Aquaculture 513 (2019) 734442

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Genetic parameters for resistance to *Aeromonas hydrophila* in the Neotropical fish pacu (*Piaractus mesopotamicus*)

Vito A. Mastrochirico-Filho[a], Raquel B. Ariede[a], Milena V. Freitas[a], Lieschen V.G. Lira[a], John F.G. Agudelo[a], Fabiana Pilarski[a], Rafael V. Reis Neto[a,b], José M. Yáñez[c], Diogo T. Hashimoto[a,*]

[a] São Paulo State University (Unesp), Aquaculture Center of Unesp, 14884-900 Jaboticabal, SP, Brazil
[b] São Paulo State University (Unesp), Campus of Registro, 11900-000 Registro, SP, Brazil
[c] Facultad de Ciencias Veterinarias y Pecuarias, Universidad de Chile, Santiago, Chile

ARTICLE INFO

Keywords:
Heritability
Animal breeding
Disease resistance
Aeromoniosis

ABSTRACT

Pacu (*Piaractus mesopotamicus*) is one of the main farmed freshwater fish species of South America. Disease outbreaks caused by *Aeromonas hydrophila* are considered one of the major bottlenecks for the development of pacu production. Genetic selection for disease resistance may represent a sustainable and effective alternative to reduce mortality and, therefore, improve productive performance of pacu. To verify whether *A. hydrophila* resistance can be included in genetic selection programs, the estimation of genetic parameters is needed. The aim of this study was to estimate variance components and heritability for *A. hydrophila* resistance in pacu, through experimental challenge performed in 36 full-sib families, resulting in the analysis of 1094 individuals. During 14 days of challenge, survival rate (TS) and time of death (TD) of fish presenting clinical signs of *A. hydrophila* infection were recorded. No influence of weight on TS or TD was detected. Genetic data were analyzed with two different univariate animal models using TS and TD. The total cumulative TS varied considerably among families (0 to 65.5%), which indicated a significant phenotypic variation related to resistance to *A. hydrophila* infection. TD ranged from 313 to 14,846 min, with an average of 1722 ± 1476 min. Low values for heritability were found for TS and TD (0.15 ± 0.05 and 0.12 ± 0.05, respectively). Our results represent the first report of genetic parameters for disease resistance in a Neotropical fish species and indicate that resistance against *A. hydrophila* in pacu might be improved through selection breeding.

1. Introduction

Pacu (*Piaractus mesopotamicus*) is a Neotropical fish species with a wide natural distribution throughout La Plata basin, covering an occupational area of influence of five countries, including Brazil, Uruguay, Bolivia, Paraguay, and Argentina. It is an omnivorous fish considered a suitable species for intensive aquaculture due to its rapid growth, low-cost feeding habits, resistance to environmental disturbances and excellent nutrient utilization (Urbinati and Gonçalvez, 2005; Volkoff et al., 2017). Therefore, pacu contributes to a large part of native fish species production in South America (Valladão et al., 2018), particularly in Brazil (IBGE, 2017). Moreover, pacu farming has been successfully established in other regions of the world, with increasing production in China, Myanmar, Thailand and Vietnam (Honglang, 2007; FAO, 2010).

Despite the increasing intensification of aquaculture in developing countries, the lack of planning accompanied by insufficient sanitary control measures has been identified as a potential limiting factor to aquaculture production. The inadequate practices in commercial fish farms, such as high stocking density, periodic management procedures of fish, inadequate nutrition and problems related to water quality have caused several disease outbreaks with significant production losses (Jansen et al., 2012; Leung and Bates, 2013).

Disease outbreaks are considered a major bottleneck for the development of the aquaculture industry (Subasinghe et al., 2001). The increasing occurrence of a large number of bacterial and viral infections in aquaculture facilities represents a serious risk to animal health, and both production and consumption of fish and shellfish products (Villena, 2003).

