TSV, IMNV

- Mortality between 15-20 day post-stocking

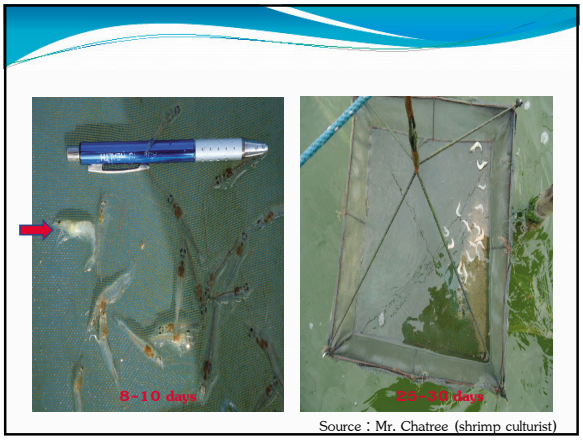




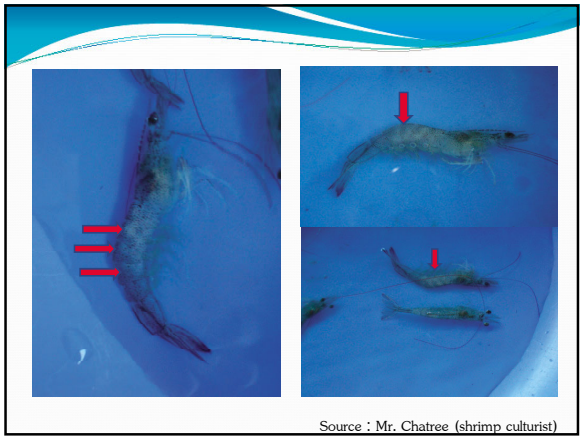
Causes of EMS

1. Mortality before 30 days

- Unhealthy postlarvae due to improperly larval rearing
  - over used of acid-producing bacteria for Vibriosis prevention
- Over using probiotic bacteria during water preparation and first 30 days (for decreasing pH and toxic form ammonia). This practice will result in shrimp molting more than normal condition leading to mortality after molting with soft-shell and whitish muscle.

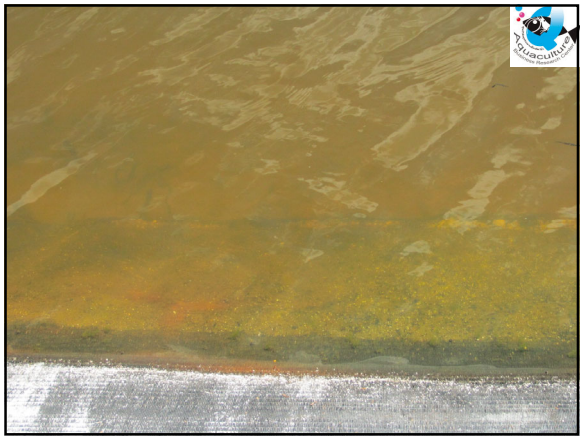


Source : Mr. Chatree (shrimp culturist)


Source : Mr. Chatree (shrimp culturist)

2. Mortality 30-50 days

- Improper water preparation e.g.
  - high transparency leading to benthic algae growth
  -low pH and alkalinity due to raining
- Inadequate aerators causing insufficient dissolved oxygen(DO) at surrounding sludge area

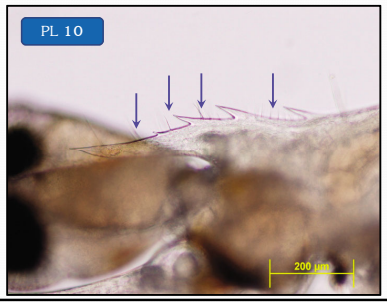


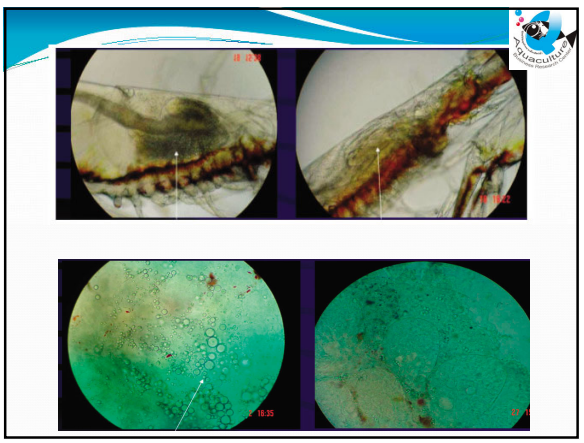

Vietnam (March-2012)
Inadequate aerators


Inadequate aerators

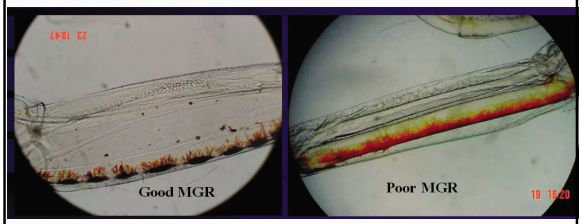
Prevention

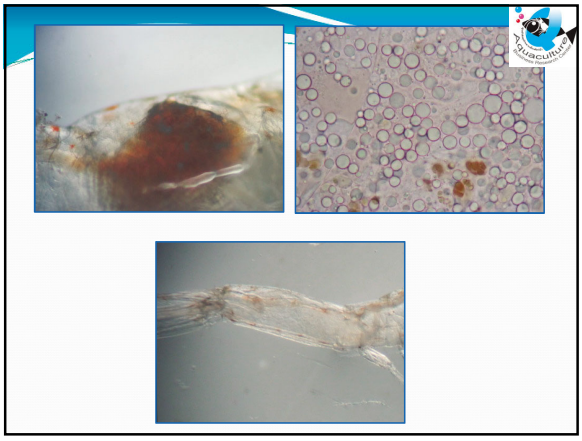
1. Use good healthy postlarvae(PL 10 or bigger)



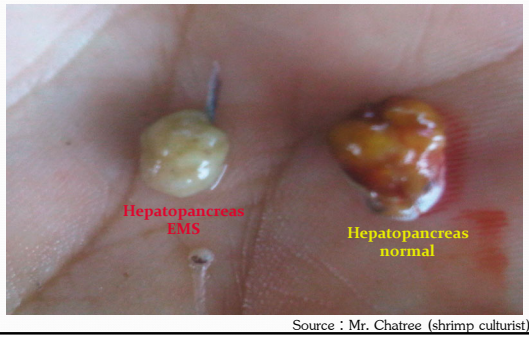


M:G ratio





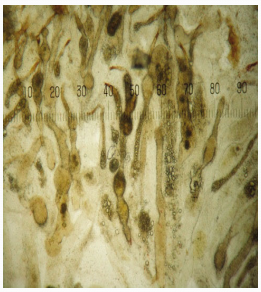
Hepatopancreas



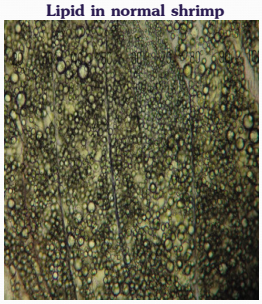
Source : Mr. Chatree (shrimp culturist)

Lipid in hepatopancreas

EMS



Lipid in normal shrimp



Source : Mr. Chatree (shrimp culturist)

Prevention(continue)

2. Use chemicals for water treatment (eliminate viral and carrier) not over using probiotic during first month post-stocking
3. pH 8.0± 0.2
4. Alkalinity should be maintained not lower than 100 mg/L(ppm)
5. Maintain DO 4.0 mg/L all the time
6. Maintain consistence water color (phytoplankton)


Thailand
Chalor Limsuwan


Chalor Limsuwan


After EMS
Be ready for bamboo shrimp


Thank you for your attention

Australian Government
Department of Agriculture, Fisheries and Forestry

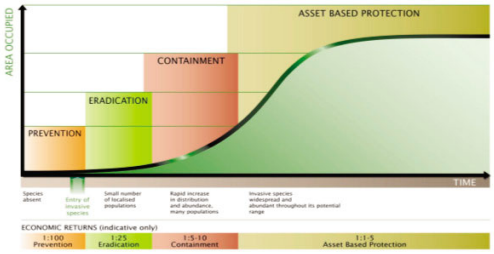
Emergency aquatic animal disease response

Ingo Ernst
Aquatic Animal Health
Animal Division
10 August 2012

What should happen next….?

What should happen next….?

GENERALISED INVASION CURVE SHOWING ACTIONS APPROPRIATE TO EACH STAGE



http://www.dpi.vic.gov.au/agriculture/pests-diseases-and-weeds/invasive

Overview

1. What is an "emergency"
2. What happens in an aquatic animal disease emergency response
3. Summary – principles of emergency response

1. What is an aquatic animal disease emergency

What is an emergency?

- Incidents that could have significant impact - economic (production or trade), environmental or human health
- Response action is needed to mitigate impacts (e.g. contain or eradicate)
- Response action aims to return industry to normal production and trade as soon as possible.

Emergency response options

Responses differ, but 3 basic possibilities:

- containment
- eradication
- mitigation and management.

Nature of the response will depend on many factors

- occurrence in closed/open systems
- distribution of the disease
- existing knowledge of the disease (e.g. epidemiology)
- tools available (e.g. diagnostics)
- potential consequences
- cost-benefit of response
- technical feasibility.

2. What happens in an aquatic animal disease emergency

Phases of an emergency response

1. Disease freedom
2. Alert
3. Incident investigation
4. Emergency response
5. Recovery

1. Freedom

- Sustainable production
- Access to markets
- Ongoing risk mitigation activities

Activities include:

- National quarantine (pre-border and border)
- Regional and farm-level biosecurity
- Surveillance programs
- Disease reporting (nationally and internationally)
- Market access through substantiating disease status.

