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# Effects of Salinity Changes on Hematological Blood Parameters and Stress Responses in Red Tilapia (*Oreochromis* spp.) Infected with *Vibrio harveyi*

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## ABSTRACT

The effect of salinity manipulation on the blood parameters and stress responses of red tilapia, *Oreochromis* spp. During infection with *Vibrio harveyi* was investigated. The fish were reared in five different salinities (0, 5, 10, 15, and 20 ppt) with three replicates for 30 days and were injected with $10^6$ CFU/mL *V. harveyi* intramuscularly in all treatments except the negative control. After infection, the fish were observed for clinical signs for 14 days, collected blood samples, and measured stress responses in 0, 2, 3, 4, 5, 6, 7, and 14-days post-infection (dpi) with *V. harveyi*, meanwhile the cortisol plasma was taken on 0, 2, 3, 4, 5, and 6-dpi. The analysis of blood parameters consisted of total erythrocyte count (RBCs), total leucocyte count (WBCs), hemoglobin (Hb) level, percentage of monocytes (Mon), lymphocytes (Lym) and neutrophils (Neu). The stress response parameters included primary responses (cortisol plasma), secondary responses (blood glucose), and tertiary responses (ventilation rate). The results indicate that salinity manipulation influenced the resistance of red tilapia after infection with *V. harveyi*.

Keywords: Hematology, *Oreochromis* spp., salinity, stress responses, *Vibrio harveyi*



## INTRODUCTION

Red tilapia (*Oreochromis* spp.) is one of the euryhaline commodities that have the highest production value in Indonesia compared to other fish species from 2015 – 2020 (Kementerian Kelautan dan Perikanan, 2022). This fish can be cultivated in a high salinity medium and is susceptible to bacterial pathogens (Vadhel *et al.*, 2017) including Aeromoniasis (Azzam-Sayuti *et al.*, 2021); Streptococcosis (Palang *et al.*, 2020) and Vibriosis (Eissa *et al.*, 2024). Luminous disease is one of the infectious diseases that cause mass mortality in brackish water and marine culture, including at the nursery and growth stages of fish (Zhang *et al.*, 2020). This disease causes high mortality and losses in aquaculture (up to 75%), including in marine teleosts (*Acanthurus sohal*) (Hashem & El-Barbary, 2013), shrimp (*Penaeus vannamei*) (Nurhafizah *et al.*, 2021), abalone (*Haliotis tuberculata*) (Cardinaud *et al.*, 2014), sea cucumber (*Holothuria scabra*) (Becker *et al.*, 2004), and seahorse (*Hippocampus kuda*) (Xie *et al.*, 2020). Furthermore, infection with *V. harveyi* also has an impact on human health (Brehm *et al.*, 2020).

This disease is caused by *Vibrio harveyi*, a curved rod (comma) shaped bacterium, measuring 1.4 – 5.0 µm in length, and 0.3 – 1.3 µm in width. It is a facultative anaerobe with polar flagella for movement (Montánchez & Kaberdin, 2020). This opportunistic pathogen forms yellow colonies in Thiosulphate Citrate Bile Salt (TCBS) agar medium, exhibits bioluminescence, and requires sodium chloride to grow (Austin & Zhang, 2006). Several extra-cellular products (ECP) have been produced from *V. harveyi* including gelatinase, caseinase, lipase, phospholipase, and hemolysins (Zhang *et al.*, 2020). All of the ECPs produced from *V. harveyi* play as virulence factors in infecting the host. Infected fish show several alterations in behavior and lesions in external and internal organs. Behavioral changes seen in infected fish include passive swimming at the bottom of the pond, loss of balance, and abrupt darting

(Yanuhar *et al.*, 2022). The organ lesions accompanying with this disease consist of skin pigmentation and ulceration, ascites, inflammation in the heart, lesions in the dorsal body and fin, and necrosis and congestion in the liver and kidney (Atujona *et al.*, 2018).

Salinity manipulation can be used as an environmental tool to control opportunistic pathogens that cause mortality in cultured organisms. Salinity increased from 0.5 ppt to 3.5 – 4.5 ppt can reduce the rate of pathogen transmission from infected fish (Clulow *et al.*, 2018). Hauton *et al.* (2000) reported that high salinities show a significant effect on the immune system of the European flat oyster (*Ostrea edulis*) by increasing the number of large granulocytes. The effect of salinity in enhancing the immune system of aquatic organisms has been reported in other studies, including grass carp after being challenged with *Flavobacterium columnare* (Fang *et al.*, 2022); Pacific oyster (*Crastorea gigas*) after infection with *V. alginolyticus* (Li *et al.*, 2022) and coastal fish (*Scatophagus argus*) during infection with *Aeromonas hydrophila* (Lu *et al.*, 2022).

