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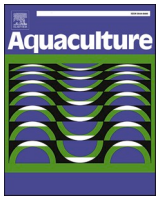

Genetic parameters of resistance to acute hepatopancreatic necrosis disease (AHPND) caused by *Vibrio parahaemolyticus* and their genetic correlations with growth traits in an Ecuadorian *Penaeus vannamei* population

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ABSTRACT

Infections with strains of *Vibrio parahaemolyticus* causing acute hepatopancreatic necrosis disease (VP_{AHPND}) in *Penaeus vannamei* have not yet caused high levels of mortality in Ecuador like those reported in Asia; however, target animals resistant to VP_{AHPND} are a goal for industrial farmers. The implementation of breeding programs to produce fast-growing and disease-resistant shrimp would be a key to addressing the expected situation. The main objective of this study was to estimate heritabilities and genetic correlations for VP_{AHPND} resistance and growth traits in an Ecuadorian population cultured under industrial conditions. A total of 3345 animals from 155 sibling families of the PMG-BIOGEMAR© genetic breeding program were individually tagged, measured for initial length and weight (only for Test-1), and tested for VP_{AHPND} in two trials: Test-1 at a concentration of 2×10^5 CFU/ml and infection by immersion and Test-2 at a concentration of 2×10^8 CFU/g and by oral infection. After 72 h, all shrimp were analysed for final weight and length, survival, and infection levels by Ct values (qPCR) of the *pirA* and *pirB* genes. Genetic parameters of growth and resistance traits were obtained using two statistical models: Linear and Threshold models. Heritabilities were medium (0.16–0.31) for growth and low (<0.09) for infection-level traits, by both methods and in both tests. In the case of survival, the heritability was low using the Linear model (0.04), and medium (0.22 and 0.26) with the Threshold model, in Test-1 and Test-2, respectively. However, the genetic correlations found between growth and survival traits were high and positive (>0.55) with both methodologies in Test-1 and low-medium and positive in Test-2 using Threshold model. The results suggest that genetic selection for growth in *P. vannamei* has a positive effect on resistance to AHPND. Furthermore, the genetic selection for growth over time in this Ecuadorian population may have induced possible resistance or tolerance to the disease.

1. Introduction

Penaeus vannamei is the most important animal species for the global aquaculture industry in terms of production (FAO, 2022). In recent years, acute hepatopancreatic necrosis disease (AHPND) has become one of the major causes of significant economic losses in this species

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(Dhar et al., 2019; Campos-Montes et al., 2020), especially in Asia-Pacific countries (Tran et al., 2013; Joshi et al., 2014; Lee et al., 2015; Lyu et al., 2020). The pathogenic agent causing AHPND has been identified as *Vibrio parahaemolyticus* (Tran et al., 2013; Han et al., 2015a; Aranguren et al., 2020), which contains the binary toxin genes *pirA* and *pirB*. Molecular diagnostic methods, such as quantitative PCR (qPCR) (Han et al., 2015a, 2015b), accompanied by an accurate histopathological examination (Lightner and Flegel, 2012; Joshi et al., 2014; Han et al., 2015a; Aranguren et al., 2020) could determine the bacterial pathogenicity in shrimp.

Over the past decade, the main method of disease eradication in the shrimp industry, particularly in Asia, has been the use of Specific Pathogen-Free (SPF) animals (Moss et al., 2012). These animals are free of one or more specific pathogens that can be easily diagnosed, physically excluded from farms, and cause a huge economic loss (Lightner et al., 2009; Moss et al., 2012). Nevertheless, this proposal does not address the root of the industrial problem as SPFs are known to be extremely susceptible to disease, even being utilized as positive controls in academic pathogen challenge tests (Aranguren et al., 2020). *Vibrio* spp., are ubiquitous microorganisms, which are part of the normal gut flora of *P. vannamei*, so they cannot be physically excluded from farm facilities.

Despite the high prevalence of pathogens in industrial estuaries, *P. vannamei* production has not ceased in Ecuador, the world's leading producer and exporter of this species, reaching more than one million ton in 2022 (CNA, 2022). According to Aranguren et al. (2020), *Vibrio parahaemolyticus* does not cause mass mortalities in shrimp farming in South America, such as those reported in Asia, although AHPND has been reported there throughout history (Nunan et al., 2014; Jun et al., 2016; Restrepo et al., 2018; Aranguren et al., 2020; Peña et al., 2020). To understand this phenomenon, it is important to consider the *modus operandi* of the industry in Latin America, particularly in Ecuador, where animals coexist with pathogens. Evidence of resistance to AHPND in Ecuadorian shrimp (Castillo-Juárez et al., 2018; Aranguren et al., 2020; Mai et al., 2021) and a new chronic phase of the disease (Aranguren et al., 2020) have been reported. Therefore, it is important to consider the origin of lines in AHPND challenge test results, not only because of the possibility of a natural tolerance selection but also because the immune system in shrimp is non-specific, thus resistance to one pathogen may confer protection against another (Cock et al., 2009; Castillo-Juárez et al., 2018; Campos-Montes et al., 2020).

The possibility of developing disease-resistant shrimp to AHPND caused by the pathogen *Vibrio parahaemolyticus* (VP_{AHPND}), through genetic improvement, is seen as a key approach to reducing economic losses in the Pacific white shrimp farming industry (Lyu et al., 2020). The development of broodstock resistant to the major economic diseases has been investigated, particularly for the Taura syndrome (TSV) and white spot syndrome viruses (WSSV) (Argue et al., 2002; Kong et al., 2003; Jiang et al., 2004; Gitterle et al., 2005; Cuéllar-Anjel et al., 2011). It should be noted that the heritability reported for WSSV resistance is low (< 0.1), and a high negative genetic correlation was found between growth and WSSV resistance, making it a challenging trait to include in breeding programs and rarely selected for by the industry (Gitterle et al., 2005, 2006a, 2006b).

Regarding AHPND resistance, several studies have shown low heritabilities in *P. vannamei*, from 0.06 to 0.18 (Castillo-Juárez et al., 2018; Campos-Montes et al., 2020; Lyu et al., 2020). Furthermore, there is no information on genetic correlation estimates between AHPND resistance and other economically important traits such as growth.