Aeromonas hydrophila is a free-living gram negative opportunistic bacterium responsible for substantial economic losses in freshwater aquaculture worldwide (Camus et al., 1998; Harikrishnan and

* Corresponding author at: Aquaculture Center of Unesp, Via de Acesso Prof. Paulo Donato Castellane, s/n, 14884-900 Jaboticabal, SP, Brazil.
E-mail address: diogo.hashimoto@unesp.br (D.T. Hashimoto).

https://doi.org/10.1016/j.aquaculture.2019.734442
Received 1 June 2019; Received in revised form 29 August 2019; Accepted 30 August 2019
Available online 05 September 2019
0044-8486/

Balasundaram, 2005; Crumlish et al., 2010; Mu et al., 2010; Plumb et al., 2011; Chen et al., 2014). Fish mortalities due to aeromoniosis have been verified in important species such as carp (Yin et al., 2009), tilapia (Ardó et al., 2008), trout (Nya and Austin, 2009), catfish (Zhang et al., 2016) and, particularly, pacu (Carraschi et al., 2012; Farias et al., 2016). Anecdotal communications of Brazilian fish farmers have reported that *A. hydrophila* infection results in around 20–30% of mortalities, although there are not an accurate number of losses related to aeromoniosis outbreaks in pacu, due to the lack of statistical data and an inefficient program of sanitary monitoring. Therefore, the control of aeromoniosis outbreaks is considered a challenging task to assist the increased production of pacu in the South America aquaculture.

Aeromoniosis diseases in fish have been generally associated with epizootic ulcerative syndrome and rotting of fish fins (Nielsen et al., 2001). Additionally, infected fish present loss of appetite and exhibit lethargic or erratic swimming (Conte, 2004). Aeromoniosis is considered a special food safety risk, due to its pathogenicity linked to human diseases, such as gastroenteritis, muscle infections, septicemia, and skin diseases (Igbinosa et al., 2012).

In order to provide novel control measures for Aeromoniosis, different studies have been carried out to better understand the host-pathogen interaction between *A. hydrophila* and pacu. For instance, the effect of competitive exclusion over intestinal microflora by means of probiotic bacteria (exogenous *Bacillus*) has been tested in pacu, but showed suppression of the immune response in diets where too much probiotic was used (Farias et al., 2016). Additionally, effects of immunostimulants have been also studied and in the experimental setting promote high survival rates, but there is no information long-lasting protection or the effects of long periods of administration (Biller-Takahashi et al., 2014). Currently, treatment for bacterial diseases in the Brazilian aquaculture industry is predominantly based on the application of commercial antibiotics (*e.g.* oxytetracycline and florfenicol) (Monteiro et al., 2018). However, the use of these substances in fish farming can contaminate the aquatic environment, result in antibiotic residue in the meat, contribute to the emergence of resistant pathogens, and impact other species of the food chain and, consequently, the consumers health (Cabello, 2006; Monteiro et al., 2018). For instance, the use of antibiotics to control *A. hydrophila* outbreaks in commercial fish farms has favored the development of bacterial strains resistant to several antibiotics in Brazilian native fish species, including pacu (Belém-Costa and Cyrino, 2006).

Many studies have also evaluated the use of vaccination as a control measure against *A. hydrophila* infection in different fish species (Yin et al., 1996; Nayak et al., 2004). However, there are still few studies related to the effectiveness of vaccination strategies in production systems and their effect on enhancing the immune response of cultivated species (Figueiredo and Leal, 2008). Additionally, despite the higher survival rates already observed in an experiment with vaccinated animals against *A. hydrophila* (Poobalane et al., 2010), this strategy is considered time-consuming, laborious and stressful for the fish.

Genetic selection for disease resistance may represent a sustainable and effective alternative to reduce mortality and thus improve productive performance in aquaculture systems (Gjedrem and Baranski, 2009; Ødegård et al., 2011; Yáñez et al., 2014a). In this regard, significant genetic variation for resistance against *A. hydrophila* has been demonstrated for different fish species, which will allow the development of genetically resistant stocks by means of selective breeding (Mahapatra et al., 2008; Sahoo et al., 2008; Ødegård et al., 2010; Xiong et al., 2017; Srisapoome et al., 2019).

To include disease resistance into the breeding objective, the estimation of genetic parameters and heritability are needed to understand whether genetic variation occurs for the analyzed trait. The aim of this study was to estimate variance components and heritability for resistance against *A. hydrophila* in pacu, using data from an experimental challenge performed in a pedigreed population from Brazil. The results presented here represent the first report of heritability for disease resistance in a Neotropical fish species and will serve as a basis for future research and implementation of alternative control measures against *A. hydrophila* and other diseases for native species of economic interest in South American aquaculture.