Stress responses can be used as indicators to evaluate the fish's condition after infection with pathogens (Shahjahan *et al.*, 2022). The stress responses that are shown include primary responses (increased the secretion of corticosteroid and catecholamine hormones) (Bonga, 1997); secondary responses (increased in glucose, lactate and heat-shock proteins) (Harper & Wolf, 2009), and tertiary responses consisting of changes in performance characteristics and behavioral patterns (Barton, 2002; Balasch & Tort, 2019). Furthermore, hematological analysis can be used to monitor the health status of fish, including erythrocyte count (RBC); hematocrit (Ht), hemoglobin concentration (Hb); leucocyte count (WBC); differential leucocyte count (DLC); and thrombocyte count (TC) (Witeska *et al.*, 2022).

Salinity is one of the major environmental factors affecting to the immune system. It is necessary to determine the effect of salinity changes on immune system of euryhaline fish infected with pathogens. However, the effect of salinity manipulation in reducing stress responses and increasing the immunity of red tilapia against infection has not been revealed before. Therefore, the objective of this study was to analyze the hematological parameters and stress responses of red tilapia after being exposed to different salinity mediums and challenged with *V. harveyi*. The output of this study could be used in conjunction with the prevention of bacterial infection using environmental factor manipulation.

## MATERIALS AND METHODS

### Ethics Statement

This study was conducted from April to May 2022 at the Fish Anatomy Laboratory School of Health and Natural Sciences, Universitas Airlangga. The research was undertaken in accordance with the Law of the Republic of Indonesia No. 18 of 2002 on the National System of Research, Development, and Application of Science and Technology. The research was conducted with the approval of the School of Health and Life Sciences, Universitas Airlangga (ethical approval: 932/UN3.1.16/KP/2022).

### Experimental Fish and Bacterial Isolates

Three hundred and sixty healthy red tilapia, *Oreochromis* spp. (10 ± 1.4 cm in mean length and 16.9 ± 0.5 g in mean weight) were collected from the Fish Hatchery Center of Kabat, Banyuwangi, East Java. They were divided into 18 glass aquariums (volume 36 L, 20 fish for each) and reared in freshwater with well aeration. The experimental fish were acclimatized for 7 days and fed with commercial pellets (30 % crude protein, 6 % lipid, and 3 % carbohydrate Matahari Sakti, Indonesia), three times a day (06.00 pm, 02.00 am and 10.00 am) and until satiation. The water quality parameters (temperature, pH, dissolved oxygen, and ammonia) were controlled to maintain optimum conditions. After 7 days, the fish were assigned to each salinity treatment with 5 ppt uplift every 3 days and reared for 30 days adaptation period.

*Vibrio harveyi* isolates were obtained from the Installation of Brackish Water Aquaculture, Jepara, Central Java inoculated and sub-cultured in TCBS agar medium (Merck, Germany). These isolates were then characterized using a biochemical test based on Barrow & Feltham, (1999).

### *Vibrio harveyi* Pathogenicity Confirmation

The pure isolate of *V. harveyi* after biochemical confirmation was reintroduced to healthy red tilapia using Koch's Postulates procedures based on Mangunwardoyo *et al*. (2016). During the pathogenicity confirmation, fish were placed in an anesthetic tank (100 ppm eugenol) for 60 sec and injected with 0.1 ml of bacterial suspension in NaCl 0.9 % solution (density, $10^6$ CFU $ml^{-1}$) intramuscularly, and 0.1 ml of NaCl 0.9 % solution for the negative control.

**Experimental Design and *In-vivo* Challenge**

This research was conducted with six experimental groups, each with triplicates. The experimental groups included rearing fish in 0 ppt for positive (PC) and negative control (NC); 5 ppt (T1), 10 ppt (T2), 15 ppt (T3), and 20 ppt (T4) for 30-day rearing period. After that, all fish being challenged with *V. harveyi* in NaCl 0.9% solution (density, $10^6$ CFU $ml^{-1}$) to all treatments and PC except NC was injected with 0.1 mL of NaCl 0.9% solution, the fish were kept for 14 days and fed with the same commercial feed (Matahari Sakti, Indonesia). After infection, the fish were returned to each aquarium and the survival rate was observed. All solid waste was removed from each aquarium once a day (every morning), and water quality parameters (temperature, pH, dissolved oxygen, and ammonia concentration) were measured every morning (06:00 – 06:30 am) and evening (04:00 – 04:30 pm) and maintained at optimum conditions (Table 1). The temperature and pH of the rearing water were measured by a water thermometer (Resun, Indonesia) and pH indicator paper (Merck, Germany), respectively. In the meanwhile, dissolved oxygen and ammonia concentration of the rearing water were obtained by a DO meter (Horiba, Poland) and SERA-ammonium/ammonia test kit (SERA, Germany), respectively. The research flow chart was represented in Figure 1.

