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Motile Aeromonas Septicemia (MAS) Disease Resistance Test by *Aeromonas hydrophila* on Triploid Striped Catfish (*Pangasianodon hypophthalmus*)

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Abstract

Motile Aeromonas Septicemia (MAS) disease caused by *Aeromonas hydrophila* is a common pathogen that attacks freshwater fish commodities, including triploid as the new strain of striped catfish. This study aimed to test the resistance of triploid striped catfish to MAS infection before being released to farmers as a new commodity to increase national striped catfish production. The test was carried out for two months at the Sukamandi Fish Breeding Research Institute, Subang by injecting *A. hydrophila* into triploid and diploid striped catfish. The challenge test was carried out by injecting the cultured *A. hydrophila* with a density of 10^7 cfu.mL-1 as much as 0.1 mL.ind-1 at fish intramuscularly in all treatments, while in the Control (-) treatment, 0.1 mL of PBS solution was injected.tail-1. The test striped catfish used for each treatment were 10 individuals measuring 9.23 – 9.65 cm, and weighing 7.1 – 9.23 g. Survival parameter data and blood description were analyzed quantitatively using Microsoft Excel 2010 and SPSS version 16.0 software with analysis of variance (ANOVA) at a 95% confidence interval. The results of re-characterization with biochemical tests showed that *A. hydrophila* were gram-negative, oxidase and catalase positive, motile, could fermentatively convert sugar, and could grow in blood media. The survival of triploid striped catfish after the test was 95%, total erythrocytes on day 7, and total leukocytes on the first day were significantly different from diploid striped catfish. Thus, it can be concluded that striped triploid striped catfish are more resistant to infection with *A. hydrophila* than diploid.

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INTRODUCTION

Aquaculture production is increasingly developing, from traditional to super-intensive, to meet the community's growing protein needs. This increase in production required environmental control with the use of large amounts of synthetic chemicals to maintain the performance of farmed fish. According to Lee and Wendy (2017); Ogbonne *et al*. (2018); and Widanarni *et al*. (2020) stated that the use of these synthetic chemicals will certainly trigger the resistance of pathogenic bacteria so that the level of attack will also be more massive. High density, seed quality, and fluctuations in the water's physical and chemical parameters also impacted the level of attack by these pathogenic bacteria. The attack of infectious bacteria generally originates from *A. hydrophila* species, especially in freshwater fish (Hardi, 2018; Purkait *et al*., 2018).

According to Sutanto (2021), the commonly developed freshwater fish commodities include striped catfish, catfish, tilapia, goldfish, gourami, and several other species because they have high economic value and are considered export commodities. Specifically, for striped catfish, there was an increase in production to meet national needs by 22.25% compared to 2017 (MMAF, 2019) and export markets including Saudi Arabia as a new destination of 300 tonnes. To maintain and increase striped catfish production, it is necessary to produce superior seeds, namely triploid strains (Hartami *et al*., 2019). Triploid fish have been shown to have advantages including fast growth, being able to adapt to environmental changes, and being resistant to pathogen attack (Piferrer *et al*., 2009).

To prove the nature of triploid striped catfish strains which are suspected of being disease-resistant, it is necessary to test various *A. hydrophila* infections which commonly attack freshwater fish, including striped catfish (Nahar *et al*., 2016; Le *et al*., 2018). This test is necessary for researchers before recommending to farmers, especially striped catfish to be developed in grow-out areas. So that the striped catfish production target can be achieved optimally with a high level of profit. The purpose of this study was to test the resistance of triploid striped catfish to Motile Aeromonas Septicemia (MAS) caused by *A. hydrophila*.

METHODOLOGY

Ethical Approval

The triploid catfish seeds used are the product of the North Regional Maritime Affairs and Fisheries Service Branch, West Java Province. Meanwhile, testing was carried out at the Sukamandi Fish Breeding Research Institute. The test fish were brought via land transportation in the morning using plastic containers with an oxygen-to-water ratio of 2:1. Upon arrival at the location, the test fish were first acclimatized for 3x24 hours before testing to avoid stress and reduce factors other than the testing factors that will be carried out. After the test is complete, the test fish are quarantined and the dead are destroyed by burying to avoid the spread of disease in the aquatic environment around the test site.