The main objective of this study was to estimate the additive genetic variation associated with resistance to VP_{AHPND} and, for the first time, its genetic correlations with growth traits, in an Ecuadorian shrimp population selected for growth (PMG-BIOGEMAR©).

2. Materials and methods

2.1. Genetic line

The animals used in this study belonged to the fifth generation (G_5) of the breeding program developed by the BIOGEMAR company of ALMAR Group (PMG-BIOGEMAR©). This project was carried out between the University of Las Palmas de Gran Canaria (ULPGC) in Spain and the ALMAR Group in Ecuador. All generations of PMG-BIOGEMAR© were evaluated by BLUP (Best Linear Unbiased Prediction) methodology, and breeders were selected based on their maximum EBVs (Estimated Breeding Values) for weight at harvest size and mated according to the Optimal Contribution Selection (OCS) method. The objective of OCS method is to achieve the maximum genetic gain while controlling the inbreeding (Meuwissen, 1997; Grundy et al., 1998; Woolliams et al., 2015).

The genetic selection procedures and mating practices employed for the preceding generations of the PMG-BIOGEMAR© (Shin et al., 2023; Martínez Soler et al., 2024) were implemented in the fourth generation (G_4) to obtain the G_5, which was used in the present experiment. In accordance with the established protocol, each selected male was mated with one or two selected females by artificial insemination over a period of six consecutive days at the genetic nucleus. These days allow for the maximum number of mature selected females to be obtained. The maturation of the selected females, the availability of the selected males and the successful spawning and development of the nauplii V resulted in a total of 159 families, comprising 129 males and 159 females. After artificial insemination procedure, each fertilised female was transferred to an individual tank for spawning. Once the nauplii V stage was reached, the same number of offspring (G_5) per female (family) were mixed and cultured in six larval tanks (one per mating day) from the nauplii up to postlarval stage 12 (PL-12), under industrial conditions at BIOGEMAR S.A. (Santa Elena, Ecuador). The seeding of the same number of descendants per female in the larval tanks minimise the variance of family size and maximise the effective population size in the breeding program (Spiess, 1989). In all the generations of the present breeding program, at the PL-12 stage, all families from all mating days are mixed and a representation is transferred to on-growing industrial estuaries at PRODUMAR S.A. Another representation is kept at the genetic nucleus in BIOGEMAR S.A., where shrimp are produced under highly controlled conditions to form part of the next elite broodstock. The EBVs for weight at harvest attained to select the elite breeders at the genetic nucleus are calculated using the evaluation of their siblings at industrial estuaries at PRODUMAR S.A., at the same age.

2.2. Pre-challenge management

At ~0.5 g of weight, 40 days after hatching (DAH), a random sample of ~3900 animals (G_5) were transferred from the genetic nucleus of BIOGEMAR S.A. to the National Centre of Aquaculture and Marine Research (CENAIM-ESPOL, Santa Elena, Ecuador), where two VP_{AHPND} challenge tests were carried out. At CENAIM-ESPOL, all families were acclimated together in two fiberglass tanks for eight days. Before the challenge tests, each shrimp was individually tagged with Visible Implant Elastomer (VIE) Tags NMT®, according to manufacturer's protocol, in different abdominal segments with a unique code designed per animal using a combination of colours and positions. For the first experiment (Test-1), a random sample of 2400 shrimps (~0.80 g) were measured for weight and length (initial weight, WI; initial length, LI) and randomly distributed into forty-eight rectangular glass tanks of forty litters each, with fifty animals per tank. For the second experiment (Test-2), performed at 98 DAH, it was not possible to measure WI and LI. In this case, a random sample of 945 shrimps (~3.5 g) were distributed from the original acclimatation tanks into forty-five tanks with twenty-one animals per tank. In both experimental tests, after tagging and distribution into tanks, a recovery period of three days was required

before infection to avoid stress. To simulate the industrial growing conditions of the company, during both tests, the shrimps were fed ad libitum every two hours with the main dry feeds used in the BIOGEMAR company, according to the mean size of the animals which is different in both tests. Seawater (35 ppt) was maintained at a constant temperature of around 30 ± 0.1 °C, with complete daily changes until the first day of infection.

2.3. Challenge tests

The challenge tests were carried out against AHPND caused by *V. parahaemolyticus* (strain BA94C2), following the protocol previously described by Domínguez-Borbor et al. (2019), with slight modification. Briefly, *V. parahaemolyticus* was plated on trypticase soy agar (2 % NaCl TSA) and allowed to grow overnight. Colonies were then subjected to PCR analysis to confirm the presence of the *pirA* and *pirB* genes using AP4 primers (Dangtip et al., 2015). Once the presence of both genes was confirmed, eight colonies were transferred to 1000 mL of 2 % NaCl TSA and incubated for five hours at 30 ± 0.5 °C with constant movement. Once the inoculum reached a concentration of 2×10^8 CFU/ml (optical density at 620 nm = 0.33), it was centrifuged at 3000*g* for 10 min, at 25 °C. The supernatants were discarded, and the bacterial pellets were resuspended in saline solution (2 % NaCl).

- Test-1 by immersion.

The infection was performed by immersion, inoculating 40 ml of the *V. parahaemolyticus* suspension in forty-five tanks, at a final concentration of 2×10^5 CFU/ml (2250 shrimps challenged). Three tanks were used as the negative control (150 shrimps). No water changes were made for the first 24 h after bacterial inoculation. After this time, 50 % of the seawater was changed daily.

- Test-2 by feeding.

Due to the low mortality achieved in Test-1, a second challenge test (Test-2) was carried out with the same population (different individuals) but using a higher final bacterial concentration and a different methodology, by incorporating the pathogen into the feed. For this approach, shrimp were fed with one dose of 2.5 % of the total biomass per tank with a bacterial concentration of 2×10^8 CFU/g. The animals were not fed for twelve hours before the infection. The seawater in the tanks was changed daily by 50 % during the entire experiment. In this case, 882 animals were tested (forty-two tanks), and three tanks were used as a negative control (63 shrimps).