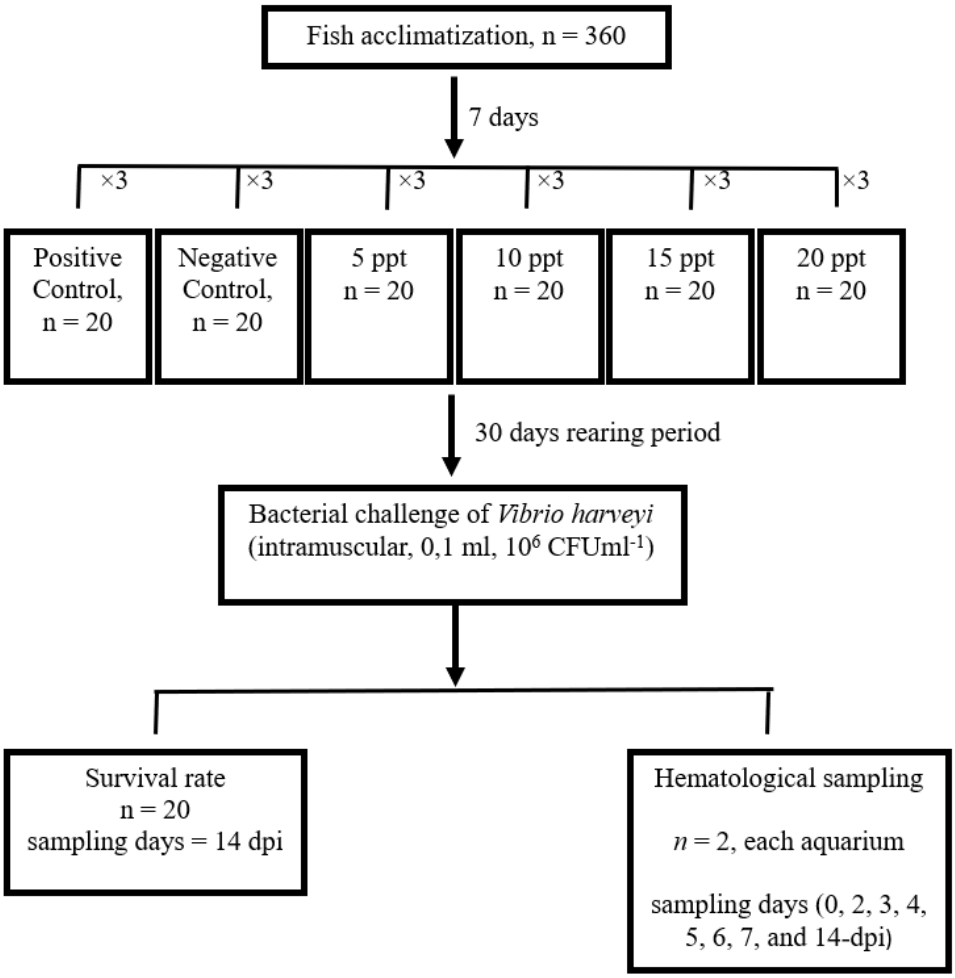


**Figure 1**. Research flow chart

**Hematological Sampling**

Sampling for blood examination was taken before (0 dpi) and after (2, 3, 4, 5, 6, 7, and 14-dpi) challenges with *V. harveyi* for stress responses (blood glucose) (Odhiambo *et al.*, 2020) and blood analyses, including total erythrocyte (RBCs), total leukocyte (WBCs), hemoglobin (Hb), percentage of monocyte (Mon), lymphocyte (Lym), and neutrophil (Neu), refers to Blaxhall & Daisley (1973), except cortisol plasma was taken on 2, 3, 4, 5, and 6-dpi with *V. harveyi*. Two fish were randomly sampled from each aquarium and moved to an anesthetic tank (100 ppm eugenol) for 60 sec. Blood was taken from two fish in each treatment from vena caudalis using a syringe and collected in an anticoagulant tube. Cortisol plasma and blood glucose were examined using the Enzyme-linked immunosorbent assay (ELISA) method according to Sadoul & Geffroy, (2019) and a glucometer (Easy Touch Glucose Test, Taiwan) refers to Bartoňková et al., (2016), respectively. Analysis of ventilation rate as tertiary stress responses was conducted on 0, 2, 3, 4, 5, 6, 7, and 14-dpi with *V. harveyi* using a hand counter every 10 minutes for 1 hour in the evening. Ventilation rates were counted and averaged from all fish from each aquarium for analysis (Flint *et al.,* 2015). The survival rate of fish was counted based on Do Huu *et al.* (2016) [Eq.(1)].

