Production results indicate that parvovirus IHHNV should be delisted as a notifiable shrimp disease

Kallaya Sritunyalucksana[1,5#], Piyachat Sanguanrut[1,#], Jiraporn Srisala[1], Jumroensri Thawonsuwan[2], Nattakan Saleetid[3], Rapeepun Vanichviriyakit[4,5], Charoonroj Chotwiwatthanakun[5,6], Timothy W. Flegel[5,7], Suparat Taengchaiyaphum[1,*]

[1]National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Yothi office, Rama VI Rd., Bangkok, 10400, Thailand

[2]Songkhla Aquatic Animal Health Research and Development Center, Aquatic Animal Health Research and Development Division, Department of Fisheries, 130/2 Pawong, Amphur Muang, Songkhla, 90100, Thailand

[3]Coastal Aquaculture Research and Development Division, Department of Fisheries, 50 Phahonyothin Rd., Lat Yao, Chatuchak, Bangkok 10900, Thailand

[4]Department of Anatomy, Faculty of Science, Mahidol University, Rama VI rd., Bangkok, 10400, Thailand

[5]Center of Excellence for Shrimp Molecular Biology and Biotechnology (Centex Shrimp), Faculty of Science, Mahidol University, Rama VI Rd., Bangkok, 10400, Thailand

[6]Mahidol University, Nakhonsawan Campus, Nakhonsawan, 60000, Thailand

[7]National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Klong Luang, Pathumthani, 12120, Thailand

#These authors with equally contribution to the work

*corresponding author

e-mail address: suparat.tae@biotec.or.th

ABSTRACT

The purpose of this study was to confirm evidence that the shrimp parvovirus IHHNV no longer has a significant negative economic impact on farmed shrimp production, and that it should be delisted as a pathogen by the World Organization for Animal Health (WOAH). Current published evidence for delisting is presented in the introduction, and experimental evidence is presented from 11 commercial ponds cultivating the giant tiger shrimp *Penaeus monodon* in Thailand. All 11 ponds gave positive PCR test results for IHHNV using 2 methods recommended for IHHNV diagnosis by WOAH. However, when an additional in-house "IHHNV Long-Amp method" (IHHNV-LA) was also used to amplify 90% of the 4 kb IHHNV genome sequence, only 4 of the 11 ponds gave positive PCR test results. Further tests using normal histopathological analysis for the presence of pathognomonic Cowdry A type inclusions (CAI), *in situ* hybridization (ISH) and immunohistochemistry (IHC) could confirm IHHNV infections in only 2 of those 4 ponds. In addition, a positive detection of CAI alone was equivalent to ISH or IHC in confirming IHHNV infection after a positive test with any of the PCR methods used. In summary, the recommended WOAH PCR methods gave false positive test results for IHHNV infection with 9/11 ponds (82%). All 11 ponds gave profitable harvests despite the confirmation of IHHNV in 2 ponds, where it was accompanied by various additional pathogens, as were all 11 ponds. Unfortunately, according to current practice, positive PCR test results with the WOAH methods can lead to rejection of traded shrimp products without assurance that they are not false positive results arising from EVE. Our results, results from others and our analysis of a recent decision of WOAH to maintain IHHNV as a listed disease suggest that WOAH must reconsider IHHNV for de-listing.

Keywords: Infectious hypodermal and hematopoietic necrosis virus (IHHNV), *Penaeus monodon*, OIE IHHNV detection method, False positive, Long-amp IHHNV detection

1. INTRODUCTION

1.1. Identity of IHHNV

The official scientific name of infectious hypodermal and hematopoietic necrosis virus (IHHNV) is now *Penstylhamaparvovirus* 1 in the family *Parvoviridae* and sub-family *Hamaparvovirinae* (Pénzes et al., 2020). Acute, catastrophic disease with mass mortality was originally reported in IHHNV-infected *Penaeus stylirostris* (Lightner et al., 1983). It was

discovered that the virus originated from grossly normal stocks of captured wild *Penaeus monodon* that were imported for experimental culture in the Americas in facilities where *P. stylirostris* was also under experimental culture. Subsequent IHHNV disease outbreaks in *Penaeus vannamei* in the Americas resulted not in mortality, but instead retarded growth accompanied by anatomical deformities (e.g., bent rostra and stunted growth) called runt deformity syndrome (RDS) (Bell and Lightner, 1984). The coefficient of variation (CV) in size for cultivated populations with RDS was typically 35-90%, with the mean shifted towards unmarketable small sizes that dominated crops and made them unprofitable. This compared to normal IHHNV-free populations with CVs of 10-30% (Bray et al., 1994; Brock and Main, 1994; Carr et al., 1996; Lightner, Donald V, 1996; Primavera and Quinitio, 2000; Pruder, 1995). The effect of IHHNV on growth of *P. monodon* is controversial, but confirmed studies (Flegel et al., 2004; Withyachumnarnkul et al., 2006) indicate that it does not cause RDS or significant negative effects on growth similar to those which occur in *P. vannamei* (more about this later).

IHHNV was among the 5 shrimp viruses listed in the first edition of the WOAH Aquatic Animal Health Code (AAHC) in 1995 and it is still listed in the current edition (WOAH, 2019a). Three distinct genotypes of IHHNV were listed in the 2019 online edition of the OIE Aquatic Manual of Diagnostic Tests for Aquatic Animals (WOAH, 2019b). These are Types 1 and 2 that are infectious viruses, while Type 3 is called non-infectious IHHNV. All are detectable by conventional PCR tests. Two primer pairs (309F/R and 389F/R) can detect all the known genotypes of IHHNV (Tang et al., 2000; Tang et al., 2007) including non-infectious Type 3 which comprises IHHNV-related DNA sequences present in the genome of *P. monodon* (Saksmerprome et al., 2011; Tang and Lightner, 2006; Tang et al., 2007). Primer set 309F/R was developed to specifically detect Types 1 and 2, but not Type 3 (Tang and Lightner, 2006; Tang et al., 2007). Type 3 has recently been revealed to be an assembly artifact that arose from scrambled sequences of an ancient form of IHHNV that sometimes occurs in a disjunctive cluster within the genome of *P. monodon* (Huerlimann et al., 2022; Taengchaiyaphum et al., 2022). Such non-retroviral virus sequences in animal genomes are called endogenous viral elements (EVE) (Feschotte and Gilbert, 2012).

The target tissues for IHHNV infection in penaeid shrimp are of ectodermal origin (e.g., the gills and sub-cuticular epidermis), and mesodermal origin (e.g., haematopoietic tissue and antennal

glands). Histological pathognomonic IHHNV lesions obtained using hematoxylin and eosin (H&E) staining are called Cowdry Type A inclusions (CAI) comprising a central, eosinophilic, intranuclear inclusion separated by a small unstained space from marginated (basophilic) chromatin (Bell and Lightner, 1984; Lightner, 1996). CAIs are an artifact of the Davidson's fixative that is recommended to preserve shrimp tissue for histological analysis and they do not occur when other fixatives such as neutral phosphate buffered fixative are used.

1.2. History of the negative economic impact of IHHNV

Although IHHNV had serious impacts first on *P. stylirostris* and subsequently on *P. vannamei* after 1983 in the Americas, it has never been associated with disease in *Penaeus monodon* cultivated by shrimp farmers in Thailand (Limsuwan and Chanratchakool, 2004). However, there have been occasional reports that have speculated on IHHNV disease in *P. monodon* from other countries in Asia. For example, one publication from the Philippines about deformed *P. monodon* during rearing of experimental broodstock (Primavera and Quinitio, 2000) speculated that IHHNV was the cause, but no histological confirmation (i.e., presence of CAI) or molecular confirmation of IHHNV infection were provided. Thus, the Philippine paper was based solely on speculation related to gross anatomical abnormalities that might have been caused by some other agent such as in abdominal segment deformity disease (ASDD)(Sakaew et al., 2008). Despite the lack of proof that IHHNV caused the abnormalities, the paper is often cited as a confirmed case of deformation caused by IHHNV. We have found no other reports of significant production losses to IHHNV from the Philippines, even since the official introduction of *P. vannamei* culture there around 2005.

The negative impact of IHHNV on cultured shrimp production in the USA dropped dramatically after the development and adoption of domesticated, specific pathogen free (SPF) shrimp stocks free of the virus (Wyban, 2007; Wyban et al., 1992). Work in Hawaii revealed that stocking PL derived from such shrimp in cultivation ponds did not result in significant RDS or other negative impact even if the shrimp became infected after stocking by residual virus from the previous culture cycle. Consistent, subsequent stocking of the same pond with IHHNV-free PL resulted in no negative impact and in a progressive decline in the number of IHHNV-positive specimens. Similarly, upon the first introduction of non-SPF *P. vannamei* for cultivation in Thailand in the

late 1990's a few cases of RDS were seen, but after the mid 2000's in Thailand upon the widespread use of IHHNV-free PL of *P. vannamei*, no cases of RDS have been reported (Flegel, 2006). In 2021, Ecuador was the world's leading exporter of cultivated *P. Vannamei*, despite the occurrence of IHHNV as evidenced by the recent news reports of Ecuadorian exports to China being rejected based on detection of IHHNV by PCR.

1.3. Ecuador's request for WOAH to review IHHNV listing

In September 2020, a report from the OIE Aquatic Animal Health Standards Commission (AAHC) responded to an official request from Ecuador to review the impact of IHHNV and to consider removing it from its list of crustacean diseases (Report of the meeting of the OIE Aquatic Animal Health Standards Commission (AAHC), Paris, 19–26 February 2020 in Section 4.6, Annex 9). The AAHC upheld the continued listing of IHHNV solely because it concluded that IHHNV fit Criterion 4b: "The disease has been shown to affect the health of cultured aquatic animals at the level of a country or a zone resulting in significant consequences e.g. production losses, morbidity or mortality at a zone or country level". If the AAHC had deemed that IHHNV did not satisfy this criterion, it would have been required to delist it.

To support their decision that IHHNV did satisfy Criterion 4b, the AAHC report cited 22 references, 8 of which were published prior to 2010 while 14 covered the period 2010 to 2020 during which the impact of IHHNV on shrimp production should have been assessed. In Supplementary Information 1, these remaining 14 cited references are examined in detail and it is shown that only 2 of them (Jagadeesan et al., 2019; Sellars et al., 2019) contained new data used to claim production losses in ponds infected with IHHNV. All the other 12 publications from 2010 onward concerned studies of IHHNV itself in terms of biology and detection and did not contain original data on production.