2. Alert

- Suspicion of an emergency disease
- Notification to responsible authority

Activities include:

- Notification to responsible authority
- Collection of samples for preliminary diagnosis
- Initial steps to prevent spread of the disease (e.g. voluntary quarantine)
- Undertake initial tracing of dangerous contacts.

3. Incident investigation

- Determine if incident relates to an emergency disease
- Activate investigation protocols
- Obtain information to inform possible response actions

Activities include:

- Preliminary diagnosis (e.g. regional laboratory) and referral to reference laboratory (e.g. national laboratory) for confirmation
- Technical advisory group activated to consider diagnostic information
- International reporting (as required/appropriate); direct contact with trading partners if there are risks
- Quarantine of affected premises
- Tracing and surveillance to determine affected areas.

4. Emergency response

- Activated when a decision is made to contain or eradicate
- Continues until disease is eradicated or contained - or a decision is made that it can't be

Activities include:

- Activation of contingency plans
- Agreement on a response plan, including a response objective
- Activate command and control structures (e.g. local, national control centres established)
- Quarantine areas declared (e.g. infected, restricted and control) and movement controls enforced
- Tracing to determine confirmed and potential locations of the disease.

4. Emergency response

Activities continued…

- Surveillance to define the extent (geographical and host ranges) of the disease; detect new outbreaks; adjust quarantine areas and monitor the progress of the response plan
- Destruction of host animals (or slaughter if suitable and safe for trade)
- Disposal of destroyed animals
- Decontamination of facilities and equipment.

5. Recovery

- Proof of freedom
- Recommence production
- Resumption of trade

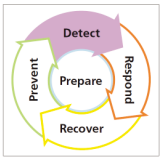
Activities include:

- Surveillance to prove freedom and success of response
- Market access negotiation
- Research and development (to support risk mitigation / disease management).

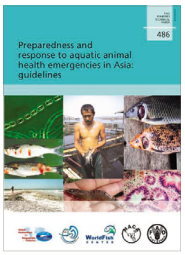
3. Summary

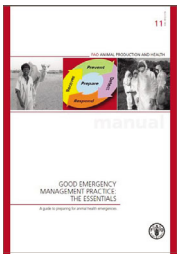
Basic principles of emergency response

- **Prevent** – Program of risk reduction measures
- **Detect** - Rapid detection and identification of the disease
- **Contain** - Early implementation of control measures to prevent spread of the disease
- **Investigate** - Rapid definition of the nature and extent of the outbreak
- **Decide** - Decision on an appropriate response objective and plan
- **Respond** – Marshal personnel and resources to implement the response plan
- **Recover** - Undertake activities to return to production and trade.

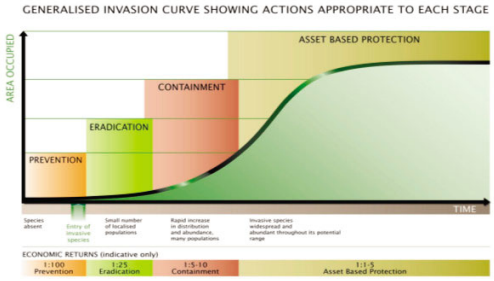


Further reading...





What should happen next....?



http://www.dpi.vic.gov.au/agriculture/pests-diseases-and-weeds/invasive

Thank you

ingo.ernst@daff.gov.au

Annex 6: AHPNS Country Presentations

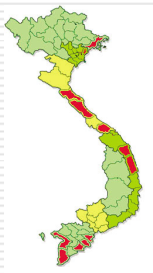
AHPNS/EMS in Shrimp in Vietnam

Pham Anh Tuan
Directorate of Fisheries (D-FISH)
MARD, Vietnam

Current Trend & Impact of AHPNS:

- First occurrence: 2010
- Early mortality: 10- 70 days after stocking
- 100% mortality
- Clinical sign: slow growth, loose shells, pale coloration
- Abnormal hepatopancreas
- Total area affected in 2012: 39,000 ha
- Mainly in intensive shrimp farms

Current Trend of AHPNS:



2010:Eastern coast of Delta (Soc Trang, Bac Lieu)
2011: Eastern coast of Delta (Soc Trang, Bac Lieu, Ca Mau)
2012: Eastern, Western Coast of Delta (Soc Trang, Bac lieu, Ca Mau, Tra Vinh), Northern (Quang Ninh, Hai Phong) and Central (Ninh Thuan, Khanh Hoa, Phu Yen, Nghe An) Region

Current Trend & Impact of AHPNS:

- AHPNS observed in PL stage
- Species affected: Black tiger and white shrimp
- End of dry season: more seriously
- Estimated loss in 2012: 5,500 Billion VND (250 Million USSD)

National Action on AHPNS:

- Task force team: MARD, PARD, Research Institutions
- Determination of primary causation:
  - The most priority
  - Participation: RIAs, CTU, DAH, IBT, IAE
- Disease surveillance
- On-farm trials to test prevention

Current investigation:

- Cypermethrin used in many shrimp ponds
- Short term test: mortality and AHPNS observed shrimp treated of Cypermethrin at 0,001 ppb
- Cypermethrin not the only, primary causation:
  - AHPNS reported from farms without Cypermethrin
  - No AHPNS observed from farms used of Cypermethrin
  - AHPNS reported from other countries where Cypermethrin banned for decades

Current investigation:

- At the PL stage:
  - Whether is AHPNDS at PL?
  - 4 samples with AHPNS
  - Samples have been collecting from different hatcheries
  - Investigation into the relation between hatchery practice & AHPNS is needed

Current Investigation:

- At growth farms:
  - AHPNS shrimp collected from Northern, Central and Southern region
  - Ponds with & without AHPNS: water quality including Cypermethrin, toxic algae analyzed
  - No evidence of relationship between toxic algae and AHPNS

Current Investigation:

- Bioassay to determine infectious causation:
- Several bioassay conducted at RIA-I, II, III.
- There is treatment with few samples indicating abnormal hepatopancreas
- No confirmation by case definition

Current investigation:

- On farm trial:
  - Several on farm trails: Tra vinh, Soc trang, Kien giang
  - To test various measures to preventing AHPNS
- Disease surveillance:
  - Conducted nationwide
  - Update situation monthly

Management Action:

- Management stragetry:
  - Seed quality control: at hatcheries & imported PL
  - Cypermethrin use in farm banned
- Recommendation to farmers:
  - Careful treatment ponds needed
  - Low stocking density
  - No use of Cypermethrin
  - Delay in farming

International Collaboration:

- Sample analysis: Prof. Donal Lightner & Prof. Tim Flegel
- Fields visit & Consultation work: FAO, WB, OIE, NACA
- Information exchanges: NACA, Malaysia…
- Financial support: FAO, OIE

Conclusions:

- More bioassay to determine whether the virus/bacteria has any causal relationship to AHPNS needed
- Review lesson learn from the success shrimp farms
- International collaboration needed

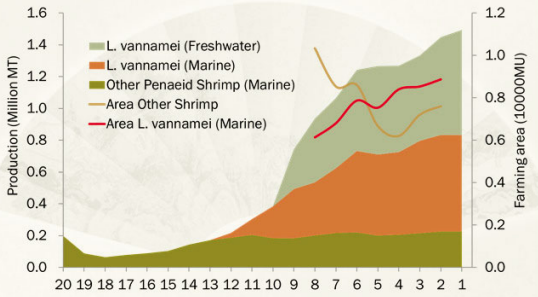
Thanks:

- Many thanks for support from FAO, OIE, NACA, WB
- Specially to Prof. Donald Lightner & Prof. Tim Flegel for technical supports

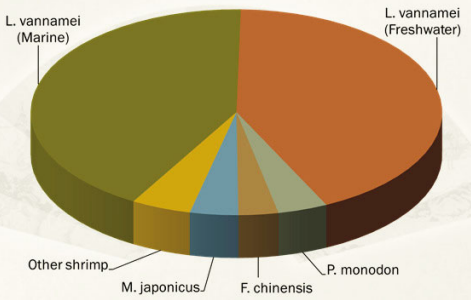
Experiences in EMS/AHPNS from China

Dr. Jie Huang
Yellow Sea Fisheries Research Institute,
Chinese Academy of Fishery Sciences

Penaeid Shrimp Farming in Last 20 Years in China



The Farmed Species and Production



History of Disease "Covert Mortality" in China

- Around 2009, a disease called "covert mortality" was noticed in some ponds farming *L. vannamei* in the southern coast of China.
- The characteristic difference of the "convert mortality" from WSD is the diseased shrimp do not appear near the shallow or swim near the surface.

Disease "Covert Mortality"

- The disease was normally found around 30 days post stocking.
- The death course happened slowly and daily. The final survival in ponds is above 20%—40%.
- The diseased shrimp show whitish or cloudy in the muscle of the abdomen. The HP may atrophy but the color is normal dark.
- The diseased shrimp do not appear near the shallow or swim near the surface. No or Rare birds appear on the diseased ponds.
- Improvement of the water quality can remit the disease.
- No known virulent virus was detected in the diseased shrimp.
- Bioassay with the disease materials does not repeat the same results.
- The disease has relationship with stocking density.
- Biological control with fish cocultivation has no effect.
- The "convert mortality" may be caused by multi facts, including stocking density, water and pond condition, and bacterial infection, etc.

History of Disease “EMS/AHPNS”

- After 2010, a disease called “early mortality” was noticed in Hainan.
- The disease characterizes with rapid and early mortality and yellow color HP.

Disease EMS

- The disease can outbreak as early as 7 days post stocking.
- The diseased shrimp do not appear near the shallow or swim near the surface. Some diseased shrimp can jump out the surface. No or Rare birds appear on the diseased ponds.
- The disease outbreaks rapidly and mortality can reach to 100% in 2—3 days.
- The disease usually outbreaks after weather change or with high growing blue green algae. But the pond has no premonition before the disease.
- The diseased shrimp appear with empty gut and stomach, yellow or discolored or small HP, swelling midgut, and slightly reddish skin.
- The known high virulent viruses, such as WSSV, TSV, and IMNV was not detected. Some virulent *Vibrio* spp. can be isolated from the diseased shrimp, but bioassays with the bacteria did not duplicate the disease features.