$$\text{Survival rate (SR)} = \frac{\text{Nt}}{\text{No}} \times 100\% \qquad \text{Eq.(1)}$$

Description:
Nt = fish number day-t (fish)
N0 = fish number day-0 (fish)

**Statistical Analyses**

All parameters including blood profiles, stress response parameters, and water quality were analyzed descriptively and compared with normal values. Survival rate was analyzed by one-way Analysis of Variance (ANOVA) and followed by the Duncan Multiple Range Test (DMRT). Significance was determined at P values < 0.05.

**RESULTS**

The results of the observation of blood parameters (Figure 2) showed a decrease in RBCs (Figure 2a) and Hb levels (Figure 2b) from 1-dpi to14-dpi, especially in T4 (20 ppt) compared to other treatments. The total WBCs (Figure 2c) showed an increasing trend in all treatments from 1-dpi until 6-dpi and remained stable until 14-dpi. There was a rise in the percentage of neutrophils (Figure 2d) to a peak at 4-dpi and 5-dpi in all treatments and then a decrease until 14-dpi. Meanwhile, the monocyte percentages (Figure 2e) increased to a maximum at 6-dpi in all treatments and decreased in the following days, except in the negative control (NC). The lymphocyte percentage (Figure 2f) showed a decline from 2-dpi to 5-dpi and then increased from 5-dpi until 14-dpi.

Observation of the stress response of red tilapia after infection with *V. harveyi* (Figure 3) presents a similar trend in all parameters (cortisol plasma, blood glucose, and ventilation rate). They all increased until reaching the maximum value in the middle of the observation and then slowly decreased until the end of the study, except for the negative control (NC). Cortisol plasma (Figure 3a), blood glucose (Figure 3b), and ventilation rate (Figure 3c) were peaked at 4-dpi, 5-dpi or 6-dpi, and 5-dpi, respectively.

The survival rate of *Oreochromis* spp. (Figure 4) varies significantly between treatments. The highest value in NC is not statistically different from T1, T2, or T3 ($P > 0.05$). However, its values are significantly different from PC and T4 ($P < 0.05$). Water quality parameters (Table 1) are optimum for *Oreochromis* spp. cultivation.

**DISCUSSION**

*V. harveyi* is a bacterial pathogen causing Vibriosis disease, leading to several changes in blood parameters and high fish mortality (Zhang *et al.,* 2020). Vibriosis in fish induces various modifications in total red blood cells (RBCs), white blood cells (WBCs), hemoglobin levels (Hb), and differential leucocytes (Qiao *et al.*, 2012). The hematological parameters and stress responses of red tilapia showed several changes after being treated in different salinity mediums and challenged with *V. harveyi*.

RBCs are the most numerous cell types essential to gas exchange in vertebrates (Shen *et al.*, 2018). In the present study, the RBC count of red tilapia after infection with *V. harveyi* decreased in all treatments (Figure 2a), with the highest decrease observed in T4 (salinity of 20

ppt) to 2.01 × $10^5$ cells $mL^{-1}$. However, when compared to the normal value of RBCs in tilapia, the value still falls within the ranges of 2.08 to 2.93 × $10^5$ cells $mL^{-1}$ (Vo *et al.*, 2022). Ruwandeepika *et al.* (2012) stated that *V. harveyi* can secrete the hemolysin toxin, which lyses the RBCs and causes hemorrhage and ulcers on the skin. Furthermore, Hernández-Cabanyero *et al.* (2022) reported that *Vibrio* infection can trigger an acute inflammatory response and a cytokine storm that leads to fish mortality. Previous studies have also reported similar results in sea bass (*Lates calcarifer*) (Pattah *et al.*, 2020) and yellowtail kingfish (*Seriola lalandi*) (Le & Fotedar, 2014).

