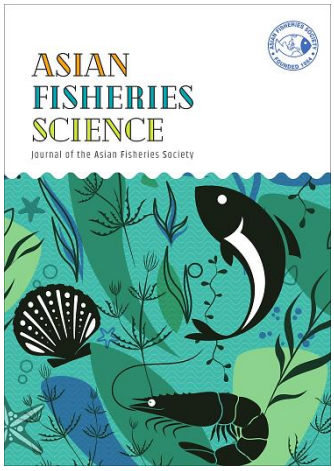

Immune Status of Bighead Catfish (*Clarias macrocephalus* Günther, 1864) Crossbreeds Between Wild and Domesticated Strains and Their Response to Challenge With *Aeromonas hydrophila*

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E-ISSN: 2073-3720
https://doi.org/10.33997/j.afs.2022.35.2.001

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Abstract

Aeromonas hydrophila is a major pathogen causing septicemic disease and high mortality in cultured bighead catfish, *Clarias macrocephalus* Günther, 1864. Crossbreeding for enhanced disease resistance may help alleviate the infection. This study aimed to evaluate the crossbreeding effects of bighead catfish from three broodstock sources, including one domesticated strain (Can Tho - CT) and two wild strains (Ca Mau - CM and Hau Giang - HG), on the innate immune response. Eight diallel crosses (excluding HG × CT) were reared in tanks for 100 days to the juvenile stage for use in this study. Forty-five bighead catfish juveniles of similar sizes, 4.5–6.1 g, were sampled from each cross to evaluate the immune response and stress indicators. Thirty fish from each cross were challenged with *A. hydrophila*. The results showed that innate immune responses of domesticated CT strains were higher than wild strains (HG and CM). Higher values of white blood cells, phagocytic activity, and lysozyme activity were observed in CT × CT and CT × CM crosses. These crosses had significantly lower cortisol levels and lower mortality rates of 40 % (CT × CT) and 43.3 % (CT × CM) after being challenged with *A. hydrophila* compared to the other crosses. The results demonstrate that domesticated CT strain and crossbreeding between CT and CM wild strains could improve the innate immune system and resistance to *A. hydrophila*. The innate immune responses of the domesticated CT strain were higher than wild strains (HG and CM).

Keywords: *Aeromonas hydrophila*, crossbreeding, disease resistance, health, innate immunity

Introduction

The bighead catfish (*Clarias macrocephalus* Gunther, 1864) is a member of the Clariidae family, has a high economic value, and is a favourite fish among Southeast Asian consumers (Na-Nakorn, 2004). Its population in the wild has declined rapidly (Ng et al., 2021), and it has been domesticated and cultured in the Philippines (Mollah and Tan, 1983), Thailand (Na-Nakorn, 2004), and Vietnam for several decades (Duong and Scribner, 2018). However, one of the challenges in farming bighead catfish is its high susceptibility to diseases (Na-Nakorn, 2004).

Disease infection is an acute problem in farmed bighead catfish (Kartikaningsih et al., 2020). The major pathogenic bacteria which commonly cause disease in *Clarias* catfish are *Aeromonas hydrophila* (Aoki, 1999; Srisapoome et al., 2019; Kozlov et al., 2021), *Flavobacterium columnare*, and *Edwardsiella ictaluri* (Boonyaratpalin and Kasornchan, 1986). However, *A. hydrophila* is reported to be more virulent, with high mortality rates, and can kill 80–100 % of fish larvae after 1 to 2 weeks of infection (Cipriano, 2001; Kozlov et al., 2021), up to 70–80 % in fry to fingerling, and 50 % mortality of grow-out stage (Sharma et al., 2018). This disease was named motile *aeromonas* septicemia (MAS) and is infectious to many freshwater fish species (Aoki, 1999).