2.4. Analysed traits

In both challenge tests, mortality was monitored every 2 h for 72 h after infection. Non-survivors were identified by their VIE tag, measured for growth-related traits (named as growth in the manuscript), and recorded in the database as 1. After 72 h of challenge, survivors were also identified by their VIE tag, recorded in the database as 2, and their final weight (WF) and length (LF) were measured. The sixth segment containing gut was removed from each animal and stored in RNAlater for DNA extraction, parental assignment, and qPCR analysis to determine the infection-level (Ct values for *pirA* and *pirB*). Six animals per tank (control and treatment) were collected to confirm the histological lesions caused by VP_{AHPND} in the hepatopancreas. All samples collected were sent to the SABE (Servicio de Acuicultura y Biotecnología de Alta Especialización, ULPGC laboratories, Gran Canaria, Spain), where the appropriate analyses were performed.

2.4.1. DNA extraction and family assignment

DNA was extracted from the sixth segment (including gut) of all animals using the BioSprint 96® device and the DNA Blood Kit (QIAGEN™), following the manufacturer's instructions. After DNA extraction, quantity and quality were measured using a NanoDrop-800™ v.3.7 spectrophotometer (Thermo Fisher Scientific). DNA samples were normalized to 30 ng/μl for sequencing and to 50 ng/μl for qPCR assay using TECAN robot (Tecan Schweiz AG. Switzerland) and Freedom Evoware® Standard v2.5 software. An external service, the Center of Aquaculture Technologies (California, USA.), was hired to perform genotyping and family assignment by single nucleotide polymorphism analysis (SNP chip array, AQUAARRAY LD™). The genealogical matrix containing all the generational information (G_0-G_5) was established by the ULPGC staff associated with PMG-BIOGEMAR©.

2.4.2. Quantitative PCR assay

iQ™ multiplex powermix (BIO-RAD®) was used to perform real-time quantitative PCR (qPCR) assay using the CFX96® Real-Time System equipment (BIO-RAD®) to detect the *pirA* and *pirB* toxin genes in shrimp after experimental VP_{AHPND} challenges. In terms of primers and probes, the following oligonucleotide sequences 5′ – 3′ were used: *pirA Forward* - TTG GAC TGT CGA ACC AAA CG / *Reverse* - GCA CCC CAT TGG TAT TGA ATG, and *pirB Forward* - ATG CAA ACC AAG ATA ACG TGT ATG / *Reverse* - ATA AGC ATA TGT TCA AAG CCG TG for primers. As probes, *pirA*-PROBE Hex-AGA CAG CAA ACA TAC ACC TAT CAT CCC GGA-MGC-Eclipse and *pirB*-PROBE 6-Fam-AAG TGA TGG GTG CTC GTA GTT GGT G-MGB-Eclipse were used. *PirA* primers and probes were obtained from OIE (2019). On the other hand, *pirB* primers and probes were designed by ULPGC and PMG-BIOGEMAR© using the reference of the known sequences (GenBank accession number CP034308.1). Primers were added at 300 nM and probes at 200 nM. Amplification was performed with an initiation denaturation at 95 °C (3 min) followed by 45 cycles of 10 s at 95 °C, and 60 s at 55 °C. The Ct (threshold cycles) values for *pirA* and *pirB* were obtained from the amplification plot using the baseline-threshold method, with the same threshold applied to all plates for comparison and recording by CFX Manager Software (Bio-Rad).

2.4.3. Histopathological analysis

The experimental shrimp were fixed by intrahepatopancreatic injection with Davidson's fixative to obtain a correct preservation. The cephalothorax was longitudinally bisected, immersed in the fixative, dehydrated (48 h) and embedded in paraffin. Sections of 4 μm (Leica Reichert Jung AUTOCUT 2055) were stained with haematoxylin and eosin (Martoja and Martoja-Pierson, 1970) for optical examination. Subsequently, the slides were scanned in a MoticEasyScan Pro digital scanner (Motic, Xiamen, China), operated by the Motic DS Assistant software (Motic VM V1 Viewer 2.0), and evaluated considering the histopathological lesions for AHPND proposed by WOAH, 2022.

2.5. Statistical analyses

Descriptive statistical analysis of the post-test data from both experiments was performed using SPSS (v25) software. To check whether the distribution of the families in the experimental tanks was random, the interaction between both factors, family and tank, was verified using SPSS (V25) software, implementing the following log-linear model:

$$f-_{ij} = \mu + \alpha_i + \beta_i + \alpha\beta_{ij},$$

where f_{-ij} is the expected frequency in row i, column j of the two-way contingency table, μ is the mean of the logarithms of the expected frequencies, α_i is the effect of category i of tank fixed factor, β_j is the effect of category j of the family factor, and $\alpha\beta_{ij}$ is the interaction term expressing the dependence of category i of the tank factor on category j of the family factor.

The evolution of survival of each family, with at least one member dead, during the 72 h of the VP_{AHPND} challenge tests, was compared using the Kaplan-Meier method of survival analysis performed by SPSS

(V25).

Regarding Test-1, heritabilities and genetic correlations for survival, growth and infection-level traits were obtained using Linear model by Restricted Maximum Likelihood method (REML) implemented in VCE software (v6.0) (Neumaier and Groeneveld, 1998; Groeneveld et al., 2010) and using Threshold mixed model with GIBBS2F90 software from BLUPF90 (Misztal et al., 2022). Survival was considered as a continuous trait when analysed by Linear model and as a categorical trait when analysed by Threshold model. For the Threshold model, estimates were obtained via Gibbs sampling using a chain of 100,000 iterations, with a burn-in period of 20,000 and a thinning interval of 80 cycles. All the fixed factors involved in the process were considered in both analyses with the following mixed model:

$$Y = Xb + Za + e \quad (1)$$

where Y is a vector of the analysed traits (survival, growth and infection-level traits); b is a vector of fixed effects (nauplii seeding day, the age of the animals, and the larval tank); a is a vector of random additive direct genetic effects; e is a vector of random residual effects, and X and Z are matrices with the corresponding effects in vectors b and a, respectively. For the Linear model, multi-trait analysis was implemented using VCE software and associated programs such as VCE-analysis (v 1.0; for processing VCE output files and automatically building the genetic parameter matrix), while for the Threshold model, bi-trait analysis for all traits was implemented using GIBBS2F90 software.