Hb is a protein in vertebrate erythrocytes that used to transport and store oxygen (Wicher and Fries, 2006). The mean values of hemoglobin levels (Figure 2b) demonstrated a decline in all treatments from the beginning to the end of the observation, similar to the RBC count. The highest decline occurred in T4 (salinity of 20 ppt) compared to all groups, including the control. RBCs function to transfer hemoglobin to the body tissues, and therefore, there is a positive correlation between hemoglobin and RBCs, leading to anemia in fish (Witeska, 2015; Yuhana *et al.*, 2019). Jun & Woo (2003) stated that *Vibrio* bacteria secrete not only hemolysin but also siderophores, which can disrupt iron uptake, especially from hemoglobin. Studies in *Salmo salar* in Chile (Ruiz *et al.*, 2016) and European sea bass (*Dicentrarchus labrax*) (Abdel-Tawwab *et al.*, 2020) have reported a decrease in hemoglobin levels after infection with *Vibrio*. As a result, these data showed that infection of *Vibrio* can affect to the RBCs and hemoglobin from infected fish at high salinity.

Leucocytes have an important role in the fish's immune system to fight against pathogens. Based on the results, the WBCs value (Figure 2c) shows an increase from 1-dpi to 14-dpi in all treatments, including the control, and is higher than the normal value (range 7.5 – 8.2 × $10^4$ cells $mL^{-1}$) (Mauel *et al.*, 2007). Group T4 (salinity 20 ppt) peaked at 6-dpi and dropped from 7-dpi to 14-dpi. This indicates that red tilapia responds to pathogen infection. The leukocytes will migrate and converge at the site of infection to defeat these pathogens (Ellis, 1977). The leukocyte levels dropped until the end of the observation. This happens because the leucocytes extravasate from the blood vessels and migrate to the infected tissue (Nourshargh and Alon, 2014). Some reports also state similar results, such as in tilapia fish (*Oreochromis mossambicus*) after infection with *V. parahaemolyticus* (Fatima *et al.*, 2022) and *Aeromonas hydrophila* (Silviana *et al.*, 2022). Zhang *et al.* (2020) state that *V. harveyi* secretes biofilm compounds that can survive and spread throughout the fish's body and enhance the fish's immune system.

Neutrophils plays vital roles in wound healing, tissue regeneration, immune system signaling, and pathogen defense (Speirs *et al.*, 2024). The percentage of neutrophils (Figure 2d) shows an increasing number after infection with *V. harveyi* until the 4-dpi and then slowly decreases until 14-dpi. The increasing neutrophils indicate that red tilapia responds to *V. harveyi* infection, and it is higher than the normal value (less than 5%) (Havixbeck *et al.*, 2016). Neutrophil cells are the first leukocyte cells that migrate directly after infection and eliminate the pathogens through several mechanisms (Mortaz *et al.*, 2018). Furthermore, Zhao *et al.* (2017) state that chemotactic signals derived from pathogens and the host rapidly recruit neutrophils from the blood to infection sites. The decreasing number of neutrophils between 5-dpi and 14-dpi suggests that neutrophil cells can control and eliminate the pathogens. This is done through various mechanisms, such as producing reactive oxygen species (ROS) and releasing toxic intracellular granules (Havixbeck & Barreda, 2015). Buchmann (2022) stated that neutrophils can use Toll-like receptors (TLRs) and pattern recognition receptors (PRRs) to trap the pathogen and be easily engulfed by phagocytic cells. This result is similar to a previous study in hybrid sturgeon (Xiao *et al.*, 2022) and humpback grouper (*Cromileptes altivelis*) (Dangeubun and Metungun, 2017) after being infected with *Vibrio*.

During the inflammatory process, monocytes help to maintain tissue-resident macrophage populations (Witeska *et al.*, 2022). The mean values of monocyte percentages (Figure 2e) slowly uplifted to a peak at 6-dpi and dropped significantly until 14-dpi, but still within normal values. The normal value of monocytes in tilapia ranged from 11 – 24% (Corrêa *et al.*, 2017). The increasing monocyte count indicates that immune responses in red tilapia have been induced after infection with *V. harveyi*.

Monocytes are the largest type of leukocyte cells and have high phagocytosis activity. They can migrate and differentiate into macrophages in the tissues (Fischer *et al.*, 2006). The antipathogenic activities of monocytes include macrophage polarization, cytokine production, antigen presentation, and phagocytosis mechanisms (Lu and Chen, 2019). At 6-dpi, the percentage of monocytes decreased considerably. This is because monocytes have a short life span in the bloodstream (10 – 20 hours), leading to fluctuations in their numbers (Grayfer *et al.*, 2018). Several studies have reported similar results, such as in ayu (*Plecoglossus altivelis*) (Lu *et al.*, 2021) and sea bass (*Lates calcarifer)* (Pattah *et al.*, 2020) when facing *Vibrio* infection.