Disease Course in a Shrimp Farm

11% total ponds diseased
4% polyculture ponds diseased
15% single culture ponds diseased

Government Supported Projects on EMS

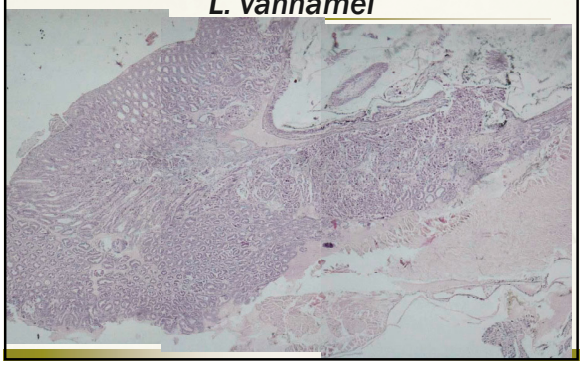
- The Special Fund for Agro-scientific Research in the Public Interest
  - Research and demonstration of the rapid diagnosis and biological control technology for the viral diseases in farmed shrimp (Grant: 201103034).
- China Agriculture Research System
  - The tasks for diseases control scientists in the Farmed Shrimp Research System (Grant: CARS-47).

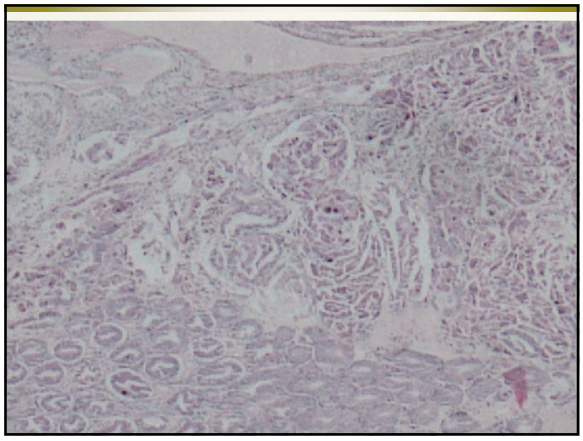
Characteristic Histopathology

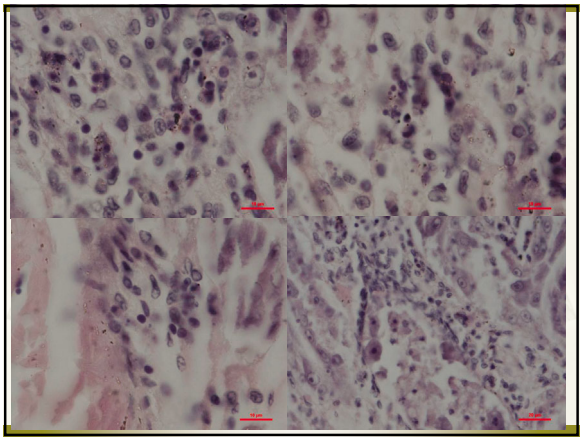
- Cytoplasmic inclusions and pyknotic nuclei were found in the cells of lymphoid organ, hemocytes, and connective tissue.
- Infiltration of large numbers of hemocytes with the cytoplasmic inclusions was found in the HP and near HP midgut. The HP tubules destruction.
- Bacterial infection follows the degeneration in hepatopancreas and near HP midgut.
- Gills and other tissues show no significant change during the infection suggests diseased shrimp do not suffer anoxia.

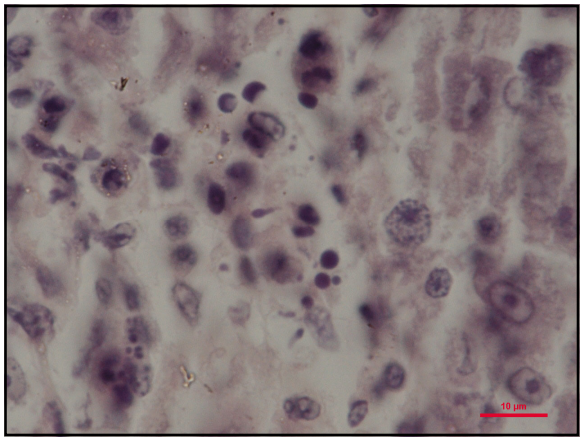
Histopathology of EMS: shrimp1

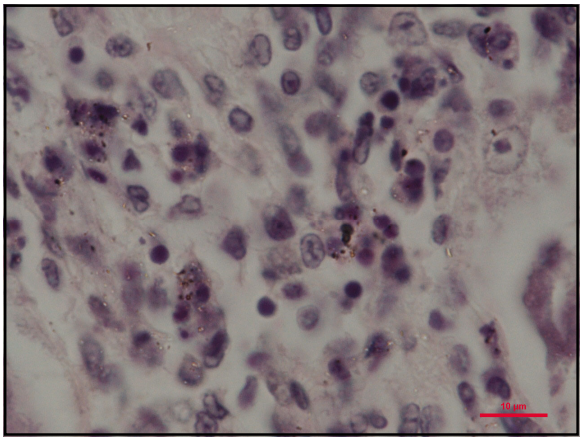
L. vannamei

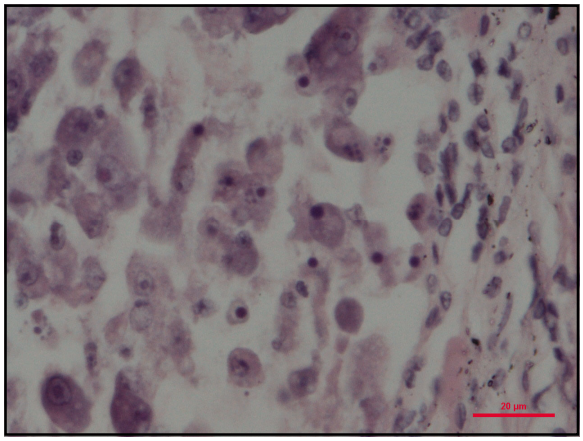




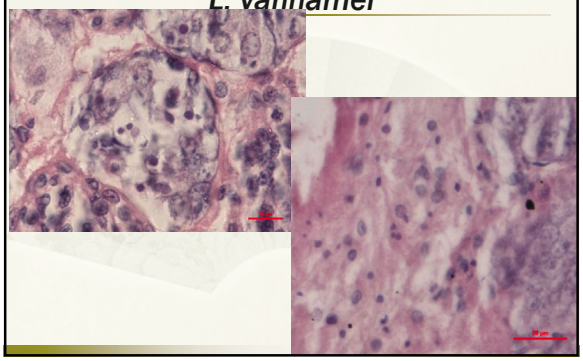




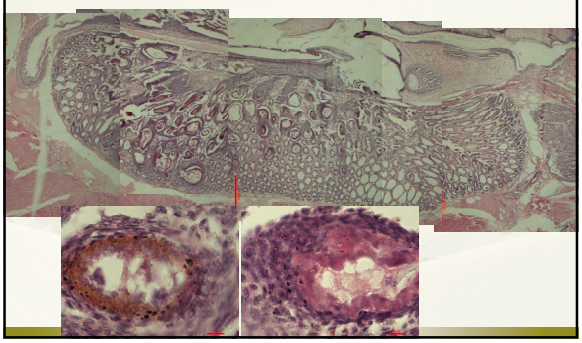


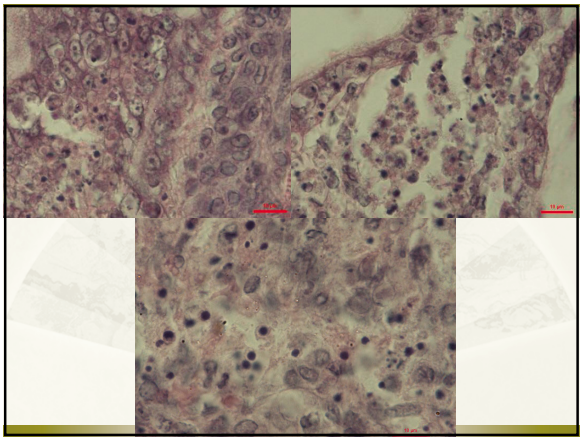


Histopathology of EMS: shrimp2
L. vannamei

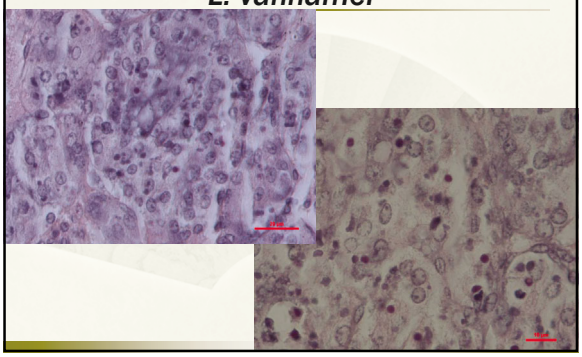


Histopathology of EMS: shrimp3
F. chinensis

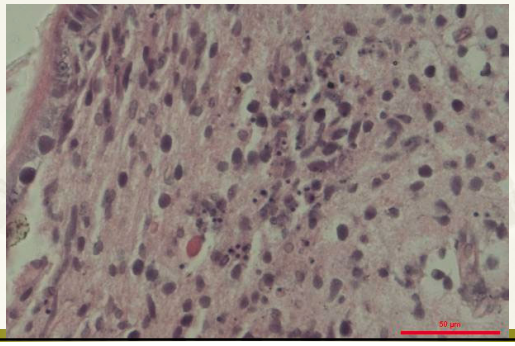




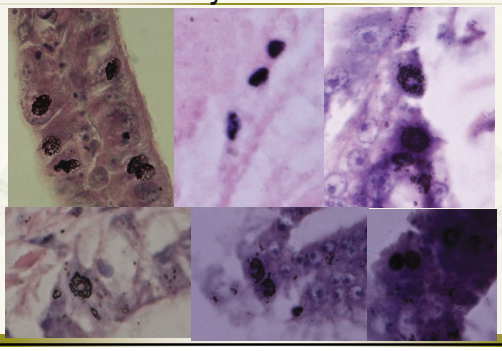
Histopathology of EMS: shrimp 4
L. vannamei



