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1 **Increased robustness of postlarvae and juveniles from non-ablated Pacific whiteleg**
2 **shrimp, *Penaeus vannamei*, broodstock post-challenged with pathogenic isolates of**
3 ***Vibrio parahaemolyticus* (*Vp*$_{\mathbf{AHPND}}$) and white spot disease (WSD)**

4

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21 **Highlights**

22 - Non-ablated *Penaeus vannamei* females produce offspring that are more resilient to
23 commonly encountered pathogens.
24 - Postlarvae from non-ablated female have a significantly higher resistance to
25 *Vp*AHPND.
26 - Juveniles from non-ablated animals have better survival to WSD than their juvenile
27 counterparts from ablated female.
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Abstract

The maturation and reproduction of Pacific whiteleg shrimp, *Penaeus vannamei*, through the practice of unilateral eyestalk ablation though common is an animal welfare concern. This study assessed the resilience of offspring from non-ablated *P. vannamei* when challenged with an isolate of *Vibrio parahaemolyticus* (*Vp*) causing acute hepatopancreatic necrosis disease (Vp_{AHPND}), and with white spot syndrome virus (WSSV). Vp_{AHPND} and WSSV challenges were conducted using PL and juveniles under controlled conditions, with both trials using four groups (i.e. shrimp from either ablated or non-ablated females and then either challenged with the pathogen or not challenged). For the Vp_{AHPND} challenge, ten replicate 20 L tanks (five replicates for each population) each containing 100 PL 17 (average weight 14 mg) in 15 ppt, 29.05 ± 0.13ºC water were challenged with 2 ml of 2.0×10^{8} CFU mL^{-1} culture of *V. parahaemolyticus*. A further ten replicate tanks (five per population) served as the corresponding non-challenged controls. The shrimp mortalities were assessed every 3 h over the following 96 h. For the WSSV challenge, individual 1.4 g (average weight) shrimp (50 individuals per population) were housed in 1 L tanks and fed 0.1 g WSSV infected tissue (av. 2.02×10^{9} WSSV). A further 50 shrimp per population served as non-challenged controls. The shrimp were maintained at 15 ppt, 26.3 ± 0.71ºC water and assessed every 3 h post-infection over the subsequent 168 h and mortalities at each time point noted. Postlarvae from non-ablated females had significantly (p = 2.4E-23) better survival (70.4%) than those from ablated females (38.8%) at 96 h post-challenge with Vp_{AHPND}. Both challenged populations had significantly (p = <1.3E-36) lower survival than the control groups. The survival of the juveniles from non-ablated females (62%) at 168 h post-infection with WSSV was not significantly higher than that of the juveniles from ablated female (48%) although the difference was significantly different at 65 to 75 h. Both challenged populations also had

64 significantly (p = <1.0E-5) lower survival rates than the control groups. The study
65 demonstrates that postlarvae and juveniles from non-ablated females are more resilient to
66 typical pathogens (Vp_{AHPND} and WSSV) and may show higher survival rates during a disease
67 outbreak.

68

69 **Key words:** eyestalk ablation, acute hepatopancreatic necrosis disease, early mortality
70 syndrome (EMS), welfare, white spot syndrome virus (WSSV)

71

72 1. Introduction

73 Recent global shrimp production statistics indicate that more than half of shrimp production
74 (i.e. nearly 4.5 million tons) comes from aquaculture. The Pacific whiteleg shrimp, *Penaeus*
75 *vannamei*, is currently the most cultured marine shrimp worldwide representing 78% of
76 global shrimp aquaculture production in 2019 (Anderson et al., 2019).

77 Maturation and reproduction of Pacific whiteleg shrimp, in most hatcheries
78 worldwide, is induced through unilateral eyestalk ablation (Chamberlain and Lawrence,
79 1981b; Zhang et al., 1997; Palacios et al., 1999a; FAO, 2003; Sainz-Hernández et al., 2008;
80 Das et al., 2015). This technique leads to more frequent and predictable peaks of ovarian
81 maturation and spawning. This facilitates the establishment of production schedules and
82 increases egg production (Chamberlain and Lawrence, 1981b; Palacio et al., 1999a; Bae et al.,
83 2013).

84 Given concerns regarding the practice of eyestalk ablation with respect to animal
85 welfare (Taylor et al., 2004; Little et al., 2018), it has been suggested that similar productivity
86 in broodstock can be realised without eyestalk ablation, through the application of husbandry
87 interventions including pre-maturation conditioning, increased stocking density and altered
88 sex ratios (Zacarias et al., 2019). Trials conducted using these practices have demonstrated

89 that rapid maturation and re-maturation of non-ablated *P. vannamei* females can be obtained
90 while maintaining similar levels of eggs/nauplii productions as ablated females (Zacarias et
91 al., 2019).

92 Growth performance and final survival of offspring produced from non-ablated
93 broodstock have been demonstrated to be similar to those from ablated broodstock in
94 larviculture, nursery and grow-out (Zacarias et al., 2019). Salinity stress tests, however,
95 suggest that non-ablated females can produce more resilient animals (Zacarias et al., 2019).

96 The global shrimp farming industry has been affected by regular outbreaks of disease-
97 causing catastrophic crop failures with severe financial losses (Cock et al., 2009; Tran et al.,
98 2013; Shinn et al., 2018b). Acute hepatopancreatic necrosis disease (AHPND), or Early
99 Mortality Syndrome (EMS) as it is more commonly known among farming communities, the
100 microsporidian *Enterocytozoon hepatopenaei* (EHP) and white spot virus disease (WSD) are
101 the top bacterial, parasitic and viral diseases impacting whiteleg shrimp production (Phuoc et
102 al., 2009; Lightner et al., 2012; Sajali et al., 2019). AHPND is caused by pathogenic isolates
103 of *Vibrio parahaemolyticus* (*Vp*), and a number of other *Vibrio* spp., that carry a plasmid
104 encoding two *Pir*-like toxins which cause progressive degeneration of the shrimp
105 hepatopancreas (Sajali et al., 2019). Infection often results in acute episodes of mortality in *P.*
106 *vannamei* postlarvae (PL) within the first 20-35 days after stocking in nursery or grow-out
107 ponds (Lightner and Redman, 2012; Tran et al., 2013; De Schryver et al., 2014), usually
108 resulting in high rates or the complete loss of stock or the need to clear out the stock (De
109 Schryver et al., 2014; Sajali et al., 2019). The collective losses attributed to AHPND alone
110 throughout a number of Asian states (i.e. China, Malaysia, Thailand, and Vietnam) and in
111 Mexico across the period of 2009 to 2016 were estimated by Shinn et al. (2018b) to be US$
112 23.58 bn.