The flaws described in the Supplementary Information for the remaining 2 publications containing data used to support a claim for production losses due to IHHNV (Jagadeesan et al., 2019; Sellars et al., 2019) are briefly summarized here. Both publications were based predominantly on positive PCR results for detection of IHHNV. The PCR results were not backed up with additional histological examination or molecular tests (e.g., *in situ* hybridization or immunohistochemistry)

156 to confirm IHHNV infections. Nor were tests done to exclude other pathogens known to be
157 associated with rearing problems such as retarded growth. In addition, the paper by Sellars et al.,
158 2019 was carried out in 1600 m^2 experimental ponds but its estimated economic "losses" were
159 based on extrapolated production to 10,000 m^2 (1 ha) ponds at production levels (9 to 14 tons per
160 ha) greatly beyond the known average of actual *P. monodon* production (6.0-7.5 t/ha) normally
161 expected in Australian culture ponds (Marín-Riffo et al., 2021; West E et al., 2019). In addition,
162 for the paper by Jagadeesan et al. (2019), it was a survey of 350 *P. vannamei* farms in India of
163 which 30 were PCR positive for IHHNV. Of these 16/30 had problems while 14/30 did not,
164 indicating that IHHNV was not significantly associated with problem ponds and that other causes
165 should have been sought but were not. In addition, publications from the interval 2010 to 2020 that
166 describe lack of significant problems linked to IHHNV were not included in the AAHC review
167 (e.g., Hernández-Ruíz et al., 2020; Mendoza-Cano et al., 2014).
168
169 We argue that if IHHNV caused significant economic losses to the current shrimp farming
170 industry, there would have been many more publications regarding confirmed production losses
171 due to IHHNV from major producing countries in Asia and the Americas during the interval 2010
172 to 2020. Our attempts to obtain hard evidence of significant production losses to IHHNV between
173 2010 and 2021 from colleagues in China, Ecuador, India, Indonesia, Malaysia, Thailand, the
174 Philippines, and Vietnam have been met with responses that IHHNV has been detected and
175 sometimes reported as obliged to WOAH but that they have no data they can provide to show
176 significant levels of related production losses. The absence of such data suggests that any losses
177 that might have occurred were not significant enough to be noted. This contention is supported by
178 a recent publication regarding the lack of IHHNV impact on farmed shrimp production in Ecuador
179 and Peru (Aranguren Caro et al., 2022).
180
181 **1.4. Study of arbitrarily selected Thai cultivation ponds positive for IHHNV by PCR**
182 In this paper we present results from 11 recently harvested Thai shrimp ponds that were
183 selected because they gave positive PCR test results for IHHNV using two WOAH PCR detection
184 methods via active surveillance but did not exhibit significant negative effects on production that
185 could be ascribed to IHHNV. However, the results also revealed significant problems might arise
186 from false positive tests for IHHNV using currently recommended WOAH methods, if additional

187 confirmatory tests are not carried out. These should not be additional PCR tests, even with
188 sequencing, but could be histopathological analysis for Cowdry A type inclusions (CAI) and/or *in*
189 *situ* hybridization (ISH) or immunohistochemistry (IHC). We hope that this information will stir
190 others to carry out similar studies and provide more evidence from production ponds to support
191 the contention that IHHNV no longer has a significant negative impact on shrimp production.
192

193 **1.5. The process of removing a WOAH-listed shrimp disease**

194 In the 11th edition of the Aquatic Animal Health Code, there were 10 listed crustacean
195 diseases. These were Taura syndrome, White spot disease, Yellowhead disease, Tetrahedral
196 baculovirosis, Spherical baculovirosis, Infectious hypodermal and haematopoietic necrosis,
197 Crayfish plague (*Aphanomyces astaci*), Spawner-isolated mortality virus disease, White tail
198 disease and Infectious myonecrosis. However, in the 12th edition (2009), the list was reduced to 7
199 by removal of Tetrahedral baculovirosis, Spherical baculovirosis, and Spawner-isolated mortality
200 virus disease. These diseases were removed not because they were no longer a threat to cultivated
201 shrimp, but because the losses from them could be easily avoided. This was achieved by simple
202 precautionary measures implemented at hatcheries that employ captured wild broodstock shrimp
203 to produce postlarvae (PL) used by shrimp farmers to stock their rearing ponds. These control
204 measures included PCR screening and elimination of the broodstock positive for the viruses and
205 washing and/or disinfection of the eggs or nauplii. Of course, if the recommended precautions are
206 not taken with captured broodstock, problems will still result. These pathogens can also be avoided
207 by the use of PL derived from specific pathogen free (SPF) broodstock (the most common current
208 practice).
209

210 We believe that a similar situation now applies to IHHNV, not only because of available
211 precautions to screen captured broodstock for IHHNV before use, but also because of the
212 availability of SPF stocks of both *P. vannamei* and *P. monodon* that are free of IHHNV. In addition
213 to this, there is strong evidence that the available SPF stocks are not only free of IHHNV but also
214 produce offspring that are highly tolerant to infection with it.
215

216 **2. MATERIALS AND METHODS**

217 **2.1. Shrimp specimens**

Juvenile giant tiger shrimp (*Penaeus monodon*) were obtained from 2 sets of shrimp cultivation ponds. These ponds were selected based on being positive for IHHNV by active surveillance using the WOAH recommended PCR methods. The first, **Pond Set 1** consisted of 4 ponds, 1 from Chachoengsao province on the eastern coast of the Gulf of Thailand and 3 from Krabi province on the southwestern Andaman Sea coast of Thailand. These were cultivated from February to August, during the optimum time for shrimp production in Thailand. Another set of 7 ponds (**Pond Set 2**) were obtained from the adjacent provinces Phuket and Phang Nga on the southwestern coast of Thailand on the Andaman Sea. These were cultivated from October to April, through the Thai winter (November to February) which is the riskiest time for shrimp cultivation in Thailand. However, this period attracts some farmers due to higher product prices.

Thirty shrimp were collected randomly from each pond at harvest to assess the range of shrimp sizes by fresh weight. For Pond Set 1, an additional 5 were collected from each pond for PCR and histological analysis. Pleopods from the 5 shrimp specimens were removed for PCR analysis, while the cephalothoraxes of the same specimens were processed using standard methods (Bell, T.A. and Lightner, D.V., 1988) to confirm IHHNV presence by normal histological analysis plus *in situ* hybridization (ISH) analysis and immunohistochemistry (IHC) analysis. For Pond Set 2, the technician mistakenly prepared 6 shrimp specimens from each pond for PCR analysis and a separate set of 3 shrimp each for histological analysis. Thus, for Pond Set 2, results from the PCR tests and histological analysis could not be directly compared, but production results could be compared between ponds confirmed or not for the presence of infectious IHHNV.

2.2. PCR detection methods for IHHNV

Three PCR detection methods for IHHNV detection were used in this study (Table 1). Two comprised WOAH-recommended methods here called the IHHNV-309 method employing primer pairs 309F/R and the IHHNV-389 method employing primer pairs 389F/R (Tang et al., 2000; Tang et al., 2007). These methods were carried out following the OIE manual (WOAH, 2019b). A third, long-amp IHHNV (IHHNV-LA) detection method consisted of a two-step nested PCR protocol that was established in our laboratory to cover 90% (~3.6 kb) of the whole genome sequence of infectious IHHNV (4.1 kb). The primers were designed from the GenBank database of IHHNV accession number AF218266. The sensitivity limit of the IHHNV-LA method (10 copies/ng total

249 DNA) was determined using a DNA plasmid containing an insert of the 3,665 bp-product from the
250 first amplification step as the template (Taengchaiyaphum et al., 2021).
251
252 **Table 1**. Primers used for detection of IHHNV by PCR in this study.

Method	Primer	Sequence (5'-3')	Product size	Reference
IHHNV-309	309F	TCCAACACTTAGTCAAAACCAA	309 bp	Tang et al., 2007
	309R	TGTCTGCTACGATGATTATCCA		
IHHNV-389	389F	CGGAACACAACCCGACTTTA	389 bp	Tang et al., 2000
	389R	GGCCAAGACCAAAATACGAA		
IHHNV-LA	First step PCR			
	3665F	CCCAGTTTCTAACTGACGAGTGAAGAGG	3,665 bp	Taengchaiyaphum et al., 2021
	3665R	CCTGACTCTAAATGACTGACTGACGATAGGG		
	Nested PCR			
	1044F	ACAGATGTCTACAATTCAAT	1,044 bp	Taengchaiyaphum et al., 2021
	1044R	AATAGAAGAAAAGTGTCCC		

253
254 **2.3. DNA extraction**
255 Pleopods were collected from 5 individual shrimp from each pond and homogenized for
256 separate analysis in DNA lysis buffer (50 mM Tris-base, 100 mM EDTA, 50 mM NaCl, 2% (w/v)
257 SDS and 100 µg/ml proteinase K). The mixtures were incubated at 56 ºC for 1 h before DNA was
258 extracted using an QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's
259 protocol. Total DNA concentration was determined by Qubit 3.0 Fluorometer (Life Technologies,
260 USA).
261
262 **2.4. Histological analysis**
263 Histological analysis was carried out by standard methods (Bell & Lightner, 1988). Briefly,
264 the cephalothorax of each shrimp sample was fixed with Davidson's fixative for 24 h and then
265 changed to 70% ethanol. The tissue was next dehydrated with a series of graded ethanol ending
266 with xylene and embedded in paraffin. Sections were cut at 4 µm and placed onto glass slides for
267 H&E staining to detect of Cowdry Type A inclusions (CAI) pathognomonic for IHHNV, for *in*
268 *situ* hybridization (ISH) analysis and for immunohistochemistry (IHC) analysis (Lightner 1996).

269 Positive control slides were prepared using archived tissue blocks of shrimp infected with IHHNV
270 and showing typical IHHNV-CAI. We consider the detection of CAI as the gold standard for
271 confirmation of IHHNV infection. The farm samples were also examined histologically for the
272 presence of lesions in the hepatopancreas (HP) caused by monodon baculovirus (MBV),
273 hepatopancreatic parvovirus (HPV) and other pathogens such as bacteria known to cause retarded
274 growth in *P. monodon* (Flegel, 2004; Flegel et al., 2006). The viruses MBV and HPV are now
275 called Penaeus monodon nudivirus (PmNV) (Yang et al., 2014) and Penaeus monodon
276 hepandhamaparvovirus (Cotmore et al., 2019), respectively. Analysis of the lesions for these 2
277 viruses was carried out as recommended by Lightner (1996). The HP of the specimens were also
278 screened for the presence of the microsporidian *Enterocytozoon hepatopenaei* (EHP) that is known
279 to infect the HP and to be associated with retarded shrimp growth (Chaijarasphong et al., 2021).
280

281 **2.5. *In situ* hybridization (ISH)**

282 The PCR products amplified from both IHHNV-309 and IHHNV-389 methods were used as
283 templates to prepare digoxigenin (DIG)-labeled IHHNV probes using a PCR DIG-labeling kit
284 (Roche, Germany). After preparation, probes were purified using a Gel/PCR cleanup kit (Geneaid,
285 Taiwan) using the protocol described by the manufacturer. The protocol for ISH has been
286 previously described (Sanguanrut et al., 2022). Briefly, slides containing adjacent tissue sections
287 to those used for histological analysis by H&E staining were de-paraffinized, rehydrated and TNE
288 buffer (500 mM Tris-Cl, 100 mM NaCl, l0 mM EDTA) before treatment with proteinase K (Sigma,
289 USA) and assay using 200 ng per slide of the DIG-labeled probes in hybridization buffer. A control
290 reaction without probe was included in a separate container. After incubation overnight at 42 ºC
291 the slides were washed, incubated with blocking buffer and further incubated with 1:500 anti-DIG-
292 AP antibody in the dark, washed and developed for signal using NBT/BCIP solution (Roche,
293 Germany) and counterstained with 0.5% Bismarck Brown Y (Sigma, USA) for microscopic
294 examination (Leica DM750, ICC50W model with a digital camera and LASV4.12 Software).
295

296 **2.6. Immunohistochemistry (IHC) analysis**

297 A polyclonal antibody to the capsid protein of IHHNV was produced in a rabbit using
298 recombinant IHHNV capsid protein and the concentration appropriate for IHC was determined.
299 The tissue sections were de-paraffinized, rehydrated with xylene and a series of graded alcohols

300 to water. They were then treated with 0.6% H_2O_2 in methanol for 15 min to quench endogenous
301 peroxidase activity followed by washing two times with 1X PBS for 5 min each. The slides were
302 incubated with blocking buffer (10% fetal bovine serum in 1X PBS pH 7.4) for 1 h, at RT and
303 replaced with rabbit anti-capsid IHHNV antibody (1:1000 in blocking buffer) (Kiatmetha et al.,
304 2018) in a moist chamber at 37 ºC for 1-2 h. The slides were washed three times to remove unbound
305 antibody with 1X PBS for 5 min each followed by incubation with horseradish peroxidase (HRP)-
306 conjugated goat anti rabbit antibody (1:500 in the blocking buffer) at 37 ºC for 1 h in a moist
307 chamber before washing three times with 1X PBS for 5 min each. The slides were incubated with
308 a VECTOR®NovaRED™ substrate kit (Vector laboratories, USA) for 5-15 min and washed with
309 water for 5 min before counterstaining with Mayer's hematoxylin, dehydrating in a series of graded
310 alcohols to xylene and covering with a cover glass.
311
312 **3. RESULTS**
313 **3.1. History of official surveillance reports of IHHNV in Thailand**
314 For general interest, the percentage prevalence of IHHNV in samples of both *P. monodon*
315 and *P. vannamei* received by the Thai Department of Fisheries (Thai DOF) from the years 2011 to
316 2021 is shown in Fig. 1 together with National production. In 2013, production dropped
317 precipitously due mostly to reduced cultivation because farmers feared acute hepatopancreatic
318 necrosis disease (AHPND) (Sanguanrut et al., 2018). Subsequent recovery to pre-2013 levels has
319 not occurred due to bankruptcy of the majority of Thai shrimp export companies during the
320 AHPND crisis, and to concomitant loss of export market share to other shrimp producing
321 countries. In any case, the graph clearly shows that the percentage of IHHNV positive samples
322 was not significantly correlated with a negative effect on shrimp production. In fact, a Pearson
323 correlation analysis (Fig. 2) showed a significant ($p = 0.015$) positive association between
324 production quantity and the percentage of positive IHHNV detections in the total number of
325 farmed shrimp specimens examined by active and passive surveillance by the Department of
326 Fisheries, the competent authority for shrimp diseases in Thailand. The result was a positive
327 correlation between the percentage of IHHNV-positive samples and total shrimp production. This
328 is opposite to the expected negative correlation if IHHNV had been the cause of serious production
329 losses.
330