Lymphocytes play an important role in the innate immunity of teleosts (Van Muiswinkel, 1993). The percentage of lymphocytes (Figure 2f) in red tilapia after infection with *V. harveyi* slowly dropped until 5-dpi, then gradually increased until 14-dpi. Nevertheless, the values were within the range of normal conditions, ranging from 30.9 to 86.5% (Corrêa *et al.*, 2017). The decline in lymphocyte percentage after infection is caused by the dominance of neutrophils in WBCs at the beginning of the infection, replacing the number of lymphocytes (Scapigliati *et al.*, 2018). From 6-dpi to 14-dpi, the percentage of lymphocytes were steadily increased. This shows that the humoral immune response of red tilapia was activated to protect the fish from recurrent infections (Scapigliati, 2013). Activation of adaptive immunity in lymphocytes requires exposure to antigens presented by major histocompatibility complex (MHC) molecules (Zapata *et al.*, 2006). Previous studies have also supported this result, such as in pompano fish (*Trachinotus ovatus*) (Do Huu *et al*., 2016) and gilthead seabream (Chaves-Pozo *et al.*, 2005) during *Vibrio* infections. Taken together, these findings suggest that *Vibrio* infection can enhance the immune response of red tilapia.

Stress responses in fish were divided into three groups, primarily, secondary, and tertiary. Secretion of cortisol in circulation was one of the primary stress responses that showed where fish in highly stressed (Peter, 2011). Releasing stress hormones affected the alterations of blood chemistry, such as blood glucose as secondary responses, and the escalation of ventilation rate was a result of the fish adaptation process during stress conditions (Petitjean *et al.*, 2019). In the present study, all fish responses (Figure 3a, 3b, and 3c) showed the same result, starting from 1-dpi to the maximum at 5-dpi or 6-dpi and then slowing the falling until 14-dpi. This occurred because the red tilapia can adapt to external stressors (salinity) and internal stressors (infection of *V. harveyi)*. *V. harveyi* released protease enzymes and several toxins (enterotoxin, cytotoxin, and endotoxin) that can damage fish tissue and act as biological stressors for the host (Austin and Zhang, 2006). According to Galhardo *et al.* (2011), secretion of the cortisol hormone affected the increase in blood glucose and ventilation rate in fish due to surviving under stress conditions. The optimum value of cortisol plasma, blood glucose, and ventilation rate in fish was 20-60 ng $mL^{-1}$ (Ellis *et al.*, 2012), 40-90 mg $dL^{-1}$ (Renitasari *et al.*, 2021), and 76-99 bpm (Iwama *et al.*, 1997), respectively. Previous studies also displayed similar results in Atlantic salmon (*Salmo salar*) (Wiik *et al.*, 1989), brown-marbled grouper (*Epinephelus fuscoguttatus*) juveniles *(*Amar *et al.*, 2018), juveniles of barramundi or Asian seabass (*Lates calcarifer*) (Talpur *et al.*, 2013; Siddik *et al.*, 2019), and Gilthead seabream (*Sparus aurata*)(Vargas *et al.*, 2018) during *Vibrio* infection. Taken together, these results indicate that several stress responses were expressed after *Vibrio* infection in red tilapia.

Environmental factors such as salinity can affect the increase in immune responses in fish after infection with pathogens and result in high survival rates (Figure 4). Exposure to high salinity can activate the immune responses and produce more active cells that fight against pathogen infections (Wen *et al.*, 2021). In euryhaline fish, environmental salinity can induce the alteration of indigenous hormones, especially growth hormone (Yada *et al.*, 1994). Furthermore, Evans and Kültz (2020) states that growth hormones can stimulate macrophage function and increase non-specific immunity. Modulation of the fish immune system after exposure to high salinity is also reported in several species, including Nile tilapia (*Oreochromis niloticus*) (Dominguez *et al.*, 2005), broad nose pipefish (*Syngnathus typhle*) (Birrer *et al.*, 2012), and sablefish (*Anoplopoma fimbria*) (Kim *et al.*, 2017). As a result, these data showed that salinity manipulation can stimulate the red tilapia immune system.

