

Histopathology of cultured juvenile *Totoaba macdonaldi* associated with bacterial isolates molecularly identified as *Vibrio* spp. during a temperature increase event

Histopatología de juveniles cultivados de *Totoaba macdonaldi* asociados con aislamientos bacterianos identificados molecularmente como *Vibrio* spp. durante un evento de aumento de temperatura

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ABSTRACT | *Totoaba macdonaldi* is an endangered species of croaker endemic to the Gulf of California, Mexico. This species has been cultured for the repopulation of wild stocks and commercialization at the Wildlife Management and Conservation Unit for the culture of *T. macdonaldi* at the Universidad Autónoma de Baja California, Mexico. A mortality episode was observed in 50 day-old juveniles, that coincided with an increase of temperature from 16 to 25.5°C that affected the aquaculture facility. Histopathological and bacteriological analysis of survivor fishes were conducted to investigate the possible causes of mortality. Gill damage including severe congestion in gill filaments, necrosis, and epithelial dissociation of lamellae were observed. Moreover, epidermal, hypodermic necrosis, as well as diffuse bacterial colonization of the dermis was detected. Severe congestion and hemolysis were detected in the heart's atrium and hepatic portal vessels. From the skin lesions, eight colonies of bacteria were isolated, these corresponded to four species of genus *Vibrio*, tentatively identified by partial sequence of the 16S rRNA gene as *Vibrio harveyi*, *V. rotiferianus*, *V. brasiliensis* and *V. shilonii*. From them, *V. harveyi* corresponded to 4 of the 8 isolates and was found in all moribund fishes. Characteristics of the tissue alterations related with these opportunistic *Vibrio* spp. suggest an acute mortality event favoured by a temperature increase. This is the first record of the histopathology of *T. macdonaldi* juveniles that suffered an unusual mortality event under an increase of temperature in cultured conditions associated with *Vibrio* spp.

Palabras clave

Sciaenidae
Bacteriología
Mortalidad
Juveniles de peces
Vibriosis
Vibrio harveyi

RESUMEN | *Totoaba macdonaldi* es una especie de corvina en peligro de extinción endémica del Golfo de California, México. Esta especie ha sido cultivada para el repoblamiento de stocks silvestres y comercialización en la Unidad de Manejo y Conservación de Vida Silvestre para el cultivo de *T. macdonaldi* de la Universidad Autónoma de Baja California, México. Se observó un episodio de mortalidad en juveniles de 50 días de edad, que coincidió con un aumento de temperatura de 16 a 25,5°C que afectó a la instalación acuícola. Se realizaron análisis histopatológicos y bacteriológicos de los peces sobrevivientes para investigar las posibles causas de mortalidad. Se observaron daños en las branquias, incluida una congestión severa en los filamentos branquiales, necrosis y disociación epitelial de las laminillas. Además, se detectó necrosis epidérmica e hipodérmica, así como colonización bacteriana difusa de la dermis. Se detectaron congestión severa y hemólisis en la aurícula del corazón y vasos portales hepáticos. De las lesiones cutáneas se aislaron ocho colonias de bacterias, correspondientes a cuatro especies del género *Vibrio*, identificadas tentativamente por secuenciación parcial del gen 16S rRNA como *Vibrio harveyi*, *V. rotiferianus*, *V. brasiliensis* y *V. shilonii*. De ellos, *V. harveyi* correspondió a 4 de los 8 aislamientos y se encontró en todos los peces moribundos. Las características de las alteraciones tisulares relacionadas con estos *Vibrio* spp. oportunistas, sugieren la ocurrencia de un evento de mortalidad aguda favorecido por un aumento de la temperatura. Este es el primer registro de la histopatología de juveniles de *T. macdonaldi* que sufrieron un evento inusual de mortalidad ante un aumento de temperatura en condiciones de cultivo asociado con *Vibrio* spp.

INTRODUCTION

As the result of overfishing and alterations of breeding and nursery areas, the endemic croaker of the Gulf of California, Mexico, *Totoaba macdonaldi* (Gilbert, 1890) has become a threatened species that was included in Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in 1976 (Pedrín-Osuna *et al.*, 2001; Minjarez-Osorio *et al.*, 2014; Findley, 2010; True, 2012; Juarez *et al.*, 2016). The culture of this species represents an important alternative for the repopulation of wild stocks and for commercial production.