2. Material and methods

2.1. Ethic statement

This study was conducted in strict accordance with the recommendations of the National Council for Control of Animal Experimentation (CONCEA) (Brazilian Ministry for Science, Technology and Innovation) and was approved by the Ethics Committee on Animal Use (CEUA number 19.005/17) of Faculdade de Ciências Agrárias e Veterinárias, UNESP, Campus Jaboticabal, SP, Brazil.

2.2. Experimental population

Data were obtained from 1094 *P. mesopotamicus* individuals belonging to 36 full-sib families, generated by a hierarchical mating scheme using 18 dams and 36 sires (approximately 1 dam for each 2 sires). The breeders were obtained from eight different commercial fish farming facilities from Brazil (Sao Paulo State) to obtain an appropriate representation of the genetic variation present in aquaculture stocks. A previous study was performed to select breeders from the commercial fish farms using multilocus markers represented by microsatellites and SNPs (single nucleotide polymorphisms) (Mastrochirico-Filho et al., 2019). The molecular data were used to evaluate parameters of genetic diversity, genetic structure and kinship analysis, whose results were applied to delineate the best matings and to represent a broad genetic base in this population base. In general, the best strategy adopted was mating unrelated individuals from different fish farms to obtain highest values of allelic richness and heterozygosity (Mastrochirico-Filho et al., 2019), and particularly to avoid inbreeding risks.

Induced spawning was performed using carp pituitary extract dissolved in saline solution (0.9% NaCl) and applied in two dosages, with a 12 h interval (first and second dosage of 0.6 and 5.4 mg/kg, respectively). Dams also received 2 ml of synthetic prostaglandin (Ciosin®, containing 0.25 mg/ml cloprostenol) at the time of the second pituitary extract dosage, according to the protocol described by Criscuolo-Urbinati et al. (2012). For sires, a single dosage was used, at the same time of the second dosage for dams, equivalent to 1.5 mg/kg of carp pituitary extract. After hatching in 20 l conical fiberglass incubators, the larvae were fed with artemia nauplii for 20 days. Gradually, the feed was replaced by 50% of crude protein. In the fingerling stage, 1.2 mm pelleted feeds were used (40% of crude protein), being gradually replaced by 2 to 3 mm pelleted feeds (36% of crude protein) provided twice daily in 60 l tanks.

Animals used in the experiment were pit-tagged when they reached a minimum of 5.0 g (SD = 1.0 g) to maintain the pedigree information during the challenge experiments. Posteriorly, fish were kept in 800 l fiberglass tanks at the Laboratory of Genetics in Aquaculture and Conservation (LaGeAC), at the Universidade Estadual Paulista (UNESP), Jaboticabal city (São Paulo State, Brazil). Prior to challenge, a subsample of the population was checked to verify the presence of *A. hydrophila* and other pathogens, such as *Flavobacterium columnare* and *Streptococcus agalactiae*, by routine microbiological tests. The mean weight of animals prior to the inoculation time was 34.1 g (SD = 25.5 g).

2.3. Bacterial challenge experiment

Bacterial challenge was performed using a strain of *A. hydrophila* isolated from an outbreak of pacu aeromoniosis in a commercial facility from the São Paulo State, by the Laboratory of Microbiology and

Parasitology of Aquatic Organisms, at the Universidade Estadual Paulista (UNESP), Jaboticabal city (São Paulo State, Brazil). The strain was cultured in Trypticase Soy Agar (TSA), Vegitone (Sigma-Aldrich) for 24 h (28 °C). The colony was then transferred to a nutrient tryptic soy broth (TSB) (Sigma-Aldrich) and cultured for 24 h (28 °C). After bacteria growth, the culture was centrifuged at 5000*g* for 10 min (4 °C, in the Eppendorf Centrifuge 5810), forming a bacterial pellet suspended in a saline solution (PBS) and washed twice.