113 The Whispovirus commonly referred to as white spot (syndrome) virus (WSSV)
114 responsible for white spot disease (WSD) infects a broad range of crustaceans inhabiting all
115 tropical aquatic environments with temperatures typically ranging from 18 to 30˚C (Lightner
116 et al., 2012; Verma et al., 2017). Infection can similarly result in high rates of mortality which
117 can reach 100% within 3-10 days of infection (Lin et al., 2011; Verma et al., 2017). Since the
118 first report of WSSV infection in Taiwan and the People's Republic of China in 1992 (Chou
119 et al., 1995), the subsequent resultant losses were estimated by Lightner et al. (2012), up to
120 the point of their report, to be in the order of US$ 8-15 bn. In the same year, Stentiford et al.
121 (2012) estimated that WSD accounts for an annual loss of almost US$1 bn.

122 The growth performance and final survival of the offspring of non-ablated shrimp is
123 not different from those of ablated shrimp, but a previous study (Zacarias et al., 2019)
124 suggests an improvement in their ability to cope with stress measured as survival after salinity
125 stress testing. Salinity stress testing, a common method used by shrimp farmers to check post-
126 larvae quality when sourcing, however, mainly relates to the ability of the PL to withstand
127 environmental stress and does not give any indication of the ability of the shrimp to withstand
128 a disease challenge. The objective of this study was to assess the resilience of postlarvae and
129 juvenile *P. vannamei* produced from ablated and non-ablated broodstock following a disease
130 challenge and test the hypothesis that non-ablated female's offspring show higher resistance
131 to disease when challenged with Vp_{AHPND} and WSSV under controlled experimental
132 conditions. Any difference in survival post-challenge would demonstrate if there is any added
133 value for farmers when sourcing PLs from ablated or non-ablated females.

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138 ## 2. Materials and methods

139 ### 2.1. Hatchery production of the two shrimp populations

140 Two postlarvae populations were produced by Syaqua Siam Co. hatchery in Surat Thani
141 Province, Thailand, one from ablated (AF) and the other from non-ablated (NAF) females
142 belonging to the same breeding batch and family. The shrimp lines were from families
143 selected using salinity tolerance as one of the selection criteria. SPF (specific pathogen free)
144 *Penaeus vannamei* broodstock with average male and female weights of 38.0 ± 2.0 and 40.0 ±
145 2.0 g respectively, were used for the production. The broodstock were all obtained from a
146 population that was tank-reared in an SPF facility with routine health checks every 10 days
147 and monthly PCR testing of the population to confirm their freedom of AHPND, CMNV,
148 EHP, IHHNV, IMNV, LSNV, SHIV (DIV1), TSV, WSSV, YHV/GAV. Four maturation
149 tanks (7×3.5×0.5 m; two tanks for males only and two tanks stocked only with females) were
150 stocked with 50 shrimp per tank (2/m^2). After one week of acclimatization, unilateral eyestalk
151 ablation (ablation of one of the shrimp's eyestalks) was performed on the females in one tank
152 (Ablated – AF) by cauterization (cutting the eyestalk with hot scissors), while in the second
153 tank, the females remained intact (non-ablated – NAF). Individual females for ablation were
154 caught with a hand net, gently lifted from the net, held in one hand and an eyestalk cauterized.
155 This procedure took less than 30 seconds per shrimp. The NAF were not specifically handled
156 to balance the stress during the trial. Ablation stress is not simply restricted to the physical
157 ablation but the whole process of capture, handling and ablation. If animals had been captured
158 and handled but not ablated, this would not reflect the actual practice and experience of NAF.

159 The rearing conditions and water quality assessments made on the broodstock tanks
160 and their feeding regime is provided in the Supplementary information section S1. One week
161 after ablation, mature females from each treatment were collected and placed in tanks

162 containing males (1 male tank for each treatment group). After 3-4 hours, the mated females
163 were collected from the male tank and placed into separate spawning tanks. Females were
164 removed from the spawning tanks after spawning and returned to their respective maturation
165 tanks. The hatch success of the two groups of eggs were 73% for the AF and 65% for the
166 NAF. Nauplii were harvested after 36 h using a net (100-micron mesh), dipped in 50 ppm
167 iodine for 60 seconds and then rinsed in running seawater for 5 minutes.

168 Six plastic tanks (500L) with an initial 300L water volume were stocked with 45,000
169 stage 5 nauplii at a density of 150 nauplii/L. Both treatments were set up in triplicate and
170 randomly distributed within a greenhouse. The rearing conditions and water quality of the
171 tanks used to rear the nauplii are provided in the Supplementary information section S2. The
172 larval diets consisted of algae (*Thalassiosira* sp.), a microparticulate feed (HiPro® from
173 SyAqua Sdn. Bhd.) and live *Artemia*. The type and amount of food was adjusted for each
174 larval stage. At the end of the larviculture period, the final survival of the PLs were 58.8 ± 5.0
175 % for the AF group and 58.8 ± 5.6 % for the NAF.

176 When postlarvae were 15-days old (PL 15), they were shipped (i.e. flight and
177 specialist couriers) to the research aquarium and challenge facilities of Benchmark R&D
178 (Thailand) Ltd in Chonburi, Thailand. To avoid bias, a double-blind approach was used
179 throughout the trial and subsequent analysis. The ablation status of the females producing
180 each group of PL (AF or NAF) was not disclosed by SyAqua Siam until the completion of the
181 challenge trials.

182 Details relating to the mandatory health checks that were conducted on the receipt of the
183 shrimp and on the maintenance of the two *P. vannamei* populations are provided in the
184 Supplementary information sections S3 and S4, respectively.

185 The trials conducted in this study used one batch of PL from AFs and another from NAF
186 shrimp. The two groups were from the same commercial broodstock and genetic line. This

187 approach has been used in similar studies (Phuoc et al., 2009; Tran et al., 2013; He et al.,
188 2017; Noble et al., 2017) which used a single batch and genetic line to avoid confounding
189 factors that could create noise in the results of the study. It is, however, important to highlight
190 that the study outcomes may also be a result of the genetic makeup of the population under
191 test.

