Aquaculture 523 (2020) 735225

Contents lists available at ScienceDirect

Aquaculture

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

Genetic (co)variation between resistance to *Aeromonas hydrophila* and growth in tambaqui (*Colossoma macropomum*)

Raquel B. Ariede[a], Milena V. Freitas[a], John F.G. Agudelo[a], Carolina H.S. Borges[a], Lieschen V.G. Lira[a], Grazyella M. Yoshida[b], Fabiana Pilarski[a], José M. Yáñez[b], Diogo T. Hashimoto[a,*]

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

ARTICLE INFO

Keywords:
Heritability
Genetic parameters
Disease resistance
Aeromoniosis

ABSTRACT

Tambaqui (*Colossoma macropomum*) is the main native fish farmed in South American continental aquaculture. One of the major pathogens affecting world fresh water aquaculture is the bacterium *Aeromonas hydrophila*, which generates several disease outbreaks and production losses in farmed tambaqui stocks. Up to date, there are no studies focusing on understanding the quantitative basis for the genetic improvement for disease resistance in tambaqui. Thus, the objective of this study was to determine the genetic parameters for resistance to *A. hydrophila* and the genetic correlation with average daily gain in juveniles of tambaqui, to determine whether these traits can be included in selective breeding programs. Estimation of genetic parameters was performed using data from an experimental challenge performed in 18 full-sib families, using a total of 576 individuals. Before bacterial challenge, all animals were evaluated for average daily gain (ADG) in the juvenile phase during 30 days. The challenge spanned 120 h (5 days) and disease resistance traits were defined as: i) binary survival (BS) and, ii) time of death (TD) of fish presenting clinical signs of *A. hydrophila* infection. The mean ADG was 0.49 g/day (SD = 0.21) in the population and heritability for this trait was moderate (h^2 = 0.37 ± 0.13). BS and TD varied considerably among families (26% to 89% and 10.7 h to 69.2 h, respectively), which indicated a significant genetic variation related to resistance to *A. hydrophila* infection. Low to moderate values for heritability were found for BS and TD (0.17 ± 0.06 and 0.23 ± 0.09, respectively). The genetic correlations between resistance to *A. hydrophila* and ADG in juveniles of tambaqui were not significantly different from zero. The significant genetic variation found for *A. hydrophila* resistance in tambaqui indicates that selecting superior genotypes is a viable approach to reducing the impact of diseases outbreaks in aquaculture.

1. Introduction

Tambaqui *Colossoma macropomum* is a Neotropical fish belonging to the order Characiformes and family Serrasalmidae (Calcagnotto et al., 2005). Special teeth and the filtering apparatus allow tambaqui be classified as unique omnivorous fish, with feeding behavior of a wide range of natural foods, such as fruits/seeds and zooplankton (Woynárovich and Van Anrooy, 2019). This species is widely distributed in the Amazon and Orinoco rivers, with natural occurrence in Brazil, Venezuela, Colombia, Peru and Bolivia (Araujo-Lima and Goulding, 1997). Tambaqui is one of the main freshwater species produced in South American aquaculture; moreover, its production is spread in several countries in Asia, including China, Indonesia, Malaysia, Myanmar and Viet Nam (Woynárovich and Van Anrooy, 2019). The global tambaqui aquaculture production reached about 142 thousand tonnes in 2016, of which 96.4% was produced by Brazil (approximately 137 thousand tonnes) (IBGE, 2017).

Economic losses due to diseases are estimated in about US$ 84 million in Brazil (Tavares-Dias and Martins, 2017). Due to the high density stocking and intensification in the tambaqui production, important pathogens have been identified in these fish, which have the potential to cause significant losses of production, especially due to the disease outbreaks caused by *Aeromonas hydrophila* (Valladão et al., 2018). This bacterium is an opportunistic pathogen that is widely distributed in freshwater environments (Camus et al., 1998), causing mortality in several important species of the aquaculture, including *Cyprinus carpio* (Ødegård et al., 2010; Jeney et al., 2011), *Oreochromis niloticus* (Ardó et al., 2008), *Ictalurus punctatus* (Pridgeon and Klesius,

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

https://doi.org/10.1016/j.aquaculture.2020.735225
Received 6 December 2019; Received in revised form 11 March 2020; Accepted 11 March 2020
Available online 25 March 2020
0044-8486/

2011), *Pangasianodon hypophthalmus* (Crumlish et al., 2010) and the Neotropical fish *Piaractus mesopotamicus* (Mastrochirico-Filho et al., 2019).

