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# Histopathological characteristics of striped catfish (*Pangasianodon hypophthalmus*) challenged with *Edwardsiella ictaluri*

Luu Tang Phuc Khang[1*], Nguyen Huu Giau[2], and Tran Thi Phuong Dung[1]

[1]*Ho Chi Minh City University of Education, Viet Nam*

[2]*SaiGon University, Viet Nam*

**Correspondence: Luu Tang Phuc Khang (email: ltpkhcmue@gmail.com )*

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**ABSTRACT**

*The present study attempted to understand the histopathological index of striped catfish (Pangasianodon hypophthalmus) challenged with Edwardsiella ictaluri, the causative agent of bacillary necrosis of Pangasius. A total of 355 healthy striped catfish juveniles was challenged with E. ictaluri (25 control; 330 infected). A total of 355 samples of the trunk kidney, liver and spleen were collected at five different time points including just prior to infection and, 24, 48, 264 and 312 hours post infection (hpi) to measurements the represented tissue damage of the fish. Results showed that many areas of diseased liver, kidney, and fish organ tissues were congested and hemorrhaged as early as 24 hpi. The number of macrophage centers in kidney and spleen increased at 24 - 48 hpi. Tissue damage (multifocal hemorrhages; necrosis) increased sharply from the period 48 - 256 hpi. The number of dead fish increased throughout the duration of infection, particularly in the period from 48 to 200 hpi. At 312 hpi, the histopathological index and the mortality rate was the highest during the experimental period.*

## 1. INTRODUCTION

Aquaculture is one of the fastest-growing food production sectors in the world, including Viet Nam. The overall Vietnamese fisheries production in the first six months of the year 2022 was estimated at 4,196.8 thousand tons, an increase of 2.5% over the same period of the previous year (VASEP, 2022). Striped catfish (*Pangasianodon hypophthalmus*), a freshwater species cultured mainly in the Mekong Delta region in Southern Viet Nam, is an important aquaculture species of high economic value. The popularity of striped catfish is due to its relatively fast growth rate, high flesh quality, tolerance to low oxygen and crowding, as well as its ability to adapt well to various culture systems. It also exhibits an efficient feed conversion rate and accepts manufactured feed (Nguyen & Do, 2010; De Silva et al., 2010; De Silva & Nguyen, 2011). In 2021, the production of striped catfish in Viet Nam reached 1.56 million metric tons and a turnover of approximately 1.61 billion USD (VASEP, 2022). There has been a phenomenal shift from the extensive to the intensive culture of carps and catfishes in the last three decades. Yet, intensive aquaculture offers an increased opportunity for the spreading of infectious diseases at all stages of production. Many factors are responsible for periodical disease outbreaks in aquaculture, such as microorganisms (54.59% bacterial, 22.6% viruses, 3.1% mycotic) and parasitic agents (19.4%) (Dhar et al., 2014). Among the bacterial pathogens *Pangasianodon hypophthalmus (P. hypophthalmus*) is most susceptible to the *Aeromonas* spp. and *Edwardsiella ictaluri* (*E. ictaluri*), which causesmotile aeromonas septicemia (MAS) and bacillary necrosis (BNP) respectively (Subagja et al., 1999; Ferguson et al., 2001). In particular caused by *E. ictaluri* is considered to be the most serious disease occurring in farmed striped catfish within Viet Nam. This disease has had an increasing impact over the last ten years and has been reported to cause 50-90% mortality of stocks during a single outbreak (Nguyen, 2014).

Histopathological changes have been widely used as biomarkers to evaluate the health of fish exposed to contaminants (Thophon et al., 2003). The gill, kidney and liver organs are responsible for vital functions, such as respiration, excretion and the accumulation and biotransformation of xenobiotics in the fish. One of the great advantages of using histopathological biomarkers for environmental monitoring is that this category of biomarkers allows for examining the specific target organs (Gernhofer et al., 2001). Furthermore, the pathological changes discovered in these organs are normally easier to identify than functional ones (Fanta et al., 2003), and serve as warning signs of damage to animal health (Hinton and Laurén, 1990, Mamun et al., 2022).