192

193 **2.2.Survival to salinity stress test**

194 Two days after the receipt of the PL at BRDTL and one day before the start of the
195 Vp_{AHPND} challenge, salinity stress tests were conducted on the two populations in
196 quadruplicate (100 PL per replicate with 6.0 mg mean individual weight). Salinity testing is a
197 routine practice within the shrimp industry to assess the robustness of each batch of PL. Each
198 batch of PL was transferred from 15 ppt seawater into a 1 L beaker with dechlorinated tap
199 water (0 ppt) for 30 mins and then transferred into another 1 L beaker with clear 15 ppt
200 salinity water. After a further 30 mins, the survival (%) of the PL in each replicate was
201 evaluated based on immobility/response after physical stimulation with a pipette.

202

203 **2.3. *Vibrio parahaemolyticus* preparation**

204 AHPND results in acute mortalities in *P. vannamei* postlarvae within the first 20-35
205 days after being stocked into grow-out ponds. This first disease challenge set out to explore
206 the resilience of each population of PL17 to Vp_{AHPND}.

207 The bacterial inoculum for the challenge was prepared by inoculating isolate
208 FVG0001 (an isolate derived from a Vp_{AHPND} mortality event in *P. vannamei* cultured in
209 Thailand and acquired through the Thai Department of Fisheries) into tryptone soya broth
210 (TSB) supplemented with 2% NaCl and cultured for 12h at 28°C, shaking at 250 rpm. Pure
211 cultures of the isolate were produced and additional cross checked for five viral pathogens

212 (IHHN, IMNV, TSV, WSSV and YHV) using OIE approved molecular methods. Thereafter,
213 the bacterial cells were collected by centrifugation at 900×g for 10 mins at 10°C and the
214 resultant bacterial pellet re-suspended in sterile seawater (15 ppt). The number of colony-
215 forming units (CFU mL^{-1}) in the suspension was then determined by measuring the optical
216 density at 600 nm (OD600), where for Vp_{AHPND}, an OD value of 1.0 corresponded to
217 approximately 2.0×10^8 CFU mL^{-1}. The bacterial cell number was then adjusted and verified
218 by viable plate counts following standard methods; cultures were pure, i.e. no contamination.
219 The presence of the pVA plasmid and the binary *Pir*-like toxin pair ToxA and ToxB was
220 confirmed using the AP4 nested PCR method of Dangtip et al. (2015) and a sub-sample of the
221 culture additional confirmed free of five viral pathogens namely IHHNV, IMNV, TSV, YHV
222 and WSSV using recognized methodologies (these are detailed in Supplementary information
223 section S3).
224

225 **2.4. Survival of shrimp postlarvae challenged with Vp_{AHPND}**

226 The Vp_{AHPND} challenge tests followed the methods described in Shinn et al. (2018a) and Sajali
227 et al. (2019). Pre-challenges were conducted to define a bacterial dose to use for the main
228 challenge – details relating to these are provided in the Supplementary information section S5.
229

230 From the pre-challenge trials, a challenge dose of 2.0 ml of a 2×10^8 CFU mL^{-1} was selected.
231 This dose resulted in 64% and 33% mortality in populations from AF and NAF respectively at
232 96 h post-infection. The main challenge was performed under the same conditions as the pre-
233 challenge. For the main challenge, the performance of each population and condition was
234 tested by using five replicate, static, aerated, 20 L tanks, with a total of 100 × PL17 per tank.
235 The groups were Population AF + Vp_{AHPND}; Population NAF + Vp_{AHPND}; Population AF –
236 control with no Vp_{AHPND} added; Population NAF - control with no Vp_{AHPND} added. The PL17

237 from both populations had average individual weight of 14 mg at the time of the challenge.
238 Water quality parameters within the challenge vessels are provided in the Supplementary
239 information section S6. A semi-randomized block design was used to allocate the test tanks.
240 The control tanks were isolated from the challenge tanks to prevent cross-contamination.
241 Shrimp mortality was assessed every 3 h continuously, 24 h d^{-1}, over the entire duration of the
242 96 h post-challenge period.
243
244 **2.5. Challenge trials using white spot syndrome virus**
245 **Virus amplification:** One week prior to starting the WSSV pre-challenge, 30 shrimp
246 juveniles from population AF were placed in two tanks (10 L; 15 ppt) in a temperature-
247 controlled disease challenge room maintained at 26 ± 0.0 ºC. Population AF was selected as
248 it was the weaker performer from the Vp_{AHPND} tests to minimise animal use (3Rs). On the first
249 day, the shrimp were fed to satiation with minced tissue from WSSV infected *P. vannamei.*
250 The infected tissue was derived from frozen (-80˚C), WSSV infected tissue acquired from the
251 Shrimp-Pathogen Interaction (SPI) Laboratory, National Center for Genetic Engineering and
252 Biotechnology (BIOTEC), National Science and Technology Development Agency
253 (NSTDA), Bangkok, Thailand, and confirmed free of six other shrimp diseases (AHPND,
254 EHP, IHHNV, IMNV, TSV and YHV) by recognised methodologies (see Supplementary
255 information S3). After exposure to WSSV infected tissue, the shrimp were fed a normal
256 commercial feed thereafter. The tanks were checked every 3 h for 168 h and any dead or
257 moribund shrimp removed. Moribund shrimp were immediately euthanised in iced water
258 (<4˚C). Euthanised or dead shrimp were then stored at -80 ºC. After 7 days, all the resulting
259 shrimp material was processed – the gills, muscle and pleopods were harvested, and
260 thoroughly macerated to ensure complete mixing of the shrimp tissues. Three random 0.5 g
261 samples were then taken and the titre of WSSV virus determined by qPCR. The macerated

262 tissue was stored in the -80˚C freezer, while the qPCR tests were being conducted and the
263 WSSV pre-tests were set-up.
264

265 **Determination of the WSSV virial titres in the shrimp tissue for challenge**