**Table 1.** Water quality parameters of rearing media during treatment

| Parameters | Observing value |
|---|---|
| Temperature (°C) | 27 – 28 |
| pH | 7 – 8 |
| Dissolved oxygen (ppm) | 5 – 7 |
| Ammonia (ppm) | 0 – 0.25 |

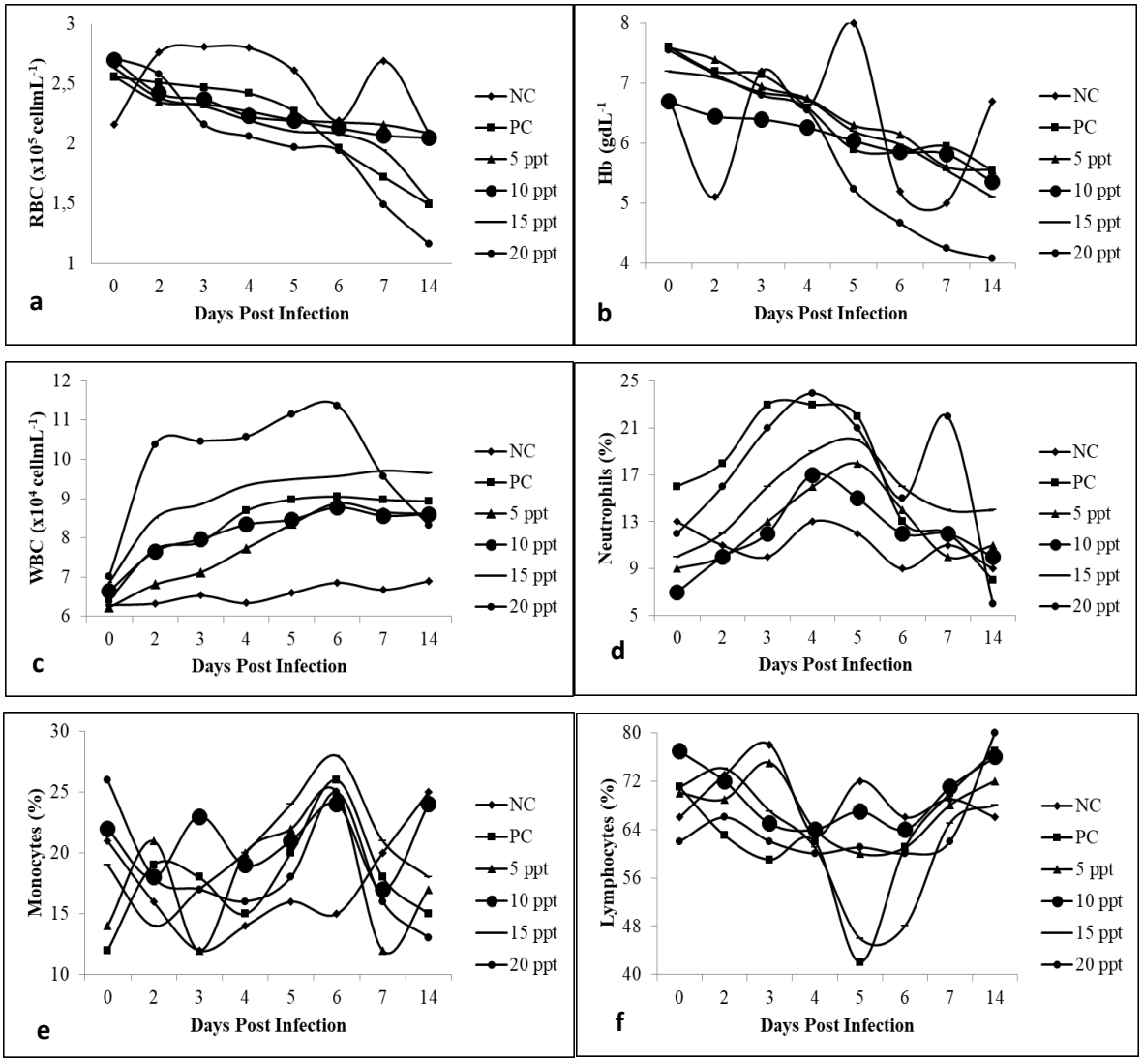


**Figure 2**. Variation of mean values for hematological parameters in red tilapia *Oreochromis* spp. reared in different salinity followed by challenges with *V. harveyi* at different sampling times. NC: negative control; PC: positive control