A number of studies have investigated BNP, but these works have only focused on describing clinical symptoms, isolating and describing the causative agent (Ye et al., 2009; Crumlish et al., 2010). At the same time, there are no studies outlining comparative data on the histopathological characteristics of *P. hypothalmus* against *E. ictaluri*. Therefore, the principal aims of this study was to understand the differences in histopathological changes of striped catfish in liver, kidney and spleen tissues. Findings from this study are expected to provide essential information over the disease manifestations at the cellular level on the internal organs of selective breeding striped catfish resistant to BNP disease caused by *E. ictaluri*.

## 2. MATERIALS AND METHOD

### 2.1. Research design

#### *2.1.1. Bacteria*

The bacterial strain *Edwardsiella ictaluri* Gly09M isolated from BNP catfish in An Giang province in 2009 and preserved in the bacterial collection of the Research Institute for Aquaculture No.2 (RIA2) was used in this research. The $LD_{50}$ was 4.9 x $10^3$ CFU/0.2 mL/fish for striped catfish 15 - 25g (Le et al., 2013). The bacteria were restored and re-isolated from healthy striped catfish three times prior to the challenge.

#### *2.1.2. Experimental fish and challenge test*

A total of 355 healthy striped catfish juveniles (15 – 20 g) were procured from the National Feeding Center for Southern Freshwater Aquaculture located in Tien Giang province. The fish were acclimatized to laboratory conditions for 15 days. All individuals in each family were stocked in 90 L tanks at a stocking density of 0.26 fish/L. The cohabitant method was used in this challenge as described in details by Nguyen et al. (2022). The cohabitant fish (16.6 ± 6.1 g/fish) were firstly infected with the bacteria *E. ictaluri* by injection at a dose $10^6$ CFU/0.2 mL per fish. Two days after the injection, the cohabitant fish were released into the tanks to rear together with the experimental fish, accounting for 35% of the total number of fish in each tank. After two days of communal rearing, suspension of *E. ictaluri* was added to the experimental tanks to retain the bacterial density ($10^5$ CFU per 1 mL of water) for disease infection. The experiment was conducted over a period of 312 hours post infection (hpi).

### 2.2. Histopathological analysis

Lethargic fish were collected and dissected for the occurrence of white spots on internal organs (liver, kidney and spleen) at the time points: before infection and, 24, 48, 264 and 312 hour post infection (hpi). A total of 25 samples of each internal organs were collected before infection, 24, 48, 264 hpi and at 312 hpi ten samples from the infected group were collected. In the control group five samples at each time points were collected. The tissues were dehydrated in ethanol series and cleared through pure chloroform. Thereafter, the samples were embedded in paraffin wax (60 – 70 °C melting point) and prepared for sectioning. The samples were cut through a microtome machine around 5 μm thicknesses and fixed on slides. Then, the slides were kept overnight in the open air for drying. Finally, the slides were stained with Hematoxylin and Eosin and mounted in DPX for long-term preservation (Coolidge & Howard, 1979). The semi-quantitative histo-pathological changes were investigated as described by Quach (2017). The measurements were performed using a light microscope under 40X magnification through a calibrated lens, obtaining numeric values which represented tissue damage per metric unit and were subsequently converted into microns (Cercadillo-Ibarguren et al., 2010).

### 2.3. Statistical analysis

The data of histopathological index of liver, kidney and spleen of striped catfish at each time point were tested. The one-way ANOVA with a significance level of 5% ($p < 0.05$) was used to compare the mean values of histopathological index at each time point. These analyses were performed on the SPSS statistical software to test the significant difference at probability $p < 0.05$. All data were presented as mean ± standard errors of mean (SE) in the actual unit of measurements.

## 3. RESULTS AND DISCUSSION

### 3.1. Clinical signs and gross pathology

Within 3 to 4 days post exposure, clinical signs commonly associated with *E. ictaluri* infection were observed in the fish in the test challenge (Figure 1).