For Test-2, all traits were also analysed by Linear model (VCE software), however, it was not possible to attain convergence for the survival trait, as a continuous trait, and its genetic correlations. Therefore, the estimation of genetic parameters was only analysed by Threshold model using GIBBS2F90 (bivariate/two-trait model) and considering it as a categorical trait. The same number of cycles as in Test-1 were used for Test-2. Geweke's diagnostic by R software was implemented to check the convergence for heritabilities and genetic correlations (results not shown). The mixed model (1) was also used in Test-2.

The genetic correlation between survival from both experiments (Test-1 and Test-2) was studied using a bi-trait Threshold model (GIBBS2F90) to assess the possibility of analysing data from both experiments together.

The correlated response was calculated on the desired trait (X) through the secondary trait (Y) according to the formula:

$$CRx/Rx = i_y\, h_y\, r_A / i_x\, h_x, \quad (2)$$

where CRx is the correlated response and Rx is the directed response of x trait, i is the intensity of the selection, h is the square root of heritability for a given trait (x and y traits), and r_A is the genetic correlation between the two traits (Falconer and Mackay, 1996).

3. Results

3.1. Challenge tests

The distribution of families between tanks in both challenge tests showed no significant association ($P > 0.05$) according to the result of the log-linear test. These results allow us to confirm that the distribution of families between thanks was totally random.

In Test-1, after 72 h of infection, 2210 animals were collected in the challenged tanks, comprising 2090 survivors and 120 deaths (94.57 % survival), while all animals in the control tanks were collected as survivors. In Test-2, 860 shrimp were collected, 405 as survivors and 455 as deaths (47.21 % survival), and all survived in the control tanks. Due to the occurrence of cannibalism, 40 animals in Test-1 and 22 animals in Test-2 failed to appear or it was not possible to read their VIE at the end of the challenge tests. Hence, they were not considered in the percentage of survival. The Kaplan-Meier plots providing information on survival over time per family (with at least one dead member) are shown in Figs. 1 and 2, showing how a subset of families perish rapidly while others tolerate and even resist infection.

3.2. Genotyping and family assignment

For Test-1, a total of 1811 challenged shrimp (81.95 %) were successfully assigned to both parents. These offspring arose from 155 sibling families, with an average of 12 offspring per family. For Test-2, a total of 652 shrimps (75.81 %) from 147 families were assigned, with an average of 5 offspring per family.

3.3. Histopathological hepatopancreas analysis

After 72 h of infection, the hepatopancreas of shrimp from challenged tanks were macroscopically pale. Microscopically, there were clear histological differences between animals from control and VP_{AHPND} -treated tanks. In Fig. 3a, samples from control shrimp display a healthy hepatopancreas structure with E cells (embryonic) surrounding the organ and showing mitotic activity. Gradually, as the E cells approach the central zone, they differentiate equally into B (secretory vacuoles), R (lipid droplets), and F (enzymatic) cells, which conform to well-developed tubules. Thus, the lumen of the tubules appears highly dilated with the characteristic star shape. In contrast, challenged hepatopancreas samples showed histopathological changes associated with AHPND in both chronic and acute states. Fig. 3b, c, and d of hepatopancreases from challenged shrimps showed typical AHPND lesions, such as hyperemia (Fig. 3b), multifocal granulomatosis, inflammatory response (advanced stage; Fig. 3c), and global necrosis and disorganization (acute phase; Fig. 3d). In general, challenged hepatopancreas samples (Fig. 3b and d) presented a decrease in functional hepatopancreatic tubules, an increase in basophilia, and severe sloughing of the epithelial cells (Fig. 3d), also associated with an acute phase of AHPND.

3.4. Phenotyping

Phenotypic results for growth and infection-level traits in Test-1 are presented in Table 1. During the three-day recovery period and the 72-h challenge test, the animals grew an average of 0.73 cm (15.5 %) and 0.40 g (20.4 %). Regarding Test-2, LI and WI were not measured, but

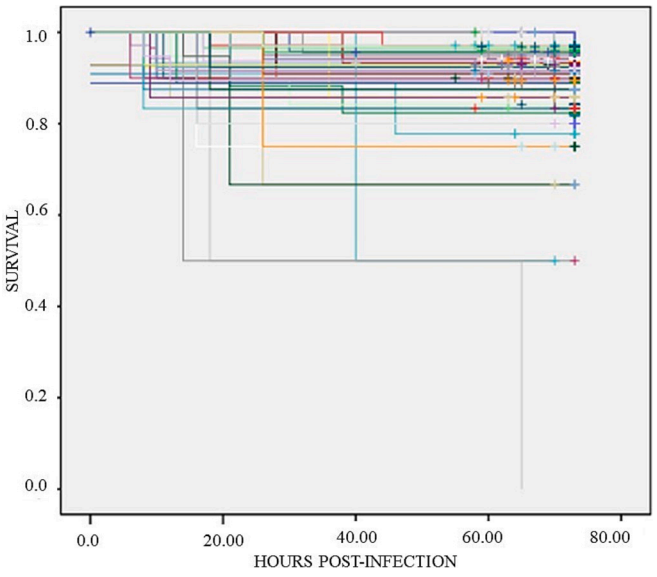

Fig. 1. Kaplan-Meier survival plot for *Penaeus vannamei* families, with at least one dead member, from the PMG-BIOGEMAR© breeding program in the VP_{AHPND} challenge Test-1. Each colour represents a different family.

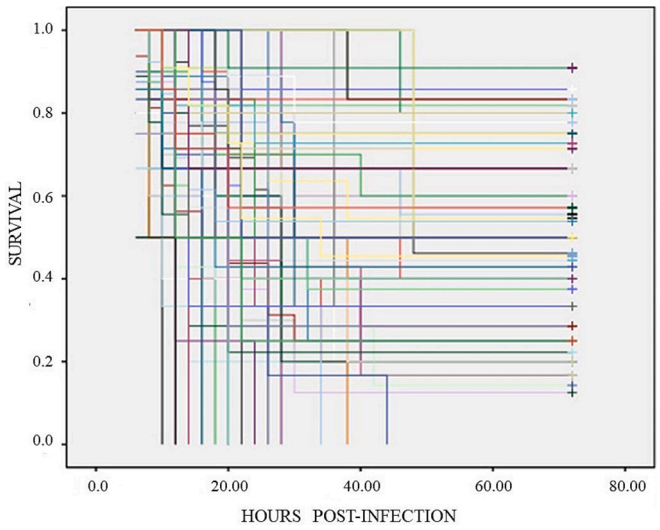

Fig. 2. Kaplan-Meier survival plot for *Penaeus vannamei* families with at least one dead member from the PMG-BIOGEMAR© breeding program in the VP_{AHPND} challenge Test-2. Each colour represents a different family.

