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Increased robustness of postlarvae and juveniles from non-ablated Pacific whiteleg shrimp, *Penaeus vannamei*, broodstock post-challenged with pathogenic isolates of *Vibrio parahaemolyticus* (Vp_{AHPND}) and white spot disease (WSD)

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Highlights

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

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

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

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

1. Introduction

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

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

Given concerns regarding the practice of eyestalk ablation with respect to animal welfare (Taylor et al., 2004; Little et al., 2018), it has been suggested that similar productivity in broodstock can be realised without eyestalk ablation, through the application of husbandry interventions including pre-maturation conditioning, increased stocking density and altered 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.

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

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

2. Materials and methods

2.1. Hatchery production of the two shrimp populations

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

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

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

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

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

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

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

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

2.2.Survival to salinity stress test

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

2.3. *Vibrio parahaemolyticus* preparation

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

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

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

2.4. Survival of shrimp postlarvae challenged with *Vp*$_{AHPND}$

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

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

from both populations had average individual weight of 14 mg at the time of the challenge. Water quality parameters within the challenge vessels are provided in the Supplementary information section S6. A semi-randomized block design was used to allocate the test tanks. The control tanks were isolated from the challenge tanks to prevent cross-contamination. Shrimp mortality was assessed every 3 h continuously, 24 h d^{-1}, over the entire duration of the 96 h post-challenge period.

2.5. Challenge trials using white spot syndrome virus

Virus amplification: One week prior to starting the WSSV pre-challenge, 30 shrimp juveniles from population AF were placed in two tanks (10 L; 15 ppt) in a temperature-controlled disease challenge room maintained at 26 ± 0.0 ºC. Population AF was selected as it was the weaker performer from the Vp_{AHPND} tests to minimise animal use (3Rs). On the first day, the shrimp were fed to satiation with minced tissue from WSSV infected *P. vannamei.* The infected tissue was derived from frozen (-80˚C), WSSV infected tissue acquired from the Shrimp-Pathogen Interaction (SPI) Laboratory, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Bangkok, Thailand, and confirmed free of six other shrimp diseases (AHPND, EHP, IHHNV, IMNV, TSV and YHV) by recognised methodologies (see Supplementary information S3). After exposure to WSSV infected tissue, the shrimp were fed a normal commercial feed thereafter. The tanks were checked every 3 h for 168 h and any dead or moribund shrimp removed. Moribund shrimp were immediately euthanised in iced water (<4˚C). Euthanised or dead shrimp were then stored at -80 ºC. After 7 days, all the resulting shrimp material was processed – the gills, muscle and pleopods were harvested, and thoroughly macerated to ensure complete mixing of the shrimp tissues. Three random 0.5 g samples were then taken and the titre of WSSV virus determined by qPCR. The macerated

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

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

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

Pre-challenges were conducted to define a dose to use for the main WSSV challenge – details relating to these are provided in the Supplementary information section S7.

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

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

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

3. Results

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

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

3.2. Vp_{AHPND} challenge

Drop counts confirmed that the growth equated to 2.35E+08 and 2.0E+08 CFU mL for the pre challenge and main challenge respectively. The PL originating from NAF had significantly ($p < 0.05$) better survival (70.4%) than PL from AF (38.8%) at 96 h post-challenge (Fig. 1; Table 1). Over the challenge period, a significant difference between the two challenged groups was observed from 9 h post-challenge onwards (Table S1). The survival of the control (i.e. un-challenged) shrimp from the NAF and AF 96 h post-challenge was not significantly different between the two populations (100 and 100% for NAF and AF, respectively) ($p > 0.05$) (Table 1; Fig. 1). The Vp_{AHPND} challenged groups, however, had significantly ($p < 0.05$) lower survival than the control groups (Table 1; Fig. 1). Supplementary data with replicate tank 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 x 10^9 copies (range 1.09-1.31 ×
369 10^9) WSSV copies / 0.1 gram ; NAF (n = 3), av. 1.40 × 10^9 (range 1.37 - 1.44 × 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.
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4. Discussion