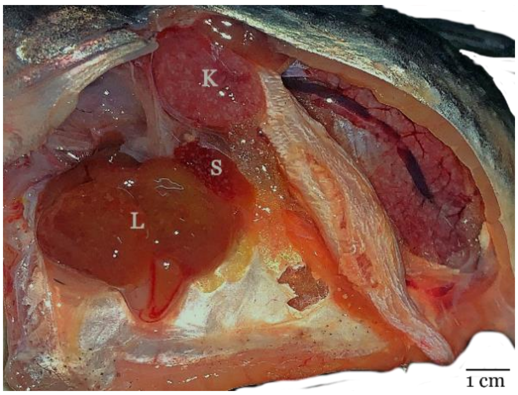


**Figure 1. Clinical signs and gross pathology of striped catfish after infection**

*White lesions were seen in the anterior kidney (K), spleen (S) liver (L) were enlarged*

Prior to infection, the fish were swimming fast, responded quickly to noise, exhibited a smooth body with no signs of injury or sores, with no bleeding in the fins or gills. Fish started to die after 5 days of cohabitation. At 48 hpi, small focal circumscribed haemorrhagic lesions were observed in the skin of fish infected with *E. ictaluri*. After 14-day post infection (dpi), the mortality rate of fish increased sharply. Fish in the challenge test showed behavioural changes including erratic swimming in a spiral motion and stopped feeding prior to mortality. Internally, the affected fish's kidney and spleen showed white lesions of 1-2 mm in diameter. Later, white lesions also occurred in the liver of infected fish (Figure 1). In addition to abdominal dropsy and fluid in the peritoneal cavity, the abdomen was enlarged. The kidney and spleen were also enlarged.

A comparison of the data provided in this study showed that the fish exposed to the bacterium showed similar behavioural and clinical signs to those described for both natural and experimental BNP infections (Ferguson et al., 2001; Crumlish et al., 2002; Crumlish et al., 2010). Bacterial recovery from the spleen and kidney samples of all infected striped catfish (both moribund and dead fish) produced bacterial colonies identified as *E. ictaluri*. The histopathology taken from moribund fish exposed to the bacteria and with gross clinical signs of BNP was also similar to that observed in the previous natural and experimental infections in BNP studies (Ferguson et al. 2001; Crumlish et al., 2002, 2010).

### 3.2. Histopathological index of liver

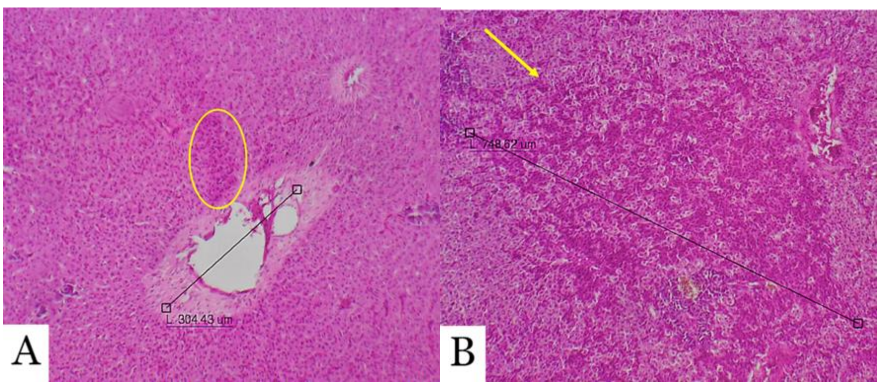


**Figure 2. Liver histopathological changes in the challenged fish**

*(A) liver haemorrhages (circle) after 24 hours; (B) area tissues of multifocal hemorrhages after 312 hours; (C) liver tissues deformed after 48 hours (arrow); (D) necrosis of liver tissues (circle)*

Observed liver histopathological changes from the striped catfish sampled after the challenge were: (i) cell adhesions were destroyed; (ii) large areas of cellular necrosis and haemorrhage were present in the liver; (iii) many areas of hepatocytes were deformed leading to necrosis and loss of structure; (iv) multiple extensive areas of necrosis were observed in the liver (Figure 2). At 24 hpi, the liver began to haemorrhage slightly and increased sharply at 48 hpi (from 231.93 ± 60.94 µm to 383.08 ± 82.26 µm). The differences were not statistically significant ($p > 0.05$). Prolonged haemorrhage lead to necrosis. At 312 hpi, the most necrotic liver tissue structure (413.40 ± 65.49 µm) resulted in fish death (Figure 3).