Disease prevention and control methods in aquaculture typically include vaccines and the use of commercial antimicrobials (Pilarski et al., 2017; Monteiro et al., 2018). However, no commercial vaccines are available for South American fish, including *A. hydrophila* in tambaqui (Valladão et al., 2018). Regarding the use of commercial antimicrobials, uncontrolled use can contaminate the aquatic environment and contribute to the emergence of resistant pathogens (Belém-Costa and Cyrino, 2006; Monteiro et al., 2018). The development of genetically resistant populations against resistance to *A. hydrophila* have been demonstrated to be feasible in several species, therefore, representing an important alternative for controlling the disease (Mahapatra et al., 2008; Sahoo et al., 2008; Ødegård et al., 2010; Xiong et al., 2017; Mastrochirico-Filho et al., 2019; Srisapoome et al., 2019).

Genetic parameters have not yet been estimated for any disease resistance trait in tambaqui, which is considered the main native species as target for the development of selective breeding programs in South America. Therefore, the objective of this work was to estimate variance components and heritability for resistance against *A. hydrophila* in tambaqui, using data from an experimental challenge performed in a pedigreed population from Brazil. Values of genetic correlation were also estimated between average daily gain (ADG) and resistance traits to test if growth performance could be used to assist indirect selection for improved resistance against *A. hydrophila* in tambaqui.

2. Material and methods

2.1. Ethics 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 019006/17) of Faculdade de Ciências Agrárias e Veterinárias, UNESP, Campus Jaboticabal, SP, Brazil.

2.2. Experimental population

The fish population analyzed in the present study was composed of 576 *C. macropomum* individuals belonging to 18 full-sib families, generated by a hierarchical mating scheme using 9 dams and 14 sires (nearly 1 dam for each 2 sires). The families were produced during the period of approximately 40 days (breeding season of 2018). The breeders were obtained from five different commercial fish farming facilities from Brazil to obtain an appropriate representation of the genetic variation present in aquaculture stocks.

Induced spawning was performed using carp pituitary extract (Danubio piscicultura LTDA) dissolved in saline solution (0.9% NaCl) and applied in two dosages, with a 12 h interval (first and second dosage of 0.5 and 5.5 mg/kg, respectively). For males, a single dosage was used, at the same time of the second dosage for females, equivalent to 2.5 mg/kg of carp pituitary extract (Pinheiro and Silva, 1988). After hatching in 20 l conical fiberglass incubators, the larvae were transferred to 250 l tanks and maintained separately until tagging. 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) and provided twice daily (commercial feed Nutripiscis Presence).

The experimental population was pit-tagged by intraperitoneal inoculation when the smallest animal reached a minimum of 5.0 g (SD = 1.0 g), to maintain the pedigree information known during the experiments. All the tagging process was performed during three days. Fish presenting morphological abnormalities were excluded of the experiments. All the experiments were carried out at the Laboratory of Genetics in Aquaculture and Conservation (LaGeAC), at the Universidade Estadual Paulista (UNESP), Jaboticabal (São Paulo State, Brazil).

2.3. Average daily gain records

Initially, growth was followed across 30 days prior the experimental challenge in the healthy juvenile fish, in order to test the correlation between ADG and resistance to *A. hydrophila*. The growth experiment initiated 15 days after the tagging process to avoid that stress and handling associated with tagging could have interference in fish performance. The 18 families were distributed using a balanced design in four 750 l tanks, with about 8 individuals from each family per tank (communal treatment tanks) and density of approximately 200 fish/m^3, similarly to conditions of the production system for this species (Brandão et al., 2004). The tanks were connected in a water recirculation system, fitted with mechanical and biological filters, external aeration system, and controlled temperature using a thermal controller connected to a heater (1.5 KW). Water quality parameters were determined daily during whole period. 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 5.0 mg/L (SD = 1.0) and pH at 7.2 (SD = 1.0) during the growth recording period.

