Current Status and Impact of Early Mortality Syndrome (EMS)/Acute Hepatopancreatic Necrosis Disease (AHPND) and Hepatopancreatic Microsporidiosis (HPM) Outbreaks on Thailand's Shrimp Farming

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Abstract

Outbreak of early mortality syndrome (EMS) or acute hepatopancreatic necrosis disease (AHPND) in whiteleg shrimp (*Penaeus vannamei*) and black tiger shrimp (*P. monodon*) was first documented in August 2011 in Eastern Thailand. The disease subsequently spread to almost all shrimp production areas in 2012 until the early part of 2016. These episodes of AHPND outbreaks consequently impacted the shrimp industry as evidenced by significant reduction in the production of farmed shrimps, shortage of raw materials for the shrimp export industry, and reduction of global shrimp supply from Thailand. Following the discovery of *Vibrio parahaemolyticus* as the causal agent of AHPND, PCR techniques subsequently became available for the rapid and accurate detection of AHPND in cultivated shrimps. The Department of Fisheries (DOF) consequently included AHPND in the National Surveillance Program focusing on the investigation of risk factors responsible for the outbreak and concomitant spread of the disease. As a result, the quality of broodstock and postlarvae (PL) and as well as farm management practices, i.e. pond bottom and water preparation, stocking density, feeds and feeding practices, and water quality fluctuations were identified as key risk factors associated with AHPND outbreaks. By and large, the DOF has undertaken mitigating measures to control and prevent further outbreaks of AHPND including the improvement of sanitation in marine shrimp broodstock and PL hatcheries, quality evaluation and disease screening of broodstocks and PL, detection of pathogens in soil and water samples, and acquisition of new broodstocks for improved genetic diversity. To date, Thailand's shrimp industry has gradually recovered from the devastating effects of AHPND since 2015.

Heptopancreatic microsporidiosis (HPM) caused by *Enterocytozoon hepatopenaei* (EHP), a spore-forming microsporidian, is another emerging disease of cultured penaeids in Thailand. EHP was first documented in farmed *P. monodon* in 2004, however, its impact was not clearly evaluated at that time. EHP was again observed in *P. vannamei* in 2014 at the same period of AHPND outbreak in Thailand. In the field, EHP could be transmitted horizontally through feeding of the EHP-contaminated feed and feces from infected shrimp. Samples collected from numerous shrimp farms showed that EHP was heavily present in both ponds with successful and failed crops indicating that EHP infection in shrimp may not be a significant contributing factor to a failed production run. *In vitro* challenge likewise showed that there was no correlation between EHP and white feces syndrome. However, EHP infection at significantly high levels could affect shrimp growth. One of the mitigating measures to control EHP infection in cultured shrimp is the reduction of contamination in hatcheries and grow-out facilities.

Despite the negative impacts of AHPND and HPM on the shrimp industry of Thailand, all parties of the shrimp sector have been working in concert to attain the projected annual shrimp production volume of approximately 300,000 metric tons (MT) in 2016.

Introduction

Thailand is one of the major marine shrimp-producing countries in Southeast Asia exporting about 85% of its annual shrimp production to many regions of the world including USA, EU, Japan, and Canada, among others. Since 1987, extensive farming of the black tiger shrimp (*Penaeus monodon*) along the 2,614 km coastline of Thailand gradually shifted to the intensive system (Tookwinas, 1999). Shrimp production has increased to more than 13 folds, i.e. from 23,566 metric tons (MT) in 1988 to about 309,862 MT in 2000 (Figure 1). The significant increase in shrimp production has been primarily attributed to the tropical climate that creates an ideal environment for intensive marine shrimp farming (Szuster, 2006).