Place and Time

This research was conducted for two months at the Sukamandi Fish Breeding Research Institute, Subang October – December 2022.

Research Materials

The material used in this research is triploid catfish which is a collection from the North Regional Maritime Affairs and Fisheries Service Branch, West Java Province. *A. hydrophila* suspension was obtained from the Fish Disease Control Research and Development Installation, Freshwater Aquaculture Research and

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Development Center, Bogor. Trypticase Soy Agar (CRITERION™) and Trypticase Soy Broth (MERCK) as bacterial growth media, PBS solution, distilled water, KIT API 20E (Biomerieux, France). The equipment used were an incubator (LZHZXY 220v), autoclave (Gea 35 Liter LS-35LJ), petri dish, vortex (G560 SI-0236 2 Shaker), centrifuge (Labnet), micropipette (Eppendorf) and 1 ml syringe.

Research Design

This test was carried out using a completely randomized design (CRD) which consisted of two treatments and four replications. The treatments used were triploid striped catfish seeds and diploid striped catfish seeds as shown in Table 1 below.

Table 1. Motile Aeromonas Septicemia (MAS) resistance test treatment design.

Treatments	*A. hydrophila* injection
Triploid	√
Control (-) triploid	–
Diploid	√
Control (-) diploid	–

Work Procedure

Test Fish Preparation

The test fish used were triploid striped catfish with an average length and weight of 9.65 ± 1.08 cm and 9.23 ± 0.51 g. This triploid catfish comes from a crossbreeding between female tetraploid and male diploid striped catfish at the Northern Regional Fisheries and Marine Service Branch, Cijengkol, West Java.

Preparation of *A. hydrophila* Bacterial Suspension

The preparation of the *A. hydrophila* suspension was carried out using bacterial cultures obtained from the Fish Disease Control Research and Development Installation, Freshwater Aquaculture Research and Development Center, Bogor. Recovery of bacterial virulence was carried out using the Koch Postulate test by injecting 0.1 mL of *A. hydrophila* suspension of 109 cfu.mL-1 intramuscularly into healthy striped catfish. The fish that had been injected with the bacteria were observed for clinical symptoms indicating the presence of *A. hydrophila* infection. Bacteria were then isolated from the liver and kidney of diseased fish which were then grown using the cup scratch method on Trypticase Soy Agar (TSA) media and incubated at 29°C for 18 hours. After that, re-characterization of the bacteria was carried out with biochemical tests (Cowan and Steel, 2003) and further tests using KIT API 20E (Biomerieux, France) to ensure that *A. hydrophila* caused the diseased fish.

Determination of the dose of *A. hydrophila* used for the challenge test was carried out through the LD_{50} test using *A. hydrophila* which had been cultured on Trypticase Soy Broth (TSB) media for 18 hours, injected intramuscularly into striped catfish at a density of 106 to 108 $cfu.mL^{-1}$ as much as 0.1 $mL.ind^{-1}$ fish. Survival was observed for 7 days after injection. LD_{50} calculation is based on Reed and Muench's (1938) method.

Challenge Test of *A. hydrophila*

The challenge test was carried out by injecting the bacterial culture *A. hydrophila* with a density of 107 $cfu.mL^{-1}$ as much as 0.1 $mL.ind^{-1}$ fish intramuscularly in all treatments, while in the Control treatment injected (-) 0.1 mL.ind-1 intramuscularly of PBS solution. 10 fish were used per treatment repetition. Changes that occur are observed and recorded for 14 days. After that, re-characterization of the bacteria was

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carried out with biochemical tests (Cowan and Steel 2003) and further tests using KIT API 20E (Biomerieux, France) to ensure that *A. hydrophila* caused the post-challenged sick fish.

Sampling

A sampling of striped catfish blood images was carried out at the beginning (D-0), namely before the challenge test, and on days 1, 7, and 14 after being challenged with *A. hydrophila*. The blood picture sampling of striped catfish was carried out at the beginning (H-0), namely before the challenge test.