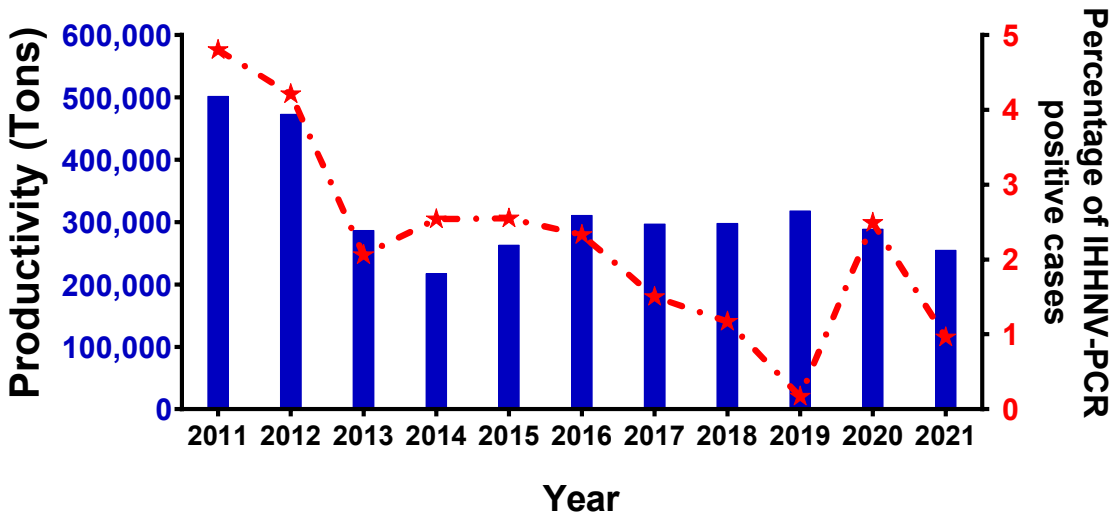

Figure 1. Thailand shrimp production (blue bars and left Y axis) for both *P. monodon* and *P. vannamei* versus the percentage of IHHNV-PCR positive cases (dotted red line and right Y axis) from 2011 to 2021 (see Supplementary Table S1). The IHHNV detection from 2011-2017 was recorded through a Thai DOF active surveillance program, whereas those in 2018-2021 were recorded through a Thai DOF passive surveillance program. The dotted line indicates the percentage (right axis) of positive IHHNV-PCR test results in the total number of samples tested for any reason by Thai DOF in each individual year.

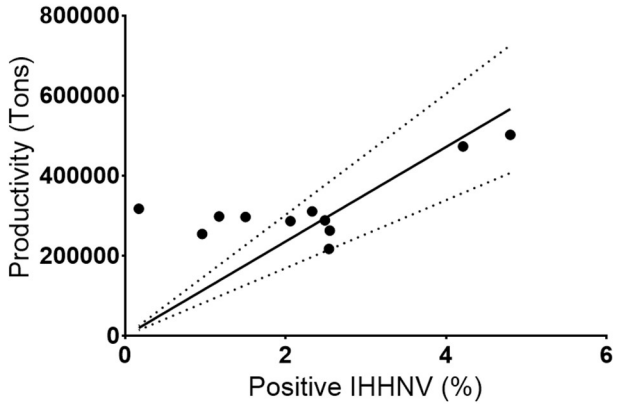

Figure 2. Correlation between percentage of IHHNV-PCR positive cases and shrimp production in Thailand from 2011 to 2021. The Pearson product-moment correlation coefficient (r) is 0.7 (p = 0.015) indicating a significant positive correlation between the yearly percentage of IHHNV-PCR positive samples and yearly shrimp production.

363 ### 3.2. IHHNV relationship to production in Pond Set 1

364 #### 3.2.1. All ponds in Pond Set 1 gave profitable harvests

365 Shrimp from all 4 culture ponds in Pond Set 1 (selected due to positive PCR test results for
366 IHHNV) were profitably harvested with total culture periods between 123 and 185 days (**Table**
367 **2**). Cultivation was carried out beginning during the months of February to August which is the
368 optimum period of year for shrimp cultivation in Thailand. The coefficients of variation for weight
369 were in the normal range from 16-25%. This contrasts with those reported for RDS at 35-90% (see
370 introduction for references). In addition, the average daily weight gain for all the ponds was
371 approximately 0.2 g, which is also normal for *P. monodon* in Thailand (Limsuwan and
372 Chanratchakool, 2004).

373

374 The size distribution for the shrimp in the 4 ponds is shown in **Fig 3**. Pond #1 (blue bars) was
375 cultivated for the normal period of approximately 4 months and the mean weight of the shrimp
376 was 25.2±8.50 g. The size-group distribution had a median of 29-35 g characterized by a tail
377 towards larger sizes. This is the opposite to RDS where the median would be below the normal
378 size and the distribution would be shifted sufficiently towards small sizes to make the whole crop
379 unprofitable. That was not the situation with any of the 11 ponds in this study. The time of final
380 harvest, and sometimes partial harvesting times, chosen by farmers are dependent on highly
381 variable market conditions. Despite these variables, the overall ADGs, shrimp sizes and CVs for
382 all the ponds (16 to 25) can be considered normal to better than normal for Thai conditions and
383 not characteristic of RDS. RDS is characterized by a mean weight at harvest of non-profitable
384 shrimp sizes and a CV of 35% or more shifted towards sizes of low-value.

385

386 **Table 2**. Shrimp growth performance and pond productivity in Pond Set 1.

Pond	Stocking density (shrimp/m^2)	Duration of culture (days)	CV (%)	Mean harvested weight (grams) (mean± SD)	Avg. Daily gain (g)
1	36	123	16	25.2±8.50	0.20
2	50	152	19	30.9±5.97	0.20
3	54	149	22	37.0±8.13	0.25
4	55	185	25	32.5±8.14	0.18

387

388

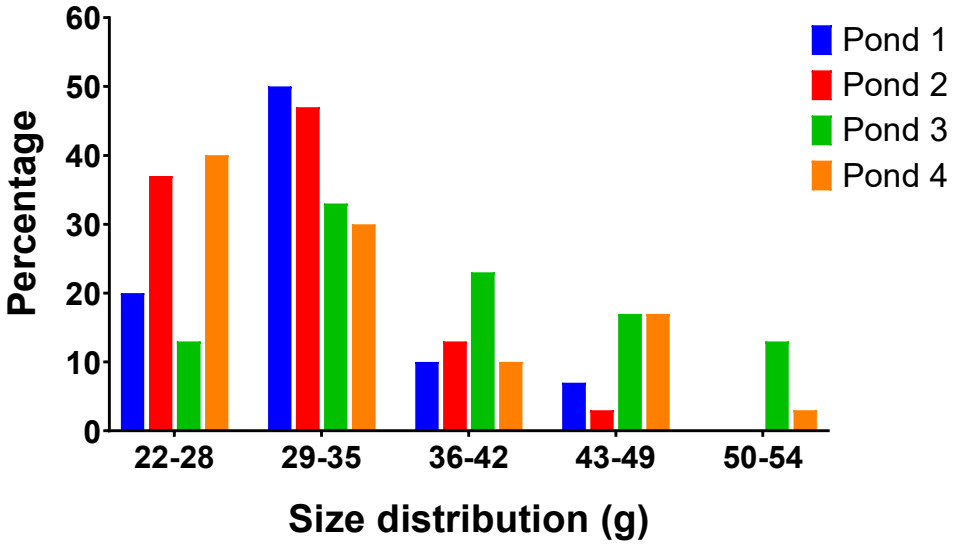

Figure 3. Bar graph showing the size distribution of the shrimp in the 4 ponds studied. The medians for all the ponds were in the range of 22 to 35g.

3.2.2. Two ponds from Pond Set 1 were PCR-positive using the IHHNV-LA method

Using a plasmid carrying the target sequence for IHHNV, the detection sensitivity of the IHHNV-LA nested PCR method was found to be 10 copies (Supplementary figure S1). Of the DNA extracts from 5 shrimp randomly collected from each of the 4 ponds in Pond Set 1, all gave positive PCR test results with both the OIE-IHHNV-309 and OIE-IHHNV-389 methods, while only 2 ponds (Pond #1 and #2) gave additional positive PCR test results with the IHHNV-LA method. Representative agarose gels of PCR amplicons from Pond Set 1 are shown in **Fig. 4** and the results are summarized for all 20 shrimp specimens in **Table 3**.

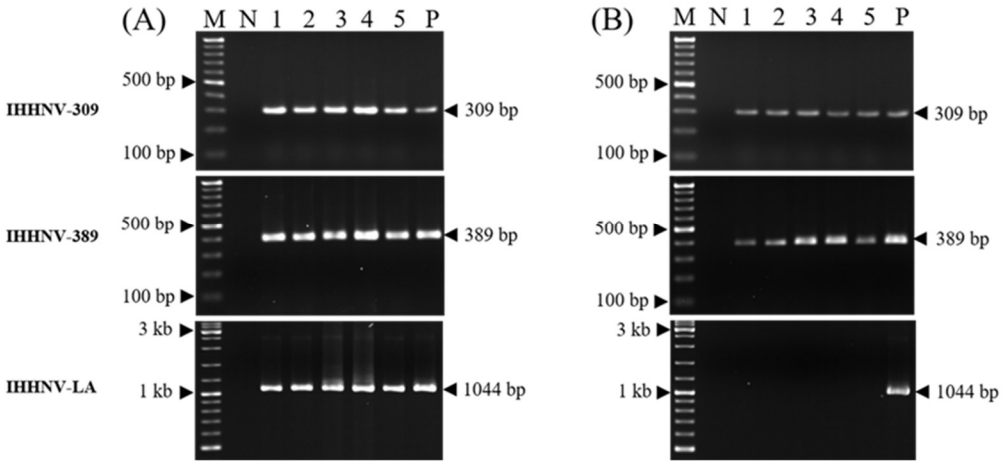

Figure 4. Example gels of PCR amplicons from shrimp specimens using 3 IHHNV-PCR detection methods. (A) Agarose gels showing positive PCR test results for all 3 PCR detection methods with the representative specimens from Pond #1. (B) Agarose gels from representative specimens from Pond #3 showing positive PCR test results with the 2 OIE methods only and negative results for the IHHNV-LA method.

Table 3. Summary of IHHNV-DNA detection by PCR and histological methods in juvenile shrimp from the 4 culture ponds. ND = not determine.

Pond #	PCR methods			Histological methods		
	IHHNV-309	IHHNV-389	IHHNV-LA	CAI	ISH	IHC
1	5/5	5/5	5/5	1/5	1/5	1/5
2	5/5	5/5	5/5	0/5	ND	ND
3	5/5	5/5	0/5	0/5	0/5	0/5
4	5/5	5/5	0/5	0/5	ND	ND

3.2.3. Histological failure to confirm IHHNV in 4/5 IHHNV-LA-positive shrimp

Shrimp from all 4 ponds (5 each, total 20) were subjected to histological examination. There were no cells of the hematopoietic tissue, the cuticular epidermis or any other IHHNV target tissues that showed Cowdry Type A inclusions (CAI), except for 1 specimen from Pond #1 that showed

1 CAI in the antennal gland (**Fig. 5c**) and 2 in the nerve cord (**Fig. 5d**). In other words, only 1 of 10 specimens positive for IHHNV with all three PCR methods showed CAI, and even then, at a very low prevalence. An example of IHHNV-CAI is shown in **Fig. 5a** from a slide of archived positive control tissue. In contrast, **Fig. 5b** shows an example of normal hematopoietic tissue seen in samples from the study ponds. Results for ISH and IHC for adjacent, serial sections from the same paraffin blocks used for histological analysis by H&E staining are given in the succeeding section.