Initially, a pilot experiment using the challenge test by cohabitation was performed, in which infected fish by *A. hydrophila* were introduced in the treatment tanks with healthy fish. However, no experimental fish died during the challenge, except the previously infected fish (cohabitants) that died within 48 h (data not shown). Therefore, the challenge test performed in this study was carried out by intraperitoneal inoculation. The LD_{50} (lethal dose in 50% of individuals) was previously tested in 60 randomly chosen individuals from the same pacu families using concentrations adjusted by optical density of the solution at 0.400, 0.600 and 0.800 at 625 nm in spectrophotometer (2100 Unico, Japan). A sample of 100 µl of the LD_{50} was removed from the inoculum to perform serial dilutions and plate counts in duplicate on Tripticase Soy Agar (TSA). Prior to the inoculation of bacteria, fish were anesthetized with benzocaine (0.1 mg/l) and weight was recorded. Fish that showed mortality by clinical signs of *A. hydrophila* infection (disequilibrium, hemorrhage, isolation of the group) were recorded. The diagnosis of *A. hydrophila* infection was confirmed by bacterial isolation from anterior kidney samples from fish that presented clinical signs. The isolation of bacteria was performed in a specific growth medium (phenol red agar and ampicillin) incubated at 28 °C for 48 h.

In the experimental design of the challenge test, fish were distributed into three communal treatment tanks of 2 m^3 (length = 2 m, width = 1 m, depth = 1 m). Approximately ten individuals from each family were randomly distributed into each treatment tank. In total, 1094 fish were used in the experiment (about 370 fish per tank). Individual fish was injected by intraperitoneal inoculation of the predefined LD_{50} of live cells of *A. hydrophila* (8×10^5 CFU/g body weight), according to protocols carried out by Mahapatra et al. (2008) and Mohanty et al. (2012). Moreover, approximately ten fish from each family were also used as control population in a separated tank (called as control tank) of 2 m^3 (length = 2 m, width = 1 m, depth = 1 m). Individuals of the control population were injected by intraperitoneal inoculation of saline solution (PBS). Each treatment and control tank was maintained with an independent water recirculation system, fitted with mechanical and biological filters, external aeration system, and controlled temperature using thermal controller connected to heaters (2 × 500 w). Water quality parameters were determined daily during 14 days of the challenge. Temperature, dissolved oxygen and pH were measured with a Multiparameter Water Quality Checker U-50 (Horiba, Kyoto, Japan). Water temperature was maintained at 30 °C (SD = 0.5), with dissolved oxygen at 5.8 mg/l (SD = 1.0) and pH at 7.0 (SD = 1.1) during the challenge period (14 days). No water was exchanged during the challenge, but the tanks were topped off to compensate for evaporation.

Fish mortality was observed during all day (24 h) in the initial three days of challenge; and in intervals of 8 h in the remaining days of challenge. Dead individuals presenting clinical signs of *A. hydrophila* were recorded and removed immediately from the tanks. Necropsy examination and microbiological tests were performed in a sub-sample of dead fish in order to confirm mortality by *A. hydrophila* and not other pathogens. All surviving fish were examined externally for clinical signs of disease.

2.4. Genetic parameters analysis

Resistance was assessed as survival to the challenge test using the following trait definitions, according to Yáñez et al. (2013):

1. Test survival (TS), which was scored as 1 if the fish died in the challenge test period and 0 if the fish survived at the end of the experiment. This trait was analyzed using a binary threshold (probit) model (THR) to account for the binary nature of the trait.
2. Time of death (TD), which was scored in minutes, ranging from the moment of the first and last event of mortality. If fish survived to the end of testing period, the time was recorded as 14,846 min. This trait was analyzed using a linear model (LIN).

Data were analyzed with two different univariate animal models as defined below:

- LIN: A linear model was used to fit the continuous variable of TD:

$$y_{ij} = \mu + t_i + a_j + e_{ij}$$

where, y_{ij} was the phenotype for the fish *j*, in tank *i*; μ was the fixed effect of the overall mean; t_i was the fixed effect of the tank *i* and covariate of weight prior bacteria inoculation; a_j was the random animal genetic effect of individual *j*; and e_{ij} was the random residual for the fish *j*.