Body weight of all animals was collected at the beginning and end of the 30 days period. During the evaluation, fish were fed by 1.2 mm pelleted feeds (40% of crude protein) twice a day. The evaluation period of 30 days was established because most of the animals increased twofold the body weight, which was enough to detect significant differences between families. Moreover, growth was not evaluated in a longer period because the tanks size (amount of fish/m^3) could have interference in fish performance affecting the real growth of the animals.

2.4. Bacterial challenge experiment

The experimental bacterial challenge was performed using a strain of *A. hydrophila* isolated from an aeromoniosis outbreak in a commercial fish farm 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 used in this study is available upon request to Dr. Fabiana Pilarski. 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 5.000 *g* for 10 min (4 °C, in an Eppendorf Centrifuge 5810), forming a bacterial pellet suspended in a saline solution (PBS) and washed twice. The LD_{50} (lethal dose in 50% of individuals) was previously tested by intraperitoneal (i.p.) inoculation in 60 randomly chosen individuals from the 18 tambaqui families using concentrations adjusted by optical density of the solution at 0.100, 0.400, 0.800 and 1.000 (CDCP, WHO, 2003) 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 body 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.

After the growth assessment period, fish were distributed into three communal treatment using tanks of 0.5 m^3 for the challenge test. Approximately ten individuals from each family were randomly distributed into each treatment tank. In total, 576 fish were used in the disease challenge experiment (about 190 fish per tank). Individual fish were injected by i.p. inoculation of the predefined LD_{50} of live cells of *A. hydrophila* (3.4×10^5 CFU/g body weight), according to protocols carried out by Mahapatra et al. (2008) and Mastrochirico-Filho et al. (2019). Moreover, approximately ten fish from each family were also used as control population in a separated tank (named as control tank) of 0.5 m^3. Individuals of the control population were injected by i.p. inoculation of saline solution (PBS). Each treatment and control tank were 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 the 5 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 28 °C (SD = 0.5), with dissolved oxygen at 5.0 mg/L (SD = 1.0) and pH at 7.7 (SD = 1.1) during the challenge period. 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 discard other pathogens. All surviving fish were examined externally for clinical signs of disease.

2.5. Statistical models

Growth and resistance were defined and analyzed using the following trait definitions and models:

1. Average daily gain (ADG), which was recorded in grams, and calculated as: $ADG = \frac{Wf - Wi}{T}$, where Wf was the weight at the end of the measurement period, Wi was the weight at the beginning of the measurement period, and T was the total time across the measurement period (30 days). This trait was analyzed using a linear model (LIN). Common environmental variance was initially considered in the analyzed model, but this variance component was not significant; therefore, we excluded of further analysis.
2. Time of death (TD), which was recorded in hours, ranging from the moment of the first and last event of mortality. If fish survived to the end of testing period, the time was defined by the last event of mortality. This trait was analyzed using a linear model (LIN).
3. Binary survival (BS), which was scored as 0 if the fish died in the challenge test period and 1 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.

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

LIN: A linear model was used to fit the continuous variables of ADG and TD:

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

where, y_{ij} was the phenotype for the fish j (ADG or TD), in tank i; μ was the fixed effect of the overall mean; t_i was the fixed effect of the tank i; 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 analyzing BS:

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

where, Y_{ij} was the phenotype (BS) 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 4.0 package (Gilmour et al., 2009). For all the models, the random animal genetic effect was assumed to be $\sim N(0, A\sigma_a^2)$, where 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 $\sim N(0, I\sigma_e^2)$, where 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:

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

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

The genetic correlations $r_{g(m,n)}$ between both trait definitions of resistance to *A. hydrophila* and ADG were calculated from the covariance components estimated by using three independent bivariate analysis (TD/BS, TD/ADG, and BS/ADG), by means of fitting linear animal models as defined above, as follows (Falconer and Mackay, 1996):

$$\boldsymbol{r_{g(m,n)} = \frac{Cov_g(m,n)}{\sqrt{Var_g m \times Var_g n}}}$$

where, $Cov_g(m,n)$ was the genetic covariance between the two traits, $Var_g m$ and $Var_g n$ was the additive genetic variance of trait m and trait n, respectively.