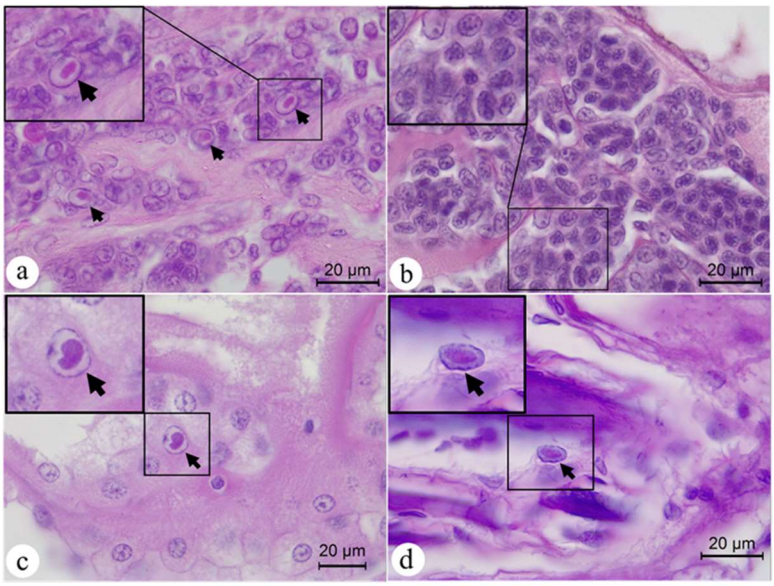

Figure 5. Photomicrographs of tissues from IHHNV positive and negative shrimp. (a) Example photomicrograph from the positive-control specimen showing IHHNV pathognomonic lesions (Cowdry Type A inclusions or CAI) (black arrows), with the upper right inclusion magnified in the insert at the upper left. (b) Example photomicrograph of normal hematopoietic tissue (i.e., no CAI) from one of the study specimens even though positive for IHHNV using the IHHNV-LA method. (c, d) Photomicrographs of antennal gland and nerve cord showing Cowdry Type A inclusions or CAI, respectively, from a single specimen (Pm 1.3) from Pond 1.

3.2.4. Positive ISH results from only 1 CAI-positive specimen from Pond #1

For confirmatory ISH tests using probes based on amplicons from the two WOAH detection methods (IHHNV-309 and IHHNV-389), samples of 5 shrimp each were examined only from Pond #1 (i.e., 5/5 specimens positive using the IHHNV-LA method) and Pond #3 (all 5 specimens negative using the IHHNV-LA method). All 5 shrimp from Ponds #1 and #3 were tested

482 and results of the ISH tests are shown in Table 2. However, photomicrographs of ISH results in
483 **Fig. 6** show only representative photos from 2 shrimp specimens from Pond #1, one that showed
484 CAI (Pm 1.3) and another (Pm 1.1) that did not. In **Fig. 6a-c**, the positive control specimen shows
485 strong ISH reactions (dark staining) in the HPT while Pm 1.1 shows no positive ISH reactions in
486 either the HPT or subcuticular epithelium (Cep), despite giving positive PCR test results with all
487 3 methods used. In contrast, positive ISH reactions can be seen in both the HPT and Cep of these
488 tissues for Pm 1.3 which gave not only positive PCR tests results with all three methods but also
489 showed CAI (although few) by H&E staining.
490
491 In **Fig. 7**, negative ISH reactions are shown for both shrimp specimens from Pond #3 that were
492 positive with both WOAH PCR methods but negative for both the IHHNV-LA method and for the
493 presence of CAI. Although the proportional numbers of IHHNV positive cells in the HPT were
494 much lower than in the Cep in Fig. 6, highly variable loads of IHHNV in different tissues of the
495 same shrimp specimen and between shrimp specimens have been previously reported for IHHNV
496 (Chayaburakul et al., 2005).
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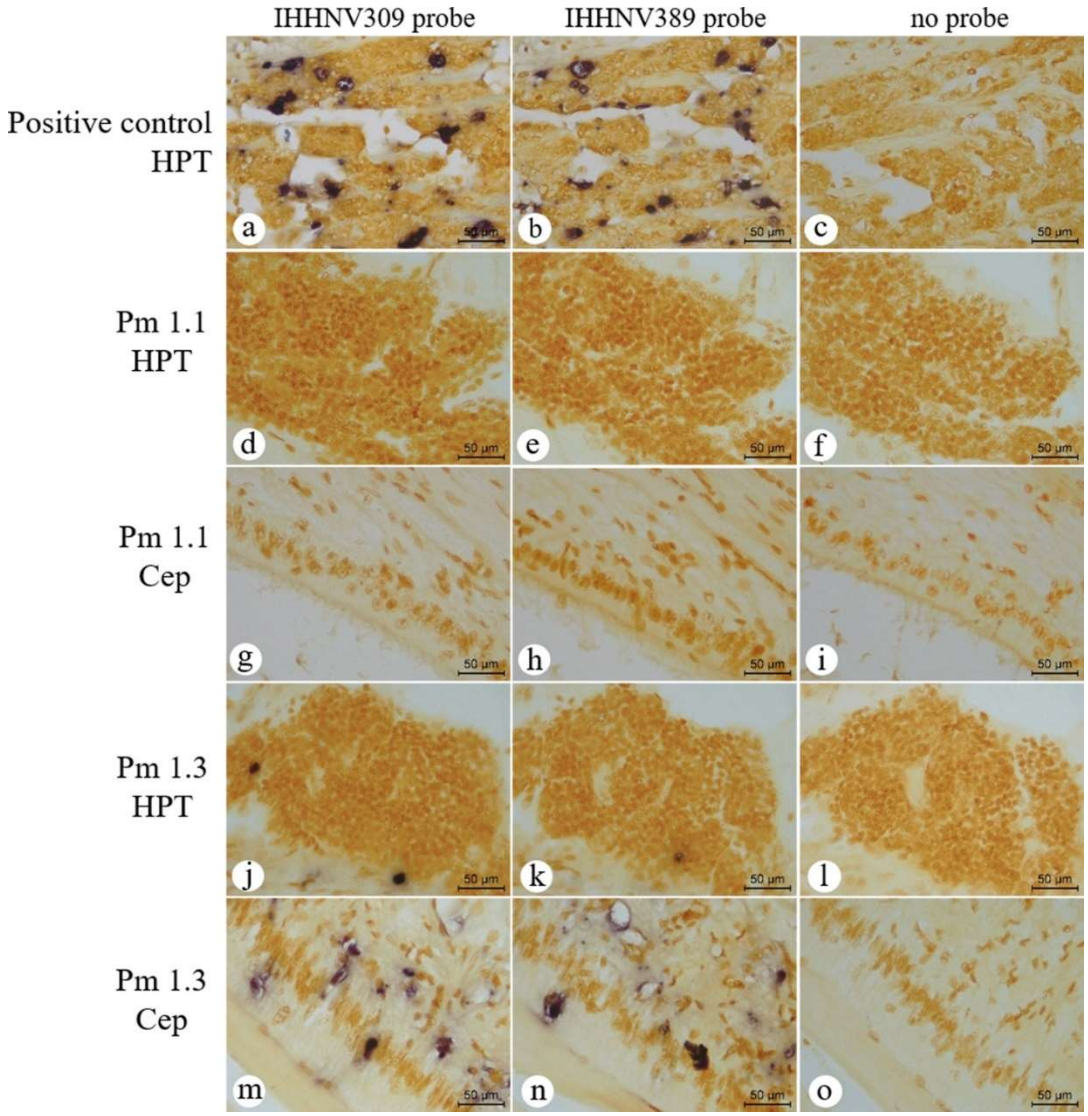

Figure 6. Representative photomicrographs of *in situ* hybridization results using IHHNV-309 and IHHNV-389 DNA probes with adjacent tissue sections from 2 shrimp specimens from Pond #1 in which all 5 specimens were positive for all 3 PCR detection methods. The no-probe controls for all tests gave negative ISH results. (a, b) Photomicrographs of positive ISH results (dark staining) for IHHNV using both ISH probes with the archived, positive-control HPT infected with IHHNV. (d, e, g, h) Photomicrographs from specimen Pm 1.1 that gave negative ISH results with both probes (no dark staining) in the HPT and Cep, respectively. (j, k, m, n) Representative photomicrographs from specimen Pm 1.3 showing positive ISH results (dark staining) for both probes in HPT and Cep.

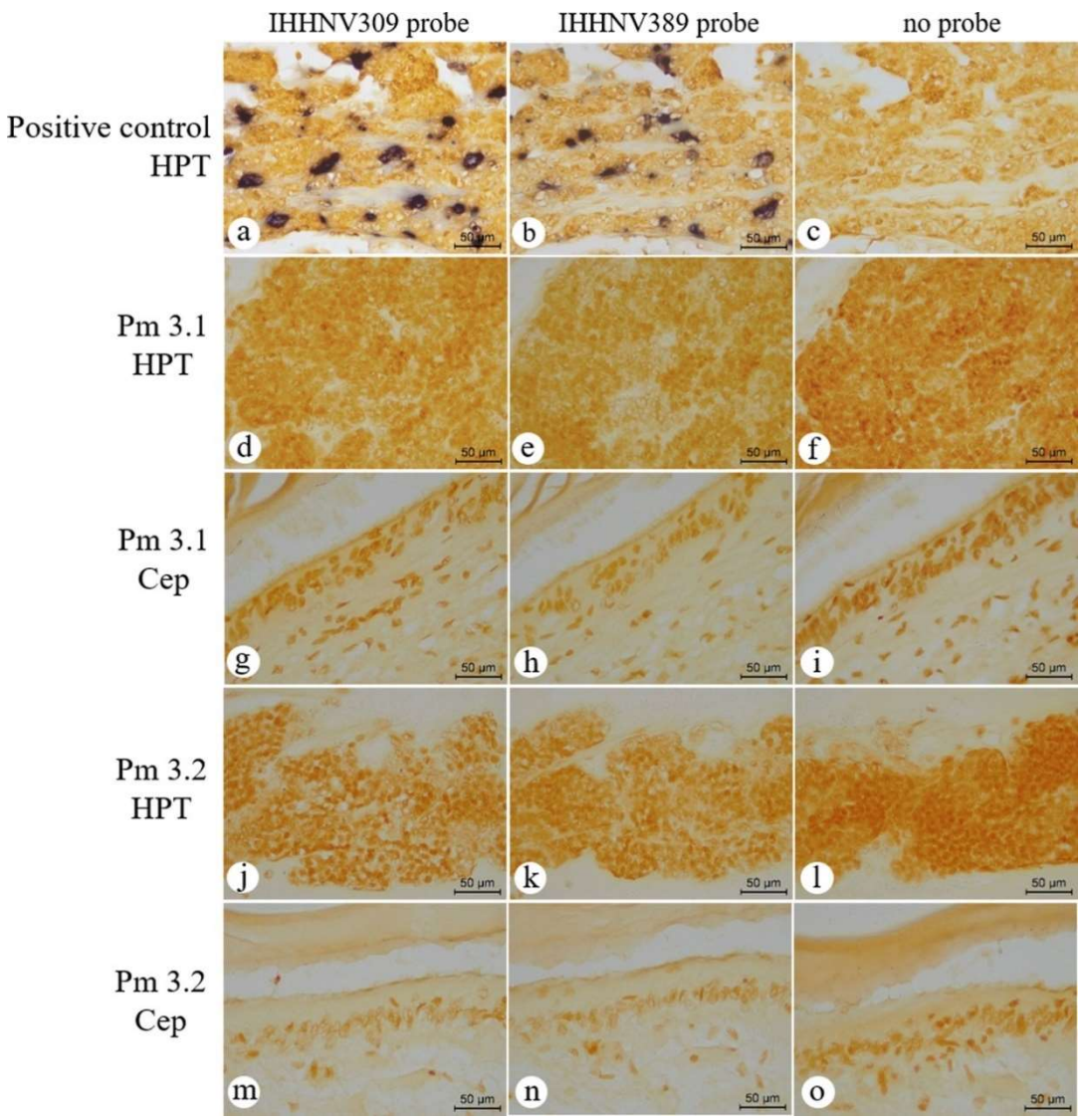

Figure 7. Representative photomicrographs of negative *in situ* hybridization results for adjacent tissue sections from 2 shrimp specimens (Pm 3.1 and 3.2) from Ponds #3 showing negative ISH reactions with both IHHNV-309 and -389 probes both in HPT and Cep tissues.