- THR: A binary threshold (probit) model was used for analysing TS:

$$Pr(Y_{ij}) = \Phi(\mu + t_i + a_j)$$

where, Y_{ij} was the phenotype (TS) for the fish *j*; $\Phi(\cdot)$ was the cumulative standard normal distribution and the other parameters as described above.

THR and LIN models were fitted using ASREML 3.0 package (Gilmour et al., 2009). For all the models, the random animal genetic effect was assumed to be $\sim N(0, \mathbf{A}\sigma_a^2)$, where $\mathbf{A}$ is the pedigree-based additive genetic kinship matrix among all the animals included in the population and σ_a^2 is the additive genetic variance. Residuals for LIN were assumed to be $\sim N(0, \mathbf{I}\sigma_e^2)$, where $\mathbf{I}$ is an identity matrix and σ_e^2 is the residual variance. For THR model, the residual variance on the underlying scale was set to 1. For both models, heritability was calculated as:

$$h^2 = \frac{\sigma_a^2}{\sigma_a^2 + \sigma_e^2}$$

where σ_a^2 was the additive genetic variance and and σ_e^2 was the residual variance.

3. Results

Descriptive statistics for each replicate tank used in the *A. hydrophila* challenge is presented in Table 1. Survival rates between the tanks ranged from 65% to 88%. Body weight (BW) of the challenged animals averaged 34.1 g (SD = 25.5 g). Fish belonging to family 1 obtained the highest BW (83.1 g, SD = 27.5 g), while family 16 presented the lowest BW (12.1 g, SD = 3.3 g). Pearson correlation coefficients (r) were calculated in order to evaluate the occurrence of correlation between BW and TS, or between BW and TD. According to the results, there was a weak correlation between BW and TS ($r = -0.050$, p-value $> .05$),

Table 1
Descriptive statistics for each replicate tank of challenge against *A. hydrophila* in pacu (*P. mesopotamicus*).

	Tank		
	1	2	3
Total number of fish	374	367	353
Average number of fish per family	10.4	10.2	9.8
Average body weight	33.9	34	24.4
Total number of dead fish	129	71	43
Final survival rate	0.65	0.81	0.88

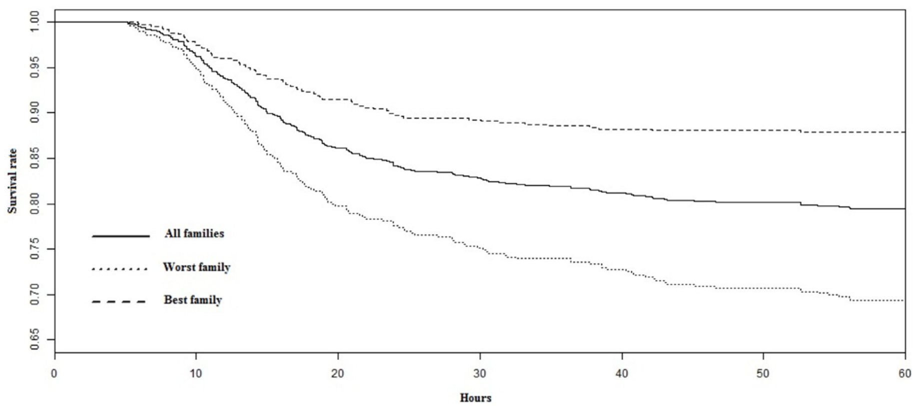

Fig. 1. Kaplan-Meier mortality curves of pacu (*P. mesopotamicus*) families infected with *A. hydrophila* during a 14 day resistance challenge. The data show the cumulative mortality corresponding to all families (black line), more susceptible families (dotted line) and more resistant families (dashed line) to infection.

and between BW and TD (r = 0.002, p-value > .05). The results show that despite the variation in BW sampled between challenged animals, there was no influence of weight on the mortality of the animals and their time to death.

All individuals susceptible to *A. hydrophila* infection had typical clinical signs caused by the bacteria infection, presenting hemorrhagic septicemia, lethargy, isolation from the group, loss of equilibrium and appetite. The total cumulative mortality across all the families reached 22.2% at the end of the test period. Mortality was detected until 14,846 min after inoculation (we considered the plateau as 3600 min), as shown by the Kaplan-Meyer analysis (Fig. 1). At the end of the challenge test (after 14 days), surviving individuals did not present clinical signs related to infection.