The phenotypic correlation $r_{p(m,n)}$ was calculated as:

$$\boldsymbol{r_{p(m,n)} = \frac{Cov_p(m,n)}{\sqrt{Var_p m \times Var_p n}}}$$

where, $Coc_p(m,n)$ was the phenotypic covariance between the two traits, $Var_p m$ and $Var_p n$ was the phenotypic variance of trait m and trait n, respectively.

Kaplan–Meier survival curves (Kaplan and Meier, 1958) were plotted for the best and worst family, as well as the average of all families in order to show the differential trajectory of mortality across the challenge.

3. Results

Descriptive statistics for each replicate tank used in the growth assessment period and *A. hydrophila* challenge is presented in Table 1. The raw data of the experiments used in this study is available upon request to the corresponding author. Body weight (BW) of the animals at the end of the growth assessment period was on average of 34.1 g (SD = 25.5 g). Fish belonging to family T01 obtained the highest BW (83.1 g, SD = 27.5 g), while family T16 showed the lowest BW (12.1 g, SD = 3.3 g). The overall mean of ADG was 0.49 g (SD = 0.21), with a minimum of 0.05 g for the family T02 and a maximum of 1.27 g for the family T05.

Fish experimentally infected with *A. hydrophila* presented clinical symptoms of aeromoniosis 5 h after inoculation, including fin erosion, group isolation and hemorrhagic septicemia. Of the 576 fish inoculated with *A. hydrophila*, 251 died during the test period, corresponding to a total cumulative mortality of 43.5%. The survival rates varied considerably between families, which indicated a considerable phenotypic variation related to resistance to *A. hydrophila* infection (Fig. 1). For instance, family T13 and T12 were considered the most susceptible and resistant families with a 26% and 89% of cumulative survival, respectively. Mortality was detected until three days after inoculation,

Table 1
Summary statistics for binary survival (BS), time to death (TD) and average daily gain (ADG) in the tambaqui (*Colossoma macropomum*) breeding population.

Variable	Tank	N[a]	N/fam[b]	Survival rate	Mean	SD[c]	Min[d]	Max[e]
BS (%)	R1	206	11.4	0.57	–	–	–	–
	R2	190	10.6	0.67	–	–	–	–
	R3	180	10	0.45	–	–	–	–
TD (hour)	R1	206	11.4	–	65.14	36.61	4.93	95
	R2	190	10.6	–	72.76	33.62	4.97	95
	R3	180	10	–	64.86	33.04	5.23	95
ADG (g)	G1	139	7.7	–	0.58	0.22	0.09	1.26
	G2	149	8.3	–	0.51	0.21	0.08	1.19
	G3	148	8.2	–	0.45	0.17	0.05	1
	G4	140	7.7	–	0.42	0.17	0.08	1.27

[a] Number of individuals;
[b] Number of individuals per family;
[c] Standard deviation;
[d] Minimum;
[e] Maximum.

reaching a plateau, as shown by the Kaplan-Meyer analysis (Fig. 2). TD to *A. hydrophila* infection ranged from 10.7 h (family T18) to 69.2 h (family T12), with an average of 32.2 h (SD = 23.2) (Fig. 1). At the end of the challenge test (after 5 days), surviving individuals did not present clinical signs related to infection. The control group showed no signs of infection and mortality.