However, outbreaks of yellow head disease (YHD) caused by yellow head virus (YHV) and white spot disease (YSD) caused by white spot syndrome virus (WSSV) since 1992 and 1994, respectively, have negatively impacted the growth and sustainability of the Thailand's shrimp industry (Booyaratpalin, 1999). In 2002, another devastating disease named monodon slow growth syndrome (MSGS) emerged and affected shrimp growing areas throughout Thailand and significantly reduced the annual shrimp production volume by approximately 36% (Flegel, 2008). The etiology of this disease was not determined but laboratory trials suggested the involvement of a filterable infectious agent (Withyachumnarnkul, *et al.*, 2004). Because of the significant economic losses brought about by MSGS to the *P. monodon* industry in Thailand, majority of the shrimp growers eventually shifted to *P. vannamei* culture since 2004.

Whiteleg shrimp was introduced to the shrimp farms in Thailand in 2003 after the significant drop of *P. monodon* production. *P. vannamei* proved to be a good candidate species for intensive shrimp farming in Thailand as evidenced by a remarkable increase in the production volume reaching nearly 600,000 MT from 2009 to 2010 (Figure 1). However, just like the other major shrimp-producing countries in the region, shrimp production in Thailand has been hampered by outbreaks of acute hepatopancreatic necrosis disease (AHPND) since 2012. Recently, an emerging infectious disease caused by the microsporidian *Enterocytozoon hepatopenaei* (EHP) has extensively spread among cultured penaeids in Thailand.

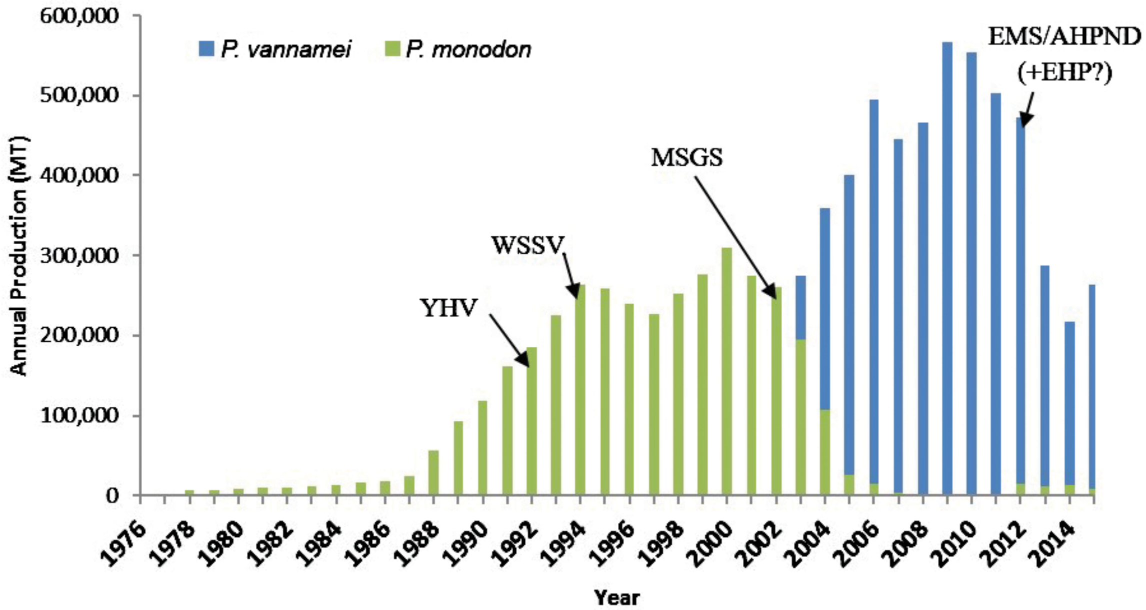

Figure 1. Annual shrimp production in Thailand from 1978 to 2015 illustrating the transition period of *Penaeus vannamei*'s dominance over *P. monodon* in terms of production volume. Black arrows point to the production years where major disease outbreaks occurred in cultured shrimps.