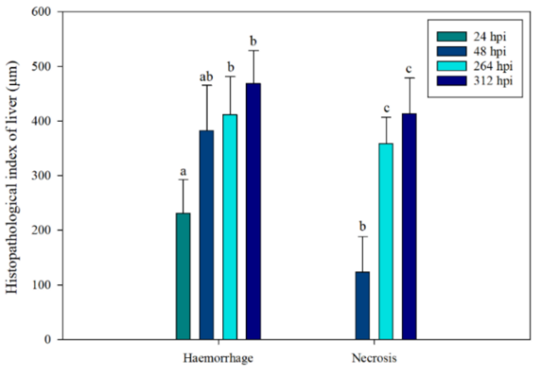


**Figure 3. Histopathological index of the liver during the challenge**

*Values are Mean ± SE, different letters indicate significant differences between treatments ($p < 0.05$).*

The liver is the largest of the extramural (outside the alimentary canal) organs. Fish liver functions similar to that of mammals. Its functions included assimilation of nutrients, detoxification, production of bile, and maintenance of the body 's metabolic homeostasis including the processing of proteins, carbohydrates, lipids and vitamins (Harder, 1975). The liver also plays an important role in the synthesis of plasma proteins, like albumin, fibrinogen, and complement factors. According to Mahdy et al. (2021), the liver is encased within a fibroconnective tissue capsule and, unlike mammalian liver, it contains no distinct lobulation, no apparent interlobular connective tissue stroma, and no typical portal triads. Rather, the most distinctive histological features of the liver include the exocrine pancreatic tissue that surrounds all of the portal veins in mature striped bass (Kibenge et al., 2021), and an anastomosing bilaminar meshwork of hepatocytes that comprises the basic functional hepatic units (Patt & Patt, 1969). The parenchyma of the organ is contained within a thin capsule of fibroconnective tissue. The parenchyma itself is primarily composed of polyhedral hepatocytes typically with a central nucleus. Glycogen deposits and fat storage are often dissolved during the routine histological process, and produce considerable histological variability. The hepatic structure normally varies(considerably in direct relationship to age, gender, available food or temperature, and with endocrine influences strongly connected to the environmentally regulated breeding conditions. The liver tissue sections of diseased pangasius revealed multiple haemorrhages on the hepatic tissues along with numerous pyknotic nuclei, dilated sinusoids, vacuolization of cytoplasm, cellular necrosis of hepatocytes. According to Ferguson (1989) the damage that takes place throughout the liver causes the liver to no longer undertake detoxification, dialysis, protein metabolism, lipids, glucid, and bile secretion. From there, the virulence of bacteria that are not eliminated accumulates in the body and reduces resistance to pathogens combined with other adverse conditions that kill fish.

These findings are consistent with previous studies that show that E. ictaluri infection can cause pathological lesions in different vulnerable fish species, including channel catfish (*Ictalurus punctatus*), brown bullheads (*Amieurus nebulosus*), ayu and zebrafish (*Danio rerio*) (Sakai et al., 2008; Areechon & Plumb, 2009; Hawke et al., 2013). Some pathological features have been studied such as infection ranging from edematous degenerating hepatocytes (Areechon & Plumb, 2009), to focal and multifocal hepatocytic necrosis, the presence of bacterial cells either in-between necrotic debris and within sinusoidal spaces (Sakai et al., 2008), or engulfed by infiltrating mononuclear cells (Iwanowicz et al., 2006).

### 3.3. Histopathological index of kidney

The observed kidney histopathological changes from the striped catfish sampled after the challenge were: (i) multiple extensive areas of necrosis were observed particularly in the anterior kidney; (ii) haemorrhages were observed in the kidney; (iii) melano-macrophage centers (MMCs) appeared in kidney tissues; (iv) the glomerulus and the nephron were congested, bleed, swelled and spread which leads to loss of kidney structure (Fig. 4). The histopathological index of fish over the challenge time was compared in Fig. 5. At 24 hpi, the kidney appeared congestive and haemorrhagic phenomena