Genetic improvement is one of the potential solutions to increase fish growth rates and fish health (Kjoglum et al., 2008; Leeds et al., 2010; Gjedrem, 2015). Previous studies have noted the feasibility of

improving disease resistance to specific pathogens by selective breeding or crossbreeding approaches in various aquaculture species (Chevassus and Dorson, 1990; Yanez et al., 2014; Houston, 2017). The selective breeding method has been more commonly applied due to relatively high additive genetic variance for resistance to different diseases in salmonid species (Yanez et al., 2014), such as three main farmed salmonids species, including Atlantic salmon (*Salmo salar* Linnaeus, 1758) (Correa et al., 2015), rainbow trout (*Oncorhynchus mykiss* (Walbaum, 1792)) (Vallejo et al., 2017), and coho salmon (*Oncorhynchus kisutch* (Walbaum, 1792)) (Barria et al., 2019). Crossbreeding of genetically different strains that showed improvement in growth performance or survival rates have been undertaken for guppy, (*Poecilia reticulata* Peters, 1859) (Shikano and Taniguchi, 2002), the Pacific blue shrimp (*Penaeus stylirostris* (Stimpson, 1874)) (Goyard et al., 2008), climbing perch (*Anabas testudineus* (Bloch, 1792)) (Ahammad et al., 2021).

In bighead catfish, Srisapoome et al. (2019) reported that heritability estimates for genetic parameters of resistance to *A. hydrophila* were low to moderate, implying a low to moderate possibility of success in improving bacterial resistance by selection. The potential of crossbreeding has been only somewhat previously explored to enhance disease resistance in fish. For example, in African catfish (*Clarias gariepinus* (Burchell, 1822)), crossbreeds between the two genetically distinct populations exhibited an increase in phagocytosis activity compared to the parental crosses but did not differ in the specific immune response to *A. hydrophila* (Wachirachaikarn et al., 2009). This current study investigates whether crossbreeding could improve the innate immune response and resistance to *A. hydrophila* in bighead catfish at juvenile stages. One domesticated and two wild strains of bighead catfish from the Vietnamese Mekong Delta, previously found to have a relatively high level of genetic diversity (Duong and Scribner, 2018), were used to produce offspring for this study.

Materials and Methods

Ethical approval

The experiments using bighead catfish in this study were approved by Can Tho University, under the approval of the project "Green technology innovation for aquaculture", No. 08/HD.VN14P6NC.2018, dated 31 January 2018.

Fish population and mating design

The current study used three bighead catfish populations from the Vietnamese Mekong Delta. The wild males and females of bighead catfish were collected in Ca Mau (CM) and Hau Giang (HG) provinces, while the cultured fish were bought from a hatchery in Can Tho (CT). Nine (3 × 3) diallel crosses within and among the three populations (16 to 18 pairs for each genetic type) were artificially produced (Table 1). These crosses were nursed in tanks for 100 days (in larval and juvenile rearing experiments reported by Duong et al. (2022)) and used for health evaluation. Unfortunately, the crossbreed of HG × CT (dam × sire) had low survival and was not used for this study.

Table 1. Nine crosses within and between three bighead catfish *Clarias macrocephalus* broodstock from Ca Mau, Can Tho, and Hau Giang used in the present study (Duong et al., 2022).

Male Female	Ca Mau (CM)	Can Tho (CT)	Hau Giang (HG)
Ca Mau (CM)	CM × CM	CM × CT	CM × HG
Can Tho (CT)	CT × CM	CT × CT	CT × HG
Hau Giang (HG)	HG × CM	HG × CT	HG × HG

Fish rearing and sampling

The rearing of fish from larval to juvenile stages was divided into two stages as follows.

Larval rearing

Three days post-hatch (DPH), larvae from the nine crosses were randomly stocked in 50-L tanks (1000 larvae per tank) and six replicates. Larvae were fed with *Moina* sp. twice a day for the first 10 days. Then, commercial feed (40 % crude protein) was added to combine with *Moina* until they could utilise 100 % artificial feed. After 40 days, the fingerlings were transferred to 500-L tanks and reared for 2 months to reach the juvenile size.

Juvenile rearing

Nine groups of fish with four replicates each were reared in a recirculating system of 36 tanks. Three hundred fish were stocked in rearing tanks and fed satiation with a commercial diet containing 43 % crude protein four times a day. After 2 months, fish from each cross were collected for an immune assay and pathogen challenge. The cross of HG × CT had low survival rates and was not used for health evaluation.