3.2.5. Immunohistochemistry (IHC) results paralleled the ISH results

The same 2 shrimp specimens each from Pond #1 (positive with all 3 PCR detection methods) and from Pond #3 (positive with both of the WOAH PCR methods but negative for the IHHNV-LA method) were also subjected to IHC analysis using an anti-IHHNV capsid protein antibody. Results obtained using the IHC method exactly matched the ISH positive and negative results obtained using the same specimens (Table 3 above). Specifically, the positive (brown

541 staining) and negative (no brown staining) IHC results for each of the specimens matched the
542 positive and negative ISH results (dark staining) for the same shrimp specimens. Only one
543 specimen, Pm 1.3 from Pond 1 gave positive IHC results, despite testing positive for all 3 of the
544 PCR methods used. These results indicate that even the IHHNV-LA method can give false positive
545 test results for the presence of infectious IHHNV.
546
547 Representative photomicrographs of IHC results from the specimens Pm 1.1 and 1.3 from Pond
548 #1 are shown in **Fig. 8**. The positive control result (dark staining) is shown in Fig. 8a while all the
549 negative controls (no antibody) show no brown staining. Representative photomicrographs of
550 negative IHC reactions for IHHNV are shown in the HPT (**Fig. 8c**) and Cep (**Fig. 8e**) from
551 specimen Pm 1.1, and these matched the negative ISH results for the same specimen (Pm 1.1) in
552 Fig. 6. Similarly, representative photomicrographs for specimen Pm 1.3 show positive IHC
553 reactions (brown) in HPT (**Fig. 8g**) and Cep (**Fig. 8i**) that matched the positive ISH results for the
554 same specimen (Pm 1.3) in Fig. 6.
555
556 Representative IHC results for specimens Pm 3.1 and 3.2 from Pond #3 are shown in **Fig. 9**. Aside
557 from the positive control, none of the shrimp specimens gave positive IHC results (i.e., no dark
558 staining) in either the HPT or Cep, and this matched the negative ISH results for the same
559 specimens shown in Fig. 7.
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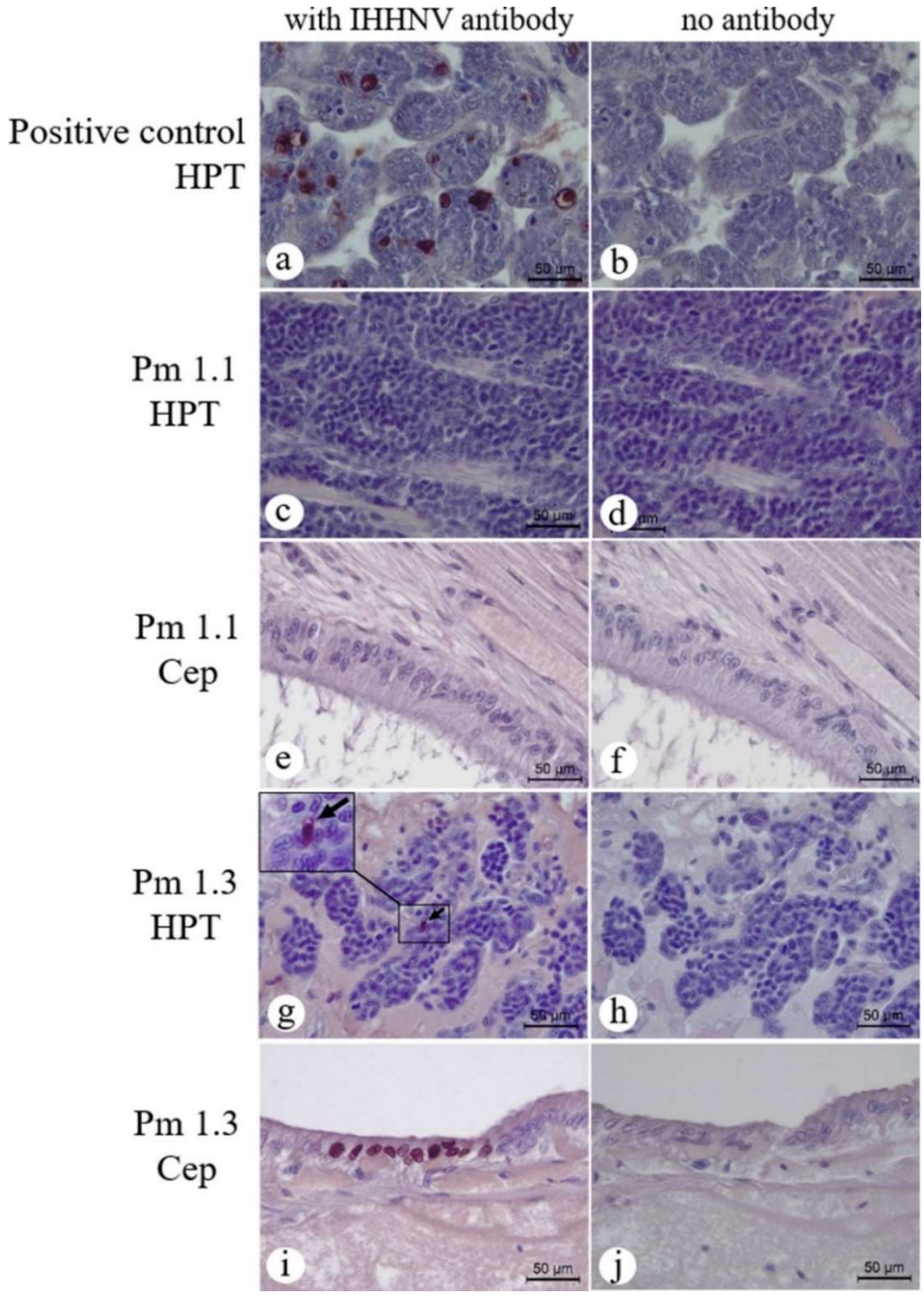

Figure 8. Representative photomicrographs of immunohistochemistry (IHC) results for the same 2 shrimp specimens (Pm 1.1 and Pm 1.3) from Pond #1 used for ISH experiments. (a, b) Archived positive control shrimp tissue infected with IHHNV and showing positive IHC staining (dark brown) in the HPT. (c, d) Negative IHC results for HPT of Pm 1.1. (e, f) Negative IHC results for Cep of Pm 1.1. (g, h) Positive IHC results for HPT of Pm 1.3. (i, j) Positive IHC results for Cep of Pm 1. 3.

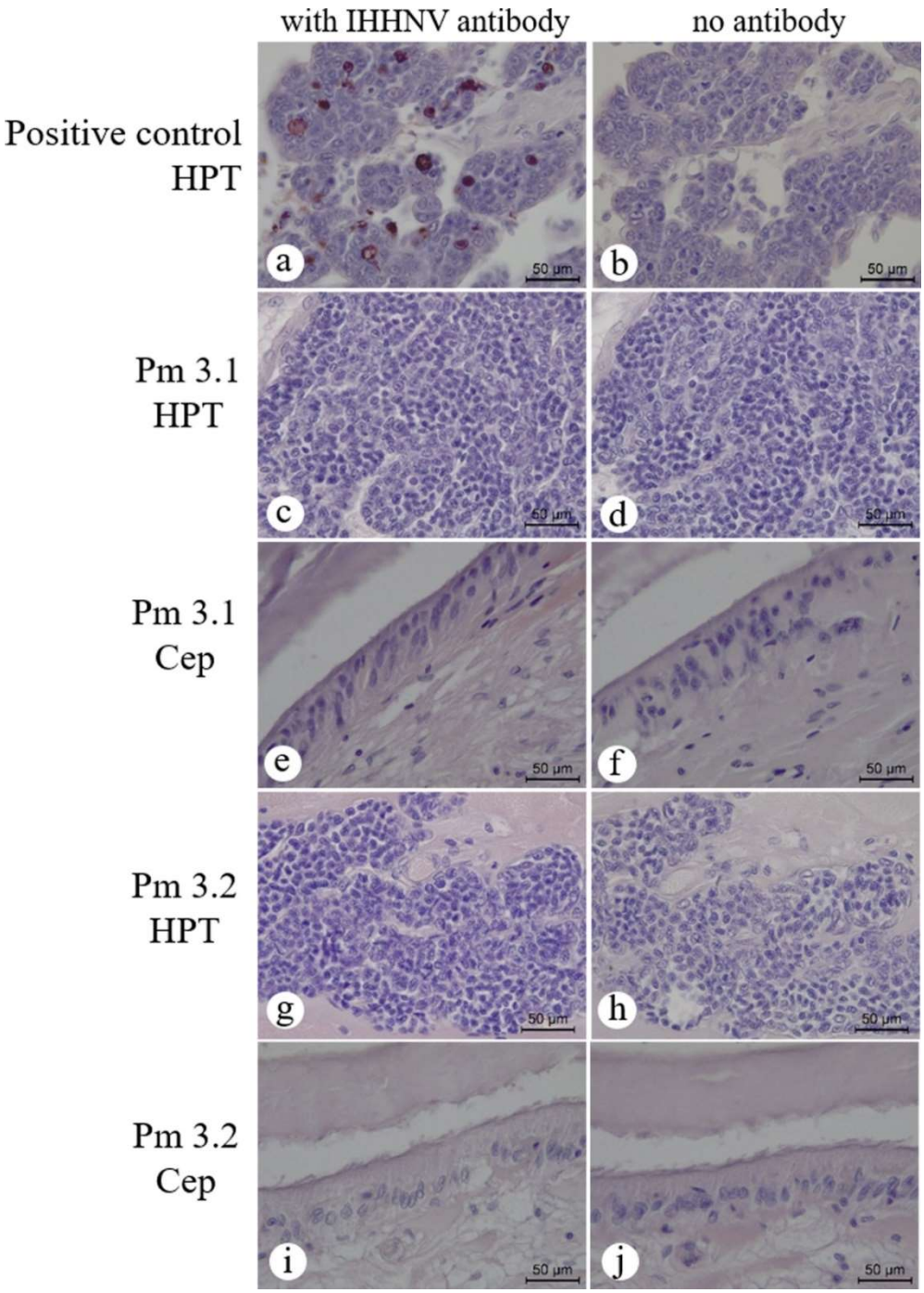

Figure 9. Example photomicrographs of IHC results from the same two specimens (Pm 3.1 and Pm 3.2) from Pond #3. (a, b) Archived positive control shrimp infected with IHHNV showing positive IHC staining (dark brown) in the HPT. All other photomicrographs for both tissues show negative IHC results (no brown staining).

634 **3.2.6. Pathogens other than IHHNV were also present in ponds from Set 1**

635 In addition to microscopic examination for CAI, all 20 shrimp specimens in Pond Set 1were
636 examined for other histopathology that might be associated with retarded growth (e.g., MBV,
637 HPV, EHP and bacteria in the HP). No signs of MBV, HPV or EHP were seen in the HP of any of
638 the 20 specimens. However, some specimens showed the presence of bacterial granulomas, some
639 of which were melanized, indicating long presence. Other anomalies seen are summarized in **Table**
640 **4**.

641

642 **Table 4.** Other histopathological signs in tissues of the 20 specimens from the 4 study ponds.
643 Totals for each pond less than 5 indicate absence of the target tissue in the microscope slide. PMC
644 indicates postmortem change from poor tissue fixation of the target organ, which makes it difficult
645 to assess pathology. Example photomicrographs of the histopatholgies are shown in
646 **Supplementary Figure 2**.