The total cumulative mortality rates varied considerably between families (0 to 65.5%), which indicated a significant phenotypic variation related to resistance to *A. hydrophila* infection (Fig. 2). For example, family 1 was considered the most susceptible family (65.5%), while no mortality was observed in family 18.

TD to *A. hydrophila* infection ranged from 313 to 14,846 min, with an average of 1722 ± 1476 min. Family 12 registered higher TD (average of 4740 min), although this family presented with a large variation in TD (± 5532 min). Families 16 and 25 showed the shortest TD, with an average value of 180 min (Fig. 2).

Significant additive-genetic variation was observed for both traits. Estimated heritabilities and variance components for TS and TD are presented in Table 2. The results show low heritability values for *A. hydrophila* resistance in pacu, which were estimated to be 0.15 (± 0.05) and 0.12 (± 0.05) for TS and TD, respectively.

Table 2
Estimates of additive genetic variance (σ_a^2), residual variance (σ_e^2), phenotypic variance (σ_p^2) and heritability (h^2) for resistance to *A. hydrophila* in pacu (*P. mesopotamicus*), measured as time of death (TD) and test survival (TS). Standard error in parenthesis.

Variance components	TD	TS
σ_a^2	0.42×10^7 (0.17×10^7)	0.18 (0.07)
σ_e^2	0.30×10^8 (0.17×10^7)	1
σ_p^2	0.34×10^8 (0.16×10^7)	1.18 (0.07)
h^2	0.12 (0.05)	0.15 (0.05)

4. Discussion

Genetic selection of plants and terrestrial animals has been practiced since agriculture began (Brander, 2007). However, breeding programs have been implemented in aquaculture only recently and > 90% of fish production is still based on genetically unselected units (Gjedrem et al., 2012). In this context, Brazil's fish biodiversity provides substantial potential for genetic improvement with 40 native species used for aquaculture (Godinho, 2007), but there is no native fish in Brazil produced commercially as result of breeding programs. The importance of pacu production and the aquaculture expansion in developing South American countries (Valladão et al., 2018) reflects the need of investment in modern biotechnology for high-level production of Neotropical fish. Therefore, one of the main results of this study is the establishment of a breeding population for pacu, representing the first steps toward the genetic improvement of Neotropical fish species.

Aeromoniosis is considered one of the main causes of mortality in

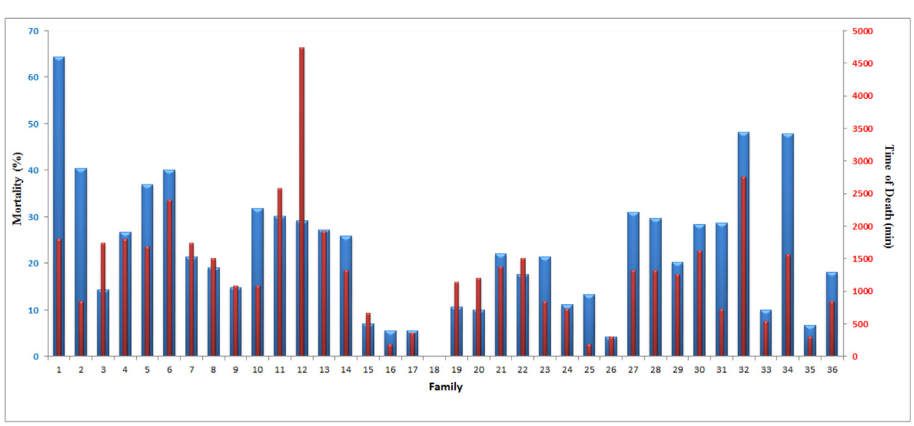

Fig. 2. Total cumulative mortality rate (%) and average of time of death (min) of pacu (*P. mesopotamicus*) families infected with *A. hydrophila* during a 14 day resistance challenge. Blue and red columns represent mortality rate and time of death, respectively, for each family of pacu infected with *A. hydrophila*.

farmed populations of pacu (Farias et al., 2016). As demonstrated by the results of the present study, superior genotypes for resistance to *A. hydrophila* infection may be an alternative to the use of vaccines and antibiotics. We have detected considerable phenotypic variation for this trait, *i.e.*, families resistant to *A. hydrophila* with no mortality and families highly susceptible with 65.5% total cumulative mortality, which suggests the possibility to improve pacu resistance through selective breeding. Moreover, in relation to TD, we found substantial differences, ranging from 313 to 14,846 min between individuals. Increasing survival time is critical to control the disease outbreak; providing more time for adequate treatment of fish or the aquatic environment.