Fish sampling for health evaluation

Forty-five fish from each of 8 crosses with relatively similar sizes (mean weight 4.5–6.1 g and total length 8.9–9.3 cm across treatments) were chosen and anaesthetised with 100 mg.L^{-1} benzocaine. The fish were then bled from the caudal vein using 1.0 mL syringes. For plasma isolation, blood samples were left for 4 h at 4 °C, centrifuged at 5000 ×g for 10 min, and the supernatant was retained as plasma. Plasma samples were stored at -80 °C before further analyses. All samples were analysed for immune parameters, including counts of white blood cells (WBCs) and red blood cells (RBCs), phagocytic activity,

and lysozyme activity.

Thirty fish from each cross mentioned above were challenged with *A. hydrophila* for evaluation of bacterial resistance.

Haematological and immunological variables

Selected blood cells parameters and indicators of humoral immunity were analysed.

Red blood cell (RBC) counting

Total RBCs were counted on a Neubauer haemocytometer after staining with Natt-Herrick solution (Natt and Herrick, 1952). First, 10 µL of each blood sample was diluted into 1990 µL of Natt and Herrick's solution and mixed gently for at least 3 min. The cell suspension was placed into the chamber and allowed to settle for 2–3 min before initiating the count under the light microscope. The RBCs were counted in 5 of the 25 small areas.

White blood cell (WBC) counting

A small drop of the whole blood was smeared on a microscope slide using a smearing slide (Cover glasses 24 x 50, Germany). The slide smear was dried quickly, fixed in methanol (95 %, M1775, Sigma) for 1-2 min, and stained with Wright-Giemsa (Rowley, 1990). Total WBC count was determined following Correa et al. (2017).

Phagocytic activity

Phagocytic activity was assayed following the method of Siwicki and Anderson (1993) with slight modifications by Soltanian and Fereidouni (2016). Briefly, 100 µL of *Saccharomyces cerevisiae* was added to 100 µL of blood samples in a 1.5 mL tube. Then, the mixture was incubated at 28 °C for 30 min after thorough mixing in the tube. After incubation, the tube was mixed gently, and 30 µL of this suspension was smeared on the glass slide. The smeared slides were air-dried and fixed with ethanol for 1 min and stained with Giemsa. The phagocytic cells were counted under the microscope. Phagocytic activation (PA) was determined by enumerating 100 phagocytes per slide. The mean PA in each slide was calculated by dividing the number of phagocytic cells with engulfed bacteria by the number of phagocytes and multiplying by 100.

Lysozyme assay

The lysozyme assay protocol was adapted from Ellis (1990) and Milla et al. (2010). Briefly, 10 µL of plasma was mixed with 130 µL of lyophilised *Micrococcus lysodeikticus* (Sigma) suspension in phosphate buffer (pH 6.2) in 96-well microplates. The difference in absorbance at 450 nm was monitored between 0 and 5 min and used to calculate lysozyme activity in units. One unit represents the amount of lysozyme that caused a 0.001 decrease in absorbance.

Challenge experiment

The challenge experiment was set up with nine treatments, including fish from eight crosses that were immersed with 10^5 CFU mL^{-1} of *A. hydrophila* and one treatment as a negative control using 30 fish from eight crosses. Each treatment was triplicated using 30 fish and 10 fish stocked per tank. The mortality and clinical signs of fish were recorded daily for 14 days after the challenge test. The head kidney was collected from moribund fish for bacterial confirmation. After 3 days of challenge test, blood samples of fish were collected to analyse cortisol levels in plasma. Plasma cortisol was analysed using a cortisol ELISA kit (DRG Instruments GmbH, Germany) and following the manufacturer's instructions.

Statistical analysis

The data were expressed as mean ± standard deviation (SD). Statistical data analysis involved one-way analysis of variance (ANOVA) followed by Tukey's posthoc multiple comparison tests. The level of significance is expressed as $P < 0.05$.