Histopathology	Pond 1 Chachoengsao	Pond 2 Krabi	Pond 3 Krabi	Pond 4 Krabi	Total	#Ponds
LO spheroids	5/5	4/4	5/5	5/5	19/19	4/4
HP bacterial lesions	0/5	PMC	3/5	0/1	3/11	1/3
Gill fouling	4/5	0/4	0/5	0/5	4/19	1/4
Gill pyknotic nuclei	2/5	3/4	4/5	0/5	9/19	3/4
ATM	4/5	PMC	0/5	0/1	4/11	1/3
Muscle lesions	0/5	4/5	1/5	5/5	10/20	¾
Total # of problems	4/6	3/6	4/6	2/6		

647

648 None of the anomalies (e.g., LO spheroids, HP lesions, etc.) listed in Table 4 gave ISH-positive or
649 IHC-positive signals using the IHHNV-specific probes. Thus, absence of ISH and IHC signals
650 indicated that the histological anomalies did not contain detectable IHHNV. Nor have such
651 anomalies been reported to be caused by IHHNV. Lymphoid organ (LO) spheroids develop in
652 response to foreign material accumulated by phagocytic hemocytes and also during viral infections
653 with Taura syndrome virus (TSV) (Hasson et al., 1999) and yellow head virus (YHV)
654 (Soowannayan et al., 2003). The spheroids caused by these pathgens are positive by ISH and IHC
655 specific for the respective viruses. Thus, we can assume that the spheroids in our specimens were
656 not formed in response to IHHNV but to some other cause.

657

658 The other problems variously exhibited in the specimens were the presence of bacterial lesions in
659 the HP, pyknotic nuclei in gills tissue, muscle necrosis and gill fouling. According to the Lightner
660 scale of disease severity (Lightner, 1996), these other problems were present at the levels from
661 "Trace" (Signs of infection/infestation by internal pathogens, parasite or epicommensals present
662 at just above diagnostic procedure minimum detection limits) or "1" ("Signs of
663 infection/infestation by pathogen, parasite or epicommensal present, but at levels that may be
664 below those needed for clinical disease"). These levels were similar to those for IHHNV in the
665 few specimens where IHHNV-CAI were observed.
666
667 The presence of LO spheroids in specimens from all ponds indicated a substantial immune
668 response to an unknown factor in all ponds and this was compounded by additional sub-clinical
669 problems that varied from pond to pond, also from undetermined causes. In addition, gill fouling
670 is a sign of low pond water quality leading to reduced ability of shrimp to maintain clean gills and
671 muscle necrosis is a sign of shrimp stress. It is important that even 1 positive specimen for any
672 pathogen in a random sample of 10 specimens is indicative of 26% or more prevalence with 95%
673 confidence in the source population (Cameron, 2002). Thus, even 1 positive sample in 6 or 5
674 specimens would indicate prevalences of 40 to 46% or more. As a result, the histological
675 information clearly indicates that conclusions about the effect of IHHNV on production from these
676 ponds based solely on PCR detection of IHHNV would be scientifically untenable.
677
678 **3.2.7. Summary for Pond Set 1**
679 In summary for Pond Set 1, all 4 ponds and all 5 sampled shrimp from each gave positive
680 PCR test results with the 2 WOAH-recommended PCR methods while only two ponds (#1 & #2)
681 gave additional positive tests using the IHHNV-LA method. However, only 1 of 5 specimens from
682 Pond #1 that gave positive PCR test results employing all three PCR methods showed the presence
683 of pathognomonic CAI lesions supported by matching positive ISH and IHC results, proving the
684 presence of infectious IHHNV. Thus, 4 out of 5 of these specimens gave false positive PCR test
685 results for infectious IHHNV using 2 WOAH-recommended detection methods and using an
686 IHHNV-LA detection method previously suggested as a possible PCR method to confirm the
687 presence of infectious IHHNV (Taengchaiyaphum et al., 2022). The work herein reveals that the
688 IHHNV-LA method can be used only as an additional control for the 2 WOAH-recommended

methods, in that a negative PCR test result with the IHHNV-LA method after positive test results with one or both of the WOAH methods would indicate that the WOAH test results were false positive results. Thus, the best tests for confirmation of IHHNV presence would be to require that positive test results with either or both the WOAH methods or with the IHHNV-LA method be followed by detection of CAI or by positive ISH or IHC signals in IHHNV target tissues. Ideally, viral purification followed by bioassays would provide absolute proof. The only pond confirmed to contain IHHNV-infected shrimp gave a profitable harvest without signs of RDS.

3.3. IHHNV relationship to production in Pond Set 2

3.3.1. Al1 ponds studied in Pond Set 2 gave profitable harvests

Similar to Pond Set 1, all the ponds in Set 2 also gave profitable harvests (**Table 5**) including Ponds 1 and 7 that showed abnormally high CV (44% and 39%, respectively). However, the mean size for Pond 1 was 29±12 g and the median (**Fig. 10**, blue arrow) was 36 to 42g indicating that the high CV was due to a wide range of sizes shifted towards large sizes (a profile opposite to the RDS profile). On the other hand, Pond 7 did have the smallest mean size (17±7 g) and smallest median size (15-21 g) (**Fig. 10**, gray arrow), fitting the profile of an RDS pond. Pond 6 (**Fig. 10**, black bar) was intermediate, with a CV of 24%, a mean size 20±5g and a median size of 15-21g (**Fig. 10**, black arrow). Like Pond 1, this does not fit the RDS profile. In summary, only Pond 7 showed a CV and size profile that corresponded with the description of RDS in terms of size distribution (i.e., there was no shrimp deformity). Despite the differences among these ponds and despite the profile of Pond 7, profitable crops were obtained by all the farm operators. This was because the cultivations were carried out during the months of October to April which is the riskiest "Thai winter season" characterized by low temperatures conducive to outbreaks of white spot disease (WSD) caused by white spot syndrome virus (WSSV). However, during this period shrimp prices are high, attracting some farmers to attempt cultivation despite the risks.

720 **Table 5**. Shrimp growth performance and pond productivity in Pond Set 2.

Pond	Stocking density (shrimp/m^2)	Duration of culture (days)	CV (%)	Mean harvested weight (g) (mean± SD)	Avg. daily weight gain (g)
1	22	160	44	29±12	0.18
2	63	164	22	33±7	0.20
3	326	166	31	32±10	0.19
4	152	166	24	28±7	0.17
5	47	120	19	32±6	0.27
6	200	174	24	20±5	0.11
7	87	132	39	17±7	0.13

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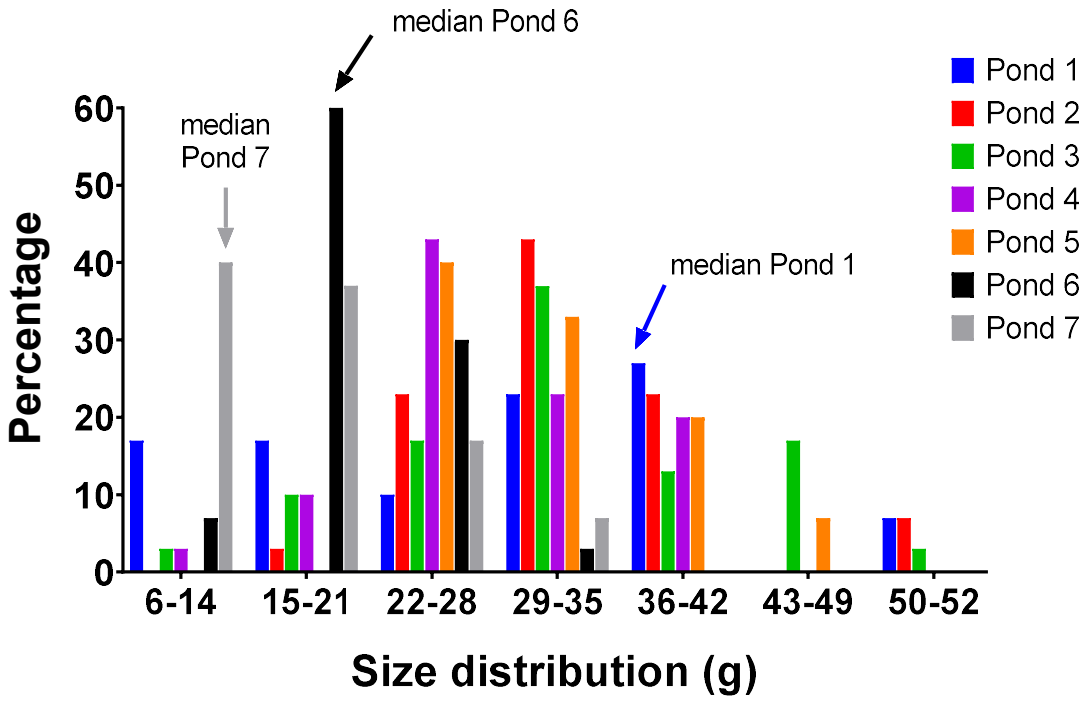

736 **Figure 10**. Bar graph showing the size distribution of the shrimp in the 7 ponds studied in Pond
737 Set 2. The median for 4 of the ponds (2 to 5) were in the range of 22 to 35 g, similar to Ponds 1 to
738 4 in Pond Set 1. Pond 7 (gray arrow) shows the smallest median size (6-14g), followed by Pond 6
739 (black arrow) (15-21g). Pond 1 is unusual with a median size of 36-42g and a mean of 29±12g
740 with a CV of 44% but shifted towards large sizes (i.e., opposite to RDS).

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744 ### 3.3.2. False-positive WOAH-PCR test results for 5/7 ponds in Set 2

745 The 6 shrimp samples individually tested for IHHNV from each of 7 ponds in Set 2 gave
746 positive test results with both WOAH-recommended methods. However, with the IHHNV-LA
747 method, only specimens from Pond #3 (1 in 6) and Pond #7 (3 in 6) gave positive test results. This
748 indicated that 5 out of 7 ponds had given false positive PCR test results for infectious IHHNV
749 using the 2 WOAH methods. For Ponds #3 and #6, the results suggested that the prevalence of
750 IHHNV was higher in Pond #7 than Pond #3.

751

752 **Table 6.** Summary of IHHNV-DNA detection by PCR and normal histological methods with Pond
753 Set 2. Five ponds (grey background) that gave negative test results with the IHHNV-LA method
754 (gray background) had given false positive test results with the 2 WOAH methods.

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Pond #	PCR methods for IHHNV		
	IHHNV-309	IHHNV-389	IHHNV-LA
1	6/6	6/6	0/6
2	6/6	6/6	0/6
3	6/6	6/6	1/6
4	6/6	6/6	0/6
5	6/6	6/6	0/6
6	6/6	6/6	0/6
7	6/6	6/6	3/6

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766 ### 3.3.3. IHHNV confirmation by histology in Pond Set 2

767 Out of the 7 ponds in Set 2, only Pond #7 gave positive test results for IHHNV by ISH,
768 IHC and presence of CAI in 2 of 3 specimens tested (**Table 7**). It is possible that if more
769 samples had been provided, that Pond #3 may have yielded some positive specimens. What
770 can be concluded, as with the results from Pond Set 1, is that positive test results using either
771 ISH, IHC or CAI can be used to confirm positive PCR tests using any of the 3 PCR methods,
772 while a negative IHHNV-LA test result may be useful in revealing some false positive WOAH-
773 PCR test results by using it as a 3rd PCR test.

774

775 **Table 7.** Summary of IHHNV-DNA detection by PCR and normal histological methods with Pond
776 Set 2. Unfortunately, due to communication problems, the results shown were from different
777 specimens than those whose results are shown in Table 6. Thus, the protocol differed from that of
778 the specimens whose results are shown for Pond Set 1 in Table 3 above where the PCR and
779 histology results came from the same specimens. Note that only Pond #7 (gray background) gave
780 positive test results for IHHNV with all three methods. ND = not determine.