The Wildlife Management and Conservation Unit for the culture of *T. macdonaldi* at the Facultad de Ciencias Marinas, Universidad Autónoma de Baja California, located in Ensenada, Mexico (FCM-UABC) has been producing this species for repopulation and commercial purposes. However, as any other fish production operation in captivity, opportunistic infectious diseases tend to become a common problem (Pillay and Kutty, 2005; Patarnello and Vendramin, 2017). Among the pathogens that cause diseases in aquatic cultured species, bacteria conform a group that requires increased attention since most of these agents are opportunists, which means that only cause disease or mortalities under stressful conditions, such as high densities, deficient water quality, temperature fluctuations and other management or environmental conditions (Toranzo *et al.*, 2005; Noga, 2010; Saravanan *et al.*, 2013).

From an economic standpoint, bacteria belonging to the genus *Vibrio* have become a prevalent problem for modern mariculture due to their role in acute mortalities of fishes in culture, which also has been related to increments in temperature (Noga, 2010; Austin and Austin, 2016; Patarnello and Vendramin, 2017; Ina-Salwany *et al.*, 2019). It is possible, that all marine fishes are vulnerable to at least one species of *Vibrio*, being some of the most frequent pathogenic species: *V. harveyi*, *V. alginolyticus*; *V. parahaemolyticus* and *V. anguillarum* (Toranzo *et al.*, 2005; Noga, 2010; Patarnello and Vendramin, 2017).

Diverse reports have highlighted the pathogenic potential of *V. harveyi* as the etiological agent of several diseases in fish (Zhang *et al.*, 2020). These diseases include, vasculitis in the Sandbar shark, *Carcharhinus plumbeus* (Grimes *et al.*, 1984); eye lesions in several fish species (Ishimaru and Muroga, 1997); gastroenteritis in the grouper, *Epinephelus coioides* (Lee *et al.*, 2002); the red drum, *Sciaenops ocellatus* (Liu *et al.*, 2003), hemorrhagic skin lesions in the European bass, *Dicentrarchus labrax* (Jale and Gülşen, 2008), the Arabian surgeon fish, *Acanthurus sohal* (Hashem and El-Barbary, 2013) and tail rot disease of sea bream, *Sparus aurata* (Haldar *et al.*, 2010), flattened lesions in the tiger puffer *Takifugu rubripes* (Mohi *et al.*, 2010), skin ulcer disease in juvenile hybrid groupers *Epinephelus fuscoguttatus* x *Epinephelus lanceolatus* (Shen *et al.*, 2017), scale drop and muscle necrosis disease in farmed barramundi *Lates calcarifer* in Vietnam (Dong *et al.*, 2017), Vibriosis in the turbot *Scophthalmus maximus* (Xu *et al.*, 2019).

The aim of this study was to carry out a histopathological and bacteriological analysis of survivor individuals of *T. macdonaldi* that could reveal the possible causes of mortality after an unusual mortality event under an increase of temperature in cultured conditions.

Mortality event and sampling

In August of 2018, a temperature increases in the tank water from 16°C to 25.5°C over 8 days was observed at the aquaculture facility of the FCM-UABC attributed to a sudden increase in water temperature during summertime. In three tanks of 8 m³ coupled to a recirculation system, unusual mortality of juveniles of *T. macdonaldi* was observed (10 fish daily during 12 days, originating 100% of mortality). Culture density was 100 organisms/m³ (normal density in the FCM-UABC). The dissolved oxygen was 6.3 mg/L (considered adequate for that density). Ammonia compounds were also within the tolerance limits of the species (0.4±0.3 mg/L) (True, 2012). In addition, fishes were provided with food three times that day as the protocol of feeding indicates (True, 2012). Three moribund juveniles of 50 days after hatching (dah) (6.5 cm of total length) that presented loss of balance, reduced swimming activity, remained lethargic at the shallow and bottom zone of the tank, had visible skin lesions and showed opercular movement, were sampled from the tanks (one per tank) at day 4 of the temperature increment. Live fish were transported in refrigerated containers with culture

water to the Laboratory of Biology and Pathology of Aquatic Organisms of CICESE, where external signs and morphologic changes were recorded during the necropsy following the protocol described by Ferguson (2006).