Status of acute hepatopancreatic necrosis disease (AHPND)

Figure 2. Photograph of a normal (A) and acute hepatopancreatic necrosis disease (AHPND)-infected shrimp exhibiting an empty gut (yellow arrow) (B). (Photo courtesy of the Department of Fisheries, Thailand)

AHPND, formerly called EMS, is an infectious bacterial disease that caused significant economic losses among cultivated shrimps in Thailand. The first outbreak of AHPND began in China in 2009 and subsequently spread to Viet Nam and Malaysia in 2010 and 2011, respectively (Tran *et al.*, 2013). In Thailand, the first reported outbreak of AHPND was in August of 2011 in a pond located in the eastern province of Thailand (Chucherd, 2013).

The Aquaculture Pathology Laboratory of the University of Arizona isolated *Vibrio parahaemolyticus* in pure culture from the hepatopancreas of shrimp infected with AHPND in 2013 (Tran *et al.*, 2013). Subsequent immersion challenge of healthy shrimps with *V. parahaemolyticus* strain isolated from the hepatopancreas of AHPND-infected shrimp led to the development of clinical signs similar to what have been previously observed in naturally-infected shrimp, indicating that indeed the *V. parahaemolyticus* strain isolated from the hepatopancreas of the diseased shrimp is the etiologic agent of AHPND (Tran *et al.*, 2013). Molecular diagnostic tests were then developed for the rapid detection of AHPND-causing *V. parahaemolyticus* (VP_{AHPND}) strains using polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) methods (Flegel and Lo, 2013, Tinwongger *et al.*, 2014; Kowai *et al.*, 2016). The Department of Fisheries (DOF) of Thailand has an ongoing collaboration with Tokyo University of Marine Science and Technology (TUMSAT) on the use of a Multiplex PCR technique to investigate and differentiate the presence of typical *V. parahaemolyticus* and VP_{AHPND} strains in shrimp samples (Tinwongger *et al.*, 2014). The causative bacterium has a specific plasmid that is responsible for the production of PirA and PirB toxins, which have been proven to be the direct cause of acute necrosis in the shrimp's hepatopancreas. The gross clinical signs exhibited by shrimps with AHPND include soft or loose shell, pale coloration and significant atrophy of hepatopancreas (Figure 2). Moreover, the infected shrimps manifested

Table 1. PCR detection of acute hepatopancreatic necrosis disease-causing *Vibrio parahaemolyticus* (VP_{AHPND}) plasmid and toxin genes in the different stages of cultured whiteleg shrimps.

Types of sample	PCR detection of			
	VP_{AHPND} plasmid		VP_{AHPND} toxin gene	
	No. of sample tested	Percentage (%) of positive sample tested	No. of sample tested	Percentage (%) of positive sample tested
Broodstock	73	0	73	0
Broodstock feces	27	19	27	0
Nauplii	53	0	53	2
Post larvae	2,174	11	1,363	5
Juvenile	1,490	28	1,261	18
Farm water	3,116	29	2,166	22
Hatchery water	542	19	23	0
Farm sediment	1,614	36	1,054	17

Table 2. Acute hepatopancreatic necrosis disease (AHPND)-positive whiteleg shrimp (*Penaeus vannamei*) postlarvae produced from the different shrimp farming areas in Thailand determined by PCR method.

Location of hatchery	AHPND detection by PCR method in *Penaeus vannamei* postlarvae		
	No. of samples tested	No. of positive samples tested	Percentage (%) of positive samples tested
Eastern provinces	120	9	7.5
Southern provinces	80	11	13.75
Total	200	20	10

Table 3. Estimated volume of annual shrimp production losses due to acute hepatopancreatic necrosis disease (AHPND) in Thailand from 2012 to 2015.

Year	Shrimp Production (MT)			Estimated annual production losses (MT)
	P. monodon	*P. vannamei*	Total	
2011	1,469	500,719	502,188	-
2012	15,219	458,012	473,231	28,957
2013	12,124	274,755	286,879	215,309
2014	13,053	204,385	217,438	284,750
2015	7,828	255,294	263,122	239,065

slow growth, empty guts, swim sluggishly or spirally along the dikes followed by mass mortality within 7 to 35 days after stocking.

