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Response of juvenile Danube sturgeon (*Acipenser gueldenstaedtii*) to *Aeromonas hydrophila*: Histopathological and hematological findings

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Abstract

The Danube sturgeon (*Acipenser gueldenstaedtii*), classified as an endangered species, is susceptible to pathogenic microorganisms in both aquaculture systems and natural habitats, potentially leading to fatal infections. This study investigated the physiological and pathological responses of juvenile Danube sturgeon to *Aeromonas hydrophila* infection. Fish were intraperitoneally injected with 0.1 mL of A. hydrophila suspension (1×10^8 CFU/mL). The experiment was conducted with three replicates per group (infected and control), each containing 10 fish. Based on the challenge test results, mortality in the infected group began on day 3 and continued progressively until day 13. Additionally, swim bladder deflation, hyperemia, and hemorrhaging in the visceral organs were observed. Hematological analysis revealed that LYM, HGB, MCH, and MCHC values were significantly higher in the control group, whereas WBC and MCV levels were elevated in the infected group. Histopathological examination of liver tissues revealed prominent melanomacrophage centers and signs of necrosis. According to a semi-quantitative scoring model, regressive changes and inflammation were significantly higher in the infected group compared to controls ($p < 0.01$). In conclusion, *Aeromonas hydrophila* demonstrates a highly pathogenic potential for juvenile Danube sturgeon, inducing rapid systemic deterioration and significant tissue damage. The findings confirm that this bacterium is capable of causing disease and lethal outcomes within a week, emphasizing the urgent need for preventive strategies in conservation and aquaculture management of this endangered species.

Keywords: Challenge test, Fish, Hematology, Histopathology, Pathogen microorganism.

1. Introduction

Aquaculture meets the protein food needs of countries with a constantly increasing growth rate since the 1970s (De Schryver *et al.*, 2008). Therefore, aquaculture and fisheries activities need to be improved in order to alleviate poverty, famine and malnutrition caused by the ever-increasing world population (Minaz and Kubilay, 2021). In this context, all biotic and abiotic impacts that

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may negatively affect fish physiology should be reduced (F. Zhang *et al.*, 2016). Biotically, there is a vital stress process that leads to disease and subsequent death of fish. Some types of bacteria, which are biotic stressor, are essential for the balance of nature without directly making fish disease. However, 125 bacterial species belonging to approximately 34 different families have been reported to cause fish diseases around the world (Öztürk and Altinok, 2014). The number of bacterial species that cause fish diseases is increasing day by day (Austin and Austin, 2016).

Stress caused by biotic and abiotic factors makes fish vulnerable to pathogenic microorganisms. Even when acting as a primary pathogen, *Aeromonas hydrophila* is often reported as a secondary invasive species (Harikrishnan and Balasundaram, 2005). The availability of primary infection is required to infect secondary pathogens that have a limited invasive capacity. *A. hydrophila* is generally considered a secondary pathogen that infects an already infected fish (Snieszko, 1974). *A. hydrophila* causes various diseases such as bacterial hemorrhagic septicemia, ulcerative conditions, abdominal distensions, fin/tail rot, exophthalmia, and epizootic ulcerative syndrome in stressed fish in freshwater (Shao *et al.*, 2004). It has a negative economic impact on the aquaculture industry worldwide as it causes various complications in cultured fish (Nayak, 2020).

There is always a need for rearing alternative species to ensure the continuity of aquaculture. In addition to the species currently cultured in general, interest in alternative species is also increasing. In this context, the fact that sturgeon is both in danger of extinction and has high economic added value makes it valuable for aquaculture (Xu *et al.*, 2023). Sturgeons are an interesting species for the ultimate consumer because they have high meat quality and valuable caviar (Song *et al.*, 2022). However, its late maturity stage and long cultivation period are worrying for aquaculture facilities. Because in this period, it is necessary to cope with abiotic and biotic stress factors (Minaz *et al.*, 2024a; Minaz and Kurtoğlu, 2024). It is possible that sturgeons may encounter pathogens such as *A. hydrophila* not only in culture facilities but also in wild nature. Therefore, the effect of pathogenic microorganisms on sturgeon will mobilize local governments. To date, there is no comprehensive experimental infection study focusing on *A. hydrophila* in Danube sturgeon (*Acipenser gueldenstaedtii*), a critically endangered species. This study is the first to simultaneously evaluate the mortality rate, histopathological alterations, and hematological responses following a controlled *A. hydrophila* challenge in this species. By addressing this gap, our research not only enhances understanding of the pathogen's impact on a conservation-priority species but also provides a reference framework for disease control strategies in aquaculture.