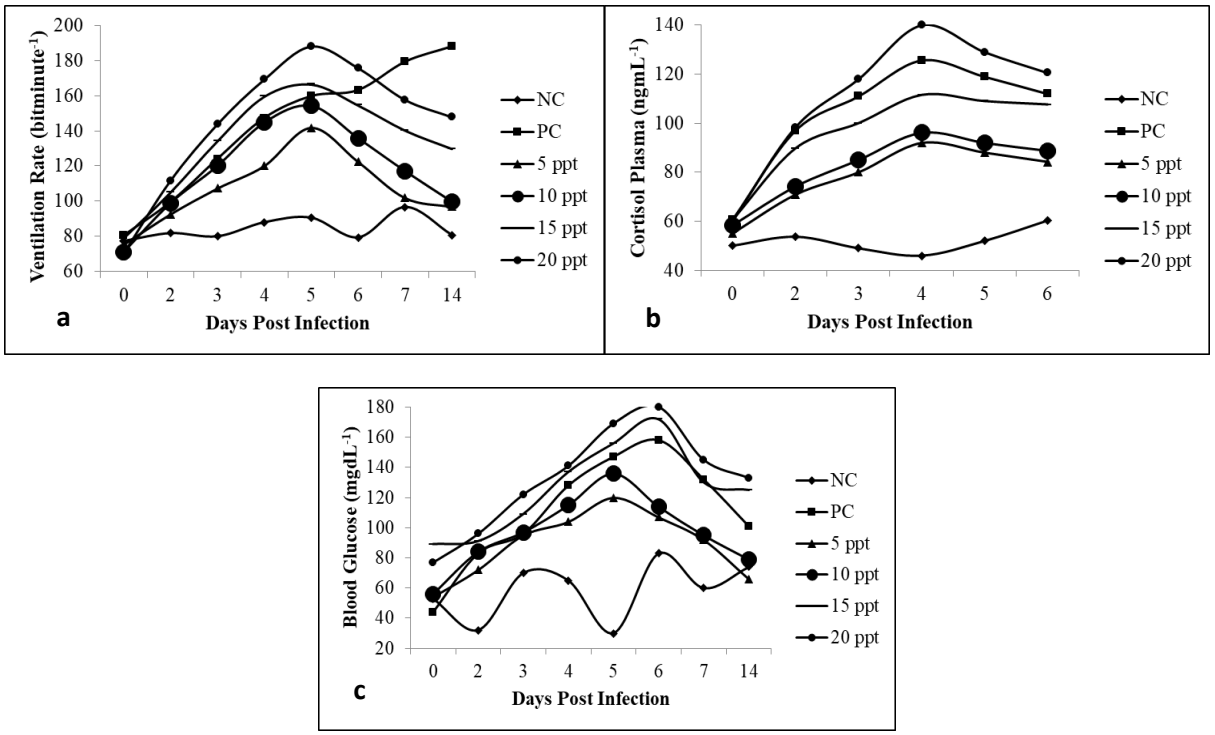


**Figure 3**. Mean values of stress responses in red tilapia *Oreochromis* spp. reared in different salinity followed by challenges with *V. harveyi* at different sampling times. NC: negative control; PC: positive control

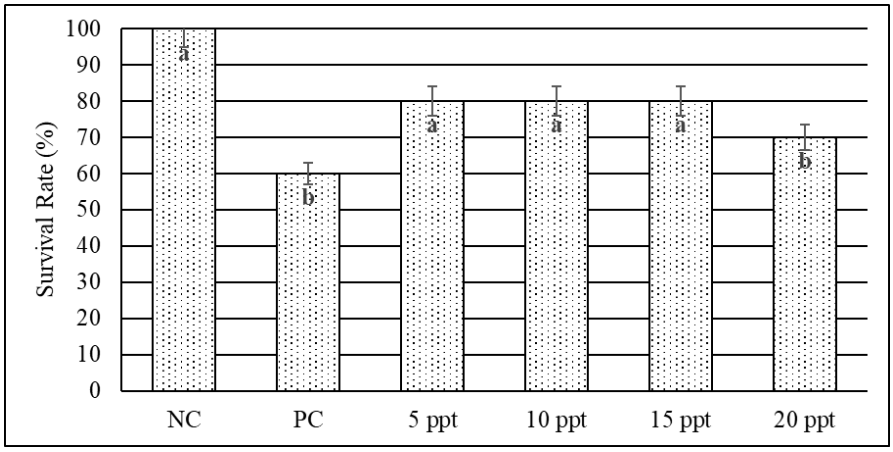


**Figure 4**. Survival rate of red tilapia *Oreochromis* spp. reared in different salinity followed by challenges with *V. harveyi*. NC: negative control; PC: positive control. Different letters indicate statistically significant between experimental groups ($p<0.05$)

## CONCLUSION

The findings of the current investigation indicated that salinity manipulation influenced the resistance of red tilapia after infection with *V. harveyi*. Low salinity (5 – 15 ppt) can increase the resistance of red tilapia infected with *V. harveyi* in terms of blood parameters and stress responses. However, further studies of co-infection with other pathogens would be needed to detect histopathological alterations in red tilapia.

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## CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

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