All stages of shrimp can be infected by VP_{AHPND}. The target species are both the whiteleg and black tiger shrimps with the former seemingly the more susceptible species to AHPND. PCR detection of VP_{AHPND} plasmids and toxin genes in whiteleg shrimp juveniles collected from farms affected by AHPND outbreaks in 2015 showed the highest number of samples positive for plasmids (28%) and toxin gene (18%) (Table 1). PCR detection of VP_{AHPND} in postlarvae samples collected from hatcheries located in the Eastern and Southern provinces of Thailand from 2013 to 2014 have prevalence rates of ca. 7.5% and 13.75%, respectively (Table 2). In addition, as shown in Table 1, VP_{AHPND} could be found in the pond and hatchery water, as well as farm sediment with prevalence rates ranging from 19-36%.

Outbreaks of AHPND which commenced in 2012 have over the past 4 years continued to cause significant losses among cultivated shrimps produced in Thailand. Notably, the cumulative volume of production losses from 2012 to 2015 totaled to ca. 768,081 MT. As shown in Table 3, the annual shrimp production volume increased from 217,437 MT in 2014 to 263,122.90 MT in 2015, indicating that the shrimp industry of Thailand has apparently recovered from the negative impacts of AHPND.

Prevention and control AHPND

Effective mitigating measures geared at curbing AHPND outbreaks in shrimp farms include strict adherence to biosecurity and good shrimp aquaculture practices. In Thailand, the following *Guidelines for good shrimp farming practices* was issued by the DOF in 2014:

- Appropriate pond preparation to remove excess organic matter from the pond bottom;
- Intensification of farm biosecurity and water treatment;
- Increase in the number of reservoirs and water filtration systems to eliminate fish and disease carriers;
- Monitoring of postlarvae quality and health condition;
- Stocking of shrimp at appropriate density according to farm capacity;
- Maintenance of good water quality in

order to prevent anoxic condition of the pond bottom, phytoplankton blooms, and water quality fluctuations. Oxygen should be maintained at acceptable levels to promote good growth;

- If necessary, useful probiotics should be applied to pond water during the course of shrimp culture. The use of probiotics (e.g. *Bacillus subtilis*) has been promoted by the Thai government since 2013 for maintaining good water quality and shrimp health;
- Monitoring of cultivated shrimps for the presence of pathogens and removal of susceptible species;
- If cultured shrimps exhibit clinical signs of AHPND, laboratory analysis should be conducted to confirm the case. In case an outbreak of AHPND occurs in a grow-out pond, termination of culture is necessitated, and thorough chlorination of the pond water should be executed.

Status of hepatopancreatic microsporidiosis (HPM)

Hepatopancreatic microsporidiosis (HPM) is caused by *Enterocytozoon hepatopenaei* (EHP), a microsporidian first discovered in *P. monodon* in Thailand in 2004 (Chayaburakul *et al.*, 2004) and later described in detail and named (Tourtip, 2005; Tourtip *et al.*, 2009). EHP infects only the tubule epithelial cells of the hepatopancreatic (HP) tissue of shrimp. EHP was also later found to infect cultured *P. vannamei* in Thailand (Tourtip *et al.*, 2009). Through the wet mount method, EHP spores could be observed in the hepatopancreas of infected shrimps stained with Phloxine B solution (2% w/v) by light microscopy at 100x magnification (Figure 3.) PCR and *in situ* hybridization methods for EHP detection were initially described by Tourtip, *et al.* (2009). The PCR detection method was later improved using nested PCR primers synthesized based on nucleotide sequences of the small subunit ribosomal RNA (ssu rRNA) gene with PCR products of 779 bp and 176 bp, respectively (Figure 4) (Tangprasittipap *et al.*, 2013). More recently, alternative *in situ* and PCR detection (Tang *et al.*, 2015), real-time PCR (Liu *et al.*, 2014) and LAMP-nanogold methods (Suebsing *et al.*, 2013) have also been described. In general, EHP reduces the growth rate and increases the size variation of cultured penaeids. The degree of EHP infection was found to be primarily influenced by the number of spores in the hepatopancreas of HPM-infected shrimp. Additionally, the higher the number of spores present in the shrimp's hepatopancreas, the greater is its impact in stunting shrimp's growth.