Histopathology of EMS and WSD: shrimp5
L. vannamei

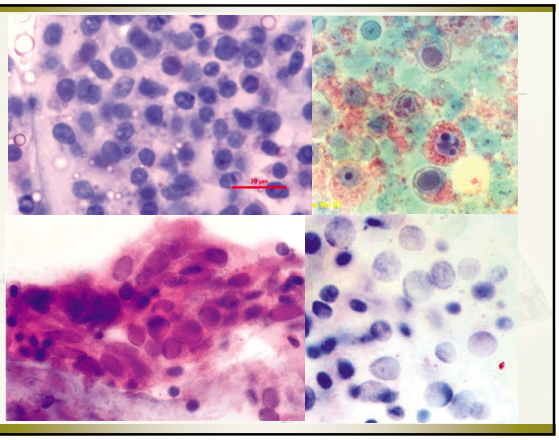


Unknown Intranuclear Refraction Crystalloids

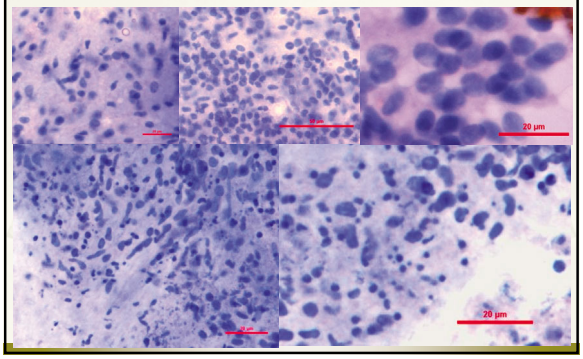


Diagnosis by Rapid T-E Staining of Smear

- Prescripts of T-E staining solution
  - Trypan blue 0.6g
  - Eosin Y 0.2g
  - Phenol 0.5g
  - NaCl 0.5g
  - Glycerol 20 mL
  - Water 80 mL
- Protocol of staining
  - Place a piece of lesion tissue on a slide and mince with a scalpel;
  - Add 1–2 drops of the T-E staining solution to the minced tissue, mix and allow to stain for 3–5 min;
  - Lay a cover glass over the stained tissue and cover with several pieces of absorbent paper. Use a thumb to squash the mince into a single layer of cells.



T-E Stained Lymphoid Organ Smear



Detection and Analysis of Known Pathogens

- Samples were tested by PCR or LAMP for at least 15 pathogens, including WSSV, IHHNV, HPV, MBV, IMNV, TSV, YHV, GAV, PvNV, MrNV, MrDV, NHP, and Spiroplasma, etc.
- Positive results for some viruses obtained in some samples. No virus was always positive in all diseased samples. Further analysis is now on-going for confirmation.
- *Vibrio parahaemolyticus*, *V. alginolyticus*, *Pseudoalteromonas* sp., *Photobacterium damselae*, *V. harveyi*, and other *V.* spp. were isolated from the samples. Challenge with *V. parahaemolyticus* proved the existence of virulence.
- Bioassay showed both filtered and unfiltered homogenate from diseased *F.* chinensis could cause 100% mortality in 8 days post challenge. The unfiltered homogenate caused more rapid mortality.

Sequencing of Random Cloning of Virus Extracts

- The tissues were homogenized and centrifuged to precipitate possible virus particles.
- The supposed virus preparation was digested with DNase and RNase to remove host nucleic acid.
- Inactive the DNase and RNase and extract the viral
- Random primer with AA at 3'-end was used to amplify possible virus nucleic acid.
- The amplified products were cloned and sequenced.
- Hundreds of sequences were obtained and analyzed by BLAST.
- 16 sequences are now selected for further preparing tests by *in situ* hybridization with slides of the shrimp tissues.

Hypothesis of the Mechanism of EMS

- Supposed virus infection in the immune system of shrimp results immune turbulence.
- The shrimp died due to the infectious immune turbulence followed by acute HP destruction and secondary infection of bacteria.
- The further confirmation research to prove the hypothesis are on-going.

Research on biological control technology

- Biological control technology by polyculture
  - Freshwater: carp, catfish, snakehead, turtle, etc.
  - Seawater: grouper, fugu, red fish, etc.
- Disease-resistant probiotics feeding
  - Collection of probiotics from gut of health shrimp.
  - Selection of the disease-resistant enhancement ability of the probiotics.
  - Addition of the probiotics into shrimp feed.
- Microorganism enhanced biofloc technology
  - Selection of microorganisms with special function.
  - Functional enhance the biofloc technology with the selected microorganism.

Possible Resolution for EMS

- Rapid diagnostic kit for on-site use.
- Disease surveillance
- Egg or postlarva quarantine.
- Polyculture technology.
- Probiotics for feed and environment use.
- Microorganism enhanced biofloc technology.
- Combination of technologies.

THANKS FOR YOUR ATTENTION!

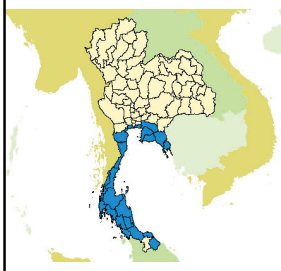
Acknowledgements

The OIE and NACA invitation. My colleagues' cooperation. The China national projects CARS-47 and 201103034 supporting for the investigation.

Early Mortality Syndrome (EMS) in Thailand: Country Report

Dr. Jiraporn Kasornchandra
Dr. Varin Tanasomwang
Dr. Putt Songsangchinda
Dr. Somkiat Kanchanakan
Ms. Janejit Kongkumnerd
Department of Fisheries, Thailand

Early Mortality Syndrome (EMS)



Shrimp farming in Thailand has been practiced more than 30 years, but develop and expand very rapidly during mid 1980s.

By 1987, *Penaeus monodon* took off in Thailand and spread quickly along the coast and its cultivation declined in 2000-2003 due to diseases and poor growth rate.

P. vannamei was first introduced to Thailand in 1997 but the cultivation was not succeeded. The development of white shrimp culture began in 2002, since then, this shrimp has become very popular and the cultivation of black tiger shrimp was replaced.

Early Mortality Syndrome (EMS)

Extensive aquaculture has been practiced for many years and moved to semi-intensive production in 70's.

Intensive shrimp farming, however, took off in 1988/9 using hatchery reared seed and formula feeds. It requires high financial and technical inputs.

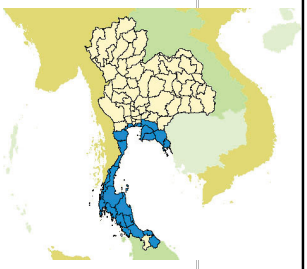
In 1998, the farm standard, CoC was applied for shrimp farms followed by the GAP farm standard in 2000.

Recently, biosecurity system has been introduced and applied for shrimp farms.

Early Mortality Syndrome (EMS)

With respect to the area of production, approximate 378,000 rai (58,154 ha) has been used for shrimp farming, of which, about 43% of the total area of shrimp farms locate in the South.

At the present, penaied shrimp contributed approximate 45 % of total mariculture production.



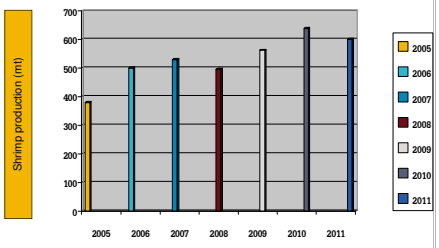
Early Mortality Syndrome (EMS)

Two species have been cultured.

: Pacific white shrimp (*Penaeus vannamei*) 99.5%

: Black tiger prawn (*Penaeus monodon*) 0.5%

Early Mortality Syndrome (EMS)

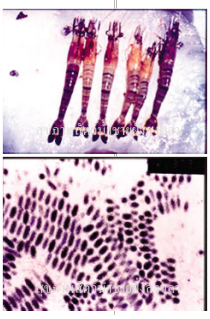


Production of farm-raised shrimp in Thailand

Early Mortality Syndrome (EMS)

Shrimp diseases status in Thailand:

1. White spot disease (WSD)**
2. Yellowhead disease (YHD)*
3. Tuara Syndrome (TS)
4. Infectious hypodermal and haematopoietic necrosis (IHHN)
5. White Feces Syndrome**



Early Mortality Syndrome (EMS)

Early Mortality Syndrome (EMS) in Thailand

Early Mortality Syndrome (EMS)

History:

Disease similar to EMS was first reported in shrimp farm located in the eastern Gulf of Thailand in late 2011. It was claimed that those farms used the same stock of PLs.

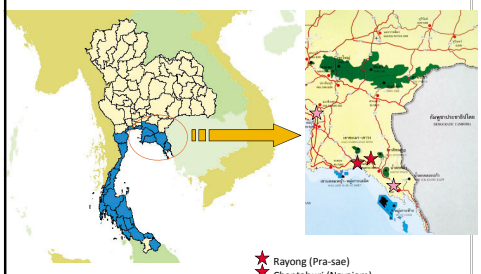
In early 2012 (January-April), EMS was reported in 3 provinces in the east coast (Gulf of Thailand): Rayong, Chantaburi and Chachoengsao provinces.

Early Mortality Syndrome (EMS)

At the present, approximate 0.7% of total shrimp ponds in Thailand have the effect from EMS, of which, mostly occur in the eastern Gulf of Thailand in Chachoengsao, Rayong, Chantaburi and Trad provinces.

Among them, Prasae in Rayong and Nayaiam in Chantaburi, are the most affected areas, approximate 10% of total shrimp ponds in that areas have been lost.