Accordingly, this study aimed to assess the susceptibility of juvenile Danube sturgeon to *Aeromonas hydrophila* infection by evaluating mortality rates, histopathological changes, and hematological parameters. We hypothesized that infected fish would exhibit significantly higher mortality and marked alterations in hematological and liver histopathological indicators compared to controls, confirming the pathogenic potential of *A. hydrophila* in this vulnerable species.

2. Material and Method

2.1. Experimental design and bacterial strain

Danube sturgeon juveniles (52.4 ± 1.8 g) were obtained from the Aquaculture Application and Research Center of Recep Tayyip Erdoğan University (Rize, Türkiye). All fish used in the experiment, including both the control and infected groups, were selected from the same stock tank to ensure uniformity. Importantly, the individuals used in this study were experimentally produced in our facility from a single broodstock origin, meaning that they share the same parental lineage. This controlled origin minimizes the likelihood of genetic variation in disease resistance, ensuring that observed differences are attributable to experimental treatment rather than heritable immunological traits. Prior to the experiment, the health status of the fish population was assessed by randomly sampling ten individuals from the stock tank and performing parasitological examinations using swab analysis and bacteriological screening via culture techniques to confirm the absence of external parasites, fungal, and bacterial infections. The experiment was conducted in the toxicology laboratory of the Faculty of Fisheries. A total of 60 fish were used, divided equally into two groups: the infected group (n = 30) and the control group (n = 30), each with three replicates containing 10 fish per tank. The fish were acclimatized in the experimental tanks for 15 days prior to the challenge test, and feeding was discontinued two days before the trial. Throughout both the adaptation and trial periods, 50% of the tank water was replaced daily. The trial was conducted under a 12 h light/12 h dark photoperiod in 100 L fiberglass tanks (⌀: 40 cm). Behavioral changes and mortality were monitored and recorded regularly. At the end of the experiment, pathological changes in the visceral organs were examined. Since fluctuations in water quality parameters can influence the growth and virulence of A. hydrophila, the following parameters were monitored throughout the study: temperature = 20.1 ± 0.9 °C, pH = 7.6 ± 0.6, and dissolved oxygen (DO) = 8.42 ± 1.14 mg/L.

In the current challenge study, *Aeromonas hydrophila* (National Center for Biotechnology Information (NCBI) accession number: MT730016.1) isolated from rainbow trout (*Oncorhynchus mykiss*) fry in fish disease laboratory was used as pathogenic bacteria. The bacteria were stored at -80 ºC in Tryptic Soy Broth (TSB, Merck) containing 30% (v/v) glycerol until experiments commenced. The 16S rRNA region was utilized for the molecular identification of the bacteria. PCR reaction was performed on the genomic DNA of the bacteria using specific primers (27 Fwd 5'-AGA GTT TGA TCC TGG CTC AG-3', 1492 Rev 5'-GTT TAC CTT GTT ACG ACT T-3'). The bacteria were subcultured at 20 ± 2 ºC for 24 hours on Tryptic Soy Agar to check for purity. Subsequently, pure colonies were inoculated into TSB and incubated for 24 hours at 20 ± 2 °C. The bacteria were then centrifuged at +4 ºC at 6000 RPM and adjusted to a 0.5 McFarland turbidity using sterile physiological saline solution (PSS). The concentration of the bacterial suspension was measured as 2.7×10^8 CFU mL^{-1} using Plate Count Agar (PCA) prior to fish infection. Bacterial suspension (0.1 mL) was injected into the fish via the intraperitoneal route using sterilized insulin syringes (Köse *et al.*, 2023). To eliminate any injection-related bias, fish in the control group were administered 0.1 mL of sterile physiological saline solution (PSS) using the same route and equipment. Both groups were handled under identical conditions, including environmental parameters, tank size, feeding regimen, and sampling protocols. This design ensured that any observed differences in response were attributable solely to the presence or absence of A. hydrophila rather than the injection procedure itself.