266 Quantitative PCR (qPCR) was used to determine the viral titre of the shrimp tissues
267 used for the main WSSV challenge. DNA from macerated *P. vannamei* gill, muscle and
268 pleopod tissue was extracted using a Qiagen DNEasy Blood & Tissue Kit (Qiagen, Hilden,
269 Germany). qPCR was performed using qPCR Green Master Mix LRox (biotechrabbit GmbH,
270 Hennigsdorf, Germany) on a Roche Lightcycler® 96 (Roche Diagnostics GmbH, Mannheim,
271 Germany). The protocol used follows that of Durand and Lightner (2002) approved by OIE
272 (OIE, 2019) for the detection of WSSV using primers WSS1011F (5'-TGG-TCC-CGT-CCT-
273 CAT-CTC-AG-3') and WSS1079R (5'-GCT-GCC-TTG-CCG-GAA-ATT-A-3'). The qPCR
274 conditions used were: an initial denaturation step of 95°C for 3 min, followed by 40 cycles of
275 95°C for 15 sec, 60°C for 30 sec, and then 72°C for 30 sec. A melting curve analysis was
276 performed to estimate the specificity of the method and used to confirm that no secondary
277 products were observed. A negative DNA template control was included in the qPCR assay
278 alongside a serial diluted plasmid DNA standard ($1 \times 10 - 1 \times 10^5$ μL^{-1}; Centex Shrimp,
279 Mahidol University, Thailand) to permit the determination of the WSSV copy number within
280 each sample. From >30g minced tissue resulting from the WSSV amplification step, the
281 WSSV titre was determined from triplicate samples to be $1.81 – 2.37\times 10^9$ WSSV/0.1 gram
282 (av. 2.02×10^9 WSSV/0.1 gram).
283 Pre-challenges were conducted to define a dose to use for the main WSSV challenge – details
284 relating to these are provided in the Supplementary information section S7.
285

286 **WSSV main challenge:** From the pre-challenges, a dose of 0.1 g WSSV-infected tissue (av.
287 2.02×10^9 WSSV/0.1 gram) was selected as it resulted in 70% mortality of shrimp at 168 h
288 post-infection. The main challenge was performed under the same conditions as the pre-
289 challenge but using a total of 200, static, aerated, 1 L vessels, each stocked with a single
290 juvenile (i.e. 50 replicates per treatment – 50 × Population AF + WSSV; 50 × Population
291 NAF + WSSV; 50 × Population AF – control not exposed to WSSV; 50 × Population NAF -
292 control not exposed to WSSV). All shrimp used for the experiment were pre-graded (1.3-1.5 g
293 size range) and had an average individual weight of 1.42 ± 0.07 g. A larger sized shrimp, i.e.
294 average weight of >1g was used rather than postlarvae so that the ingestion of the WSSV-
295 infected material presented to each shrimp could be confirmed. As shrimp cannibalise their
296 dead counterparts, to ensure that each shrimp received the same dose of WSSV, it was
297 necessary to house them in individual vessels. Water temperature, salinity, pH, alkalinity,
298 unionized ammonia and nitrite were within the following ranges: 26.33 ± 0.73 ºC, 15.0 ppt,
299 8.40 ± 0.14, 147.0 ± 5.2 mg/L $CaCO_3$, 0.04 ± 0.01 mg/L and 0.1 ± <0.01 mg/L respectively. A
300 semi-randomized block design was used to allocate the test tanks in the challenge room. As
301 with the Vp_{AHPND} challenge, the control treatments were isolated to prevent cross-
302 contamination. The experimental vessels were inspected every 3 h continuously, 24 h d^{-1}, over
303 the entire duration of the 168 h post-challenge observation period and any dead or moribund
304 shrimp removed. Moribund shrimp were euthanized in pre-iced water where necessary, and
305 then all removed shrimp stored in a -80 ºC freezer. After 168 h post-infection, the gills,
306 pleopods and muscle were harvested from a random sample of shrimp from each population
307 of shrimp and then analysed by qPCR to confirm the presence of WSSV and to determine the
308 titres of WSSV.
309

2.6. Disposal of experimental materials

On completion of each trial, all surviving shrimp were humanely euthanized in pre-iced water (<4˚C), and subsequently incinerated together with other remaining dead shrimp collected during the trials.

2.7. Ethics statement

These trials were reviewed by and conducted under the approval of the University of Stirling Animal Welfare and Ethical Review Body (AWERB; ref. no. (18 19) 191) and BRDTL AWERB which included external independent assessors (ID. B-TH-NON-2020-106). All members of BRDTL directly involved in the study hold licences for the use of "Animals for Scientific Purposes" issued by the Institute for Animals for Scientific Purpose Development, National Research Council of Thailand. The BRDTL laboratories and challenge facilities are registered with the relevant Thai authorities and have been inspected as required under current Thai legislation.

2.8. Statistical analysis

One-way ANOVA followed by a Tukey test (Zar, 2010) was used to compare survival to salinity stress test in significance level of 0.05. Normality and homogeneity were tested using Shapiro-Wilk and Levene tests, respectively. Percentage data were transformed to arcsine square-root prior to analysis. The data are presented as mean ± standard error.

The survival of the experimental shrimp was assessed using a Mantel-Cox log rank test conducted in Excel Windows 365 to conduct pairwise comparisons of the survival distributions between each set of samples using shrimp mortality (or their removal from the challenge) as the time to event. The time stratified Cochran-Mantel-Haenszel test was used to

333 calculate the number of observed and expected events at each time point to derive summary
334 survival probabilities across all time points where there was a response (i.e. a shrimp
335 mortality). The approach follows that used in other similar Vp_{AHPND} challenge-based
336 evaluations with *P. vannamei* (see Shinn et al., 2018a; Sajali et al., 2019a). All comparisons
337 were conducted at a significance level of 0.05.

338

339 **3. Results**

340 3.1. **Salinity stress tests and the survival rate of the shrimp post-larvae**

341 No significant survival difference (p = 0.13) between the two populations was observed after
342 the salinity stress tests. The PL from NAF and AF had 96.5 ± 1.84 and 99.75 ± 0.25 %
343 survival, respectively.

344

345 3.2. **Vp_{AHPND} challenge**

346 Drop counts confirmed that the growth equated to 2.35E+08 and 2.0E+08 CFU mL for the pre
347 challenge and main challenge respectively. The PL originating from NAF had significantly (p
348 < 0.05) better survival (70.4%) than PL from AF (38.8%) at 96 h post-challenge (Fig. 1; Table
349 1). Over the challenge period, a significant difference between the two challenged groups was
350 observed from 9 h post-challenge onwards (Table S1). The survival of the control (i.e. un-
351 challenged) shrimp from the NAF and AF 96 h post-challenge was not significantly different
352 between the two populations (100 and 100% for NAF and AF, respectively) ($p > 0.05$) (Table
353 1; Fig. 1). The Vp_{AHPND} challenged groups, however, had significantly ($p < 0.05$) lower
354 survival than the control groups (Table 1; Fig. 1). Supplementary data with replicate tank
355 mortality are shown in Table S2. Terminal disease testing using the AP4 nested PCR of a

356 random selection of moribund shrimp from each population confirmed that mortality was
357 death due to Vp_{AHPND} . No samples, however, were evaluated by histopathology.