Bacteriological analysis

In aseptic conditions, the skin wounds were cleaned 3 times with sterile seawater to remove organic material before bacteriological sampling. The lesions were then cut with a sterile scalpel, the sterile loop was introduced into the lesion and the sample was inoculated in general Zobell marine agar (HIMEDIA® Laboratories) and selective Thiosulfate Citrate Bile Sucrose agar (TCBS Difco™). The media were incubated at 25°C ± 1°C (Precision 2EG) for 24 h. Following the protocol described by Garcia-Mendoza *et al.* (2019), the bacteria were isolated by their morphotype, dominance, and color. Furthermore, the phenotypic characterization was complemented with Gram staining, catalase, and oxidase tests.

Molecular identification

The DNA extraction of bacteria was performed using the kit Qiagen (QIAamp DNA) following manufacturers' recommendations. After this, two oligos (fD1 and rP2) were used for the amplification of the 16S rRNA gene (1500 pb) by polymerase chain reaction (PCR). The PCR mixes were prepared at 25 µL where the components and concentrations of the mixture were: 1x buffer with 1.5 mM MgCl₂, 100 µM de dNTPs, 1µM forward and reverse oligo (Eton Bioscience y Alpha DNA), 1 U de Taq DNA polymerase (APEX™) and 700 ng/µL of DNA. The reactions were run using a thermocycler (Apollo model ATC 201) following the amplification conditions mentioned by Devereux and Wilkinson (2004). The detection of the PCR products was carried out by electrophoresis in a 1.2% agarose gel using a 1.5 µL of the molecular marker of 100 a 1013 pb. The gel was stained with GelRed Biotium®. The amplified products were partially sequenced using the Sanger method at Eton Bioscience, Inc., San Diego, CA. The analysis of the sequences were performed in MEGA7 software and Geneious Prime. The sequences were analyzed and compared in the GenBank database by the BLASTn using at least 700nt. The sequences that showed the highest percentage identity was taken as the putative species.

Histology analysis

The organisms were fixed in Davidson's solution, which has been used in the laboratory with good results for fish for 7 days, followed by decalcification in a formic acid-formalin solution for 10 days to have a better decalcification of bone tissue and finally transferred to 70% alcohol until analysis. Before processing, the areas of the skin lesions were dissected and placed separately in histological cassettes. All samples were dehydrated in a graded series of alcohol, cleared in benzene, and embedded in paraffin. The samples were cut with a LEICA RM2255 microtome to obtain serial sections of 5-6 µm and were mounted on gelatinized slides. Finally, the slides were stained with hematoxylin-eosin (H&E) (Bancroft and Layton, 2013) and observed with a microscope coupled with a digital camera.

RESULTS

All three fish had lesions on the skin located near the dorsal fin. Additionally, one fish showed a wound on the cephalic region. The wounds were characterized by a loss of scales, slight depigmentation, and erosions of the skin. Ocular opacity was also recorded in one of the specimens (Figure 1). The gills were slightly pale at the ends of the gill filaments of animals. In the abdominal cavity, apparent alterations were not observed on the liver, spleen, intestine, or stomach. Fish had a notorious amount of undigested food in the stomach and intestine.

Eight colonies were isolated from the skin lesions of the fish. The phenotypic and biochemical characteristics of bacterial isolates are shown in Table 1. According to the partial sequence of the 16S rRNA gene (~700 nt) of these bacteria, they corresponded to the *Vibrio* genus, tentatively assigned to the species:

V. harveyi, *V. brasiliensis*, *V. shilonii* and *V. rotiferianus*. From the identified species, *Vibrio harveyi* is of importance since 4 of the 8 isolated and sequenced colonies corresponded to this species (Table 1).