WF, LF, and the level of infection of the 860 animals tested are shown in Table 2. Values are presented as mean ± standard error (SE); minimum (Min), maximum (Max) and standard deviation (SD) (Tables 1 and 2). Concerning infection levels, the animals presented a mean Ct value of 36.59 ± 0.08 for *pirA* and 37.38 ± 0.10 for *pirB* genes, with a minimum of 8.09 for *pirA* and 11.62 for *pirB* in Test-1 and a mean of 34.00 ± 0.18 for *pirB* and 33.55 ± 0.18 for *pirA*, with a minimum of 15.07 for *pirB* and 13.79 for *pirA* in Test-2.

3.5. Heritabilities and genetic correlations

Heritabilities and genetic correlations estimates for growth, survival, and infection-level traits in *P. vannamei* under the VP_{AHPND} challenge test by immersion (Test-1), obtained by Linear model, are shown in Table 3; the results obtained by Threshold model are presented in Table 4. It should be noted that heritabilities for growth traits, are very similar, medium (0.15–0.25), with both methods, and its genetic correlations are very high (> 0.93) (Tables 3 and 4). For survival, the results are slightly different between both methods. In the case of Linear model, the heritability for survival is low, 0.04 ± 0.03, and it is important to highlight the positive and very high genetic correlations between

Table 1
Phenotypic results for growth and infection-level traits (mean ± standard error, minimum, maximum, and standard deviation) for *Penaeus vannamei* from VP_{AHPND} Test-1 (n = 2210) with infection by immersion. Measurements are in centimeters for length and grams for weight.

	Mean ± SE	Min	Max	SD	CV
LI	4.72 ± 0.01	2.20	7	0.59	12.53
LF	5.44 ± 0.01	1.50	8.1	0.65	11.88
WI	0.81 ± 0.01	0.18	2.1	0.26	32.33
WF	1.20 ± 0.01	0.18	3.33	0.37	30.90
Ct *PIRA*	36.59 ± 0.08	8.09	40.26	4.53	12.39
Ct *PIRB*	37.38 ± 0.10	11.62	40	3.81	10.19

Abbreviations: **LI:** Initial length; **LF**: Final length; **WI**: Initial weight; **WF:** Final weight; **SE**: Standard error; **Min:** Minimum; **Max:** Maximum; **SD:** Standard deviation; **CV**: Coefficient of variation.

Table 2
Phenotypic results for growth and infection-level traits (mean ± standard error, minimum, maximum, and standard deviation) for *Penaeus vannamei* from VP_{AHPND} Test-2 (n = 860) with infection by feeding. Measurements are in centimeters for length and grams for weight.

	Mean ± SE	Min	Max	SD	CV
LF	8.66 ± 0.03	2.30	10.50	0.91	10.51
WF	3.91 ± 0.04	0.60	9.00	1.22	31.47
Ct *PIRB*	34.00 ± 0.18	15.07	40	5.15	15.15
Ct *PIRA*	33.55 ± 0.18	13.79	40	5.17	15.40

Abbreviations: **LF**: Final length; **WF:** Final weight; **SE**: Standard error; **MIN:** Minimum; **MAX:** Maximum; **SD:** Standard deviation; **CV:** Coefficient of variation.

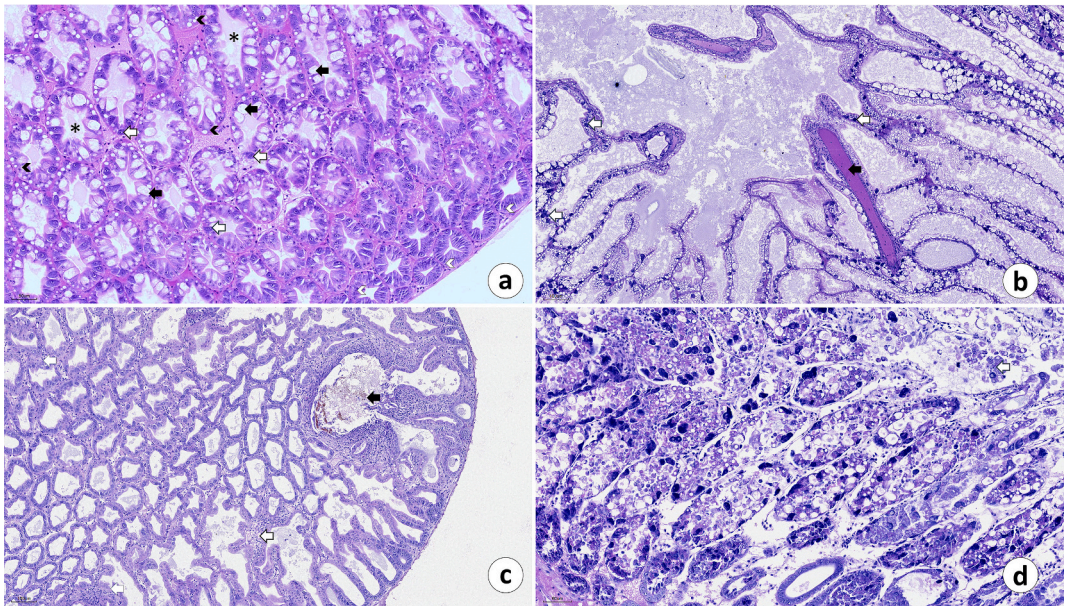

Fig. 3. Photomicrograph of a longitudinal section of *Penaeus vannamei* hepatopancreas from control tanks (a) and with different levels (Ct values) (b, c, and d) of VP_{AHPND} infection. a: a large number of B cells (black arrow), F cells (white arrow), R cells (black arrowhead) and E cells with mitotic activity (white arrowhead), and well-developed and dilated tubules (asterisks). b: early stage of infection with hyperemia (black arrow) and basophilic cells (white arrow). c: advanced stage of AHPND with granulomas (black arrow) and intratubular hemocytic inflammation (white arrow). d: acute phase with almost complete necrosis, sloughing, and multifocal melanisation. Scale bar as follows: (a) 50 μm, (b) 100 μm, (c) 100 μm, and (c) 60 μm.