Survival Rate

The survival rate of the striped catfish was calculated on the 14th day after being challenged with *A. hydrophila* based on Rejeki *et al*. (2019) using the following formula:

$$SR\ (\%) = \frac{Nt}{No} \times 100$$

Descriptions:

SR = Survival rate (%)
Nt = Number of fishes at the end of the observation (individual)
No = Number of fishes at the beginning of observation (individual)

Post-Challenge Blood Picture of *A. hydrophila*

Blood sampling of striped catfish was carried out for blood picture parameters which were carried out at the beginning (D-0) before the challenge test and on days 1, 7, and 14 after being challenged with *A. hydrophila*. The observed blood images were total erythrocytes and total leukocytes. For total erythrocytes, the measurement was carried out according to Blaxhall and Daisley (1973) by sucking blood using a red grain pipette up to a scale of 0.5 and then diluting it with Hayem's solution up to a scale of 101. Both ends are closed parallel and then shaken to form a number 8 for 3-5 minutes. After that, the first drop of blood was discarded and then the blood was dripped into the hemocytometer and covered with a cover glass. The number of red blood cells can be calculated using the following formula.

$$\sum \text{erythrocytes (cell.mm}^{-3}) = \sum \text{counted cells} \times \frac{1}{\text{chamber volume}} \times \text{dilution factor}$$

While total leukocytes, measurements were made according to Blaxhall and Daisley (1973) by sucking blood using a pipette containing grains up to a scale of 0.5 then adding Turk's solution up to a scale of 11, and stirring for 3-5 minutes with a figure eight motion. After that, the first two drops were discarded. Blood was dripped on a hemocytometer and covered with a covered glass to be observed and counted for the number of white blood cells under a microscope. The following is a formula for calculating the number of white blood cells.

$$\sum \text{leukocytes (cell.mm}^{-3}) = \sum \text{counted cells} \times \frac{1}{\text{chamber volume}} \times \text{dilution factor}$$

Clinical Symptoms

The clinical symptoms observed included changes in fish behavior such as changes in swimming patterns, feeding behavior, and microscopic changes in the anatomy of external organs and internal organs (Hardi, 2018).

Data Analysis

Survival parameter data and blood counts were analyzed quantitatively using Microsoft Excel 2010 and SPSS version 16.0 software with analysis of variance (ANOVA) at 95% confidence intervals. If there are differences between treatments, then the analysis is continued with Duncan's test. Parameter data of clinical symptoms were analyzed descriptively.

RESULTS AND DISCUSSION

Characterization of Bacteria

Results of re-characterization with biochemical tests of *A. hydrophila* after recovery of virulence are presented in Table 2.

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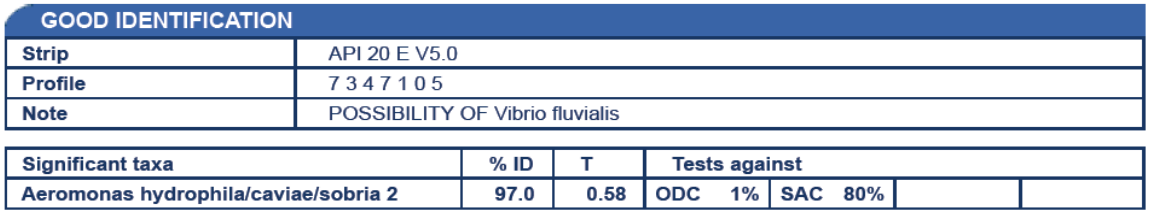
Table 1. Re-characterization of *A. hydrophila*.

Characterization	*A. hydrophila*
Gram +/-	-
Oxidase	+
Catalase	+
Motility	+
Oxidative/Fermentative	Fermentative
Blood Media	+

Based on the data in Table 2, it can be seen that the results of the re-characterization of *A. hydrophila* with biochemical tests showed that *A. hydrophila* are Gram-negative, oxidase and catalase positive, motile, can fermentatively break down sugar and can grow in blood media. The results of the re-characterization using the API 20E KIT follow-up test of *A. hydrophila* after recovery of virulence are presented in Figure 1.