All studies performed to date examining disease resistance to *A. hydrophila* have used intraperitoneal inoculation (Mahapatra et al., 2008; Ødegård et al., 2010a; Xiong et al., 2017), even though challenge by cohabitation might be better at reproducing the immune response to natural infection; providing more reliable resistance data for genetic evaluation. In the current study, we previously performed an unsuccessful challenge test by cohabitation, and then the final experiment was carried out using intraperitoneal inoculation. However, because *A. hydrophila* is considered an opportunistic pathogen whose infections frequently appear when there is a deficiency of the immune system (Harikrishnan and Balasundaram, 2005), future experiments to optimize challenge tests by cohabitation need to be developed using immunological stressors in pacu, such as environmental changes (*e.g.*, temperature oscillation).

Our results demonstrated low but significant heritability for TS (0.15 ± 0.05) and TD (0.12 ± 0.05), which are intermediate values compared to similar experiments in other species. For instance, lower values have been found for *A. hydrophila* resistance for rohu carp (*Labeo rohita*) and common carp (*Cyprinus carpio*), with values of 0.02 and 0.04, respectively (Mahapatra et al., 2008; Ødegård et al., 2010); nevertheless, moderate heritabilities of 0.27, 0.33 and 0.39 have also been reported in *Clarias macrocephalus*, *Megalobrama amblycephala* and rohu carp, respectively (Mahapatra et al., 2008; Xiong et al., 2017; Srisapoome et al., 2019).

Selective breeding for disease resistance is challenging because these traits are difficult to measure directly on selection candidates (Sonesson and Meuwissen, 2009; Yáñez et al., 2014b). Individuals submitted to challenge test cannot be included in the breeding nucleus as the subject could die during the experiment or could be an eminent threat of disease to the entire population. Therefore, some studies have attempted to correlate resistance to *A. hydrophila* with other indirect traits, such as innate immune parameters (Sahoo et al., 2008, 2011; Srisapoome et al., 2019), growth performance (Xiong et al., 2017), and molecular markers (Ariede et al., 2018). The inclusion of molecular marker information for increasing the accuracy of genetic evaluations, whether using marker-assisted selection or genomic prediction approaches (Barría et al., 2018; Yoshida et al., 2018), is a promising alternative to be applied in pacu.

In conclusion, challenge experiments of *A. hydrophila* showed significant genetic variation in TS and TD between pacu families, which can provide the genetic basis for the improvement of *Aeromonas* resistance in pacu. Currently, all Neotropical fish production is still being carried out with non-selected products, particularly related to disease resistance. Therefore, our results represent a pioneering study about genetic improvement in Neotropical species, which may help decrease the occurrence of *A. hydrohila* outbreaks in aquaculture facilities, supporting the reduction of antibiotic use and resulting in the production of a sustainable and safe fish.

Author contributions

Conceptualization: VAMF and DTH; Formal analysis: VAMF, RVRN and JMY; Funding acquisition: VAMF and DTH; Investigation: VAMF, RBA, MVF, LVGL and JFGA; Methodology: VAMF, RBA, MVF, LVGL, JFGA and FP; Project administration: DTH; Supervision: DTH; Roles/Writing – original draft: VAMF and DTH; Writing – review & editing: VAMF, RBA, MVF, LVGL, JFGA, FP, RVRN, JMY and DTH.

Funding

This work was supported by São Paulo Research Foundation (FAPESP grant 2016/21011-9 and 2016/18294-9), National Council for Scientific and Technological Development (CNPq grant 311559/2018-2 and 140740/2016-3), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES - Finance Code 001). No potential conflict of interest was reported by the authors.

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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