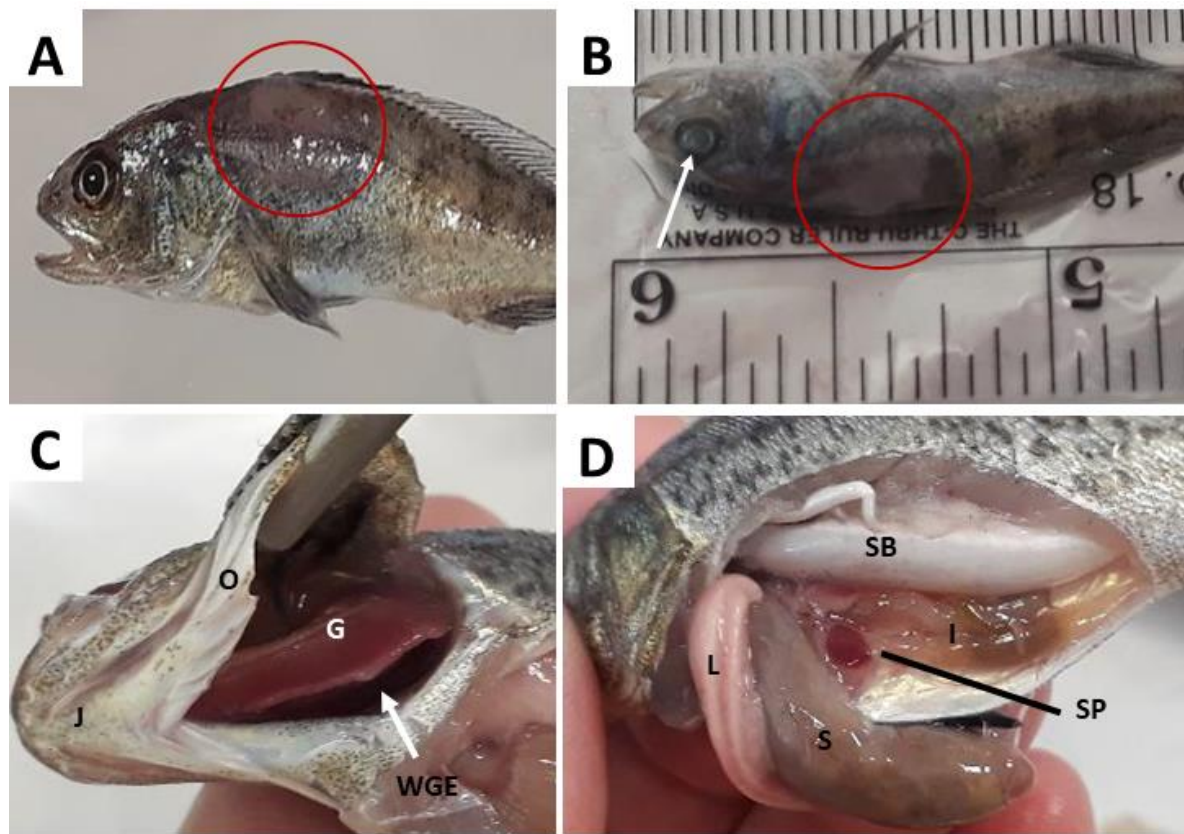


Figure 1. Clinical signs in juveniles of *T. macdonaldi* of 50 dph. A and B. Skin lesion near the dorsal fin. C. Opercular cavity. D. Abdominal cavity where no evident lesions are observed. Abbreviations: G, gills; I, intestine; J, jaw; L, liver; O, operculum; S, stomach; SB, swim bladder; SP, spleen; WGE, white gill ends. Red circles demark the skin lesion and the white arrow shows an eye opacity.

Table 1. Resume of the bacterial identification analysis of the eight isolated colonies from the skin lesions. The table shows, for each strain, the ID, morphology, species determined by Blastn analysis (Species), nucleotide identity to the closest species (% identity), Gram staining (Gram), oxidase test (O), catalase test (C) and lesion and bacterial presence.

Identification	Colony morphology	Molecular identification		Biochemical analysis		
		Species	identity (%)	Gram	O	C
007-18	Cocobacillus	<i>Vibrio harveyi</i>	99.57	-	+	+
008-18	Short rod	<i>Vibrio brasiliensis</i>	97.65	-	+	-
009-18	Short rod	<i>Vibrio rotiferianus</i>	99.71	-	+	+
010-18	Cocobacillus	<i>Vibrio harveyi</i>	99.71	-	+	+
011-18	Short rod	<i>Vibrio shilonii</i>	99.72	-	+	+
012-18	Cocobacillus	<i>Vibrio harveyi</i>	99.43	-	+	+
013-18	Short rod	<i>Vibrio rotiferianus</i>	98.71	-	+	+
014-18	Short rod	<i>Vibrio harveyi</i>	99.20	-	+	+

At the histological level, the wounds of the three fishes were characterized by necrosis of the epidermis and the hypodermis (Figure 2A). At the hypodermis, light bleeding was detected, consisting of leukocytes

and erythrocytes. Besides this infiltrate, a couple of rod-shaped bacteria were identified in this zone and over the scales of the samples (Figure 2B). On the other hand, the dermis did not evidence signs of inflammation such as thickening of the tissue. However, in several sections, the loss of continuity of the tissue by the presence of bacterial nodules was observed (Figure 2C).