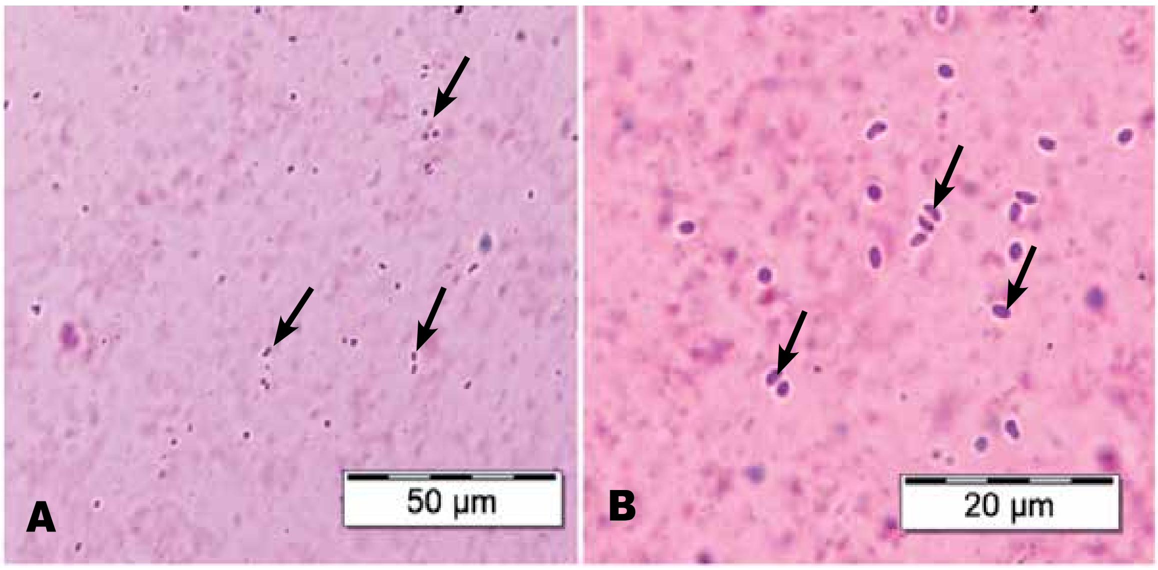

Figure 3. Spores (black arrows)of *Enterocytozoon hepatopenaei* stained with Phloxine B solution (2% w/v) examined by light microscopy at 40X (A) and 100X (B) magnification (Source: Sritunyalucksana, K. Biotec Thailand).