358

359 ### 3.3. **Survival of shrimp juveniles following WSSV challenge**

360 The survival of the shrimp from NAF (62%) at 168 h post-infection was higher than that of
361 the shrimp from AF (48%) but the difference was not significantly different (Table 2). There
362 were, however, significant differences between the two populations at 65 to 75 h post-
363 challenge (see Table S1; Fig. 2). No significant difference was observed in the survival of
364 non-challenged animals from both groups 168 h post-challenge (98 and 98% for NAF and
365 AF, respectively) ($p > 0.05$; see Table 2). The WSSV challenged groups, however, had
366 significantly ($p < 0.05$) lower survival than the control groups (Table 2; Fig. 2). Terminal
367 disease testing of a random selection of moribund and dead shrimp from each population
368 confirmed death due to WSSV infection (AF (n = 3), av. 1.21×10^9 copies (range $1.09\text{-}1.31 \times$
369 10^9) WSSV copies / 0.1 gram ; NAF (n = 3), av. 1.40×10^9 (range $1.37 - 1.44 \times 10^9$) WSSV
370 copies / 0.1 gram). Terminal sampling of five shrimp from each of the two non-challenged
371 control groups were tested by qPCR and were negative (i.e. below detectable limits). In
372 addition, shrimp from the challenge groups surviving the challenge at 168 h post-challenge
373 were sampled and archived at -80˚C, they were not however analysed as their survival does
374 not necessarily mean that they were free of infection but rather that they survived the
375 challenge doses they were exposed to.

376

377

378

379 ## 4. Discussion

380 Although unilateral eyestalk ablation facilitates the establishment of production schedules and
381 increased nauplii production in commercial shrimp hatcheries, it is not a good welfare
382 practice (Little et al., 2018). Furthermore, it has long been recognized that ablation can also
383 cause physiological imbalance and compromise the immunological health of broodstock
384 (Palacios et al., 1999ab; Sainz-Hernandez, et al., 2009; Bae et al., 2013; Treerattrakool et al.,
385 2014; Das et al., 2015). Ablation can also lower the nutritional reserves of the offspring
386 (Wickins and Lee, 2002; Racotta et al., 2003) possibly decreasing their chance of survival
387 during disease outbreaks. This study and previous study (Zacarias et al. 2019), confirms that
388 ablation has an impact not only on the female broodstock, but that negative effects are carried
389 on through to the offspring. Eliminating ablation will require hatcheries to accept that this
390 can be done without significant impact on their production and profitability and that there
391 may be additional benefits in adopting a non-ablation approach. Zacarias et al. (2019) have
392 demonstrated that it is possible to use NAF under commercial conditions and achieve similar
393 productivity to AF and that the final survival and growth performance in larviculture, nursery
394 and grow-out of their offspring is also similar to AF.

395 In the study presented here, PLs from NAF and AF treatments displayed similar survival rates
396 after salinity stress testing, indicating equivalent robustness of the employed experimental
397 animals against this commercially used quality check method. Nonetheless, different survival
398 rates between NAF and AF were observed following experimental challenges with two key
399 shrimp pathogens. Under challenge with Vp_{AHPND}, the survival of the challenged PL from
400 NAF was significantly higher than the PL from AF at 96 h post-challenge. The trial supports
401 the hypothesis posed by Zacarias et al. (2019), that ablation can negatively affect offspring
402 quality in terms of their physiological status.

403 When the same two populations of shrimp were challenged with WSSV, there was no
404 statistical difference ($p > 0.05$) between the two challenged groups at the conclusion of the
405 experiment (168 h post-challenge) although the level of significance was close ($p = 0.09$). At
406 intermediate times (54 h and 75 h post-challenge) the NAF population survival was
407 significantly higher than that of the AF. The higher survival of juveniles from NAF, although
408 not statistically significant, suggests that there may be some slight disadvantage of ablation on
409 the offspring's ability to withstand a WSSV challenge but that the current experimental
410 design was inadequate to demonstrate this.

411 Eyestalk ablation has been reported to compromise the immune system of broodstock shrimp
412 (Sainz-Hernandez, et al., 2009; Bae et al., 2013 and Treerattrakool et al., 2014). It can,
413 therefore, be hypothesized that the overall improvement of survival in offspring from non-
414 ablated *P. vannamei* broodstock to AHPND and WSSV observed in this study is evidence of
415 enhanced "robustness" within the stock. The mechanisms that lead to this improvement could
416 be multifarious and most likely linked to enhancement in the immune status of the offspring
417 from non-ablated broodstock. However, as no measurements of immune response were
418 conducted in this study the mode of action for enhanced robustness remains to be confirmed.

419 The results presented here were obtained under laboratory-controlled conditions. If, however,
420 the potential of NAF offspring to better survive a Vp_{AHPND} and WSSV outbreak was to be
421 confirmed in commercial scale scenarios, the economic impact to farmers would certainly be
422 significant. Indeed, if farmers were to stock their nursery tanks/ponds with PL from NAF,
423 significant improvements in the survival of stock compared to PL from AF when shrimp are
424 exposed to Vp_{AHPND} within the first days of stocking are likely. Similarly, a higher rate of
425 survival of juveniles from NAF parents stocked in grow-out ponds may be observed in the
426 first days of WSSV exposure. Vp_{AHPND} infections can result in the complete loss of stock (De

427 Schryver et al., 2014; Sajali et al., 2019), which has been estimated to have resulted in
428 accumulated losses of ca. US$ 23.58 bn in 8 years (2009-2016) across Vietnam, Thailand,
429 Malaysia, China and Mexico (Shinn et al., 2018b). Lightner et al. (2012) also reported losses
430 of US$8 – $15 bn due to WSSV. The higher survival observed in PL and juveniles from NAF
431 might, therefore, reduce the levels of loss and bring economics benefits to farmers and other
432 actors in shrimp value chains.

433 In conclusion, these results contribute to the current discussion around the opportunity and
434 incentives to move beyond the use of eyestalk ablation as a management practice and towards
435 adoption by the sector of higher welfare production standards. A further benefit of this, as
436 these results show, is that there is compelling economic argument of the benefits of non-
437 ablation as results now confirm growth performance and survival under normal conditions are
438 not compromised and in fact survival in response to typical pathogens (Vp_{AHPND} and WSSV)
439 is likely to be higher in PLs and juveniles from non-ablated animals. Validation at the farm
440 level of the current study's findings alongside in-depth study of the mechanisms responsible
441 for the results observed here is now needed.