Significant additive-genetic variation was observed for both trait definitions of *A. hydrophila* resistance and also for ADG. Estimated heritabilities for the all traits studied here are presented in Table 2. The results showed low to moderate heritability values for *A. hydrophila* resistance in tambaqui, which were estimated to be 0.17 (± 0.06) and 0.23 (± 0.09) for BS and TD, respectively. Heritability for ADG was also considered moderate with value of 0.37 (± 0.13).

The phenotypic correlations between the two trait definitions of *A. hydrophila* resistance and ADG were positive and low, with values of 0.17 and 0.19 for the correlation between TD and ADG, and BS and ADG, respectively. The genetic correlations of ADG with the two trait definitions of *A. hydrophila* resistance were not significantly different from zero. Phenotypic and genetic correlations between BS and TD were high and positive (0.90 and 0.92, respectively) (Table 2).

4. Discussion

In South America, there is limited commercial production of native species resulting from fish genetically selected from selective breeding programs. In tambaqui, which is the main freshwater native species produced in Latin American aquaculture, some initial efforts have been addressed to evaluate genetic parameters for growth-related traits, including weight gain, standard length, head size, height and width (Marcos et al., 2016; Mello et al., 2016; Ariede et al., 2018; Perazza et al., 2019). Disease resistance traits are also relevant to be included into the breeding goal of genetic improvement programs for tambaqui, particularly resistance against *A. hydrophila* (Ariede et al., 2018). Therefore, one of the main results of this study was the quantification of the magnitude of the genetic variation for resistance against *A. hydrophila* infection in an experimental breeding population of tambaqui. The most resistant family had a survival close to 90%, while the most susceptible family showed a survival of 26%. The variation in survival rates across families was within the range of those shown by previous studies in others species, such as *Cyprinus carpio* (60% to 20%), *Megalobrama amblycephala* (80% to 10%), and *Piaractus mesopotamicus* (100% to 40%) (Jeney et al., 2011; Xiong et al., 2017; Mastrochirico-Filho et al., 2019).

In the present study, the estimation of heritability against *A. hydrophila* demonstrated low to moderate and significant values for BS (0.17 ± 0.06) and TD (0.23 ± 0.09). Although we used a low sample size (due to the low number of families) to calculate genetic parameters, the standard deviation of our heritability estimates were relatively low, which indicates that these values are statistically reliable for the analyzed population. However, the precision of the heritability values must be considered with moderation and a higher number of families/family size is still necessary to be analyzed in future studies to corroborate the heritability values against *A. hydrophila* resistance in tambaqui.

The heritability values herein presented were similar to those previously reported against *A. hydrophila* in others fish species, such as *Clarias macrocephalus*, *Megalobrama amblycephala*, *Labeo rohita* and *Piaractus mesopotamicus*, ranging from 0.12 to 0.39 (Mahapatra et al., 2008; Xiong et al., 2017; Srisapoome et al., 2019; Mastrochirico-Filho et al., 2019). Nevertheless, lower values have also been found for *A. hydrophila* resistance in 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). The significant genetic variation