Table 3
Heritabilities (diagonal), phenotypic correlations (below diagonal) and genetic correlations (above diagonal) obtained by Linear model (multi-trait) for growth, survival, and infection-level traits in *Penaeus vannamei* under *V. parahaemolyticus* infection by immersion (2×10^5 CFU/ml) (Test-1) (mean ± standard error).

	LI	LF	WI	WF	SUR	Ct *PIRA*	Ct *PIRB*
LI	**0.15 ± 0.04**	0.93 ± 0.04	0.97 ± 0.02	0.96 ± 0.04	0.95 ± 0.22	0.78 ± 0.31	0.46 ± 0.28
LF	0.72	**0.23 ± 0.04**	0.94 ± 0.03	0.99 ± 0.01	0.88 ± 0.16	0.43 ± 0.58	0.21 ± 0.25
WI	0.85	0.76	**0.22 ± 0.05**	0.95 ± 0.03	0.75 ± 0.47	0.45 ± 0.34	0.28 ± 0.26
WF	0.73	0.87	0.83	**0.23 ± 0.04**	0.99 ± 0.03	0.30 ± 0.48	0.21 ± 0.24
SUR	0.03	0.06	0.04	0.04	**0.04 ± 0.03**	−0.56 ± 0.80	−0.46 ± 0.52
Ct *PIRA*	0.09	0.05	0.06	0.08	0.1	**0.01 ± 0.01**	1.00 ± 0.00
Ct *PIRB*	0.11	0.08	0.08	0.09	0.11	0.71	**0.03 ± 0.02**

Abbreviations: **LI:** Initial length; **LF**: Final length; **WI**: Initial weight; **WF:** Final weight; **SUR:** Survival.

Table 4
Heritabilities (diagonal) and genetic correlations (above the diagonal) obtained by Threshold model (bi-trait) for growth, survival, and infection-level traits in *Penaeus vannamei* under *V. parahaemolyticus* infection by immersion (2×10^5 CFU/ml) (Test-1) (mean ± standard error).

	LI	LF	WI	WF	SUR	Ct *PIRA*	Ct *PIRB*
LI	**0.16 ± 0.00**	0.96 ± 0.02	0.97 ± 0.00	0.95 ± 0.01	0.72 ± 0.08	0.42 ± 0.11	0.26 ± 0.04
LF		**0.22 ± 0.00**	0.95 ± 0.00	0.99 ± 0.00	0.55 ± 0.05	0.22 ± 0.14	0.04 ± 0.04
WI			**0.24 ± 0.00**	0.95 ± 0.00	0.57 ± 0.10	0.29 ± 0.12	0.03 ± 0.02
WF				**0.25 ± 0.00**	0.55 ± 0.03	0.05 ± 0.07	0.06 ± 0.03
SUR					**0.22 ± 0.03**	−0.00 ± 0.18	−0.02 ± 0.10
Ct *PIRA*						**0.02 ± 0.00**	0.95 ± 0.03
Ct *PIRB*							**0.05 ± 0.00**

Abbreviations: **LI:** Initial length; **LF**: Final length; **WI**: Initial weight; **WF:** Final weight; **SUR:** Survival.

survival and the growth traits studied (from 0.75 to 0.99). Using Threshold model and considering survival a categorical trait, the heritability is higher: 0.22 ± 0.03, and the genetic correlation with growth traits continues to be positive and high (from 0.55 to 0.72). Heritability for the infection-level traits is very low (0.01–0.05) using both methodologies, and their genetic correlations with growth traits are low to high (0.03–0.78), although, these correlations are not very consistent due to their high standard errors (Tables 3 and 4).

The heritabilities for LF and WF in Test-2 are medium (0.24 ± 0.01 and 0.31 ± 0.00, respectively) and their genetic correlation is very high (0.98) (Table 5). In this case, the heritability for survival is slightly higher, 0.26 ± 0.01, but similar to that obtained by Threshold model in Test-1. Convergence was not achieved for genetic correlations between survival, growth, and infection-level traits using the Linear model or between survival and WF using Threshold model in Test-2. The results obtained by Threshold model are shown in Table 5. Genetic correlation between survival and LF is positive, and between infection-level and growth traits is high and positive (> 0.40).

According to the merit calculation of indirect selection (Eq. 2), selecting AHPND-resistant animals, infected by immersion (Test-1) and analysed by Linear model, through WI and LI improves efficiency by 75 % and 84 %, respectively. However, when the results are analysed using the Threshold model, the efficiency of the direct selection (survival) is 37 % higher than indirect (through WI and LI) for Test-1. In that case, growth traits are the secondary traits while resistance to AHPND is the desire trait.

The possibility of combining the two experiments to improve estimation of the genetic parameters of resistance to VP_{AHPND} was also evaluated by Threshold model, and the genetic correlation between both survivals was 0.30.

Table 5
Heritabilities (diagonal) and genetic correlations (above diagonal) obtained by Threshold model (bi-trait) for growth, survival, and infection-level traits in *Penaeus vannamei* under *V. parahaemolyticus* infection by feeding (2×10^8 CFU/g) (Test-2).

	LF	WF	SUr	Ct *PIRB*	Ct *PIRA*
LF	**0.24 ± 0.01**	0.98 ± 0.01	0.18 ± 0.05	0.57 ± 0.04	0.67 ± 0.04
WF		**0.31 ± 0.00**	NC	0.44 ± 0.06	0.40 ± 0.05
SUr			**0.26 ± 0.01**	0.24 ± 0.11	0.42 ± 0.05
Ct *PIRB*				**0.04 ± 0.00**	0.88 ± 0.02
Ct *PIRA*					**0.09 ± 0.01**

Abbreviations: **LI:** Initial length; **LF**: Final length; **WI**: Initial weight; **WF:** Final weight; **SUR:** Survival.