2.2. Hematological assessment

At the end of the challenge period, five fish were selected from each groups for hematological analysis at the end of challenge period. Fish were anesthetized with chamomile oil based on our latest procedure (Ak *et al.*, 2022). A 2.5 mL syringe were used to take blood samples from the caudal vein of fish. Blood samples were stored into the EDTA K3 tubes until analyzed. The erythrocyte (RBC), leukocyte (WBC), hematocrit (HCT), and hemoglobin (HGB), mean corpuscular volume (MCV) mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) in each blood sample were analyzed by an automatic hematological assay (Prokan6800VET). The calibration of automatic hematological assay was maintained according to fish blood before study (Er *et al.*, 2024).

2.3. Histological examination

A total of three fish in each group were randomly selected and euthanized by clove oil at the of study. Liver tissues were sampled for histological examination. Then, samples were placed in the neutral buffer solution (10%) for fixation. After 24 hours, samples were transferred to the ethanol (50%) and stored in the room temperature until analyzed. Samples were washed with water to remove ethanol and then treated by alcohol (80%, 90%, and 99.9%, respectively) and xylene (99.9% for 2 times) series. After exposure of alcohol and xylene series, liver samples were kept in paraffin one night (about 8-10 hours) at +65 °C. Tissues were submerged and blocked into the paraffin in the last step before staining. Paraffin-blocked tissues were cut at 5 µm thickness with a microtome and placed on the slides. Slides were stored at +65 °C during about 10-12 hours for removal of paraffin. In the staining stage, slides were treated again by alcohol (96% and 99.9% during 1 min), and stained with hematoxylin and eosin series then kept into the xylene (99.9% for two times) series. After this staining stage, slides were covered by an Entellan™ and cover-slip. Finally, slides were examined a light microscope (Minaz *et al.*, 2024b). In addition to qualitative observations, a semi-quantitative scoring system was applied to evaluate histopathological alterations based on a method (Bernet *et al.*, 1999) (Table 1). Histopathological changes were grouped into five categories: circulatory, regressive, progressive, inflammatory, and neoplastic alterations. In this study, only regressive changes and inflammation alterations were observed and scored. Each lesion was assigned an importance factor (ranging from 1 = minimal to 3 = marked), and a lesion score on a scale from 0 (no change) to 6 (very severe). The final score for each lesion was calculated by multiplying the importance factor by the lesion score. For each tissue, the total score for a reaction pattern was obtained by summing the values of all related lesions. This approach allowed for a more objective and quantitative assessment of tissue damage resulting from *A. hydrophila* infection.

Table 1. Semi-quantitative histopathological evaluation system used for liver tissue.[a]

Organ	Reaction pattern	Alteration	Importance factor	Score value	Index
Liver	Regressive changes	Necrosis	IF_1= 3	SV_1= 0-6	LI_{RC}
	Inflammation	Activation of RES	IF_2= 1	SV_2= 0-6	LI_I

[a] The importance factor (1–3) reflects the affected organ, type of reaction pattern, and nature of the alteration. The score value represents the severity of each alteration, rated on a Likert scale from 0 (no change) to 6 (very severe).

2.4. Statistical analysis

All data are presented as the means ± standard deviation (SD). It was decided whether the hematological data sets showed normal distribution according to the Kolmogrow Smirnov test. Student-t test and Mann-Whitney U test were applied in comparison of control and treatment group for parametric and non-parametric distribution, respectively. Differences were considered statistically significant when the calculated p value was <0.05. All analyses were performed in SPSS software (Version 23, IBM Corp., Armonk, New York, USA).