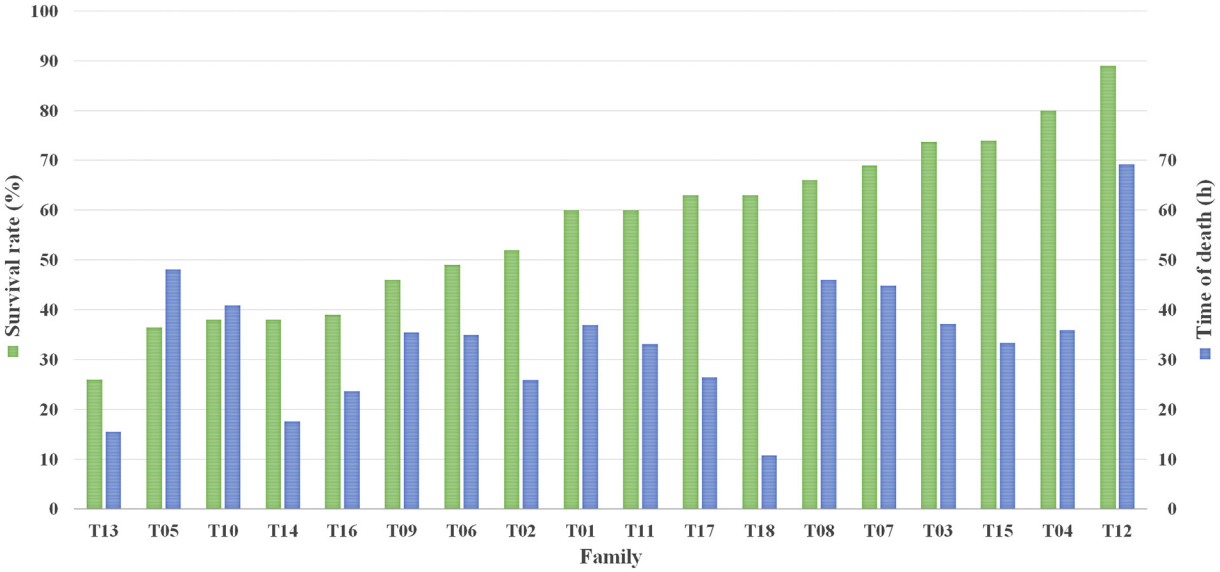

Fig. 1. Survival rate (%) (green bars) and average time of death (hour) (blue bars) for the tambaqui families infected with *Aeromonas hydrophila* during a 5 days experimental challenge. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