GOOD IDENTIFICATION	
Strip	API 20 E V5.0
Profile	7 3 4 7 1 0 5
Note	POSSIBILITY OF Vibrio fluvialis

Significant taxa	% ID	T	Tests against			
Aeromonas hydrophila/caviae/sobria 2	97.0	0.58	ODC 1%	SAC 80%		

Figure 1. API 20E KIT advanced test results.

Based on the data in Figure 1, shows that the bacteria that cause fish to get sick in the Koch Postulates test, namely 97%, is *A. hydrophila*. Following the characteristics of the bacteria reported by Mangunwardoyo *et al*. (2010); and Nahar *et al*. (2016) using biochemical tests stated that *A. hydrophila* was gram (-), oxidase (+), catalase (+), motile, able to ferment sugar and live in blood media. Camus *et al*. (1998) stated that *A. hydrophila* belongs to a facultative bacterium that can utilize nutrients found in water so that it can survive for long periods without a host. The same characteristics of fish infected with *A. hydrophila* were described by Menanteau-Ledouble *et al*. (2016), and Kusnadi *et al*. (2019) using a biochemical test method. While the application of the Koch Postulate test aims to determine the species that infect the test biota when the pathogenicity test research is carried out (Suada and Suniti, 2014; Mangunwardoyo *et al*., 2010; Salem *et al*., 2020; Setiadi and Wadjdy, 2021).

Research related to testing the resistance of triploid and diploid fish to pathogen infections has been carried out by several researchers, especially on salmon. However, the specific resistance of triploid catfish to MAS pathogen infection may be limited in this study. In theory, increasing the number of chromosomes in fish will have a positive correlation with increasing the body's immune response in resisting pathogen attacks. This also depends on the fish species, rearing environment, nutritional quality of feed, and type of infecting pathogen (Weber *et al*., 2013; Dixon *et al*., 2016; Chalmers *et al*., 2016). Meira *et al*., (2023) stated that the phenotype of diploid *Astyanax lacustris* fish is the same as triploid fish, so there may be no differences regarding resistance to infection by the same pathogen.

Lethal Dose 50% (LD_{50})

The results of observations on the LD50 test of *A. hydrophila* in striped catfish are presented in Table 3.

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Table 3. Calculation results of the LD50 test for *A. hydrophila* in striped catfish for 7 days.

Density (cfu/mL)	Dead	Survive	Death ratio	Accumulation			
				Dead	Survive	Death ratio	% of Death
10^8	13	1	13/14	22	1	22/23	95.65
10^7	8	6	8/14	9	7	9/16	56.25
10^6	1	13	1/14	1	20	1/21	4.76

Based on the data in Table 3, it can be seen that the number of bacteria injected in the striped catfish challenge test with a density of 10^7 cfu.mL-1. To get the right dose in the bacterial infection challenge test stage, an LD50 experiment was carried out (Rejeki *et al*., 2016). The duration of the test is 7 days or LD50 for 96 hours, while the in vivo test is carried out for 14 days (Setiaji *et al*., 2009). The results of determining the LD50 of *A. hydrophila* infection in African catfish were 105 cfu/ml (Setiaji *et al*., 2009; Wulandari *et al*., 2014 and Rejeki *et al*., 2016), up to 106 cfu/ml (Triyaningsih *et al*., 2014), in snakehead fish of 10^5 cfu/ml (Olga, 2012). This shows that triploid striped catfish require a higher dose of 107 cfu/ml to reach the LD_{50}.

Clinical Symptoms

Clinical symptoms observed in triploid and diploid striped catfish as a result of a challenge test with *A. hydrophila* were decreased appetite, slow swimming movements, inflammation (Figure 2a), and necrosis on the back of the fish (Figure 2b), and hemorrhage in the kidneys and liver (Figure 2a) Figure 2c).