4. Discussion

This study allowed us to estimate the heritability of resistance to $VP_{AHPND,}$ measured by survival and infection-level and, for the first time, its genetic correlation with growth traits that are the most selected for in industrial *P. vannamei* genetic breeding programs.

4.1. *Is the current Ecuadorian Penaeus vannamei line resistant or tolerant to VP_{AHPND}?*

The density of bacterial cells in the water of the challenge tanks in Test-1 is consistent with the density used in other experiments carried out by immersion in the same species and at similar ages (Han et al., 2015a; Castillo-Juárez et al., 2018; Aranguren et al., 2020). Furthermore, this is the density observed in industrial estuaries in Ecuador during an outbreak of *V. parahaemolyticus*, which should be considered to directly apply the results obtained in this work to the industry in the country.

It is difficult to find VP_{AHPND} challenge tests in the bibliography that do not immediately show high mortality, perhaps because populations from Asia are extremely susceptible. However, Aranguren et al. (2020) reported a *P. vannamei* line, imported from Ecuador, which exhibited 72.1 % survival after a VP_{AHPND} challenge immersion test (1×10^5 CFU/ml) with a virulent strain. Other authors also previously observed an increase in the survival of genetic lines coming from Ecuador compared with other origins, in terms of resistance to AHPND (Castillo-Juárez et al., 2018).

The acute histological changes observed in the hepatopancreas of the challenged shrimp, such as sloughing and reduction of all epithelial

types, have not been reported in any other disease and are considered representative diagnostic lesions of AHPND (Pantoja and Lightner, 2014). Non-specific lesions such as the inter-tubular inflammation and melanisation found, have also been reported in association with AHPND (Pantoja and Lightner, 2014; Manan et al., 2015). The granulomatous reaction and epithelial necrosis observed here, only in certain regions of the organ, are associated with a chronic phase of this disease, as described initially by Aranguren et al. (2020). These authors also detected a percentage of shrimp with no histopathological lesions after infection, indicating possible tolerance or resistance to the disease. All these histopathological changes associated with AHPND in the hepatopancreas of shrimp confirm the presence of both toxins causing the disease, despite the almost complete absence of mortality in Test-1.

Han et al. (2015a) reported that the positive Ct value for *pirA* causing AHPND in *P. vannamei* is roughly limited to 36.67 (5.8×10^5 copies per mg of tissue), which is close to the mean value obtained in Test-1 for this toxin. However, as these binary toxins are normally restricted to the hepatopancreas and intestine, removing DNA from the sixth segment, which contains muscle as well as intestine, may have slightly increased the Ct values in the present experiment.

The low mortality (~5 %) attained in Test-1 may have affected heritability estimates of survival, so it was necessary to perform a second VP_{AHPND} challenge test, using the same population but at a higher level of infection, to achieve around 50 % of mortality. Joshi et al. (2014) reported that the concentration of pathogenic bacteria could be critical in causing mortality in shrimp. Hence, to obtain a more accurate AHPND survival heritability estimation an increase in the bacterial dose of up to 10^8 was applied. In addition, the method of infection had to be changed to oral (Gitterle et al., 2006a), which affects the survival rate of *P. vannamei* (Soto and Lotz, 2001; Gitterle et al., 2005). This last option would not imply an exact reproducibility of the conditions under which the animal can become infected in Ecuadorian industrial estuaries since they do not consider important external physical barriers for shrimp, such as the exoskeleton (Gitterle et al., 2006a).

It appears that there is no phenotypic association between growth and resistance or tolerance against AHPND, given the weak phenotypic correlation observed in Test-1 for both survival and level of infection traits. However, it is not possible to draw a similar conclusion in Test-2, as the growth traits were measured only after infection, which may be highly affected by the disease in that 50 % mortality test.

4.2. Is resistance to VP_{AHPND} a heritable trait in this *P. vannamei* population from Ecuador?

Heritability is one of the most important parameters in breeding programs for any character, expressing the proportion of total variance that is attributable to the effects of genes (Falconer and Mackay, 1996). Additive genetic variance has been shown to be low-moderate for AHPND resistance in *P. vannamei* and varies depending on the population, the statistical strategy applied, whether it is analysed as time of death or binary survival (Wang et al., 2019), and whether it is measured by pedigree relatedness or molecular markers (Lyu et al., 2020). In this sense, when it is attained by a binary code, pedigree relatedness, and the restricted maximum likelihood statistical method, it shows low heritability: 0.06–0.18 (Castillo-Juárez et al., 2018), 0.09–0.14 (Campos-Montes et al., 2020), and 0.12 (Lyu et al., 2020), like those obtained in Test-1, with the same methodology. On the other hand, this heritability shows moderate values (> 0.20) when analysed by Threshold models in both experiments (Test-1 and Test-2). These results are in line with Wang et al. (2019), who presented medium heritabilities using Threshold model (Bayesian approach) and considering survival a binary trait. The Threshold model are the most theoretically acceptable methods for the analysis of categorical traits (Gianola, 1982; Gianola and Foulley, 1983; Harville and Mee, 1984), under the assumption of a continuous normally distributed underlying liability scale for the traits.

The present study is a pioneer in the estimation of the heritability of VP_{AHPND}-infection levels measured by the presence of the toxic plasmid genes *pirA* and *pirB* (Ct values) by qPCR. The latter method is implemented in the BIOGEMAR S.A. company to identify outbreaks in industrial estuaries by sampling both water and animals. However, very low heritabilities were found for these Ct values. To the best of our knowledge, this genetic information could not be compared with other studies on shrimp as they have not been published to date. These heritabilities were estimated for other diseases such as IHHNV (Shin et al., 2025) and WSSV (Trang et al., 2019), which were estimated to be moderate (0.18).

The low to moderate heritabilities obtained may indicate that direct selection for VP_{AHPND} resistance would require an increase in the financial cost to genotype a large number of candidates shrimp in the population to achieve the proposed objectives. It would also require regular controlled challenge tests, which could lead to outbreaks in estuaries if not carried out with adequate biosecurity.