Results

RBCs and WBCs counts

The RBCs of bighead catfish were not significantly different, except in the CT × CM crossbreed. They ranged between 2.55 ± 0.31 to 3.14 ± 0.14 × 10^6 cells mm^{-3} (Table 2). The number of RBCs in the CT × CM crossbreed (3.14 ± 0.14 × 10^6 cells mm^{-3}) was highest and significantly different from that of the CM × CT crossbreed and HG × HG parental cross (2.55 ± 0.31 and 2.6 ± 0.15 × 10^6 cells mm^{-3}, respectively) ($P < 0.05$).

Table 2. Red and white blood cell counts of eight crosses from three bighead catfish *Clarias macrocephalus* broodstock obtained from Ca Mau (CM), Can Tho (CT), and Hau Giang (HG).

Crossbreeds	Red blood cells (× 10^6 cells mm^{-3})	White blood cells (× 10^5 cells mm^{-3})
CT × CT	2.91 ± 0.15^{ab}	2.04 ± 0.15^{b}
CT × CM	3.14 ± 0.14^{b}	2.09 ± 0.11^{b}
CT × HG	2.86 ± 0.24^{ab}	1.74 ± 0.26^{a}
CM × CM	2.93 ± 0.16^{ab}	1.82 ± 0.19^{ab}
CM × CT	2.55 ± 0.31^{a}	1.75 ± 0.22^{a}
CM × HG	2.80 ± 0.19^{ab}	1.64 ± 0.18^{a}
HG × HG	2.60 ± 0.15^{a}	1.84 ± 0.21^{ab}
HG × CM	3.01 ± 0.40^{ab}	1.96 ± 0.22^{ab}

Values are means ± SD of different crossbreeds (n = 45). Within the columns, values with the same superscripted letters are not significantly different ($P > 0.05$).

The WBCs of bighead catfish crosses ranged from 1.64 ± 0.18 to 2.09 ± 0.11 × 10^5 cells mm^{-3} (Table 2). The fish from CT × CT and CT × CM crosses showed higher numbers of WBCs (2.04 ± 0.15 and 2.09 ± 0.11 × 10^5 cells mm^{-3}), and were significantly different from the other crossbreeds. Fish of CM × HG showed the lowest value of WBCs (1.64 ± 0.18 × 10^5 cells mm^{-3}).

Lysozyme activity

Lysozyme activity of bighead catfish crosses ranged from 129 ± 5.07–235 ± 27.16 U mL^{-1} (Fig. 1). Lysozyme activity values of CT × CT and CT × CM were 228 ± 19.15 and 235 ± 27.16 U mL^{-1}, respectively and were significantly higher than the other crosses. Meanwhile, fish of HG × CM had the lowest value of lysozyme activity (129 ± 5.07 U mL^{-1}), which was smaller by 1.8 folds than that of CT × CM.

Phagocyte activity

Phagocyte activity of the eight crosses ranged from 43 ± 2.42 to 57 ± 2.9 %. Of which CT × CM showed higher phagocyte activity (57.0 ± 2.9 %), which was not significantly different from CT × CT (53.2 ± 2.23 %), but significantly different from the other crosses (Fig. 2).

The fish of CM × CT and HG × HG showed lower phagocyte activity of 44.2 ± 3.82 and 43.5 ± 2.42 %, respectively.

Cortisol level

After challenged with *A. hydrophila*, cortisol levels of fish from the eight crosses were ranged from 4.78 ± 1.6–12.56 ± 1.26 ng mL^{-1} (Fig. 3). Fish of CT × CT and CT × CM indicated lower values of cortisol of 5.63 ± 0.66 and 4.78 ± 1.26 ng mL^{-1}, respectively and were significantly different from the other treatments with the exception of CM × HG and HG × CM. In contrast, the cortisol of CM × CT was significantly higher compared to the other crosses.