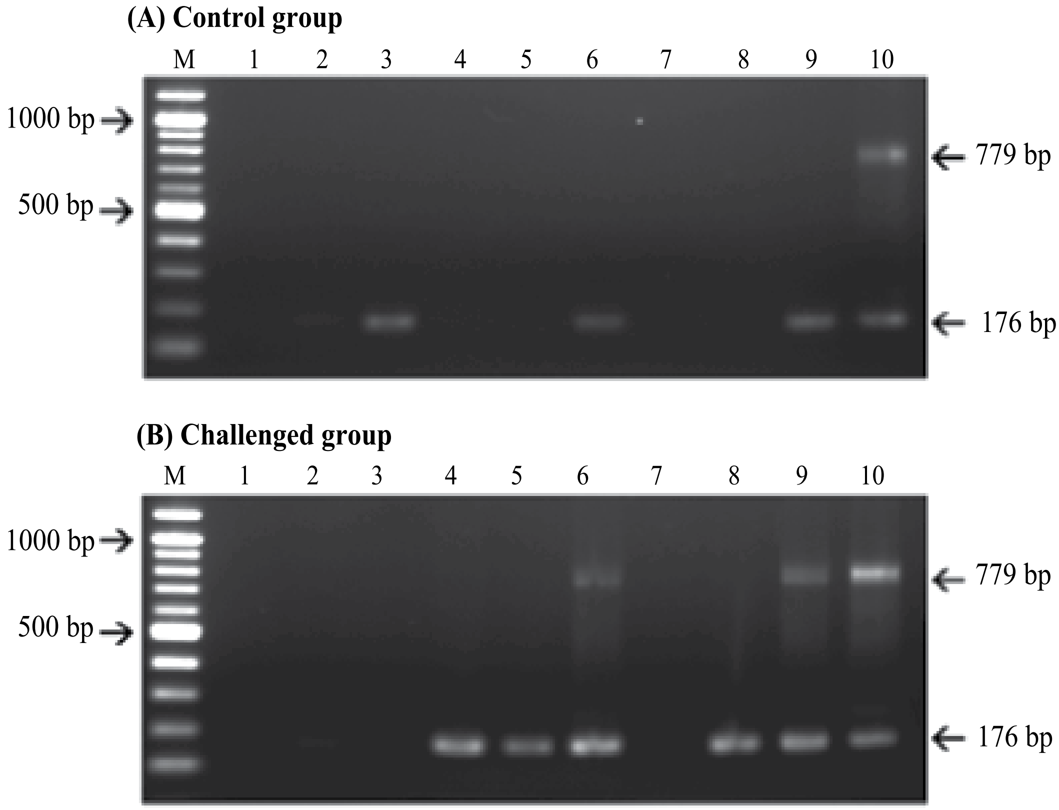

Figure 4. Agarose gels showing nested PCR microsporidian-specific amplicons using 100 ng of total DNA template from hepatopancreatic tissue obtained from *Penaeus vannamei* not challenged (A) or orally challenged (B) with the microsporidian (source: Tangprasittipap *et al.*, 2013).

Prevention and control of hepatopancreatic microsporidiosis (HPM)

At present, there is no available drug that could be practically used to treat HPM in cultured penaeids. It should be noted that EHP spores have thick walls and are not easy to inactivate. Thus, if a hatchery or grow-out farm gets infected with EHP, drastic decontamination measures should be strictly executed before restocking. In such case, disinfection of rearing water with 200 ppm chlorine has been suggested to eliminate EHP in the water. EHP-infected broodstocks could vertically transmit the spores to their offspring. Moreover, EHP-infected postlarvae could horizontally transmit the spores to the rearing water and consequently infect the yet uninfected stocks. Thus, the first and most important step that every farmer should do to avoid outbreaks of EHP infection is to ascertain that broodstock maturation facilities and hatchery facilities are clean. Because EHP has been detected in live polychaete worms and mollusks that are used as feed for broodstocks, Thai farmers have been encouraged to regularly monitor the presence of EHP in these aforementioned organisms by PCR method before use. Disinfection of eggs using chlorinated seawater has been likewise suggested. Importantly, hatchery operators have been advised to regularly clean or disinfect the rearing water and other paraphernalia such as nets, air tubes, and others, and to strictly observe biosecurity in the area.

When an outbreak of HPM occurs in a grow-out farm, farmers have been advised to contain the used water for proper disinfection prior to release. This is because effluents containing the spores released into the waterways may inadvertently contaminate the adjacent and nearby shrimp farms. Thus, to avoid and prevent the spread of EHP infection, the entire farm should be thoroughly disinfected before restocking. Liming of the pond bottom to increase the soil pH to 12 has been suggested during the pond preparation. Additionally, farmers are encouraged to maintain a bio-secure farm or closed system.

Scientific studies and programs for AHPND and HPM

In Thailand, several studies and programs have been carried out to prevent and control, and as well as mitigate the negative impacts of AHPND and HPM. These studies and/or programs include:

1. Promotion of the use of specific-pathogen-free (SPF) broodstock in order to eliminate VP_{AHPND} and EHP contamination;
2. Selective/genetic breeding program to increase *P. vannamei*'s resistance to diseases and reduction of the use of inbred lines from copy hatcheries;
3. Establishment of new shrimp farming technology to reduce the accumulation of organic wastes in the grow-out pond; and
4. Promotion of recirculating or closed systems for shrimp farming.