3. Results

3.1. Survival rates and pathological observation

The challenge test stopped on day 13 due to all individuals died in the infected group. At the end of the study, the survival rate in the control group was over 90% (Figure 1). In the infected group, the first lost was observed on day 3 and mortality gradually increased thereafter. In contrast, first dead in control group was maintained on day 8. Pathological observations in freshly dead fish on the last day are presented in Figure 2. The swim bladder inflation was noticed in external observation for each individual. This was proven when the fish was opened. Hemorrhage and significant vascularization were observed in visceral organs (liver, stomach, intestine and kidney).

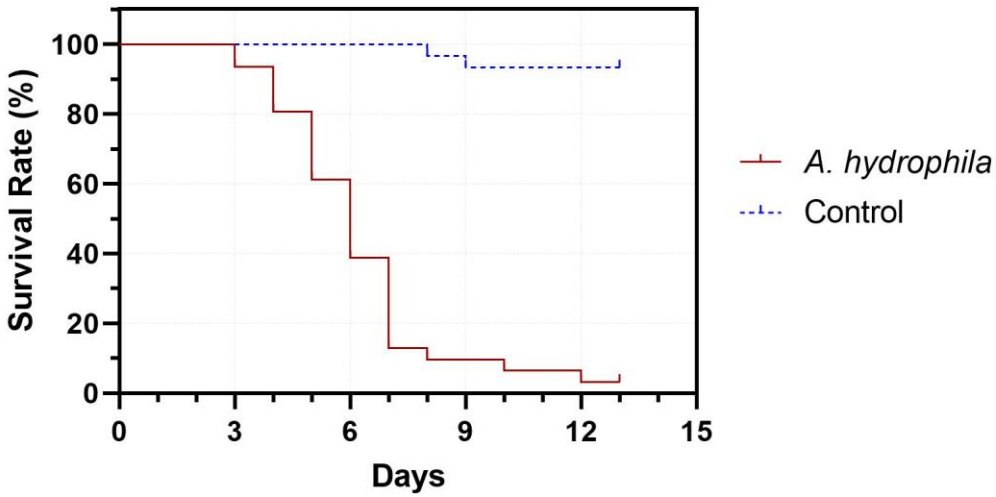

Figure 1. Survival rate of control and infected group.

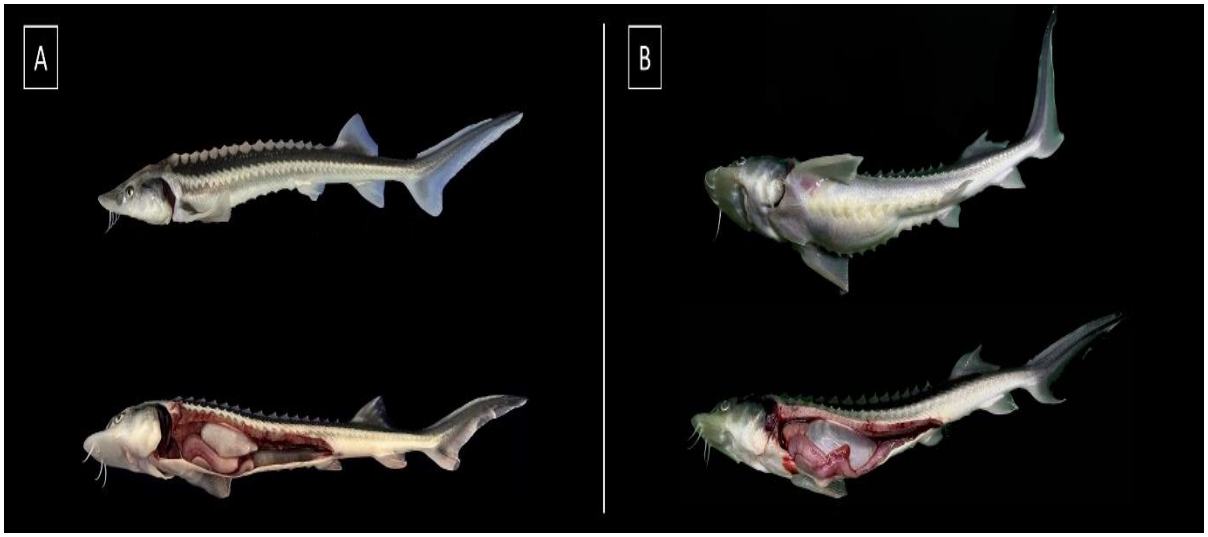

Figure 2. Pathological observation of fish in (A) Control (Kayış *et al.*, 2024), and (B) Infected group.