Fish mortality after the challenge test

The bacterial challenge test induced significantly higher cumulated mortalities of 73.3 ± 5.7 % in CM × CT and 70 ± 9.8 % in CT × HG treatments, while the mortality rates were lower in other crossbreeds (40 ± 10 %–63.3 ± 5.8 %) (Fig. 4). Fish from CT × CT and CT × CM showed lower mortalities of 40 ± 10 % and 43.3 ± 5.8 %, respectively, significantly lower than other crosses, except for CM × CM and HG × CM.

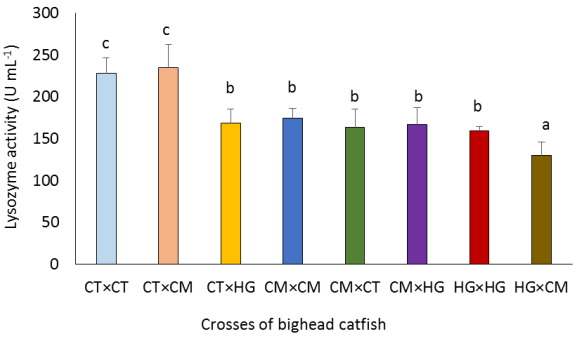

Fig. 1. Lysozyme activity of eight crosses from three bighead catfish *Clarias macrocephalus* broodstock obtained from Ca Mau (CM), Can Tho (CT), and Hau Giang (HG).

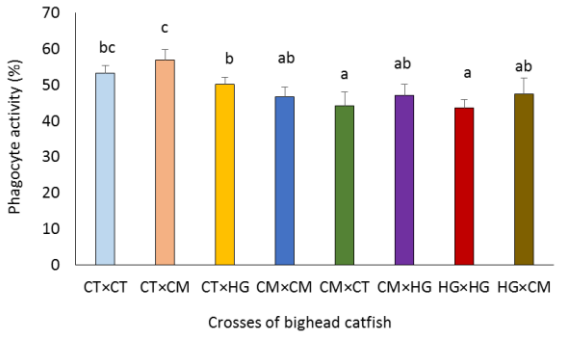

Fig. 2. Phagocyte activity of the eight crosses from three bighead catfish *Clarias macrocephalus* broodstock obtained from Ca Mau (CM), Can Tho (CT), and Hau Giang (HG).

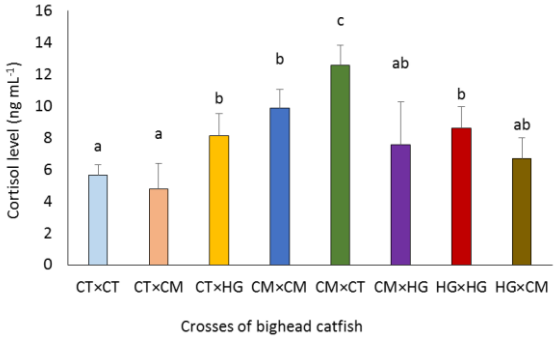

Fig. 3. Cortisol levels of eight crosses from three bighead catfish *Clarias macrocephalus* broodstock obtained from Ca Mau (CM), Can Tho (CT), and Hau Giang (HG).

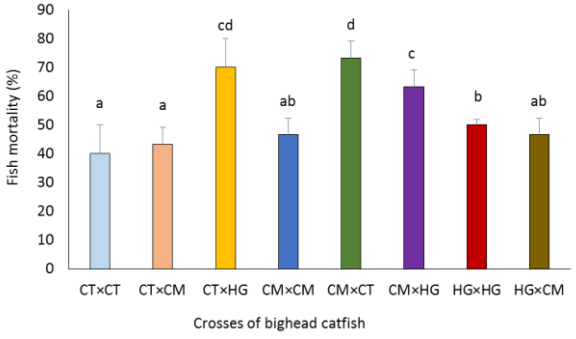

Fig. 4. Mortality in the eight crosses of bighead catfish *Clarias macrocephalus* after challenged with *Aeromonas hydrophila*.