Way forward

The productivity of marine shrimp farming has been used as index to monitor the improvement of shrimp production in Thailand after reported production losses caused by AHPND (Figure 5). Since the first outbreak of AHPND in August 2011, the cumulative impact of AHPND on shrimp production volume over a period of 1 year, i.e. from August 2011 to July 2012 (1st phase) was not apparently pronounced based on the production data generated. On the contrary, the biggest impact was documented from August 2012 to February 2014 (second phase) as evidenced by a remarkable decrease of shrimp production volume particularly observed in February 2014. Additionally, from March 2014 to December 2015 (3rd phase) the production data showed an increasing trend, i.e. from 2.9. to 7.45 MT/1,000,000 PL, indicating that the shrimp industry of Thailand has gradually recovered from the devastating impacts of AHPND (Figure 5).

By and large, despite the negative impacts of AHPND and HPM on the shrimp industry of Thailand, all parties of the shrimp sector have been working in concert to attain the projected annual shrimp production volume of approximately 300,000 MT in 2016. Importantly, to realistically achieve the goal of maximizing the productivity and sustainability of the shrimp industry in Thailand, researchers from both the government and private sector should collaboratively act to address pressing issues related to the impact and adaptation of shrimp farming technology on climate variations,

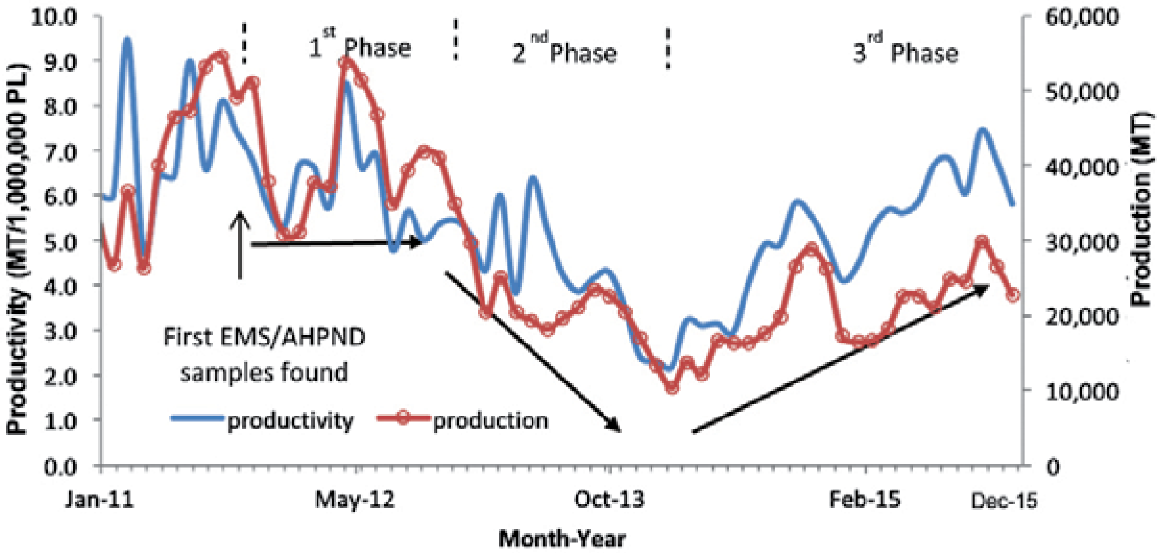

Figure 5. Productivity of shrimp farming compared with the monthly production volume during the course of acute hepatopancreatic necrosis disease (AHPND) outbreaks in Thailand from January 2011 to December 2015.

development of carrying capacity models for sustainable shrimp farming, and establishment of multitrophic aquaculture system for land-based coastal aquaculture.

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