(146.90 ± 42.32 µm). Congestive phenomena is considered the body's first response to pathogens due to the special stimuli that cause capillaries to strengthen a larger-than-normal amount of blood to lead to inflammatory foci. Under the influence of bacterial toxins, blood capillaries rupture or due to increased capillary permeability, causing cells in the congestive region to escape and mix with organ blood cells causing haemorrhage. Prolonged haemorrhages from 24 to 48 hpi will cause the inflamed swollen kidney tissue to lose its structure and lead to necrosis. Thereafter, the kidneys lose their function. The sampled kidneys shows multifocal haemorrhages and necrosis increased with the experiment time. Necrosis of kidney tissues strongly increased at 48 – 312 hpi from 189.41 ± 45.20 µm to 304.74 ± 40.25 µm. The differences between histopathological index of kidneys prior and during the challenge was statistically significant ($p < 0.05$).

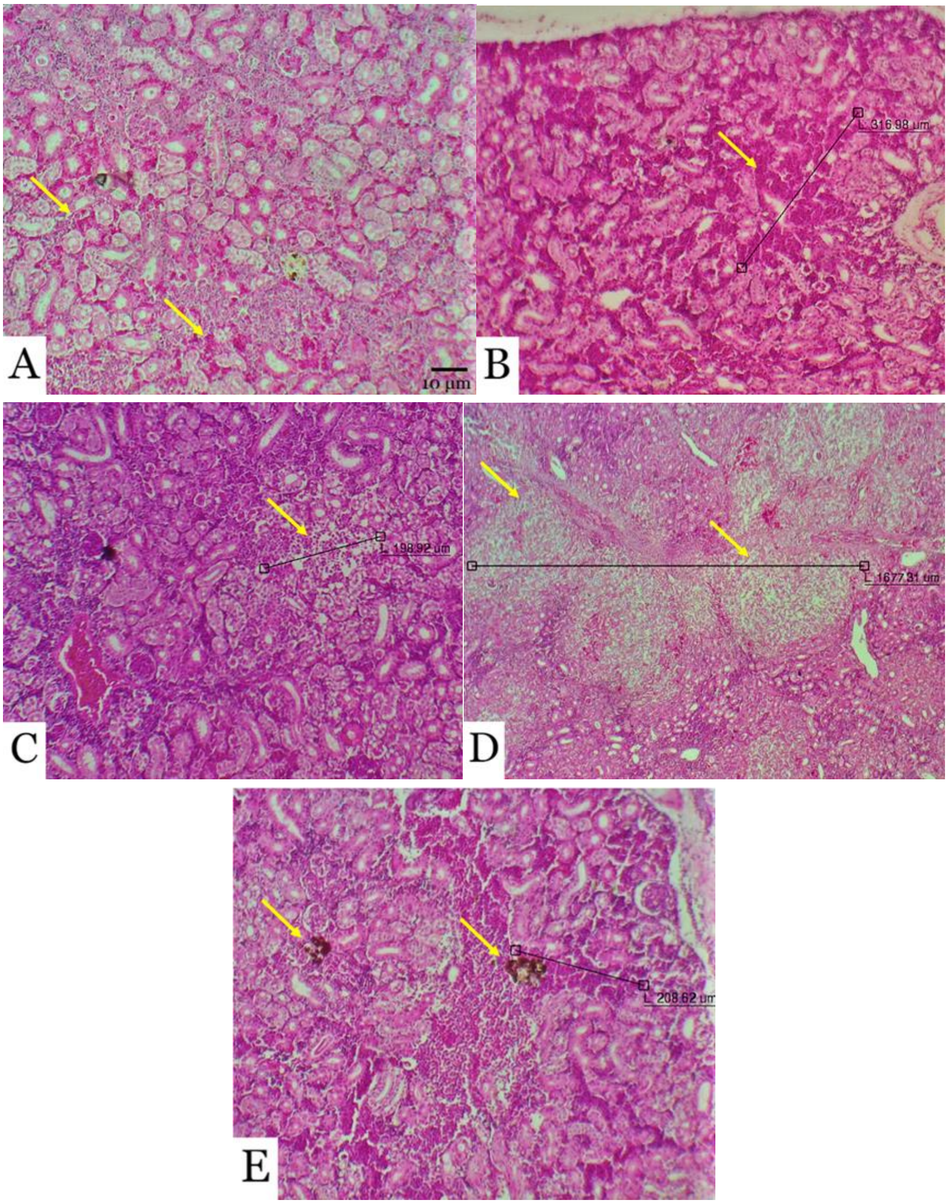


**Figure 4. Kidney histopathological changes in the challenged fish**

*(A) haemorrhages of kidney tissues (arrow) after 24 hours; (B) multifocal haemorrhages of kidney tissues (arrow) after 48 hour; (C) kidney tissues started deforming after 48 hours (arrow); (D) necrosis of kidney tissues (narrow); (E) melano-macrophage centers (MMCs) appeared in kidney tissues (arrow) after 24 hpi*

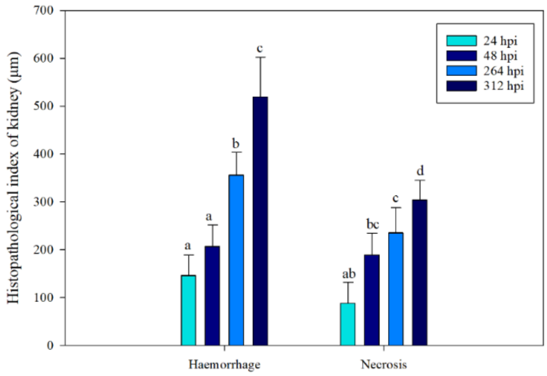


**Figure 5. Histopathological index of kidney prior and during the challenge**

*Values are Mean ± SE, different letters indicate significant differences between treatments (p < 0.05).*

Kidneys play the most important part in maintaining the water-salt balance. The primary task of a freshwater fish kidney is to produce copious dilute urine to counteract the passive influx of water across the gills and integument. *E. ictaluri* also significantly harmed the trunk kidney. Renal histopathology revealed acute degeneration of hematopoietic tissues, and destruction of Bowman's space, glomerular necrosis, tubular necrosis, and infiltration of leukocytes were detected. Invasion and multiplication of pathogenic bacteria can damage the kidney and lead to glomerulonephritis. The