Discussion

The physiological status of fish can be indicated by their haematological parameters (Chauhan et al., 2014). This study found that the RBC counts from the seven different crosses were quite similar, and only the crossbreed of CT × CM showed a significant increase in RBC counts compared to that of CM × CT and HG × HG crosses. The enhancement of RBC counts suggests that the blood has a high oxygen-carrying capacity, which is typical of fish capable of aerial respiration and high activity (Baleta and Bolaños, 2019), and oxygen absorption within living cells (Akinwande et al., 2016). Srisapoome et al. (2019) also determined the haematocrit of bighead catfish and reported that it was not different among 36 families in a selection program. The study addressed that the heritability of haematocrit was low and implied that it is difficult to improve this trait by selection.

White blood cells can be used as a health indicator to evaluate fish immune responses (Tavares-Dias et al., 2007). This study revealed that the WBC counts were significantly higher in CT × CT and CT × CM crosses. The domesticated strain (CT × CT) showed higher WBC counts than wild strains (CM × CM and HG × HG). The WBCs are important immune cells that attack foreign invaders or infectious pathogens. Therefore, increasing WBCs in fish indicate the enhancement of fish immune responses (Akinwande et al., 2016). The other crosses, including CT × HG, CM × CM, CM × CT, CM × HG, HG × HG, and HG × CT showed similar and normal ranges of WBC counts for bighead catfish juveniles. The results indicate the good health of the experimented fish after 3 months of rearing under tank conditions.

Phagocytes in blood present an important mechanism in fish's innate immune response with the role of preventing infectious diseases. The process involves the internalisation, killing, and digestion of invading microorganisms (Panigrahi et al., 2005). The findings described in this study illustrate an increase of phagocytic activity in crosses within domesticated (CT × CT) and domesticated × wild (CT × CM and CT × HG) populations compared to those of wild × wild and wild × domesticated crosses (CM × CM, CM × HG, CM × CT, HG × HG, and HG × CM). These results agree with the study of Savich and Vosnyj (1979). They found higher haemoglobin concentrations in the blood of crossbreeds with Amur wild common carp (*Cyprinus carpio* Linnaeus, 1758), and increased growth and survival rates (cited by Svobodova et al., 2008).

Wachirachaikarn et al. (2009) studied African catfish (*Clarias gariepinus* (Burchell, 1822)) in Thailand and observed that the crossbreeds of the two domesticated strains were genetically divergent for phagocytosis activity relating to the innate immune system, but not for survival and growth. Srisapoome et al., (2019) reported bighead catfish had low to moderate heritability values for immune traits, including bactericidal activity, lysozyme activity, and alternative complement activity (ACH50). Likewise, the improvement of phagocytic activity in the domesticated crossbreeds found in this study may be supported by the increase of WBCs, which aid the fish to fight against pathogens.

The innate immune system is the first line of defence in fish and comprises many components existent in the body before pathological infection (Magnadottir, 2006). Lysozyme is an anti-microbial protein associated with the front-line innate immunity of invertebrates. This enzyme breaks the bond in the cell wall of Gram-positive and Gram-negative bacteria (Saurabh and Sahoo, 2008; Marsh and Rice, 2010). This study indicated high levels of lysozyme activity in all crosses except the crossbreed of HG x CM. Higher lysozyme activity was found in CT × CT and CT × CM crosses and significantly differed from other crosses. In contrast, Srisapoome et al. (2019) reported a low heritability of lysozyme activity in bighead catfish and suggested that this trait has a low additive genetic variation. However, the variety of lysozyme activity may depend on the immune status of the fish at blood collection (Roed et al., 2002) and on measuring conditions such as incubation time and temperatures (Chiayvareesajja et al., 1999). Other authors have noted the genetic variation of immunological and physiological parameters and found a correlation among salmon immune responses and survival rates in challenge tests.

Some immune parameters, including lysozyme activity, the haemolytic activity of serum (Roed et al., 1993), antibody titre, and serum IgM levels (Lund et al., 1995) were recorded as heritability immune in salmon. In the present study, the enhancement of immune parameters in domesticated crossbreeds compared to wild crossbreeds can be explained by genetic adaptation to captive conditions. This highlights the complexity of the mechanisms involved in the immune response and many factors that may be associated with disease resistance.