3.2. Histological evaluations

Histological examination was only performed for liver tissues (Figure 3). While mild melanomacrophage centers were observed in the control group, this sign is severe in the infected group. In addition, mild necrosis was observed in the infected group (Table 2). According to a statistically developed scale, regressive changes and inflammation in liver tissues were significantly higher in the infected groups ($p<0.01$) (Table 3).

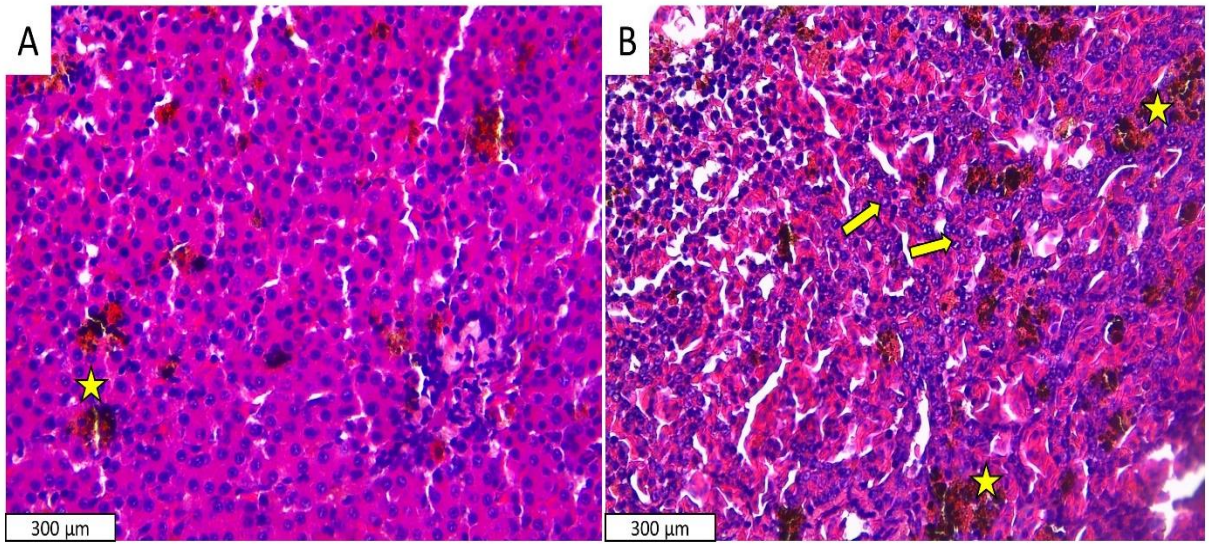

Figure 3. Histological signs in the liver tissues of Danube sturgeon juveniles. A: Control group, B: Infected group, Arrows: Necrosis, Stars: Melanomacrophage centers.

187 **Table 2**. Severity of different histopathological changes in liver tissue of Danube sturgeon.

Tissues	Symptoms	Severities	
		Control	Infected Group
Liver	Necrosis	-	+++
	Melanomacrophage centers	++	++++

(-): None, (+): Mild, (++): Moderate, (+++): Severe, (++++): Very severe.

188

189 **Table 3**. Reaction pattern indices of liver histological alterations in control and infected groups.
190 Abbreviations: RC – Regressive Changes, I – Inflammation, OI – Overall Organ Index.

		RC	I	OI
Liver	Control	0.53±0.22[a]	3.27±0.32[a]	3.80
	Infected Group	1.74±0.36[b]	8.11±0.54[b]	9.85
	t value	-6.483	-12.318	

[ab] Significant differences between groups for each reaction pattern depending on T-test ($p<0.01$).

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192 **3.3. Hematological comparisons**

193 Table 1 showed hematological parameters of fish in both control and infected group. Accordingly,
194 LYM, HGB, MCH, and MCHC were significantly higher in the control group ($p<0.01$). On the
195 other hand, WBC and MCV were significantly higher in the infected group ($p<0.05$). There was
196 no significant difference between groups for RBC ($p>0.05$).