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Pond #	Histological methods for IHHNV		
	H&E CAI	ISH	IHC
1	0/3	0/3	0/3
2	0/3	0/3	0/3
3	0/3	0/3	0/3
4	0/3	ND	ND
5	0/3	ND	ND
6	0/3	ND	ND
7	2/3	2/3	2/3

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792 **3.3.4. Pathogens other than IHHNV were present in ponds from Set 2**

793 The 3 specimens examined by PCR, ISH and IHC in Pond Set 2 (Table 7) were also examined
794 histologically in H&E-stained tissue sections for the presence of pathologies other than IHHNV
795 (**Table 8**). Because of the small number of shrimp sampled, even 1 positive specimen in 3 indicated
796 a very high prevalence (64% or more) in the source population. Similar to Pond Set 1, all samples
797 from every pond (total 3 x 7 = 21) showed the presence of lymphoid organ spheroids indicative of
798 an immune response by phagocytic cells (Hasson et al., 1999) but not a response to IHHNV
799 because of the negative ISH and IHC reactions in the LO to both types of IHHNV probes.

800

801 Present in the HP were granulomas suggestive of bacterial infections (4/7 ponds), hemocyte
802 aggregation (4/7 ponds) indicating an immune response and cell sloughing (2/7 ponds) similar to
803 that caused by acute hepatopancreatic necrosis disease (AHPND). In addition, Ponds 1, 2 and 3
804 gave high estimated prevalences for HPV and Pond 7 gave a high prevalence for MBV, both of

805 which are known to be associated with retarded growth in *P. monodon* (Flegel et al., 2002; Flegel,
806 2006). Pond 6 also had a median of relatively small shrimp sizes, but was negative for IHHNV by
807 all tests and also negative for HPV and MBV, clearly showing that retarded growth can arise from
808 causes other than IHHNV. In contrast, Pond #3 positive for IHHNV by the IHHNV-LA method
809 might have been confirmed for IHHNV by histology if more samples had been taken, but it showed
810 a normal to better than normal size profile. Each pond exhibited from 2 to 4 of these 6 histological
811 anomalies. Clearly, any proposal regarding the shrimp production in these study ponds based
812 solely on IHHNV-PCR detection results and ignorant of the presence or absence of other possible
813 pathologies would be scientifically unsupportable. This is especially important when one considers
814 that HPV and MBV are both known to cause retarded growth in *P. monodon* (Flegel et al., 2002;
815 Flegel, 2006). Overall, the histological analysis of the shrimp in Pond Set 2 was worse than that
816 in Pond Set 1 probably due to off-season cultivation.
817
818 **Table 8.** Other histopathological signs in shrimp tissues from 7 ponds of Pond Set 2. Note that
819 samples (grey background) from Ponds 1, 2 and 3 gave high estimated prevalences for HPV (gray
820 background) and from Pond 7 gave a high estimated prevalence for MBV (gray background), both
821 of which are known to be associated with retarded growth in *P. monodon*. Example
822 photomicrographs of the histopathologies are shown in Supplementary Figures 3.

Histopathology	Pond 1 Phuket	Pond 2 Phuket	Pond 3 Phuket	Pond 4 Phuket	Pond 5 Phuket	Pond 6 Phuket	Pond 7 Phang Nga	Total	# Ponds
LO spheroids	3/3	3/3	3/3	3/3	3/3	3/3	3/3	19/21	7/7
HP bacterial lesions	0/3	0/3	0/3	1/3	0/3	1/3	1/3	3/21	3/7
HP hemocyte aggregation	0/3	0/3	1/3	1/3	3/3	1/3	0/3	6/21	4/7
HP sloughing cells	0/3	0/3	0/3	0/3	3/3	0/3	1/3	4/21	2/7
HPV	2/3	1/3	1/3	0/3	0/3	0/3	0/3	3/21	3/7
MBV	0/3	0/3	0/3	0/3	0/3	0/3	1/3	1/21	1/7
Total # problems	2/6	2/6	3/6	3/6	3/6	3/6	4/6		

823
824 According to the Lightner scale of disease severity (Lightner, 1996), these other problems were
825 present at the levels from "1" ("Signs of infection/infestation by pathogen, parasite or

epicommensal present, but at levels that may be below those needed for clinical disease") to "2" ("Moderate signs of infection/infestation as shown by low to moderate numbers of parasite or epicommensal, or by number and severity of pathogen caused lesions. Prognosis is for possible production losses and/or slight increases in mortality if no treatment (if treatable) or management change is applied."). In contrast, the IHHNV lesions (when present) were at the "Trace" level.

3.3.5. Summary for Pond Set 2

In summary for Pond Set 2, all 7 ponds and all 6 sampled shrimp from each gave positive PCR test results with the 2 WOAH-recommended PCR methods while only two ponds (#3 and #7) gave additional positive tests using the IHHNV-LA method. These results alone were sufficient to reveal that Ponds #1, #2, #4, #5 and #6 (5/7 = 71%) had given false positive PCR test results for IHHNV using the 2 WOAH methods. In addition, Pond #3 with 1 in 6 positive and Pond #7 with 3 in 6 positive by the IHHNV-LA method revealed that many shrimp in each of the ponds were not infected with IHHNV. For the 3 additional specimens from each pond examined histologically IHHNV infection could be confirmed in only 2 specimens from Pond #7, leaving the status of Pond #3 (1 in 6 positive with the IHHNV-LA test) open to question due to the low number of samples tested by histology.

As with the results from Pond Set 1, any specimen giving positive test results using all three PCR test methods was not sufficient to confirm an IHHNV infection. However, a positive test with any one of the three PCR methods together with a positive test with any one of the three histological methods was sufficient to confirm the presence of IHHNV infection in a pond. In addition, a positive test result with either or both WOAH PCR methods but a negative result with the IHHNV-LA method served as a negative test result for the presence of IHHNV infection. In addition to the PCR results, the histological analysis in Pond Set 2 revealed more serious pathologies including HPV and MBV infections plus sloughing of HP tubule epithelial cells characteristic of AHPND, probably due to off-season cultivation. Overall, the results for Pond Set 2 paralleled those from Pond Set 1.

857 **4. DISCUSSION**

858 ***4.1. IHHNV false positive PCR results frequently arise from EVE***

859 ISH reactions for IHHNV were negative for 3/5 specimens from Pond #1 that gave PCR
860 positive results for IHHNV using all 3 methods. It was possible that positive PCR results but
861 negative ISH and IHC results in those 3 specimens arose because the viral loads were too low to
862 give ISH and IHC signals. This was our former belief for shrimp positive for viral pathogens
863 detectable only with nested PCR and negative with histochemical methods. However, the results
864 in this study for both Pond Set 1 and Pond Set 2 revealed a very good correlation between positive
865 ISH and IHC results with results from normal histological analysis using H&E staining to detect
866 CAI pathognomonic for IHHNV. An absolute, final answer to the question of infectivity would be
867 obtained only by preparation of purified viral extracts followed by bioassays with SPF shrimp.
868 Lack of transmission would indicate the absence of infectious IHHNV in the extracts.
869

870 We believe it is most likely that the positive PCR results that could not be confirmed by positive
871 results for CAI, ISH and IHC arose from endogenous viral elements (EVE) of IHHNV that contain
872 the primer target sequences as has previously been reported (Saksmerprome et al., 2011) and as
873 discussed in the Introduction. The presence of EVE for both IHHNV and white spot syndrome
874 virus (WSSV) in shrimp has been confirmed (see Flegel, 2020 for a review).
875

876 EVE with high sequence identity to infectious IHHNV were recently found (Taengchaiyaphum et
877 al., 2022) in pseudochromosome 7 (PC7) of a published draft genome of *P. monodon*
878 (Uengwetwanit et al., 2021). Some of these EVE showed 100% sequence identity to infectious
879 IHHNV Type 1 with sequences that matched 100% with existing types of IHHNV at GenBank
880 and especially with the targets of the IHHNV-309 and -389 methods. PCR tests with archived
881 DNA from the genome project using the IHHNV-309 and -389 methods gave positive test results.
882 However, the same DNA was negative for IHHNV using the IHHNV-LA method. Additional PCR
883 testing confirmed that the targets for the methods were in the genomic material and not in
884 contaminating IHHNV DNA. Thus, a DNA extract from the specimen used for sequencing would
885 have given a positive PCR test result for infectious IHHNV, even though it was not infected.
886

887 Similar EVE in mosquitoes have been shown to be protective against their homologous viruses
888 (Suzuki et al., 2020). Thus, eliminating EVE in breeding stocks to avoid false positive PCR test
889 results and avoid trade restrictions might have a negative impact on farmed shrimp production if
890 the eliminated EVE are protective. The same arguments may apply to EVE for other shrimp viruses
891 such as TSV and WSSV that are also included in the OIE list of controlled crustacean diseases.
892 These precautions regarding interpretation of PCR test results have been discussed previously
893 (Alday-Sanz et al., 2020; Saksmerprome et al., 2022).
894
895 In the end, we were forced to accept from our results herein that other than using sets of 7 to 10
896 overlapping primers pairs to cover the whole IHHNV genome (Chayaburakul et al., 2005;
897 Saksmerprome et al., 2011), it would be impossible to be certain using only 1 to 3 primer sets
898 whether amplicons arose from IHHNV or EVE derived from it. Instead, we now believe that
899 obtaining a positive test result using any of the PCR methods recommended by WOAH (or any
900 equivalent) must be confirmed by a histological test (CAI, ISH or IHC). One might propose that
901 the IHHNV-LA method be used as a temporary check to screen WOAH positive specimens if
902 histological analysis is not possible. This might reveal about half of the potential false positives
903 and reduce unjust rejection or destruction of at least some farmed shrimp products. On the other
904 hand, of the 10 shrimp that gave positive PCR test results with the IHHNV-LA method from Pond
905 set #1, only 1/10 showed CAI, so there would still be a significant risk of rejections based on false
906 positive test results.
907
908 ***4.2. Histopathology is required to confirm IHHNV detection by PCR***
909 This study was carried out using shrimp (*P. monodon*) cultivation ponds that had been
910 selected solely on the basis that sampled shrimp specimens had given positive test results using
911 two WOAH-recommended PCR methods for detection of IHHNV infections. Such positive PCR
912 test results are often used to justify the rejection of imported shrimp products and result in serious
913 negative economic impacts. Here we have shown that rejections based solely on the use of the
914 recommended WOAH-PCR methods singly or combined are unjustified. With 11 ponds tested in
915 the study, presence of IHHNV could be confirmed in only 2 ponds (2/11 = 18%) with 1 pond open
916 to question (3/11 = 27%). This suggests that economically important decisions might be made
917 based on false positive PCR test results 73 to 82% of the time.

As early as 2011 (Saksmerprome et al., 2011; 2022), it was revealed that fragments of the genome of existing types of IHHNV occur commonly in the shrimp genome and may collectively cover almost the whole genome (Taengchaiyaphum et al., 2022). Thus, individual specimens may potentially give false positive PCR test results for the presence of IHHNV for any target sequence. Since around 2012, these viral genome fragments have been referred to as endogenous viral elements (EVE) (Feschotte & Gilvert 2012). Recent, analysis of the whole genome of *P. monodon* has revealed clusters of scrambled EVE from IHHNV on chromosome 7 that can give false positive PCR test results (Taengchaiyaphum et al., 20220). EVE of extant types of white spot syndrome virus (WSSV) have also been reported in *P. monodon* (Utari et al., 2017). Warnings regarding the propensity of EVE to give false negative PCR and RT-PCR test results (Saksmerprome et al., 2011, 2022; Alday-Sanz et al., 2020) for viral infections have been largely ignored.

Of the 3 PCR methods used in this study, none alone or in combination with the other PCR methods were sufficient to confirm the presence of IHHNV infections. Due to the existence of highly variable EVE profiles in individual shrimp specimens, confirmation required additional histological examination using H&E-stained tissues, ISH or IHC. Thus, we recommend that the WOAH diagnostic manual for shrimp viral diseases be modified to specify that no viral infection can be confirmed in shrimp by one or more positive PCR test results without additional histological analysis to demonstrate the detection of pathognomonic lesions or positive ISH results or positive IHC results.

4.3. Histopathology required to determine the true impact of IHHNV infections

In the introduction section, we reviewed the history of IHHNV infection in *P. monodon* and showed that valid reports reveal no significant negative impact on farm production (Flegel et al., 2004; Withyachumnarnkul et al., 2006). This was also the conclusion recently reported for cultivated *P. vannamei* in Ecuador and Peru (Aranguren et al., 2022). For Pond Set 1 in this study, only Pond #1 contained shrimp with confirmed IHHNV infections, and its production did not differ significantly from that of the other 3 ponds in Set 1 that were negative for IHHNV. None of the ponds exhibited gross signs associated with IHHNV (e.g., RDS).