442

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447

448

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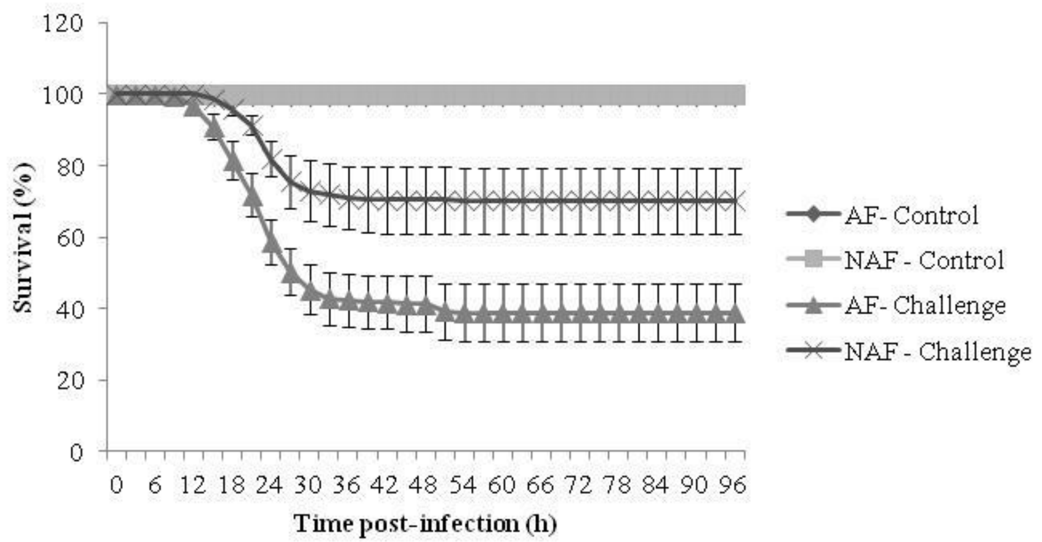
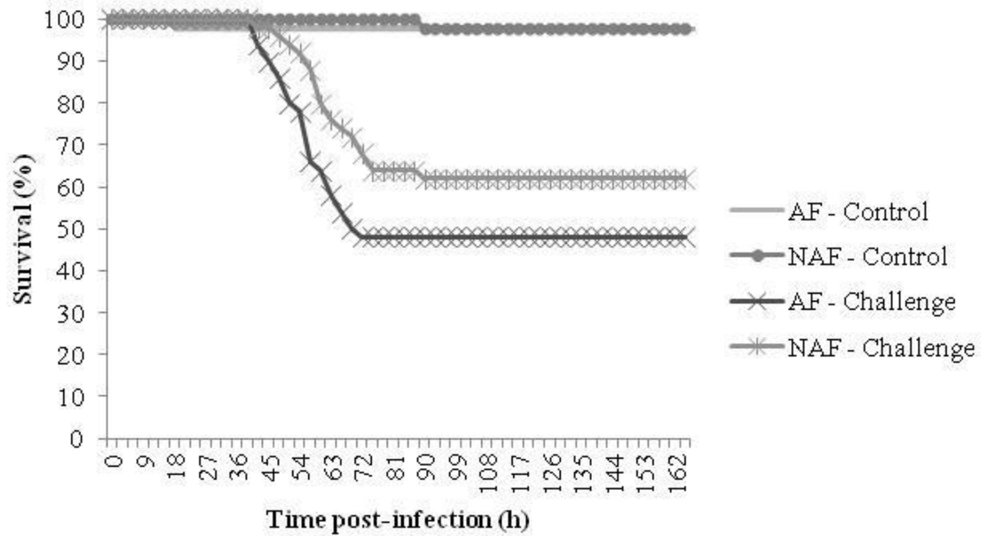
549

550 **Figures**



551

552 Figure 1: Survival of non-challenged and *Vibrio parahaemolyticus*-challenged *Penaeus vannamei* postlarvae
553 (PL17) originating from non-ablated female (NAF) and ablated female (AF) broodstock.
554



555

556 Figure 2: Survival of non-challenged and WSSV-challenged *Penaeus vannamei* juveniles originating from non-
557 ablated female (NAF) and ablated female (AF) broodstock.
558

559

560

561

562 ## Tables

563

564 Table 1. Summary of the statistics following analysis of the mortalities by Mantel-Cox log rank tests at the end
565 of the challenge (96 h post-challenge).

	AF - Control	NAF - Control	AF - Challenge	NAF - Challenge
AF - Control				
NAF - Control	0.08			
AF - Challenge	6.32E-92	5.88E-95		
NAF - Challenge	1.34E-36	6.47E-39	2.40E-23	

566 AF – Ablated female; NAF – Non-ablated female; E – Exponential

567

568 Table 2: Summary of the statistics following analysis of the mortalities by Mantel-Cox log rank tests 168 hours
569 post-challenge with WSSV at the end of challenge (168h post challenge).

	AF - Control	NAF - Control	AF - Challenge	NAF - Challenge
AF - Control				
NAF - Control	0.99			
AF - Challenge	4E-08	1.85E-08		
NAF - Challenge	1.05E-05	6.55E-06	0.09	

570 AF – Ablated female; NAF – Non-ablated female; E - Exponential

571

572 **Supplementary data**

573 Table S1. P values observed at each time point when *Penaeus vannamei* from non-ablated (NAF) and ablated
574 (AF) broodstock challenged with *Vibrio parahaemolyticus* or with WSSV were compared.

	Observed P values between NAF&AF for each challenge			Observed P values between NAF&AF for each challenge	
Time (h)	***Vp* AHPND**	**WSSV**	**Time (h)**	***Vp* AHPND**	**WSSV**
0	NS	NS	84	2.40E-23	0.06
3	NS	NS	87	2.40E-23	0.06
6	0.16	NS	90	2.40E-23	0.09
9	6.15E-05	NS	93	2.40E-23	0.09
12	2.93E-08	NS	96	2.40E-23	0.09
15	1.86E-12	NS	99		0.09
18	3.07E-15	NS	102		0.09
21	7.44E-16	NS	105		0.09
24	5.22E-17	NS	108		0.09
27	3.09E-19	NS	111		0.09
30	7.03E-21	NS	114		0.09
33	2.45E-20	NS	117		0.09
36	2.28E-20	NS	120		0.09
39	2.20E-20	NS	123		0.09
42	8.59E-21	NS	126		0.09
45	8.59E-21	0.32	129		0.09
48	6.95E-23	0.099	132		0.09
51	2.40E-23	0.08	135		0.09
54	2.40E-23	0.04	138		0.09
57	2.40E-23	0.05	141		0.09
60	2.40E-23	0.01	144		0.09
63	2.40E-23	0.06	147		0.09
66	2.40E-23	0.04	150		0.09
69	2.40E-23	0.03	153		0.09
72	2.40E-23	0.02	156		0.09
75	2.40E-23	0.03	159		0.09
78	2.40E-23	0.06	162		0.09
81	2.40E-23	0.06	165		0.09
84	2.40E-23	0.06	168		0.09

575

576 AF – Ablated female; NAF – Non-ablated female; NS – Not significant; E - Exponential

577

578
579

Table S2. AHPND mortality of Pacific white shrimp (*Penaeus vannamei*) postlarvae from non-ablated (NAF) and ablated (AF) broodstock per replicate tank (n= 5).