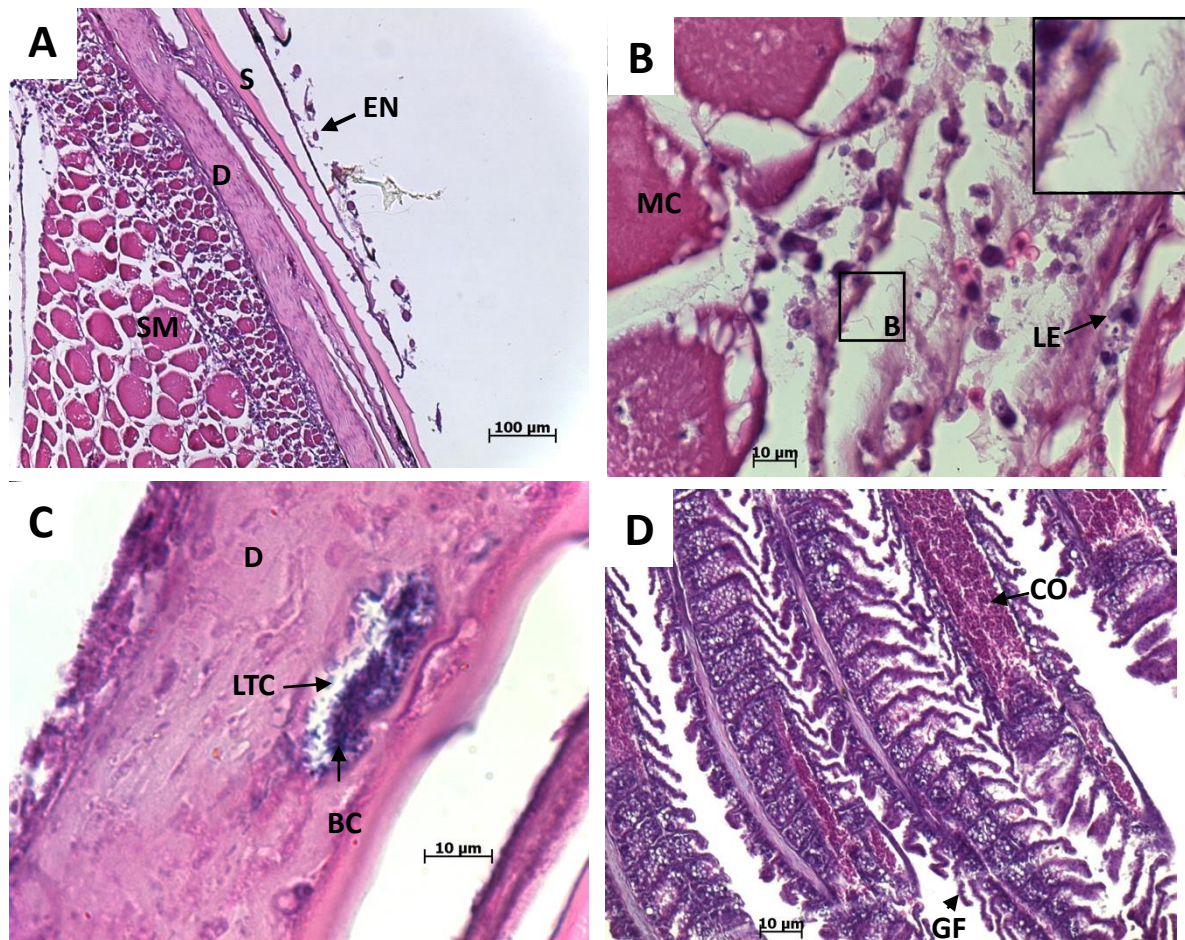


Figure 2. *Totoaba macdonaldi*. Histopathological observations in the skin and gills. A, B and C. Skin alterations. D. Gill alterations. Abbreviations: B, bacteria; BC, bacterial colonization; CO, congestion; D, dermis; EN, epidermal necrosis; GF, gill filament; LE, leukocyte; LTC, loss of tissue continuity; MC, muscular cell; SM, skeletal muscle; S, scale. Hematoxylin-eosin.