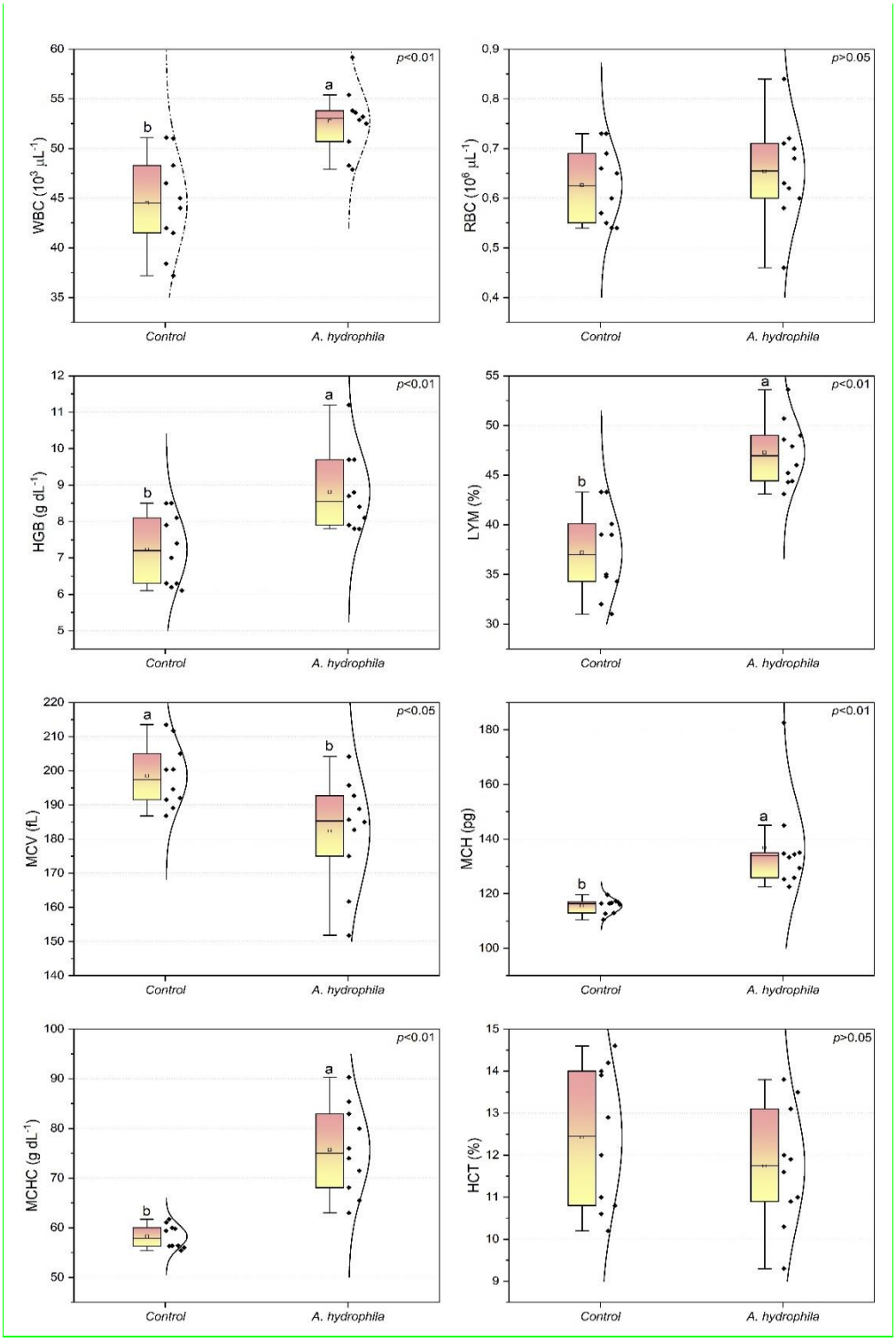

Figure 4. Hematological parameters of fish in control and infected group. [ab]: significant difference between groups (Student-t test)