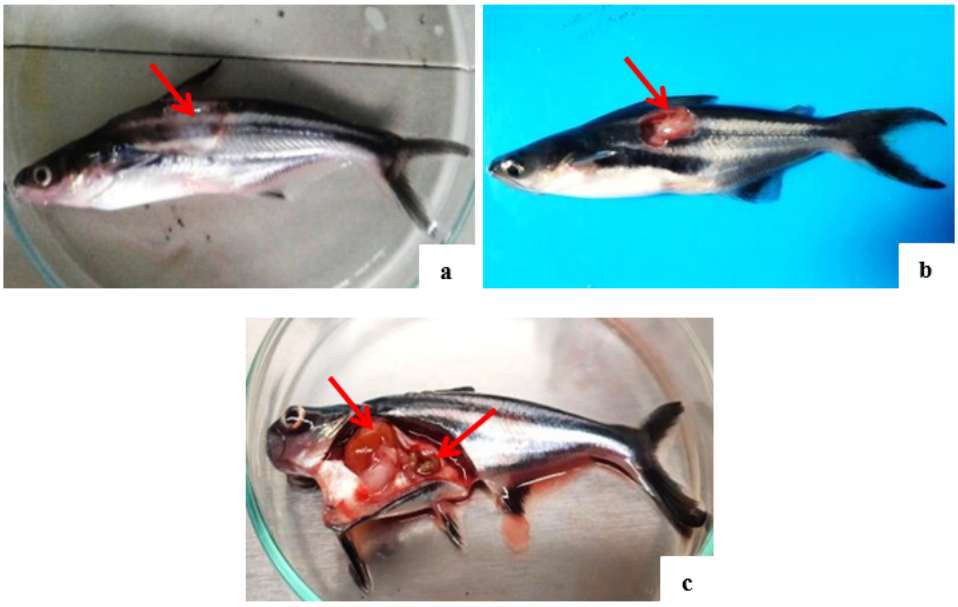

Figure 2. Clinical symptoms of triploid striped catfish after being challenged by *A. hydrophila*
Description : (a) inflammation, (b) necrosis, (c) hemorrhage of the liver and kidneys.

Fish infected with *A. hydrophila* until day 7 of the challenge test will show symptoms of changes in swimming patterns, changes in anatomical pathology, decreased blood hemoglobin levels, and death (Hardi, 2018). There is a decrease in appetite, organ damage, peeling skin, and ulcers (Wulandari *et al*., 2014; Triyaningsih *et al*., 2014; Wamala *et al*., 2018) wounds on the injection site, enlarged abdomen, protruding eyes, flaky fins, and liver and kidney damage (Weir *et*

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al., 2012; Rejeki *et al*., 2016), besides that blood clots were also found in the internal organs of fish (Mangunwardoyo *et al*., 2010; Nahar *et al*., 2016).

Survival Rate

The survival rate of triploid and diploid striped catfish after *the A. hydrophila* challenge after 14 days of observation is presented in Figure 3.

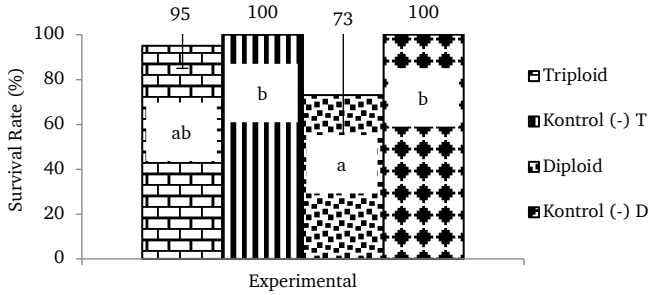

Figure 3. Survival of triploid and diploid striped catfish challenged by *A. hydrophila*. Different letters in the diagram indicate significantly different values ($p<0.05$).

Based on the data in Figure 3, it is known that the Diploid treatment was significantly different ($p<0.05$; 75% survival) from the Control (-) T and D treatment, but not significantly different ($p>0.05$) from the Triploid treatment. Whereas the Triploid treatment was not significantly different ($p>0.05$; 95% survival) from the Control (-) T and D treatment.

The first death of striped catfish treated with triploid occurred on the 4th day after being challenged with *A. hydrophila*, with two individuals. After that, for 14 days of observation, there was no death (Figure 4). Whereas in the Control (-) T treatment there was no death until the last day of observation.