949 In Pond Set 2, only Pond #7 was fully confirmed to contain shrimp infected with IHHNV and the
950 shrimp size distribution for this pond was abnormal and growth resembled that of *P. vannamei*
951 exhibiting RDS deformities, which *P. monodon* did not exhibit. If this pond had been tested for
952 IHHNV by PCR only and not subjected to histological analysis, an unwary examiner might have
953 concluded that the abnormal size distribution was caused by IHHNV. However, this conclusion
954 would not only be contrary to the history of IHHNV in *P. monodon* but also ignorant of the
955 presence of LO spheroids, bacterial lesions, HP cell sloughing and the presence of MBV
956 (associated with retarded growth in *P. monodon*). The co-occurrence of these 4 pathologies in
957 addition to IHHNV indicated a very poor overall health status for the shrimp in Pond #7. Failure
958 to consider such issues and failure to carry out a full health analysis but instead to focus simply on
959 PCR test results can result in flawed publications claiming negative impacts on shrimp production
960 caused by any pathogen. Misuse of PCR detection as the single criterion in assigning IHHNV as
961 the cause for problems with cultivated shrimp has been particularly frequent. This is most
962 important when shrimp mortality is part of the disease expression profile since death is not a
963 characteristic of IHHNV in shrimp other than *P. stylirostris*. Thus, Pond #7 from Pond Set 2 is an
964 excellent example of the perils involved in using PCR detection alone to implicate IHHNV as the
965 cause of disease expression without backup histological analysis.
966
967 Another good example is the mud crab *Scylla paramamosain* that tolerates the virus WSSV
968 without signs of disease for very long periods in the laboratory (106 days),. However, some
969 specimens eventually die from necrosis of the hepatopancreas which WSSV does not infect
970 (Somboonna et al., 2010). Lacking prior knowledge regarding its tolerance to WSSV, an unwary
971 diagnostician testing a moribund specimen of this mud crab for WSSV by PCR only might come
972 to the erroneous conclusion that its death was caused by WSSV. Thus, the practice of making
973 conclusions about impact based solely on PCR detection results for any single pathogen without
974 supporting evidence from histological analysis of overall health may result in incorrect estimations
975 of a pathogen's impact followed by unsound WOAH listing.
976
977 ***4.4. Seedstock impacts on production must also be considered***
978 In this study, comparison of the harvest outcomes for the shrimp ponds studied in Pond Set
979 1 and Pond Set 2 could not be made without considering farm management issues including details

980 related to cultivation season, farm location and infrastructure, source of seedstock (post larvae),
981 feed and water management, etc. Already addressed was the issue of poorer health of the shrimp
982 in the off-season farms, but other management details were not investigated in our study. However,
983 from Pond Set 2, the presence of HPV in Ponds #1, #2 and #3 and MBV in Pond #7 suggested that
984 viral infections might have occurred from the surrounding environment after pond stocking.
985 However, our previous experience has shown that these viruses are most often introduced by PL
986 used to stock the ponds. In our experience, farms that use genuine SPF stocks free of HPV and
987 MBV do not develop these infections in rearing ponds. Instead, unscreened PL derived from non-
988 SPF, captured broodstock or false-SPF-derived PL from unscrupulous PL suppliers are available
989 in Thailand at lower prices than genuine SPF-derived PL, and low prices may attract some unwary
990 farmers. These examples reinforce the importance of the need for farmer awareness of the need
991 to use SPF-derived PL and recommended biosecurity measures during cultivation.
992
993 ***4.5. The WOAH reporting for shrimp viruses needs to be modified***
994 As stated in the Introduction, it is generally recognized in Thailand that IHHNV has had
995 no significant negative impact on the production of cultivated *P. monodon* or *P. vannamei* since
996 around 2010. Thus, it is not normally tested for or reported by farmers. On the other hand, samples
997 from shrimp farms are sometimes submitted to the Thai competent authority to be tested for other
998 reasons such as clearance before export. If such shrimp samples are tested for IHHNV by PCR for
999 any reason and give a positive result, Thailand's WTO obligations require that WOAH be notified,
1000 because IHHNV is listed crustacean disease. Clearly, a report to WOAH based solely on PCR
1001 detection using the WOAH recommended method with the specimens described here for Pond #3
1002 in Pond Set 1 would have constituted an unintended false positive report. In addition, the confirmed
1003 detection of IHHNV from Pond #1 would also be reported, but the report would not be
1004 accompanied by a note indicating that detection was not associated with any significant impact on
1005 production. This is important, because a key characteristic of a WOAH-listed disease is that it a
1006 significant, negative economic impact on production.
1007
1008 This is a very important issue, given the current knowledge showing that shrimp are capable of a
1009 specific, adaptive immune response based on nucleic acids that can give rise to EVE that are
1010 heritable if they occur in germ cells (Flegel, 2020; 2022). As a result, shrimp that survive a viral

1011 infection give rise to a vast variety of offspring, each with an individual EVE profile such that
1012 protective EVE combinations become naturally selected in nature over time or can be purposefully
1013 selected in shrimp breeding programs (Alday-Sanz et al., 2020). The eventual result is that disease
1014 outbreaks caused by the relevant pathogens decline over time as a result of genetic tolerance and
1015 development of effective prevention protocols such that the negative economic impact may decline
1016 sufficiently to justify WOAH delisting. In order to monitor this decline, it would be necessary that
1017 obligatory country reports of listed shrimp pathogens be accompanied by an estimate of any
1018 production losses associated with each report. The current WOAH position appears to be "once
1019 listed, perpetually listed". One might propose that listing does no harm, even if there is no negative
1020 economic impact. However, this is a naïve position. For example, the cost of unnecessary PCR
1021 testing for a listed pathogen can be high and the losses arising from tests that result in unjust
1022 rejection of products can also be very high.
1023
1024 ***4.6. Current information supports arguments for IHHNV delisting by WOAH***
1025 As presented in the Introduction section, delisting of IHHNV is another way to avoid the
1026 possibility of negative economic impacts from wrongfully rejected products or shrimp stock
1027 destruction due to false positive PCR detection results. There is building evidence that IHHNV
1028 should be removed from the crustacean diseases listed by WOAH. This issue has already been
1029 presented in the introduction section and the results presented here on the lack of a significant
1030 negative impact of IHHNV infection on *P. monodon* in Thai production ponds supports the
1031 proposal that IHHNV no longer has a significant negative impact on shrimp production.
1032
1033 It is true that Thailand and other countries still report on the presence of IHHNV by PCR testing
1034 as WOAH requires its members to do for listed diseases. However, the detections in Thailand are
1035 not associated with RDS or any other signs of disease but this information is not solicited by the
1036 reporting system which apparently assumes that any report indicates a disease outbreak. We
1037 believe that the lack of a request for accompanying impact information is a significant omission in
1038 the OIE reporting system. This is because it does not provide an avenue to communicate valuable
1039 data as to whether the negative impact of a listed pathogen is increasing, decreasing or negligible.
1040 This is particularly important for crustaceans and insects that have been shown to develop high
1041 tolerance to viral pathogens over time (Flegel, 2020; Flegel, 2022).

1042

1043 To gain a clear picture of the economic impact of IHHNV on shrimp cultivation from 2010 to
1044 present, it is necessary to collect detailed information from major shrimp cultivation countries to
1045 determine whether the presence of IHHNV (i.e., by PCR detection) is or is not associated with
1046 significant economic losses. The ideal way to do this is to work together with shrimp farmers,
1047 academics and competent authorities from producing countries. In addition, any academic
1048 publications from 2010 to the present that may have been published in local journals and in
1049 languages other than English should be collected and pooled, together with brief summaries in
1050 English, so that a common consensus can be reached. In Thailand, we can clearly state that there
1051 has been no significant impact of IHHNV on farmed shrimp production of *P. vannamei* or *P.*
1052 *monodon* since at least 2010, despite its frequent detection by PCR. We believe that the same is
1053 true for other major producers.

1054

1055 It is important to understand that we do not believe IHHNV is innocuous and should be ignored or
1056 not included in the list of pathogens that should be excluded from specific pathogen free (SPF)
1057 shrimp. The situation is the same as that for the viruses MBV and BP that were formerly listed by
1058 OIE but were later removed from the list (WOAH, 2008). They were removed because full
1059 understanding of their biology allowed for their exclusion from hatcheries and from post-larvae
1060 supplied to shrimp farmers. However, producers that use wild, captured broodstock to produce PL
1061 must be aware that those delisted pathogens must be excluded from hatcheries by screening
1062 broodstock for its absence before use.

1063

1064 The same is true for IHHNV. The current, high quality SPF stocks of *P. vannamei* that are not
1065 infected with IHHNV or other known pathogens produce offspring that can achieve average daily
1066 weight gains of 0.3 to 0.4 g or more per day under ideal culture conditions, and they are unaffected
1067 by IHHNV infection. The weight gain is well beyond that possible with offspring obtained from
1068 captured, wild broodstock. This achievement has resulted from a combination of innovations in
1069 breeding, selection, feed and management. Thus, no pathogen can simply be ignored and assumed
1070 to have no possible negative impact on production. At the same time, no manageable shrimp
1071 pathogen should be maintained as a perpetual dire threat when the evidence clearly shows that it
1072 is not. Good examples are recent reports of the occurrence IHHNV in exotic *P. vannamei*

1073 cultivated in Korea and proposed to have arisen from imported shrimp from retail outlets (Kim et
1074 al., 2011; Kim et al., 2012; Kim et al., 2020; Park et al., 2020). First of all, their PCR-based studies
1075 presented no histopathological evidence to confirm IHHNV pathology or absence of pathology
1076 from other known or unknown pathogens. Since Korea does not have native *P. vannamei* the ponds
1077 must have been stocked with imported PL or PL derived from *P. vannamei* cultivated in Korea.
1078 However, no evidence was presented to prove that the broodstock used to produce the PL or the
1079 PL used to stock the shrimp ponds were free or IHHNV or other pathogens. Rational aquaculture
1080 management requires that shrimp farmers take precautions to ensure that major known pathogens,
1081 including IHHNV, are absent from the PL they use to stock their cultivation ponds. This can be
1082 achieved either by testing before stocking or by using PL from a supplier with a certified long
1083 history of providing PL free of those pathogens.
1084

1085 **4. CONCLUSIONS**

1086 Our research has shown no significant negative impact of IHHNV on production from the sampled
1087 ponds studied in Thailand. These results correspond to the lack of reliable reports since 2010 on
1088 significant negative impacts of IHHNV on commercially farmed *P. monodon* or *P. vannamei* or
1089 on the world capture fishery for those species, even in areas to which IHHNV has been
1090 translocated. Furthermore, reports claiming losses focus largely or solely on PCR detection results
1091 without confirmation by histological analysis or bioassays and without investigation for other
1092 pathogens or evaluation of actual farm losses. Further, recent reports that claim losses to IHHNV
1093 in hatcheries, experimental shrimp or on farms could have been easily avoided by simple
1094 biosecurity combined with use of IHHNV-negative broodstock and/or stocking of PL free of
1095 IHHNV. At the same time, we have found no reliable reports of significant losses to IHHNV from
1096 commercial cultivation ponds of the world's major shrimp producers since 2010. A thorough
1097 investigation regarding any significant negative impact on farmed shrimp production from IHHNV
1098 is needed to reassess whether it fits the criteria required to be listed by WOAH. It is our opinion
1099 that the massive expenditures required for needless PCR testing and the current losses suffered via
1100 trade restrictions justified by WOAH listing are now the only significant negative economic
1101 impacts of IHHNV.
1102
1103

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Conflicts of interest

None

Ethics statement

This work followed Thailand's laws for ethical animal care under the Animal for Scientific 83 Purposes ACT, B.E. 2558 (A.D. 2015) under project approval number BT-Animal document no. BT37/2563) from the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand.

Author contributions

KS, PS, JS, JT, NS, ST Methodology, Investigation, Data curation, Writing-Original draft preparation, KS, RV, CC, TWF, ST Conceptualization, supervision, manuscript writing and final approval of the manuscript.

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