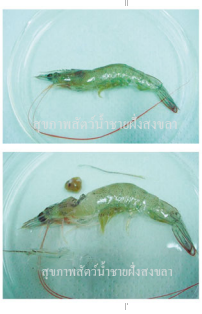
Early Mortality Syndrome (EMS)



Early Mortality Syndrome (EMS)

The disease that affects *P. vannamei* appears during the first 15-30 days of stocking ponds with PLs. In severe cases, mortality can approach 100%.

Clinical signs consist of soft or loose shell, pale coloration, lethargy and anorexia. Affected shrimp show an abnormal hepatopancrease mostly shrunken or atrophy.



Early Mortality Syndrome (EMS)

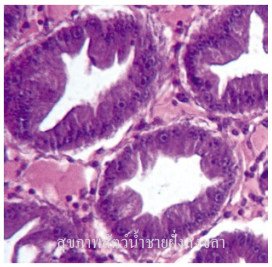
Histological finding

Pathology appears to be limited to hepatopancreas
Probably 3 stages involved:

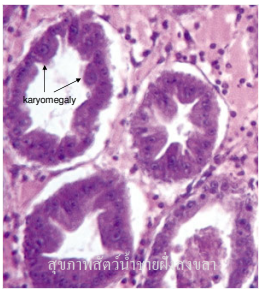
Stage 1: Acute progressive degeneration of HP, lack of mitotic activity in E cells and dysfunction of B, F, and R cells
Stage 2: Marked inflammatory response (hemolytic infiltration)
Stage 3: Secondary bacterial infection

Early Mortality Syndrome (EMS)

Stage 1:Lack of E-cell mitosis



Early Mortality Syndrome (EMS)

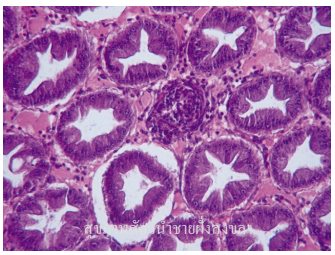


Stage 1:

:- Karyomegaly (giant nuclei)

:- Marked reduction of fat storage cell vesicles (R- cell), lack of secretory cells (B-cell) and highly basophilic cells (F-cell)

Early Mortality Syndrome (EMS)



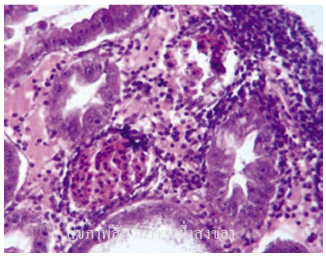
Stage 1:

Progressive degeneration of hepatopancreas

(no mitotic activity in E cells and few or no B, F, R cells)

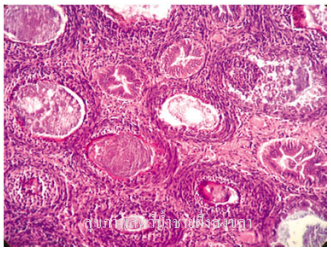
Early Mortality Syndrome (EMS)

Stage 2: Marked Inflammatory Response (Hemocytic infiltration)



Early Mortality Syndrome (EMS)

Stage 3: A severe 2° bacterial infections caused by opportunistic bacteria. Affected shrimp die from HP dysfunction and bacteria infection



Early Mortality Syndrome (EMS)

What is the possible cause of EMS?

1. Infectious agent???

:-Prachumwat *et al*. (2012) using the bacterial shotgun, they demonstrated the presence of groups of bacteria that never found for causing disease in shrimp. Three groups of bacteria have been reported: Burkholdiales (*Ralstonia*), Sphingononadales (*Delftia* or *Pseudomonas*) and Actinomycetales (*Leifsonia*, *Rhodococcus*).

:-Dr. Flegel reported the isolation of bacteriophage from the affected shrimp and his study is ongoing.

:-Virus testing has been done by injection of filtrate from the affected shrimp. No dead shrimp were observed after post-injection.

2. Toxins ???
3. Others???

Early Mortality Syndrome (EMS)

Case definition for EMS (devised by Dr Lightner and Dr Flegel)

Histopathological changes of hepatopancreas
- Lack of E-cells mitosis
- Lack of B, F and R cells
- Enlarged HP nuclei
- Sloughing of HP cells and hemolytic infiltration
- Secondary bacteria infection

Early Mortality Syndrome (EMS)

National level actions and management plans:

Government action:

1. DOF has taken an action on passive surveillance of EMS.
2. Communication on disease situation between officers and shrimp farmers at the farm level.
3. Close collaboration among officials, researchers, universities and Thai shrimp farmers association to control the disease.
4. Give the advise to farmers on health management in shrimp farm.
5. Provide diagnostic service.
6. Public awareness

Early Mortality Syndrome (EMS)

National level actions and management plans:

Private Sector action: Thai Shrimp Farmer Association

1. Communication on disease situation at the farm level
2. Set up teams for research purpose
3. Close collaboration among officials, researchers, universities and Thai shrimp farmers association for researches, health management and control of EMS
4. Provide funding for researches.
5. Hold a seminar regarding EMS including shrimp farm management throughout the country
6. Public awareness

Early Mortality Syndrome (EMS)

Lessons and experiences learned in dealing with EMS at the national level

1. Strengthening private sectors and government cooperation on disease communication
2. Information sharing among stakeholders and researchers
3. Research Fund Agencies give attention to the EMS
4. Increasing public awareness

Early Mortality Syndrome (EMS)

Conclusion and way forward

1. The causative agent for EMS has not yet be confirmed.
2. Case definition describes the histological changes of hepathopacreas
3. The possible of disease sequence

Lack of mitotic E-cell → Dysfunction of B,R and F cells → Karyomegary

→ Marked inflammatory response in HP → 2° Bacterial infections

→ Atrophy of HP → Shrimp die

Early Mortality Syndrome (EMS)

Conclusion and way forward (continue).....

4. Shrimp pond management is highly recommended.
5. Stock only healthy PLs.
5. Disinfection the disease pond as quick as possible.
6. Inform the officers or Shrimp Farm Assoc. when suspected disease occur.

Early Mortality Syndrome (EMS)

Way Forward.....

1. Identify the primary cause of EMS
2. If the primary cause is the etiological agent (bacteria, virus) we can purpose this new agent to be controlled under the Animal Epidemic Act for its regulation at national level
3. Immediate action;
   - 3.1 The EMS will be an issue of discussion in the Committee for aquatic disease control under the Animal Epidemic Act of the DOF
   - 3.2 Public awareness through media
   - 3.3 The EMS will be integrated into the Notional Active Surveillance System
   - 3.4 Researches
4. Set up contingency plans

Early Mortality Syndrome (EMS)

An investigation of outbreak in cultured White-Leg Shrimp, *Litopenaeus vannamei* in Peninsular & Sabah, Malaysia

By

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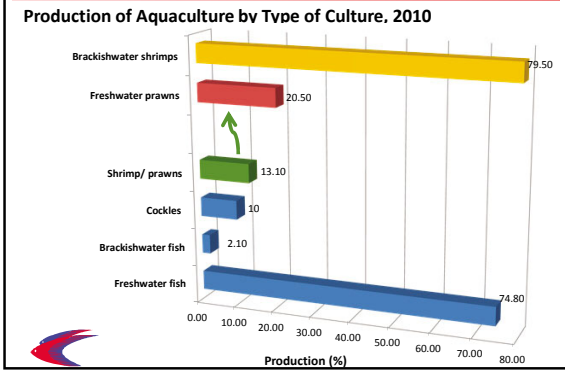
[2]National Prawn Fry Production and Research Centre, FRI P. Sayak Kg. Pulau Sayak, 08500 Kota Kuala Muda, Kedah,Malaysia

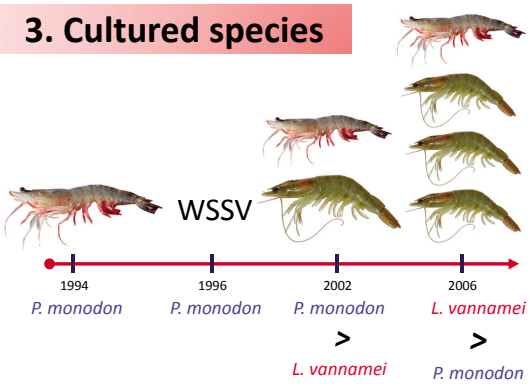
1. Introduction: shrimp farming



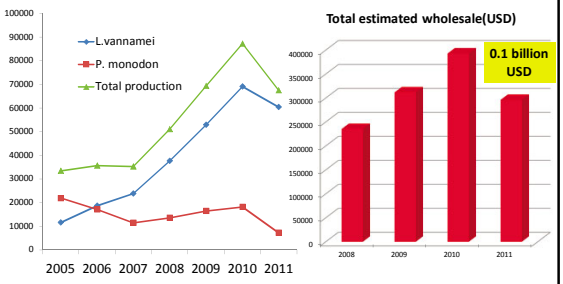
2. Cultured Area

- Earthen ponds
- HDPE lining
- Intensive culture systems (>100pc PL/m2)

3. Cultured species



4. Total Production



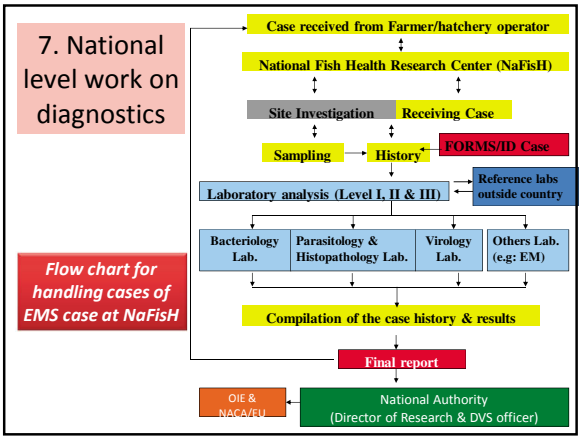
Total production of cultivated shrimps in Malaysia during year 2005 to 2010.