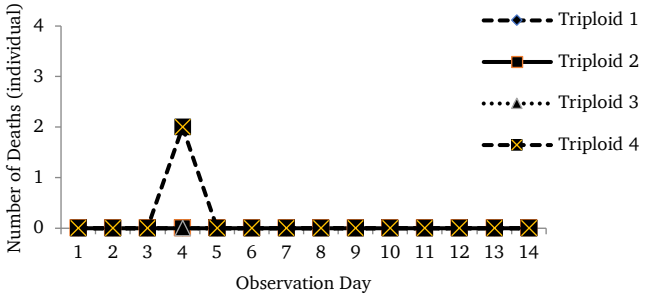

Figure 4. Mortality pattern after the challenge of triploid striped catfish with *A. hydrophila*.

The death of the striped catfish treated with Diploid treatment first occurred on day 4 after being challenged with *A. hydrophila*, namely 1 in the Diploid 1 treatment. Death continued until day 6 after being challenged with *A. hydrophila* (Figure 5). Whereas in the Control (-) D treatment there was no death until the last day of observation.

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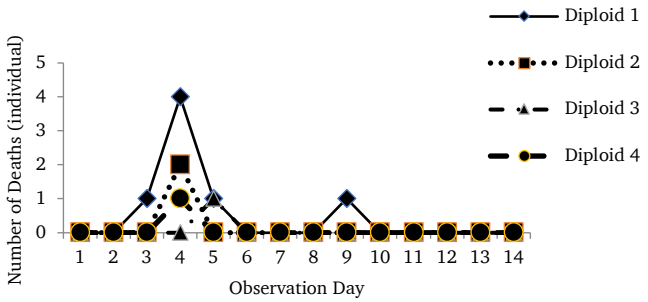

Figure 5. Mortality pattern after post-challenge diploid treatment of *A. hydrophila*.

The occurrence of death in test fish infected with *A. hydrophila* depended on the length of time the exposure was carried out (Ikpi and Offem, 2011; Walczak *et al*., 2017; Le *et al*., 2018; and Kusnadi *et al*., 2019). Most of these deaths occur due to injuries and dysfunctions in fish's external and internal organs infected with pathogenic bacteria as described in the previous clinical symptoms. The mortality rate of fish due to infection with *A. hydrophila* bacteria ranges from 50-100% (Olga, 2012; Agustina *et al*., 2017), 0-100% (Wulandari *et al*., 2014), 80-100% within 2 weeks (Christy *et al*., 2019), 10 - 70% for 2 weeks (Zubaidah *et al*., 2019). This shows that the virulence level of these bacteria in infected fish is very high.

Blood Appearance

Data on the total erythrocytes of triploid and diploid striped catfish at baseline (H-0) and after being challenged by *A. hydrophila* on days 1, 7, and 14 are presented in Table 4.

Table 4. Total erythrocytes of triploid and diploid striped catfish challenged by *A. hydrophila*.

Treatments	Total Erythrocytes ($\times 10^6$ sel.mm^{-3})			
	Day-0 (beginning)	Post-challenge Day		
		1	7	14
Triploid	1.90 ± 0.00^{b}	1.13 ± 0.25^{a}	1.40 ± 0.42^{b}	1.30 ± 0.45^{ab}
Control (-) Triploid	1.90 ± 0.00^{b}	1.70 ± 0.00^{b}	2.10 ± 0.00^{c}	1.00 ± 0.00^{a}
Diploid	1.20 ± 0.00^{a}	1.08 ± 0.39^{a}	0.83 ± 0.22^{a}	1.20 ± 0.62^{ab}
Control (-) Diploid	1.20 ± 0.00^{a}	1.50 ± 0.00^{b}	1.70 ± 0.00^{b}	1.80 ± 0.00^{b}

The data show the mean ± standard deviation of total erythrocytes ($\times 10^6$ cells.mm^{-3}). Different superscript letters in the same column are significantly different ($p<0.05$).

The data in Table 4 shows that the total erythrocytes at the start before the challenge test, the Triploid treatment, and Control (-) T were significantly different ($p<0.05$;1.90 ± 0.00 × 106 cells.mm-3) with the Diploid and Control (-) D (1.20±0.00 ×106 sel.mm-3). On day 1 post-challenge, the Triploid and Diploid treatments were significantly different from the Control (-) T and D treatments. Furthermore, on the 7th day post-challenge the Triploid treatment was significantly different from the Diploid treatment, but not significantly different from Control (-) D. Whereas on the 14th-day post-challenge, the triploid and triploid treatments were not significantly different ($p<0.05$).