4. Discussion

Sturgeon is an economically valuable, endangered cold-water fish species, primarily due to its highly prized caviar (Tavakoli *et al.*, 2021). Therefore, Danube sturgeon faces biotic pressure from *A. hydrophila* both in its natural environment and in aquaculture facilities. This challenge test demonstrated the susceptibility of Danube sturgeon to A. hydrophila infection, as evidenced by the onset of mortality beginning on day 3, accompanied by clinical signs such as swim bladder deflation, hyperemia, and hemorrhaging in visceral organs. Although *A. hydrophila* causes serious economic losses to aquaculture facilities worldwide, there is currently no commercial vaccine against infections caused by this pathogenic bacterium (Nayak, 2020). Wounds formed on fish body surface, especially during processes such as transfer, make the fish vulnerable to infections by opportunistic *A. hydrophila*, which can lead to death (D. Zhang *et al.*, 2016). In the present study, the intraperitoneal injection (IP) method was chosen to ensure consistent delivery of the bacterial dose and to represent a worst-case infection scenario, where host defenses are bypassed and the pathogen is directly introduced into the peritoneal cavity, resulting in rapid systemic exposure (Harikrishnan *et al.*, 2011; Swain *et al.*, 2008). Although immersion is considered to mimic natural infection pathways more accurately (Agriandini *et al.*, 2021; He *et al.*, 2021), it generally results in lower infection efficiency and delayed onset of disease compared to IP injection (Sun *et al.*, 2022). Therefore, IP injection is commonly used in experimental infections to evaluate the maximum pathogenic potential of a microorganism under controlled conditions. An optimized feeding protocol and appropriate nutritional supplements can enhance the immune response of sturgeon against *A. hydrophila* infection. For example, the use of chitosan as an additive moderates the growth rate, antioxidant activity, immunity, intestinal morphology and resistance against *A. hydrophila* of juvenile hybrid sturgeon (Li *et al.*, 2023; Xu *et al.*, 2023). The use of galactooligosaccharide for a similar hybrid species was also successful in the *A. hydrophila* challenge test (Xu *et al.*, 2022). Since feed supplements may have negative effects on fish and reduce bacterial resistance (Yousefi *et al.*, 2020), it is important to conduct a feasibility study for supplementary diet material in advance.

Internal septicemia and hemorrhage observed in diseased fish at the end of the challenge test represent typical symptoms of motile *Aeromonas* septicemia (MAS), which often includes visceral hyperemia, ascites, and extensive internal organ damage. These clinical signs are consistent with previous descriptions of MAS and are further discussed in relation to our histopathological findings

(Stratev and Odeyemi, 2017). In the current study, hemorrhages in fish visceral tissues resulted from *A. hydrophila* infection. *A. hydrophila* caused hemorrhagic septicemia in Danube sturgeon, causing low mortality and severe hyperemia and hemorrhage in internal organs (Timur *et al.*, 2010). In a previous study, *A. hydrophila* was isolated from sturgeon fish with swim bladder inflation (Ture *et al.*, 2018). Similarly, *A. hydrophila* has been reported to be isolated from sturgeon fish with swim bladder inflation (Kayiş *et al.*, 2017). In a report prepared for Atlantic sturgeon (*Acipenser oxyrinchus*), swim bladder inflation in fish with hyperinflationary swim bladder syndrome (HISB) was associated with the production of hydrogen gas by an anaerobic bacterium isolated from the intestine (Mohler, 2003). According to another approach, swim bladder inflation occurs in fish due to various reasons such as the supersaturation of gas, bacterial infections, and sudden changes in water temperature and pressure (Austin and Austin, 2016).

Histopathological changes reveal the general health status of fish when faced with stressful factors. In the current study, melanomacrophage centers in the liver are more severe than the control. Macrophages are an innate immune indicator with the ability to perform phagacytosis, which is found even in healthy individuals. Therefore, they take part in treatments such as post-inflammatory repair, tissue regeneration and elimination of old cells (Agius and Roberts, 2003). However, the abundance of macrophages represents the fish's response to a stressor. Phagocytosis of bacteria and viruses by macrophages is critical for a non-specific defense mechanism of the host (Monobe *et al.*, 2014). Reactive oxygen species (ROS) obtained by the respiratory burst of activated macrophages after phagocytosis cause the bacteria to die by oxidative effect on their protein structures (Gordon, 2016; Herb and Schramm, 2021). The present study also demonstrated the necrosis potential of *A. hydrophila* in the liver of Danube sturgeon juvenile individuals. Similar to our study, *A. hydrophila* injected intraperitoneally caused similar lesions in the liver of fish (Moustafa *et al.*, 2020). As another challenge method, bacterial infection via immersion and anal intubation can also cause results such as the deformed nucleus and nuclear migration in the liver tissue (Sun *et al.*, 2022).