trunk kidney is vulnerable to hazardous by-products of the bacterium since it is a filtering organ. According to Shah et al. (2017), granulomas are formed consisting of layers of white blood cells such as macrophages or lymphocytes that surround the area of necrotic tissue and damaged cells. These cells are not removed from the body but fibrosis and calcification form granulomas. This is also a characteristic histopathological manifestation in some fish with white spot disease. Huizinga et al. (1979) found extensive necrosis in the mid-kidney of largemouth bass (*Micropterus salmoides*) infected with *Aeromonas hydrophila*. Shotts et al. (1972) found that channel catfish that had been given injections of *Aeromonas hydrophila* displayed an increase in lymphoid cells and some necrosis of the epithelial cells lining their tubules in the trunk kidney. Mawdesley-Thomas (1969) also found generalized necrotic changes in the kidney of goldfish with furunculosis. Klontz et al. (1966) reported that the most significant histopathology of rainbow trout infected with *Aeromonas salmnicida* was the change produced in the hematopoietic tissue. This hematopoietic tissue destruction was caused by *E. ictaluri* oligo-polysaccharide (O-PS) (Santander, 2014).

Similarly, Suanyuk et al. (2013) found that both striped catfish (*Pangasianodon hypophthalmus*) and hybrid catfish, *Clarias macrocephalus* × *Clarias gariepinus* when infected with *E. ictaluri* had haemorrhagic and granulomatous inflammation in the kidney, while mild to severe bacterial laden leukocytic cell infiltration were observed in channel catfish, zebrafish, hybrid catfish, and Nile tilapia (*Oreochromis niloticus*) (Soto et al., 2013; Suanyuk et al., 2013), the inflammatory cell infiltrates in zebrafish were composed of macrophages, neutrophils, eosinophilic granular cells, and fewer lymphocytes (Hawke et al., 2013). Whilst, variable degenerative changes turning into focal, multifocal or diffuse tubular, glomerular, and interstitial necrosis varying in intensity were evident in channel catfish, brown bullheads, Nile tilapia, zebrafish, and striped catfish (Petrie-Hanson et al., 2007; Soto et al., 2013).