6. Chronological orders

Date		Location
1	Dec 2010	Johor
2	March 2011	Pahang
3	*April 2011	Perak
Received official report on EMS	Sept.2011	AQ division in DoF HQ
4	Dec 2011	Penang
5	May 2012	Kedah
6	June 2012	Sabah

* NaFisH received 2 reports on white faeces & slow growth of *L.vannamei*

7. National level work on diagnostics

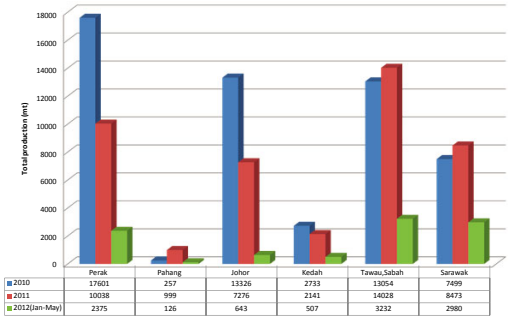


Flow chart for handling cases of EMS case at NaFisH

8. Methodology Approaches & Results

Phase	Period	Type of investigation
I (Confirmation of diseases)	Sept – Dec 2011	Case History
		Gross observation
		Hemolymph Anti-clotting time
		Bacteriology, Virology & Histopathology
II (Finding on the associated factors with EMS)	Jan – Mac 2012	Virology (IMNV, TSV, PvNv, NHBP, TSV & IHHNV)
		Cross sectional study on chemical parameter of water quality with special reference to Day Of Culture (DOC) and Unionized Ammonia (NH_3)
		Detection of Paralytic Shellfish Poison (PSP), ELISA & HPLC
III (Awareness of EMS & Control Measure by R&D)	April – Dec 2012	Awareness on EMS
		Application of Fermentation, AC & FT at farm
		Investigation of EMS in Sabah

Total production in affected state



Total production of cultivated *L.vannamei* in affected state in Malaysia during year 2010, 2011 & 2012(Jan –May)

Location	Mortality period(DOC)	DOC upon sampling	Source of PL/ Health status	Survival rate (%)	Total production (mt)
Perak					
Farm 1	27 & 40	40	Hatchery 1 / unknown	7	unknown
	30	43	Hatchery 2 / unknown	27	unknown
Farm 2	20 & 47	47	Hatchery 3 / SPF	unknown	unknown
	20	48	Hatchery 3 / SPF	unknown	unknown
Farm 3	Unknown	40	Hatchery 4 / SPF	unknown	unknown
Farm 4	30	33	Hathcery 5 /unknown	unknown	unknown
Pahang					
Farm 1	unknown	52	Hatchery 4 / SPF	44	3
	unknown	56	Hatchery 4 / SPF	44	3
	unknown	56	Hatchery 4 / SPF	27	3
Farm 2	45	65	Hatchery 3 / SPF	83	8
	Unknown	48	Hatchery 3 / SPF	25	2
	36	54	Hatchery 4 / SPF	14	0.5
	31	52	Hatchery 4 / SPF	34	2
Farm 3	40	84	Hatchery 4 / SPF	94	6
	40	85	Hatchery 4 / SPF	33	2
	40	89	Hatchery 4 / SPF	24	2
	40	89	Hatchery 4 / SPF	27	3
Penang					
Hatchery 1	unknown	unknown	Penang	unknown	unknown
Farm 1	48	50	Hatchery 1	40	unknown
	48	50	Hatchery 1	40	unknown

Location	Mortality period(DOC)	DOC upon sampling	Source of PL/ Health status	Survival rate (%)	Total production (mt)
Kedah					
Farm 1	30	30	Hatchery 5 / SPF	10	unknown
	30	30	Hatchery 5 / SPF	10	unknown
Farm 2	31	31	Hatchery 5 / SPF	10	unknown
	31	31	Hatchery 5 / SPF	10	unknown
Tawau, Sabah					
Farm 1	50	120	Hatchery 3 / SPF	96	8
	50	120	Hatchery 3 / SPF	69	8
	50	120	Hatchery 3 / SPF	52	6
Farm 2	50	120	Hatchery 3 / SPF	89	7
	50	120	Hatchery 3 / SPF	87	7
	50	120	Hatchery 3 / SPF	87	7
	50	120	Hatchery 3 / SPF	82	8
Farm 3	50	122	Hatchery 3 / SPF	26	1
	50	119	Hatchery 3 / SPF	16	1
	50	119	Hatchery 3 / SPF	8.2	0.9
	50	120	Hatchery 3 / SPF	3.6	0.3
Farm 4	Slow growth after 50	126	Hatchery 3 / SPF	80	5

8. Methodology Approaches & Results

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I	Sept – Dec 2011	Case History
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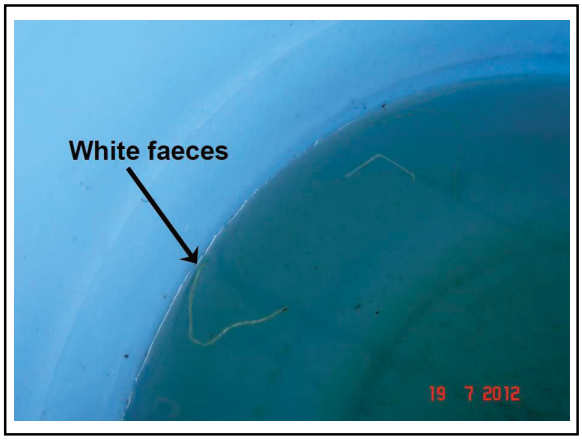
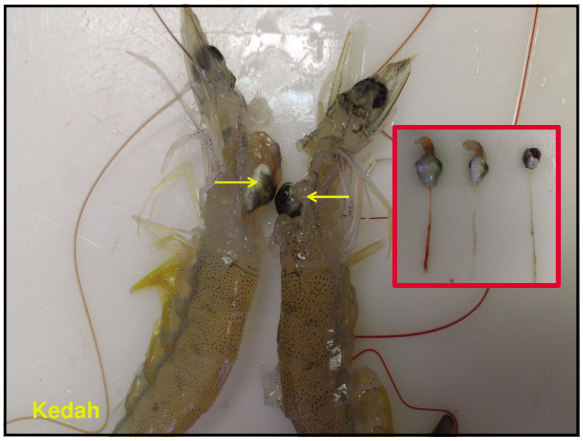
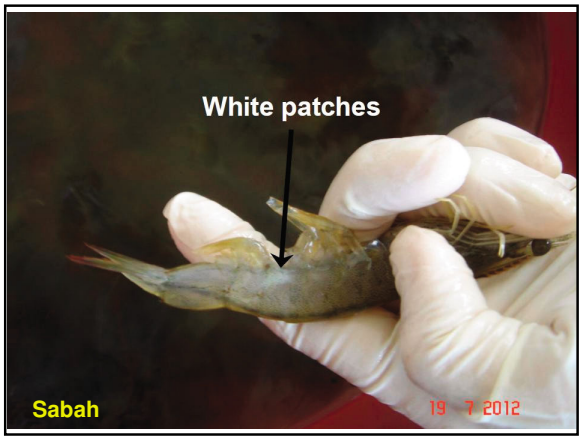
III	April – Dec 2012	Awareness on EMS
		Application of Fermentation, AC & FT at farm
		Investigation of EMS in Sabah







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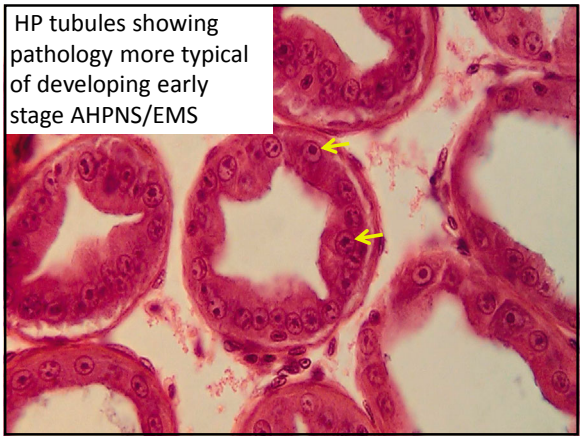
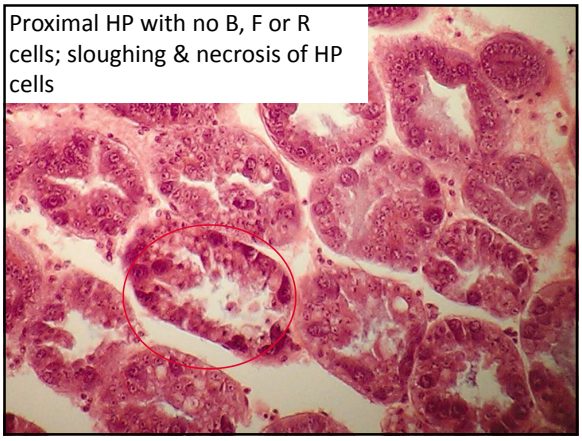
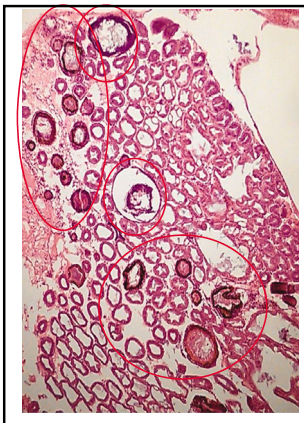
HP tubules showing pathology more typical of developing early stage AHPNS/EMS



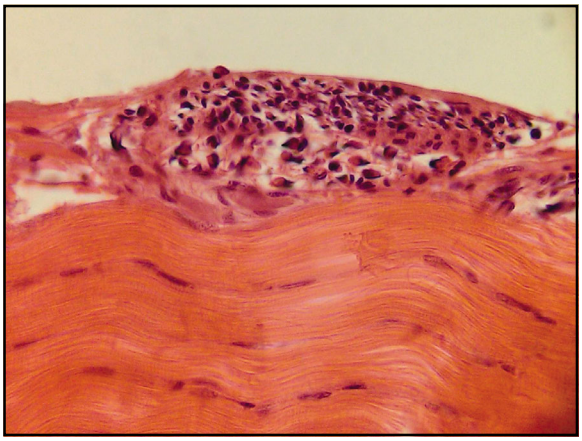
Proximal HP with no B, F or R cells; sloughing & necrosis of HP cells