	AF - Control					**NAF – Control**					**AF - Challenge**					**NAF - Challenge**				
Hours	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
3	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
6	99	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
9	99	99	100	99	100	100	100	100	100	100	100	99	100	99	100	100	100	100	100	100
12	99	99	100	99	100	100	100	100	100	100	100	99	91	99	95	100	100	100	100	100
15	99	99	100	99	100	100	100	100	100	100	100	94	79	95	87	97	99	100	100	98
18	99	99	100	99	100	100	100	100	100	100	97	82	70	89	69	94	97	99	99	90
21	99	99	100	99	100	100	100	100	100	100	89	74	63	79	54	89	94	94	98	82
24	99	99	100	99	100	100	100	100	100	100	77	60	49	66	42	76	89	87	93	65
27	99	99	100	99	100	100	100	100	100	100	69	50	38	61	34	65	88	82	91	52
30	99	99	100	99	100	100	100	100	100	100	66	44	35	55	27	60	88	80	90	47
33	99	99	100	99	100	100	100	100	100	100	65	43	32	51	23	59	86	80	90	45
36	99	99	100	99	100	100	100	100	100	100	65	43	31	50	23	58	84	80	90	43
39	99	99	100	99	100	100	100	100	100	100	65	43	31	49	22	57	84	80	90	42
42	99	99	100	99	100	100	100	100	100	100	65	43	30	49	22	57	84	80	90	41
45	99	99	100	99	100	100	100	100	100	100	65	43	29	49	21	57	84	80	90	41
48	99	99	100	99	100	100	100	100	100	100	65	43	29	49	21	57	84	80	90	41
51	99	99	100	99	100	100	100	100	100	100	65	33	29	49	21	57	84	80	90	41
54	99	99	100	99	100	100	100	100	100	100	65	32	27	49	21	56	84	80	90	41
57	99	99	100	99	100	100	100	100	100	100	65	32	27	49	21	56	84	80	90	41
60	99	99	100	99	100	100	100	100	100	100	65	32	27	49	21	56	84	80	90	41
63	99	99	100	99	100	100	100	100	100	100	65	32	27	49	21	56	84	80	90	41
66	99	99	100	99	100	100	100	100	100	100	65	32	27	49	21	56	84	80	90	41
69	99	99	100	99	100	100	100	100	100	100	65	32	27	49	21	56	84	80	90	41
72	99	99	100	99	100	100	100	100	100	100	65	32	27	49	21	56	84	80	90	41
75	99	99	100	99	100	100	100	100	100	100	65	32	27	49	21	56	84	80	90	41
78	99	99	100	99	100	100	100	100	100	100	65	32	27	49	21	56	84	80	90	41
81	99	99	100	99	100	100	100	100	100	100	65	32	27	49	21	56	84	80	90	41
84	99	99	100	99	100	100	100	100	100	100	65	32	27	49	21	56	84	80	90	41
87	99	99	100	99	100	100	100	100	100	100	65	32	27	49	21	56	84	80	90	41
90	99	99	100	99	100	100	100	100	100	100	65	32	27	49	21	56	84	80	90	41
93	99	99	100	99	100	100	100	100	100	100	65	32	27	49	21	56	84	80	90	41
96	99	99	100	99	100	100	100	100	100	100	65	32	27	49	21	56	84	80	90	41

586 **Supplementary information**

587 **S1. Water quality and maintenance of the broodstock**

588 The water temperature and salinity of the broodstock tanks were maintained at 28.0-30.0 ºC
589 and 30.0 ± 1.0 ppt, respectively. A daily water exchange was applied (50-100%). Photoperiod
590 followed a natural regime by exposure to ambient sunlight through translucent roof panels in
591 the maturation room. The broodstock were fed five times a day with squid (2 feeds) and on
592 polychaete worms (3 feeds) at between 2-5% body weight (adjusted based on actual
593 composition). The polychaetes were obtained from a source that is SPF for all major shrimp
594 pathogens of concern. All "fresh" feeds were kept frozen with samples tested by PCR for all
595 major pathogens before being approved for use.

596

597 **S2. Water quality and maintenance of the nauplii**

598 Water temperature, dissolved oxygen, pH, ammonia, nitrite and alkalinity were 28.5 ± 0.7 ºC,
599 5.4 ± 0.2 mg/L, 7.8 ± 0.1 mg/L, 0.1 ± 0.0 mg/L, <1 mg/L and 160.2 ± 39.5 mg/L $CaCO_3$
600 respectively. Salinity was gradually adjusted from 30.0 ppt to 15 ppt from postlarvae 5 at a
601 rate of 1 ppt/day. Approximately 30% of the water volume was exchanged daily when the
602 animals reached the postlarvae stage.

603

604 **S3. Mandatory health checks following the receipt of shrimp**

605 The disease challenge trials were conducted within the aquarium and disease challenge
606 facilities of Benchmark R&D (Thailand) Ltd (BRDTL) in Chonburi, Thailand. A total of
607 20,000 SPF *P. vannamei* postlarvae 15-day-old (PL_{15}), half of which were derived from NAF
608 and the other half from AF, were used for the disease challenge trials.