Data on the total leukocytes of triploid and diploid striped catfish at baseline (H-0) and after being challenged with *A. hydrophila* on days 1, 7, and 14 are presented in Table 5.

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Table 5. Total leukocytes of triploid and diploid striped catfish after the challenge of *A. hydrophila*.

Treatments	Total Leukocytes ($\times 10^4$ sel.mm^{-3})			
	Day-0 (beginning)	Post-challenge Day-		
		1	7	14
Triploid	0.40±0.00^{b}	1.23±0.43^{c}	1.05±0.30^{b}	0.60±0.24^{b}
Control (-) Triploid	0.40±0.00^{b}	0.40±0.00^{ab}	0.10±0.00^{a}	0.20±0.00^{a}
Diploid	0.30±0.00^{a}	0.68±0.15^{b}	0.80±0.24^{b}	0.70±0.12^{b}
Control (-) Diploid	0.30±0.00^{a}	0.10±0.00^{a}	1.10±0.00^{a}	1.00±0.00^{c}

The data show the mean ± standard deviation of total leukocytes ($\times 10^4$ cells.mm-3). Different superscript letters in the same column are significantly different ($p<0.05$).

The data in Table 5 shows that the total leukocytes at baseline before the challenge test, the Triploid treatment and Control (-) T were significantly different ($p<0.05$; 0.40 ± 0.00 × 104 cells.mm-3) with the Diploid and Control (-) D (0.30±0.00 ×104 sel.mm-3). On day 1 post-challenge, the Triploid treatment (1.23 ± 0.43) showed the highest score and was significantly different from the other treatments ($p <0.05$). Whereas on the 7th-day post-challenge, the total leukocytes of the Triploid and Diploid treatments were not significantly different ($p>0.05$) but significantly different from the Control (-) T and D treatments. Furthermore, the 14th day after the challenge showed a decrease in the total leukocytes of striped catfish in all treatments.

The impact of infection with *A. hydrophila* can be observed from the picture of fish blood because it is one of the factors of the immune system such as total leukocytes, total erythrocytes, differential leukocytes, hematocrit, and hemoglobin (Nahar *et al*., 2016; and Hardi, 2018). Total erythrocytes tended to decrease after being challenged due to the large number of blood cells that broke down/lyzed due to the presence of hemolysin produced by *A. hydrophila* compared to the ability of fish to produce new red blood cells (Mangunwardoyo *et al*., 2010). However, the opposite occurred in leukocyte cells which increased post-challenge due to their role as a non-specific immune system to localize and eliminate incoming pathogens as a characteristic of the first phase of infection, stress, and leukemia (Wulandari *et al*., 2014). An increase in the total number of leukocytes and a decrease in the total number of erythrocytes also occurred in this study, which began on the first day to the seventh day after the challenge test. However, it tended to be stable on the fourteenth day which indicated that the triploid striped catfish were able to control the infection until it recovered.

CONCLUSION

Based on the observations of several parameters of the study conducted, it can be concluded that triploid striped catfish are more resistant to *A. hydrophila* infection. Clinical symptoms of infection caused by A. hydrophila in test fish can be seen from inflammation, necrosis, and hemorrhage of the liver and kidneys. Judging from the increase in the total number of erythrocytes and leukocytes as an illustration of the immune response, both diploid and triploid striped catfish infected by *A. hydrophila* showed no differences. Therefore, further research is needed to understand why triploid striped catfish have a better survival rate of 22% difference compared to diploids.

CONFLICT OF INTEREST

The authors of this article declare that there is no conflict of interest among all authors upon writing and publishing the manuscript.

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AUTHOR CONTRIBUTION

Each author has contributed as follows; Prama Hartami designed the research and prepared test biota in the form of triploid and diploid catfish, Eva Ayuzar and Salamah analyzed the data obtained, Lilis Nurjannah carried out the necessary tests and collected data, Odang Carman, Alimuddin, and Muhammad Fakhri corrected and revised the manuscript so that it was suitable for scientific writing, Muhammad Rafi as a field officer who assisted with the activities required during the research.

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