The cortisol levels of fish were measured as stress indicators following exposure to *A. hydrophila*. The result revealed dramatically different cortisol levels among parental crosses and crossbreeds. The cortisol level was significantly higher in CM × CT crossbreed than in other crossbreeds, while levels were low in CT × CT and CT × CM. However, further information about the relationship between cortisol levels or stress indicators and crossbreeding of fish is mostly lacking in the available literature. In contrast, selection programs successfully improve the stress tolerance of fish. Fevolden et al. (1991) used a family selection method to establish two lines of high and low cortisol responses for Atlantic salmon (*S. salar*) and rainbow trout (*O. mykiss*). The offspring (F1) were vaccinated against *Vibrio anguillarum*, as a stressor, and their post-stress reactions were measured. The

stress responses, including glucose and cortisol levels, were significantly different between the two lines in F1 generation of Atlantic salmon, while there was no difference between the two lines in rainbow trout. These results support the evidence of a genetic component to the stress response. Therefore, selective breeding based on the correlation between stress, innate immunity, and disease resistance is considered an attractive method of improving disease resistance in fish (Cnaani, 2006).

The crossbreeding approach has also been applied to improve growth performance, survival rate, and disease resistance in some important aquaculture species (Odegard et al., 2011; Houston, 2017; Barria et al., 2019). The present study showed variations in crossbreeds mortalities (40–73.3 %) after being subjected to *A. hydrophila*. The results demonstrate a general trend that crosses from maternal domesticated CT (i.e., CT × CT and CT × CM, except CT × HG) showed improved disease resistance over wild × wild and wild × domesticated crosses. The crosses with better disease resistance were also found to have lower cortisol levels and higher innate immune system values (the number of RBCs and WBCs, lysozyme activity, and phagocyte activity).

Disease resistance has been a targeted trait for many genetic improvement programs (Houston, 2017). However, few publications show the successful improvement of disease resistance in *Clarias* catfish by using the crossbreeding approach, except for Prarom (1990), who reported that crosses from different strains of *C. macrocephalus* improved resistance to *A. hydrophila* infections. However, Na-Nakorn et al. (1995) reported that the resistance to *A. hydrophila* of *C. macrocephalus* did not improve by mass selection. A further study showed low heritability of resistance to *A. hydrophila* based on the binary survival (alive/dead) trait (Srisapoome et al., 2019). In the present study, bighead catfish resistance to *A. hydrophila* was possibly due to the complexity of the mechanisms involved in the immune system. Perhaps in the crossbred, the immunological parameters, including WBCs, phagocytic activity, lysozyme, and cortisol levels, function more effectively in response to pathogens. The results from this study support the use of crossbreeding to produce more resistant bighead catfish seeds, which can help mitigate and potentially control the problems associated with the pathogens.

Conclusion

The immune response of bighead catfish, *Clarias macrocephalus,* from eight crosses within and among domesticated strains (Can Tho - CT) and two wild strains (Ca Mau - CM and Hau Giang – HG) showed an enhancement to the innate immune system in the domesticated strain (CT × CT) and the crossbreed of CT × CM. These two crosses (CT × CT and CT × CM) showed an enhanced immune response through an increased white blood cells, lysozyme activity, phagocytic activity, and resistance to the pathogen *A. hydrophila*. These findings highly recommend promoting CT × CT and CT × CM crosses to help control one of the most common diseases affecting bighead catfish culture. Further studies should evaluate the advantages of crossbreeding for genetic improvement of bighead catfish with different breeding goals, such as adaptation to climate change.

Acknowledgements

This study was funded in part by the Can Tho University Improvement Project VN14-P6, supported by a Japanese Official Development Assistance (ODA) loan. The authors would like to thank Nigel K. Downes for language editing of the revised manuscript.

Conflict of interest: The authors declare that they have no conflict of interest.

Author contributions: Bui Thi Bich Hang: Conceptualisation, investigation, data analysis, writing and revising manuscript. Duong Thuy Yen: Conceptualisation, investigation, editing, funding acquisition.

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