### 3.4. Histopathological index of slpeen

At 24 hpi, the spleen also showed extensive confluent areas of necrosis within the parenchyma. Large areas of haemorrhages were observed in spleen. Here, extensive and prolonged bleeding leads to necrosis of the spleen. At the period 24 - 48 hpi, melano-macrophage centers appeared in spleen tissues (Figure 6). The histopathological index of the spleen over the challenge time is seen in Figure 5. Areas of haemorrhages and necrosis increased with the experiment time, quite early at 24 hpi (313.09 ± 52.42 µm; 76.39 ± 28.74 µm and reached 582.84 ± 84.84 µm (haemorrhages) and 407.25 ± 65.15 µm (necrosis). The differences between the histopathological index of the spleen at 312 hpi were statistically significant ($p < 0.05$).

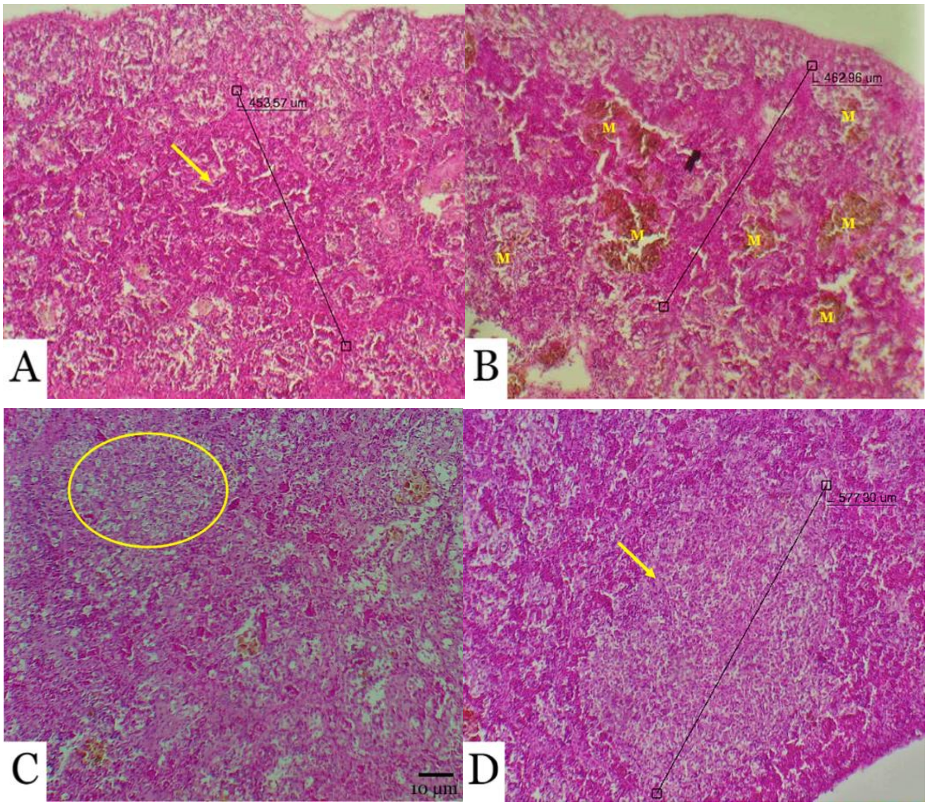


**Figure 6. Spleen histopathological changes in the fish after the challenge**

*(A) haemorrhages of spleen tissues (arrow) at 24 hpi; (B) melano-macrophage centers (M) appeared in spleen tissues at 24 – 48 hpi; (C) necrosis of spleen tissues (circle); (D) area necrosis of spleen tissues spread out*

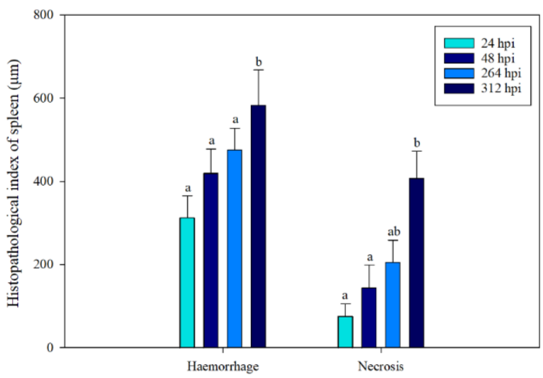


**Figure 7. Histopathological index of spleen prior and during the challenge**

*Values are Mean ± SE, different letters indicate significant differences between treatments ($p < 0.05$).*