Hematological profiles are very rapid tests that show the response of fish during stress (Minaz and Kurtoğlu, 2024). WBC values in the blood increase rapidly when fish are under stressful conditions. This increase serves as evidence that fish are stimulating their non-specific immune sys-tem, which may enhance their resistance to toxicity (Minaz *et al.*, 2022). Increased WBC levels in fish have also been previously observed in Erythrocytic necrosis virus (ENV) infected fish

(Haney *et al.*, 1992). Similarly, *A. hydrophila* infection haematologically increases WBC levels in fish (Harikrishnan *et al.*, 2003). Homeostatic processes in fish are significantly prolonged due to stress caused by biotic reasons (Pickering, 1981). This supports that WBC levels were higher in the infected group at the end of the challenge period in our current study. HGB level in infected fish was lower than in control. The hemoglobin in the blood may decrease due to swelling of the RBC and poor mobilization of hemoglobin from the spleen and other hemopoeitic organs (Scott and Rogers, 1981). Thus, the decreased WBC level in the infected group was likely due to hypochromic microcytic anemia caused by the bacteria. According to another approach, the decrease in HGB level in the blood can be attributed to the apoptosis of blood cells by bacteria (Aiswarya and James, 2016). Hematocrit levels increased as a result of lack of oxygen in the blood (Kirk, 1974; Swift and Lloyd, 1974). Significant differences in RBC indices indicate the macrocytic anemia response of fish to a hypoxic state and stress (Tort and Torres, 1988). The increase in MCV level and decrease in MCH-MCHC levels in the infected group are consistent with previous similar challenge test (Haniffa and Mydeen, 2011).

This study has certain limitations that should be acknowledged. First, only the intraperitoneal injection method was used to induce infection, which may not fully reflect natural exposure routes such as immersion or cohabitation. Second, the investigation was limited to a single tissue (liver) for histopathological evaluation; therefore, potential systemic effects on other organs such as the kidney or spleen were not assessed. Lastly, the study focused solely on juvenile Danube sturgeon from a single genetic origin, which may limit the generalizability of the findings across different age classes or genetically diverse populations.

5. Conclusions

This study evaluated the mortality, hematological, and histopathological effects of *Aeromonas hydrophila* infection in juvenile Danube sturgeon, a species of high economic value and conservation concern. The results confirmed that sturgeons are susceptible to *A. hydrophila* infection, with mortality beginning on day 3 and reaching 100% by day 13. Infected fish exhibited swim bladder deflation, hyperemia, and hemorrhage in visceral organs, along with altered hematological parameters and severe hepatic lesions including necrosis and increased melanomacrophage centers. These findings highlight the pathogenic potential of *A. hydrophila* in endangered sturgeon populations under both aquaculture and natural conditions. However, this

susceptibility should not discourage the cultivation of Danube sturgeon, as resistance to infection can potentially be enhanced through nutritional immunostimulants, improved water quality, and selective breeding strategies. Given the ecological and economic importance of sturgeon, future studies should explore long-term impacts of sublethal infections, immune gene expression profiling, and comparative assessments of different infection routes (e.g., immersion vs. injection). Additionally, broader surveillance studies on the prevalence of *A. hydrophila* in wild and cultured sturgeon populations could provide valuable data for risk management. Ultimately, improving our understanding of host-pathogen interactions in sturgeon will support the development of sustainable aquaculture practices and conservation strategies for this vulnerable species.

Ethical Declaration

Current study was checked and approved by the Ethical Local Committee of the Recep Tayyip Erdogan University (Decision no: 2023/05).

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