- Multifocal septic
- Hemocyte encapsulated
- Melanized HP tubules

The terminal stage of AHPNS/EMS



Location	Diagnosis results		
	Bacteriology	Virology (PCR)	Pathology
Perak	*Vibrio* spp *Photobacterium damsela*	7/64 +ve IHHNV	Early & terminal stage AHPNS/EMS
Pahang	*Vibrio* spp	0/110 –ve IHHNV & 0/20 –ve IMNV (Realtime PCR)	Early & terminal stage AHPNS/EMS
Penang	-	0/22 –ve IHHNV	Early & terminal stage AHPNS/EMS
Kedah	*Vibrio* spp	No sample	Terminal stage AHPNS/EMS&WSV
Sabah	*Vibrio* spp	0/41 –ve IMNV, PvNv & NHBP (IQ Plus) 0/3 –ve TSV(IQREAL) IHHNV (on going)	Early & terminal stage of AHPNS/ EMS

8. Methodology Approaches & Results

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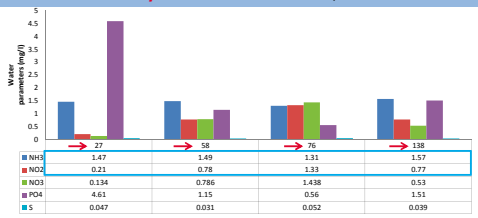
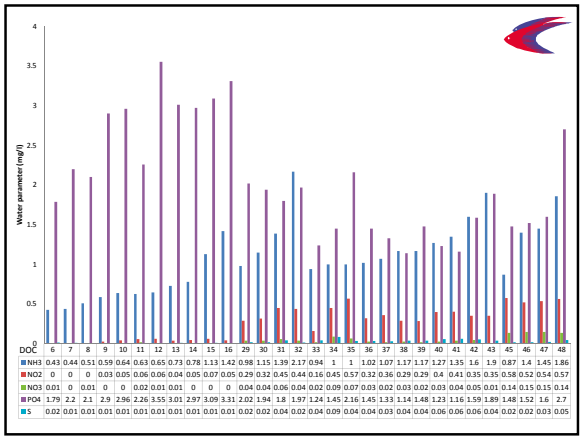
III	April – Dec 2012	Awareness on EMS
		Application of Fermentation, AC & FT at farm
		Investigation of EMS in Sabah

Cross-sectional study on chemical parameters of water quality in **different day of culture in one farm**, Perak.



Cross-sectional study on chemical parameters of water quality in **different days of culture in five farms**, Perak

DOC	Farm	Pond	Water Quality Parameters NH3	NO2	NO3	PO4	S
32	F1	B4	1.27	0.582	0.9	1.83	37
33	F2	AA	2.06	2.96	0.41	1.88	40
42	F3	2	1.14	0.196	0.27	1.46	39
58	F4	A3	1.2	1.468	1.58	0.16	53
68	F5	B3	1.31	0.037	0.08	1.32	70



Plankton — Pahang

Plankton Group	Inlet	C4-07	C5-01	Outlet
Chlrophyceae				
Chlorella				
Cynophyceae				
Oscillatoria elongata			+++++	
Baccilariophyceae (diatom)				
Coscinodiscus sp				+
Nitzchia sp	+			
Actinoptychis sp			+	
Chaetoceros (filamen)		+		
Dinophyceae				
Ceratium	+++++		+++++	
Peridinium	+		+++	
Noctiluca	+			

Occurrence of dinoflagellates in S.E.A

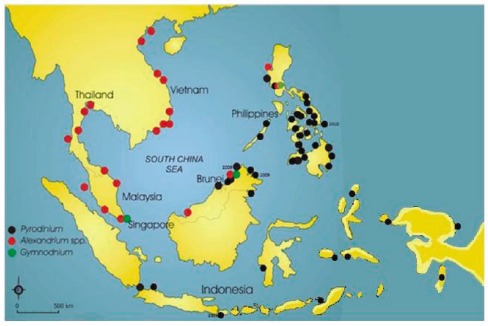


Fig. 2. Occurrence of *Pyrodinium bahamense* var. *compressum*, *Alexandrium* spp. and *Gymnodinium catenatum* in Southeast Asia.

Table 1. Occurrence of *Pseudo-nitzschia* species in the Southeast Asia.

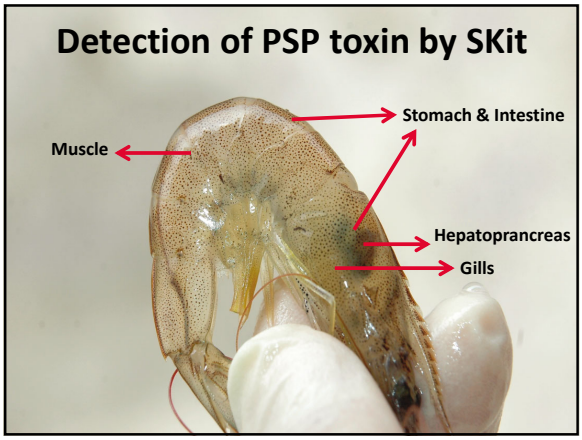
Species	Country Malaysia	Thailand	Indonesia	Vietnam	Philippines
Pseudo-nitzschia americana	—	—	—	+[b]	—
Pseudo-nitzschia brasiliana	+[b,th]	+[g]	+[g]	+[b]	+[c]
Pseudo-nitzschia caciantha	—	—	—	—	+[c]
*Pseudo-nitzschia calliantha***	+[b]	+[f]	—	+[b]	—
Pseudo-nitzschia cuspidata	—	—	—	+[b]	—
*Pseudo-nitzschia delicatissima***	+[b]	+[f]	—	+[b]	—
Pseudo-nitzschia dolorosa	+[d]	—	—	—	—
*Pseudo-nitzschia fraudulenta** *	—	—	—	+[b]	—
Pseudo-nitzschia cf. *granii*	—	—	—	+[b]	—
Pseudo-nitzschia heimii	—	+[f]	—	—	—
Pseudo-nitzschia inflatula	—	+[f]	—	+[b]	—
Pseudo-nitzschia micropora	+[b]	+[f]	—	+[b]	+[c]
*Pseudo-nitzschia multistriata** *	+[b]	—	—	+[b]	—
Pseudo-nitzschia pungens	+[dh]	—	+[g]	+[b]	+[a,c]
*Pseudo-nitzschia pseudodelicatissima** *	—	—	—	—	+[c]
Pseudo-nitzschia cf. *sinica*	—	+[f]	—	+[b]	—
Pseudo-nitzschia subpacifica	—	+[f]	—	—	—

Sources: [a]Bajarias *et al.* 2006, [b]Larsen and Nguyen 2004, [c]Yap-Dejeto *et al.* 2008, [d]Lim 2010, [e]Lundholm *et al.* 2002, [f]Prissholm *et al.* 2002, [g]Sidabutar *et al.* 2000, [h]Su 2010.
**Toxic species.

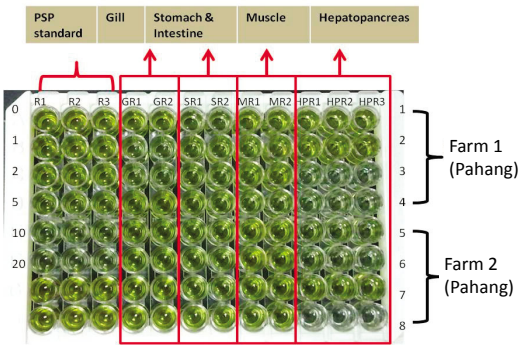
8. Methodology Approaches & Results

Phase	Period	Type of investigation
I	Sept – Dec 2011	Case History
		Gross observation
		Hemolymph Anti-clotting time
		Bacteriology, Virology & Histopathology
II	Jan – Mac 2012	Virology (IMNV, TSV, PvNv, NHBP & IHHNV)
		Cross sectional study on chemical parameter of water quality with special reference to Day Of Culture (DOC) and Unionized Ammonia (NH_3)
		Detection of Paralytic Shellfish Poison (PSP), ELISA & HPLC
III	April – Dec 2012	Awareness on EMS
		Application of Fermentation, AC & FT at farm
		Investigation of EMS in Sabah

Detection of PSP toxin by SKit



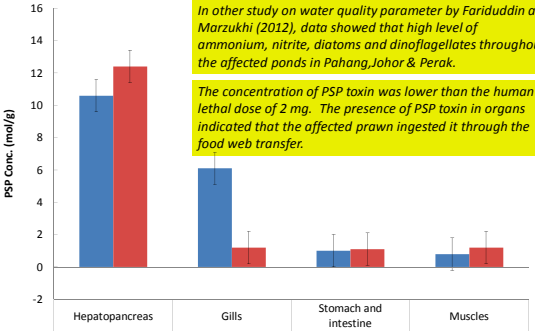
ELISA for Paralytic Shellfish Poison (PSP)



Environmental factor(s):- Paralytic Shellfish Poison (PSP) Toxin

In other study on water quality parameter by Fariduddin and Marzukhi (2012), data showed that high level of ammonium, nitrite, diatoms and dinoflagellates throughout the affected ponds in Pahang,Johor & Perak.