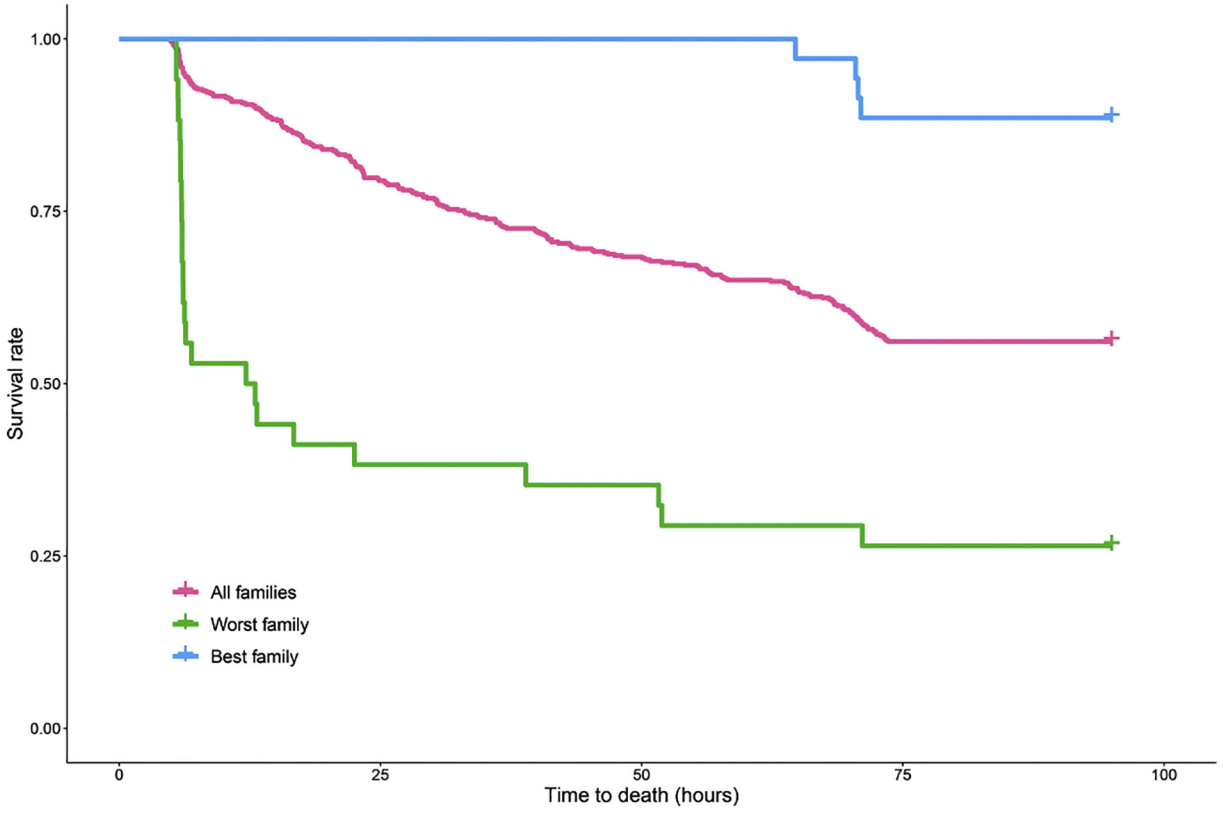

Fig. 2. Kaplan–Meier survival curves for the best and the worst family and an average of all families during an experimental challenge with *Aeromonas hydrophila* in a *Colossoma macropomum* breeding population.

Table 2
Phenotypic (above diagonal) and genetic correlations (below diagonal) between binary survival (BS), time to death (TD) and average daily gain (ADG); heritabilities (h^2) (principal diagonal) for resistance against *Aeromonas hydrophila* estimated from a *Colossoma macropomum* breeding population. Standard errors in brackets.

Variable	BS	TD	ADG
BS	0.17 (0.06)	0.90 (0.01)	0.19 (0.06)
TD	0.92 (0.05)	0.23 (0.09)	0.17 (0.07)
ADG	0.04 (0.38)	0.19 (0.35)	0.37 (0.13)

found in this study indicates the viability of genetically improve resistance against *A. hydrophila* in tambaqui through genetic selection.

The results of heritability for ADG during the juvenile stage also revealed a moderate and significant value (0.37 ± 0.13). Similarly, previous studies in tambaqui have also demonstrated moderate to high levels of heritability for this same trait, with values of 0.49, 0.41 and 0.40 for different ages (12, 20 and 24 months, respectively) (Mello et al., 2016; Perazza et al., 2019). Additional studies still need to be performed in order to evaluate the magnitude and direction of the genetic correlation or re-ranking of EBVs for growth-related traits between the phases of juvenile and harvest time in this breeding population.

In the present study, we detected a genetic correlation not significantly different from zero between ADG and *A. hydrophila* resistance traits in tambaqui, similarly to the results found for the genetic relationship between growth traits and resistance to bacterial cold-water disease in trout *Oncorhynchus mykiss* (Silverstein et al., 2009); and body weight and resistance to *Piscirickettsia salmonis* in Atlantic salmon and rainbow trout (Yáñez et al., 2014; Bassini et al., 2019). Our results suggest that selection for *A. hydrophila* resistance will not negatively affect weight gain in tambaqui, specifically in the juvenile phase analyzed in this study. In contrast, a similar study identified high values for the genetic correlation between resistance to *A. hydrophila* and growth traits, including body weight, length and height in *Megalobrama amblycephala* (Xiong et al., 2017), which could also facilitate indirect selection for disease resistance when selecting for rapid growth in this species.

In conclusion, the presence of significant genetic variation for resistance against *A. hydrophila* in the tambaqui experimental breeding population studied here indicates that genetic improvement for this trait is plausible, and therefore, a feasible alternative to control the disease in aquaculture systems. The results of this study also suggest that selection for *A. hydrophila* resistance does not affect negatively weight gain during the juvenile phase in tambaqui, and *vice versa.* However, others experiments need to be performed during different growth periods to conclude that bacterial resistance does not affect growth and genetic improvement can be made simultaneously for both traits.

Funding

This work was supported by São Paulo Research Foundation (FAPESP grant 2017/19717–3, 2019/10662–7 and 2018/08416–5), National Council for Scientific and Technological Development (CNPq grant 311559/2018–2 and 422670/2018–9), 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.

Acknowledgement

We thank Daiane Vaneci da Silva for assisting in the preparation of the bacterial cultures and microbiological tests.

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