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

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

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

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

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

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

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

Acknowledgement

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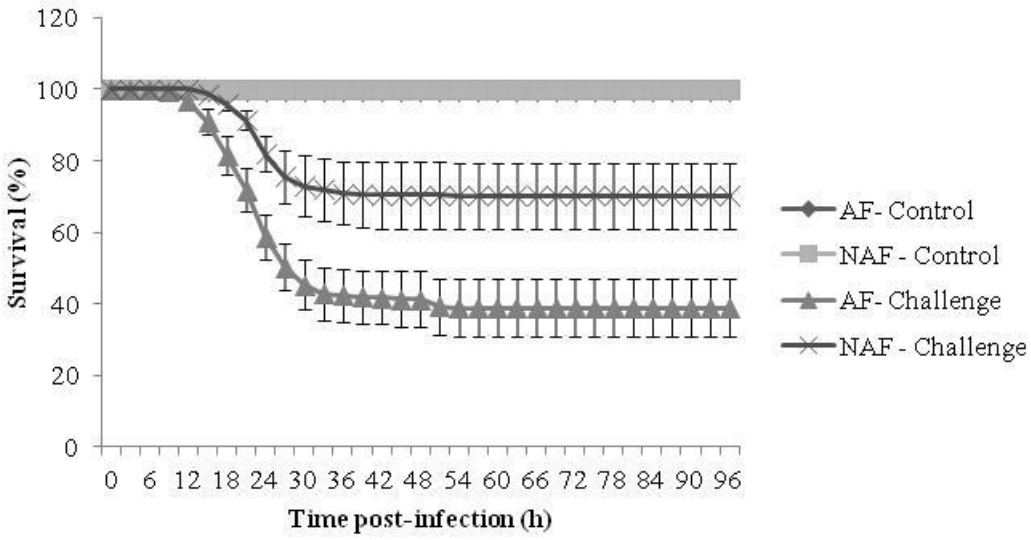
550 **Figures**



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.

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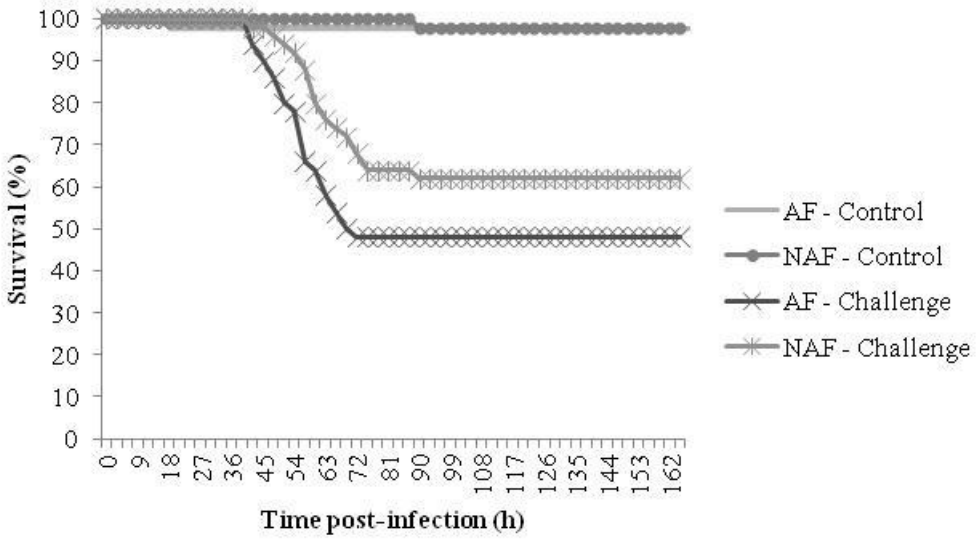

555

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.

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Tables

Table 1. Summary of the statistics following analysis of the mortalities by Mantel-Cox log rank tests at the end 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	

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

Table 2: Summary of the statistics following analysis of the mortalities by Mantel-Cox log rank tests 168 hours 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	

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

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

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

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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

Supplementary information

S1. Water quality and maintenance of the broodstock

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

S2. Water quality and maintenance of the nauplii

Water temperature, dissolved oxygen, pH, ammonia, nitrite and alkalinity were 28.5 ± 0.7 ºC, 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 $CaCO_3$ respectively. Salinity was gradually adjusted from 30.0 ppt to 15 ppt from postlarvae 5 at a rate of 1 ppt/day. Approximately 30% of the water volume was exchanged daily when the animals reached the postlarvae stage.

S3. Mandatory health checks following the receipt of shrimp

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

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

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

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

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

S5. *Vp*$_{AHPND}$ pre-challenge tests

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

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

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

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

S7. WSSV pre-challenge tests

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

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