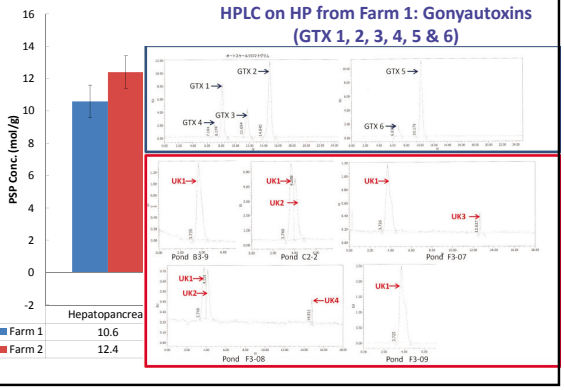
The concentration of PSP toxin was lower than the human lethal dose of 2 mg. The presence of PSP toxin in organs indicated that the affected prawn ingested it through the food web transfer.



	Hepatopancreas	Gills	Stomach and intestine	Muscles
Farm 1	10.6	6.1	1	0.8
Farm 2	12.4	1.2	1.1	1.2

Paralytic Shellfish Poison (PSP) Toxin (GTX, STX & C-toxin)

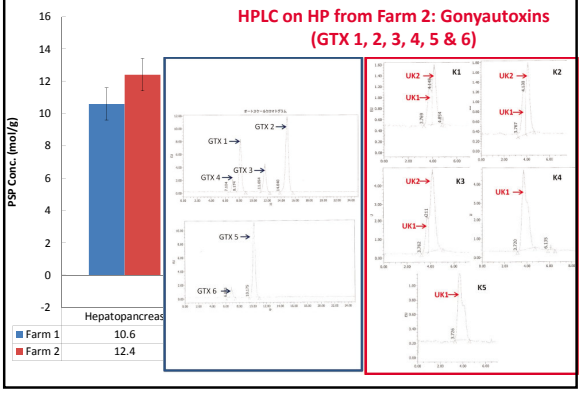
HPLC on HP from Farm 1: Gonyautoxins (GTX 1, 2, 3, 4, 5 & 6)



	Hepatopancrea
Farm 1	10.6
Farm 2	12.4

Environmental factor(s):- Paralytic Shellfish Poison (PSP) Toxin

HPLC on HP from Farm 2: Gonyautoxins (GTX 1, 2, 3, 4, 5 & 6)



	Hepatopancreas
Farm 1	10.6
Farm 2	12.4

8. Methodology Approaches & Results

Phase	Period	Type of investigation
I	Sept – Dec 2011	Case History
		Gross observation
		Hemolymph Anti-clotting time
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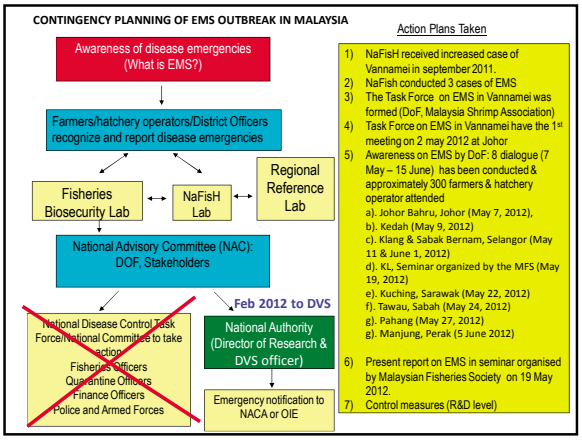
9. National level actions

- Increased Ammonia
- Increased Diatom /dinoflaggelates
- Pathology's result
- Affected prawn with postive PSP

1. 1st meeting on EMS (after received report from Stakeholders) **Sept.2011**
2. Conducted R&D on EMS (FRI P.Sayak (Water Quality) & NaFisH(Diseases) **Oct.2011- March 2012**
3. 1st report on EMS & Press conference **30 March 2012**
4. 1st meeting on Technical EMS Task Force (proposed budget USD70,000 on R&D) **2 May 2012**
5. Awareness program on EMS (focusing on pond preparation, <100 pc/m² & farm management) **7 May – 5 June 2012**
6. 2nd meeting on Technical EMS Task Force (proposed details study on HDPE lining in pond & applications of Bio-floc) **19 June 2012**



10. National Level Epidemiological

- *L. vanammei* has never been conducted since 2002
- Epidemiological study based on reported cases and R&D (Phase III)
  - Application of F, AC & FT (Perak)
  - Application of HDPE Lining (Selangor)
  - *Application of beta-defender(Sabah)

Epidemiological study: Application of F & AC (Perak)

Location	Control measure used	Pond	Stocking density (pcs/m2)	Mortality 10-30 DOC (%)	Survival rate (%)	Total production (mt)
Perak						
Company 1	Activated carbon (AC)	1	125	15 (DOC26)	60	3.3 (DOC95)
		2	110	<1	On going	On going
	Diagnosis (Histology)		DOC 20	DOC 40	DOC 60	DOC 80
		1 & 2	No EMS pathology	No EMS pathology	On going	
	Fermentation (F)	3	125	<1	On going	On going
		4	125	<1	On going	On going
	Diagnosis (Histology)		DOC 20	DOC 40	DOC 60	DOC 80
		3 & 4	No EMS pathology	No EMS pathology	On going	

Epidemiological study: Application of FT (Perak)

Location	Control measure used	Pond	Stocking density (pcs/m2)	Mortality 10-30 DOC (%)	Survival rate (%)	Total production (mt)
Perak						
Company 2	Fish Tonic(FT)	1	100	-	On going	On going
		2	100	-	On going	On going
	Control	3	100	-	On going	On going
	Diagnosis		DOC 0	DOC 10	DOC 20	DOC 30..100
	a. Pathology *(Histology)*	1	On going	On going	On going	On going
	b. Virology *(IMNV, PvNv & NHP) by IQplus & (IHHNV) by primer 389*		On going	On going	On going	On going
		2	On going	On going	On going	On going
	c. Bacteriology *(hemolymph)*		On going	On going	On going	On going
	d. WQ	3	On going	On going	On going	On going
	e.Plankton					

Epidemiological study: Application of Tripotence & OTC (Kedah)

Location	Control measure used	Pond	Stocking density (pcs/m2)	Mortality 10-30 DOC (%)	Survival rate (%)	Total production (mt)
Kedah						
Company 3	Tripotence	1	70	-	On going	On going
	Oxytetracycline (DOC15-20)	2	70	50 (DOC36)	On going	On going
	Diagnosis		DOC 35	DOC 40	DOC 60	DOC 70..100
	a. Pathology *(Histology)*	1	On going	On going		
	b. Virology *(IMNV)IQplus*		DOC 70	DOC 80	DOC 90	DOC 100
	c. Bacteriology *(hemolymph)*	2	On going			
	d. WQ					

10. National Level Epidemiological

- *L. vanammei* has never been conducted since 2002
- Epidemiological study based on reported cases and R&D (Phase III, April–Dec 2012)
  - Application of F, AC & FT (Perak)
  - Application of HDPE Lining (Selangor)
  - *Application of beta-defender(Sabah)

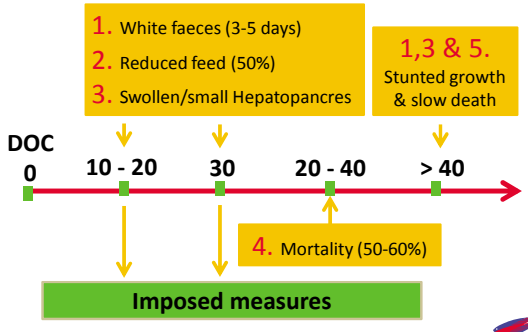
11. International collaborations to solve the problem

- Awareness given by private company
- Confirmation of EMS pathology (Dr. Lightner)
- Confirmation of toxin by HPLC (Prof. Kadamo & Dr. Takata)

13. Lessons learned and experiences gained in dealing with EMS at the national level

Phase I	Perak (Pond)	Kedah (Pond)	Penang (Hatchery)	Penang (Pond)	Pahang (Pond)	Sabah (Pond)
Identify problem a). Mortality	20 – 40 DOC	30-20 DOC	unknown	50-60 DOC	40 - 89 DOC	> 50 DOC
b). Clinical signs	White faeces, Whitish muscle, reduced feed	White faeces, Whitish muscle, reduced feed	Slow growth	White faeces, Whitish muscle, reduced feed	White faeces, Whitish muscle, reduced feed	White faeces, Whitish muscle, stunted growth, soft body
Results a).Bacteriology	*Vibrio* spp	*Vibrio* spp	*Vibrio* spp	No grow	No grow	*Vibrio* spp
b). Histopathology	• Idiopathic • Early & terminal stage of EMS	• Early & terminal stage of EMS •WSSV	•Idiopathic	• Early & terminal stage of EMS	• Early & terminal stage of EMS	Early & terminal stage of EMS
Presumptive Diagnosis	1. 30-Day mortality syndrome, (AHNDS), EMS		unknown	2. Slow Death & EMS(?)		
Phase II (Associated factors)	**2. Environmental factor(s):** - **Ammonia & ~~Nitrate~~ impacts** - **Toxin from microorganisms/feed/diatom/ dinoflagellate**					

15. Conclusions



15. Conclusions

Associated factors of outbreaks

- **50 – 100% (80%)of prawns were under stress**

(e.g: the shorter DOC or smaller prawn exposed to higher ammonium level for long term would not be able to tolerate such stress and reduced the immunity in prawn)

•Affected shrimp positive with PSP Toxin

(a). Microorganisms

(e.g: toxins produced by bacteria or toxins detected in some organisms that do not ingest dinoflagellates)

(b). Feed (mycotoxin)

(c). Diatoms & dinoflagellates

16. Way forward

- The CA should have more control on the introduction of super growth or high achiever stock
- Epidemiological study on cultivated shrimp should be active program & consistent by DOF Malaysia
- Be more precautious in using probiotic
- Awareness among the FO(s) at state level will help the farmer directly & also diagnostic centre (first hand information)
- Awareness given to target group will create a proactive responses to improve the reporting & R&D works (cross infectious between species)
- Collaboration among the regional country focusing on various aspect of AHPNS/EMS

Acknowledgements

- Farm managers (Perak, Pahang, Sabah, Kedah & Penang)
- NACA & DAFF

Thank you