4.3. Could it be a competitive advantage to indirectly select for resistance to VP_{AHPND} through growth?

Growth traits are the most economically desirable for industrial shrimp farmers (Gjerde and Korsvoll, 1999; Zhang et al., 2017; Zenger et al., 2019), which have been reported to be highly heritable, cost-effective, and easy to measure (Castillo-Juárez et al., 2015). For the current population, the heritability of growth traits increased from medium to high according to the genetic selection process, from the first to the fourth generation (Shin et al., 2023; Martínez Soler et al., 2024). The growth heritabilities obtained in these experiments were slightly lower, due to a different time of measurement, as higher heritabilities are shown at older ages in this species (Campos-Montes et al., 2012). These minor variations may also be attributable to the quantity of samples utilized.

The genetic correlations between growth traits (length and weight) were very high, in line with those reported by other authors in this species (Pérez-Rostro and Ibarra, 2003; Zhang et al., 2017; Shin et al., 2023). Very high and positive genetic correlations were also found between the two infection-level traits: the Ct values for genes *pirA* and *pirB*. Indirect correlated genetic responses are valuable when dealing with the two most important traits in the industry: growth and disease resistance. The high and positive genetic correlations obtained between AHPND resistance and growth traits before infection (WI and LI), in Test-1, together with the low-moderate heritabilities shown by the former, lead to the consideration of indirect selection, where selecting a correlated trait could achieve more rapid progress than selecting the desired trait itself (Falconer and Mackay, 1996). Based on this, it may be more cost-effective to select for resistance via a trait that is highly heritable, easier to measure, and more interesting to farmers, such as growth. The efficiency of indirect selection through growth for resistance against VP_{AHPND} in the present population, is significantly elevated when heritabilities are estimated through the observed scale; however, analysis of the data with the underlying scale reveals that indirect selection does not provide higher genetic progress than direct selection. Nevertheless, the high and positive genetic correlation identified suggests the presence of a competitive advantage, as selecting for growth will result in an enhancement of resistance to VP_{AHPND}, at the sizes studied. This is a salient consideration in the context of the high financial cost of direct selection in animal production for any disease.

For infection-levels, the selection via growth traits would improve the response; however, the high standard error obtained leads to a lack of confidence in these results. In Test-1, analysed by Linear model, the genetic correlation between weight measured after infection and survival is very high and positive (0.99). This apparent overestimation could be attributable to the measurement of the trait after infection, the low heritability observed for survival and the high SE. Consequently, this approach is not used in the calculation of indirect selection. In Test-2, the genetic correlations between weight and survival were not

convergent, which could be because weight was measured after infection, and it is highly affected by the disease. It is therefore important to measure growth traits before infection to obtain a reliable estimate of this genetic correlation. In the present study, it was not possible to measure them due to a shortage of personnel during the initial sampling of Test-2. In this sense, the genetic correlation between final length and survival should be considered with caution since this growth is highly influenced by the bacteria. It is important to highlight the high and positive genetic correlations obtained between Ct values and growth traits. This indicates a lower level of infection is associated with higher growth in the species.

For viral diseases, such as WSSV or TSV, this option cannot be considered due to the medium to high negative genetic correlations (from −0.46 to −0.64) reported between growth and resistance (Argue et al., 2002; Gitterle et al., 2005) in this species. These differences may be due to the pathogen, as in the case of bacterial disease rather than viruses in fish, genetic correlation with growth traits is positive (Standal and Gjerde, 1987; Gjedrem et al., 1991). Therefore, genetic correlations between growth and disease resistance in aquatic species may depend on the nature of the pathogen.

The positive genetic correlations obtained between growth and resistance traits could lead to the consideration that genetic selection for weight, carried out over four generations in this Ecuadorian population of *Penaeus vannamei* (PMG-BIOGEMAR©), and its genetic evaluation in industrial estuaries where shrimp coexist with pathogens, could have reduced their susceptibility to AHPND caused by *V. parahaemolyticus*. Nevertheless, given that the present experiment was conducted at small sizes, it is crucial to establish future experiments with harvest size to analyse the genetic correlations between both sizes and resistance against AHPND. The coexistence of the shrimp with pathogens may have exerted an influence on the behaviour of the present genetic line for two reasons: firstly, the shrimp of the genetic nucleus (BIOGEMAR S.A.) of the present breeding program are evaluated with the information of the shrimp from industrial estuaries (PRODUMAR S.A.), where they coexist with pathogens; secondly, the base population established at the beginning of the present breeding program comes exclusively from the industry of Ecuador. To reinforce these findings, future experiments should include measuring initial weight and length in a challenge test where 50 % mortality is reached, alongside the utilization of shrimp of harvest size.

5. Conclusion

The results demonstrate that there is a positive genetic correlation between VP_{AHPND} resistance and growth traits in the present Ecuadorian *Penaeus vannamei* population. These findings suggest that genetic selection for growth under industrial conditions has a beneficial impact on susceptibility or tolerance to this disease, at small sizes.

CRediT authorship contribution statement

Marina Martínez Soler: Writing – original draft, Methodology, Investigation. **Hyun Suk Shin:** Supervision, Investigation. **Álvaro Lorenzo-Felipe:** Software, Methodology. **María Jesús Zamorano Serrano:** Supervision. **Pedro Luis Castro:** Supervision, Methodology. **Laura Cristina Pachón Mesa:** Project administration, Conceptualization. **Jenny Antonia Rodríguez:** Methodology, Investigation. **Cecilia Tomalá:** Methodology, Investigation. **Stanislaus Sonnenholzner:** Methodology. **Roberto Carvalheiro:** Supervision, Software, Methodology. **Wagdy Mekkawy:** Supervision, Software, Methodology. **Luis Fernando Aranguren:** Supervision. **Eduardo Reyes Abad:** Visualization, Project administration, Funding acquisition. **Juan Manuel Afonso López:** Validation, Supervision, Methodology, Investigation, Conceptualization.

Author statement

Juan Manuel Afonso López, corresponding author, declares that the information reported in this paper is original.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The data that has been used is confidential.

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