609 Upon receipt at BRDTL, the PL were handled in accordance with local standard
610 operating procedures for the receipt of new stock on site, i.e. the exterior of the transport bags
611 were sprayed with 70% alcohol, then the PL were passed through a 100-micron mesh nylon
612 bag and then surface-disinfected (15-20 sec dip) in a separate vessel containing 0.1 mg/L
613 P.V.-DINE 125® (povidone iodine). The mesh bag and PL were then dipped for 15-20 secs in
614 a second vessel containing pre-treated conditioned 15 ppt seawater to rinse the shrimp. The
615 PL were subsequently assigned to three separate 200 L static aerated holding tanks, each
616 stocked in 180 L of pre-treated, dechlorinated 15 ppt seawater; stock was held under
617 quarantine conditions while mandatory disease testing was conducted. For testing, a pooled
618 sample of 150 PL (taken randomly from the holding tanks) per population were screened for

619 seven key shrimp diseases, namely: Vp_{AHPND} by nested PCR; the fungal microsporidian
620 *Enterocytozoon hepatopenaei* (EHP) and WSSV tested for by qPCR using OIE (2019)
621 approved methodologies; for infectious hypodermal and haemotopoietic necrosis virus
622 (IHHNV), infectious myonecrosis virus (IMNV), Taura syndrome virus (TSV), and, yellow
623 head virus (YHV) by iiPCR test kits (GeneReach Biotechnology Corporation, Taichung,
624 Taiwan). Following the confirmation of freedom from all seven diseases, the remaining
625 shrimp were kept in aerated, static tanks (200L) until the first disease challenge.
626

627 **S4. Maintenance of the two populations of *P. vannamei***

628 During the holding period, daily 20% water exchanges were performed using 15 ppt water
629 (water pre-treated with 50 mg/L chlorine over a 24+ h period and the residual chlorine driven
630 off by vigorous aeration). The absence of chlorine was confirmed using an orthotolidine-
631 based chlorine test kit (Monitor®; Pet Wonderland Group, Thailand). Water temperature,
632 salinity, dissolved oxygen, pH, alkalinity, unionized ammonia and nitrite were within the
633 following ranges: 27.5 ± 0.1 ºC, 15.0 ppt, 7.3 ± 0.1 mg/L, 8.40 ± 0.14 mg/L, 161.5 ± 4.9 mg/L
634 $CaCO_3$, 0.04 ± 0.1 mg/L and 0.1 ± <0.01 mg/L respectively. During the culture phase, the
635 shrimp were fed three times daily (08:00 am; 12:00 pm midday and 16:00 pm) with two types
636 of commercial shrimp feed: for the first 30 days, the animals were fed TNT 400-600 (Charoen
637 Pokphand Co., Bangkok, Thailand) at a rate of 20 – 15% of total biomass; from day 30
638 onwards, the shrimp were fed Starbird 5093 S shrimp feed (Charoen Pokphand Co., Bangkok,
639 Thailand) at a rate of 10% body biomass per day.
640

641 **S5. Vp_{AHPND} pre-challenge tests**

642 The volume of bacterial suspension required to be added to each vessel for the main
643 challenge was determined by a pre-challenge to assess the pathogen virulence by shrimp
644 mortality using seven concentrations (0.1, 0.45, 0.8, 1.15, 1.5, 1.85, and 2.2 ml of a 2.0×10^8
645 CFU mL^{-1}, respectively) and selecting the bacterial concentration required to give ca. 60-70%
646 mortality 96 h post-infection. One day before the pre-challenge, 42 replicate, static, aerated,
647 20 L tanks, each containing 5 L of 15 ppt clear seawater were set up in a temperature-
648 controlled disease challenge room maintained at 29.05 ± 0.13ºC. A total of 100 PLs per tank
649 were used, with three replicates per dose. The pre-challenge was done for both populations of
650 PL; the average weight of the PL at this stage was 10 mg. The initial volume of water in each
651 tank was 5 L then at 24 h and 48 h post-challenge, an additional 3 L and 2 L of water was

652 added respectively to a final volume of 10 L to maintain water quality. At 72 h post-challenge
653 50% water was exchanged. Shrimp mortality was assessed every 3 h, continuously over the
654 96 h post-challenge period. Shrimp were fed TNT 400-600 (Charoen Pokphand Co.,
655 Bangkok, Thailand) at 20% of the biomass following the same feeding regime as the PL held
656 in the holding tanks.
657

658 **S6. Water quality in the *Vp*$_{\mathbf{AHPND}}$ challenge tanks**

659 Water temperature, salinity, pH, alkalinity, unionized ammonia and nitrite were within the
660 following ranges: 29.05 ± 0.13 ℃, 15.0 ppt, 7.5 ± 0.0 mg/L, 155.0 ± 6.7 mg/L $CaCO_3$, 0.03 ±
661 0.01 mg/L and 0.1 ± <0.01 mg/L respectively.
662

663 **S7. WSSV pre-challenge tests**

664 The amount of WSSV infected tissue derived from the WSSV amplification for the main
665 WSSV challenge was determined from a pre-challenge assessing the virulence and mortality
666 of shrimp using three amounts (i.e. 0.1 g, 0.15 g or 0.2 g shrimp^{-1}) of tissue (av. 2.02×10^9
667 WSSV/0.1 gram). The main aim was to determine the amount which resulted in 60-70%
668 mortality 168 h post-infection. The pre-challenge was performed under the same conditions
669 intended for the main challenge. One day before the pre-challenge 30 static, aerated, 1 L tanks
670 each containing 0.4 L of 15 ppt clear seawater were set up in a temperature-controlled disease
671 challenge room maintained at 26.3 ± 0.71℃. Ten single juvenile shrimp (average weight 1.5 ±
672 0.1 g) replicates were used per assessment dose of tissue. The pre-challenge was performed
673 on shrimp taken from population AF as these had a significantly shown higher mortality in
674 the *Vp*AHPND challenge and were regarded at this stage as the "weaker" population. For the
675 infection step, WSSV macerated tissue from the pre-amplification step held at -80˚C was
676 prepared by adding 50 µL of red food grade dye to each 1 g of minced shrimp tissue for 10
677 minutes before being weighed and allocated to the experimental tanks. The shrimp were not
678 fed for 24 h prior to the start of the experiment. Shrimp were infected by weighing out the
679 relevant amount of tissue and added to each vessel. For the infection step, the relevant amount
680 of infected tissue was placed into the tank and the aeration to the tank switched off (pre-test
681 dose range was 0.1-0.2 g WSSV infected tissue shrimp^{-1}). Shrimp consumption of the entire
682 ration was confirmed by the presence of the red tissue passing into the stomach and intestine
683 of the shrimp and the absence of any remaining free tissue in the experimental tank. The
684 aeration was then switched back on, typically within 15 min. The shrimp were then

685 maintained and monitored regularly. After 24 h, additional 0.4 L water was added to each
686 experimental vessel. At 48, 72, 96, 120 and 144 h post-challenge, 50% of the water in each
687 vessel was replenished. From day 2 of the challenge, the shrimp were maintained on the same
688 feeding regime as the stock held in the main holding tanks. Shrimp mortality was assessed
689 every 3 h continuously over the 168 h post-infection period.
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