The spleen is the main erythropoietic tissue in fish. The spleen is covered by a thin, fibrous capsule with little evidence of contractile ability. Red pulp is an extensive, interconnecting system of splenic cords and sinusoid capillaries (open capillaries), consisting mainly of erythroid cells and thrombocytes, and usually comprises the majority of the splenic parenchyma (Dyková et al., 2022). The melanomacrophage (MM) is prevalent in spleen. The MM is a phagocyte containing varying amounts of pigment, including melanin (black-brown), hemosiderin, ceroid or lipofuscin (yellow-pink to golden brown) localized in vacuoles. The MM and MMC were also found in the kidney and liver. The MMCs were thought to be a scavenger structure but their role in the immune system is ambiguous. Chronically stressed fish, including those that are unhealthy, tend to have more and larger MMC. The size and number of MMC also increase with fish age. Of all the organs inspected, the spleen had one of the worst injuries. Numerous researchers have noticed bacterial infection-induced cellular degeneration in the spleen (Mawdesley-Thomas, 1969; Ferguson & McCarthy, 1978; Huizinga et al., 1979; Liu et al., 2021). Wolke (1975) thought that severe hematopoietic and structural connective tissue necrosis of the spleen was the most prominent lesion. Since haematopoiesis is one of the functions of the spleen (Grizzle & Rogers, 1976), injury or damage to the spleen could cause the inability to produce erythrocytes and leucocytes resulting in anaemia and leucopoenia. Histopathology of liver, kidney and spleen of natural infected fish was validated with severe pathologies such as macrophage aggregation with hepatitis, degeneration of hematopoietic tissues and granulomatous splenitis (Loch et al., 2017; Mamun et al., 2022). This was consistent with clinical manifestations of fish, including reduced response, appetite or stopped eating.

Likewise, in previous work, splenic necrosis was evident in channel catfish, ayu, zebrafish, and striped catfish (Sakai et al., 2008; Hawke et al., 2013), vacuolations due to depletion of white pulp in channel catfish, brown bullheads, and hybrid catfish (Iwanowicz et al., 2006; Jarboe et al., 1984; Suanyuk et al., 2013) and the depletion of red pulp (Blazer et al., 1985), changed erythrocyte distribution caused by haemorrhage (Areechon & Plumb, 2009). Moreover, Newton et al. (1989) and Baldwin and Newton (1993) denoted that the splenic parenchyma was moderately congested and that the white pulp was hypocellular in channel catfish. Soto et al. (2013) found that macrophages and occasionally neutrophils containing bacteria were present within the hematopoietic tissue in channel catfish, ayu, zebrafish, and Nile tilapia.

According to Camp et al. (2000) the number of macrophage centers in fish increased significantly from 24 to 48 hpi, and multifocal haemorrhagesand necrosis areas of liver, kidney and spleen increased slowly. MMCs in the kidney and spleen increased over the experiment duration. This can be explained by the mobilization of the immunological parameters in the fish's immune response, which help to increase survival in the early stages of infection. At this time MMCs decreased from yellow-brown to dark brown and the number was dense on the tissue. At 312 hpi, the histopathological index was highest, so that the mortality rate was the highest during the experimental period.

## 4. CONCLUSION

This study demonstrated that there were significant differences in the histopathological index, specifically damage tissues measurements (hemorrhages; necrosis) of striped catfish exposed to bacteria *Edwardsiella ictaluri*. Many areas of the diseased liver, kidney, and fish organ tissues showed congested, hemorrhaged, and necrotized. The number of macrophage centers in the kidney and spleen increased during the time period 24 to 48 hpi. Tissue damage (multifocal hemorrhages; necrosis) increased sharply from the pre-infective stage, 24 hpi, 48 hpi and the largest area of damaged tissue was seen at 312 hpi. Besides the assessment of the immune responses, the histopathological index can also be used to assess the impact of *E. ictaluri* through the duration of infection.

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