The gills showed moderate congestion of the blood vessels of the gill filaments, degeneration of the chloride cells, and a relative increase of mucous cells at the base of the gill filament (Figure 2D and Figure 3A). Also, in the lamella, degeneration changes which included, dissociation of the epithelial cells of the lamella that gave a “disordered look”, and a complete loss of the normal structure were observed (Figure 3 B and C). The spleen and heart’s atrium showed some changes such as a relative decrease of red pulp and the predominant presence of white pulp in the spleen, where white cells were observable (Figure 3D). Another observation was numerous erythrocytes, which were present in the blood and portal vessels of the liver, and to a greater proportion in the heart’s atrium (Figure 4 A, B & C). In the last two (portal vessels and heart’s atrium), the erythrocytes presented signs of hemolysis, nuclear diffusion, and cellular remnants around them (Figure 4 B & D).

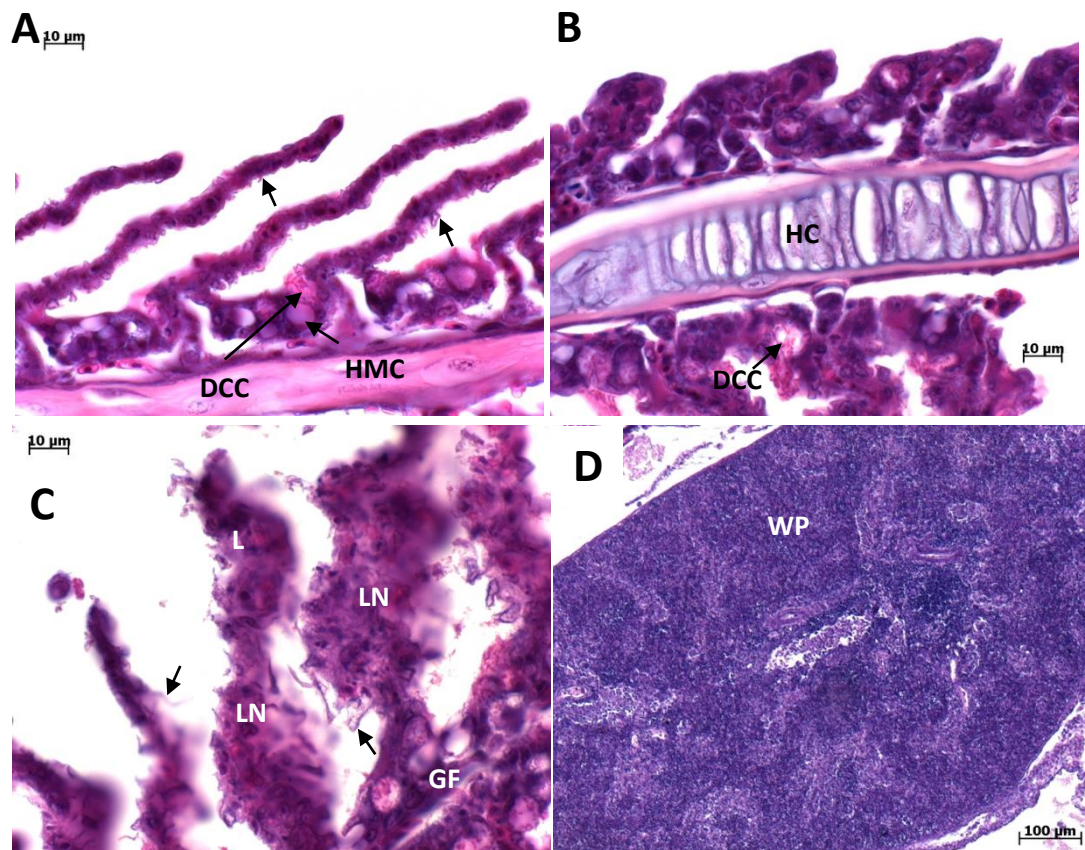


Figure 3. *Totoaba macdonaldi*. Histopathological observations in the gills and spleen. A, B & C. Gill alterations. D. Spleen alteration, predominant presence of white pulp. Abbreviations: DCC, degeneration of chloride cells; GF, gill filament; HC, hyaline cartilage; HMC, numerous mucous cells; L, lamella; LN, lamellar necrosis; WP, white pulp. Black arrows show lamellar epithelial cells dissociation. Hematoxylin-eosin.

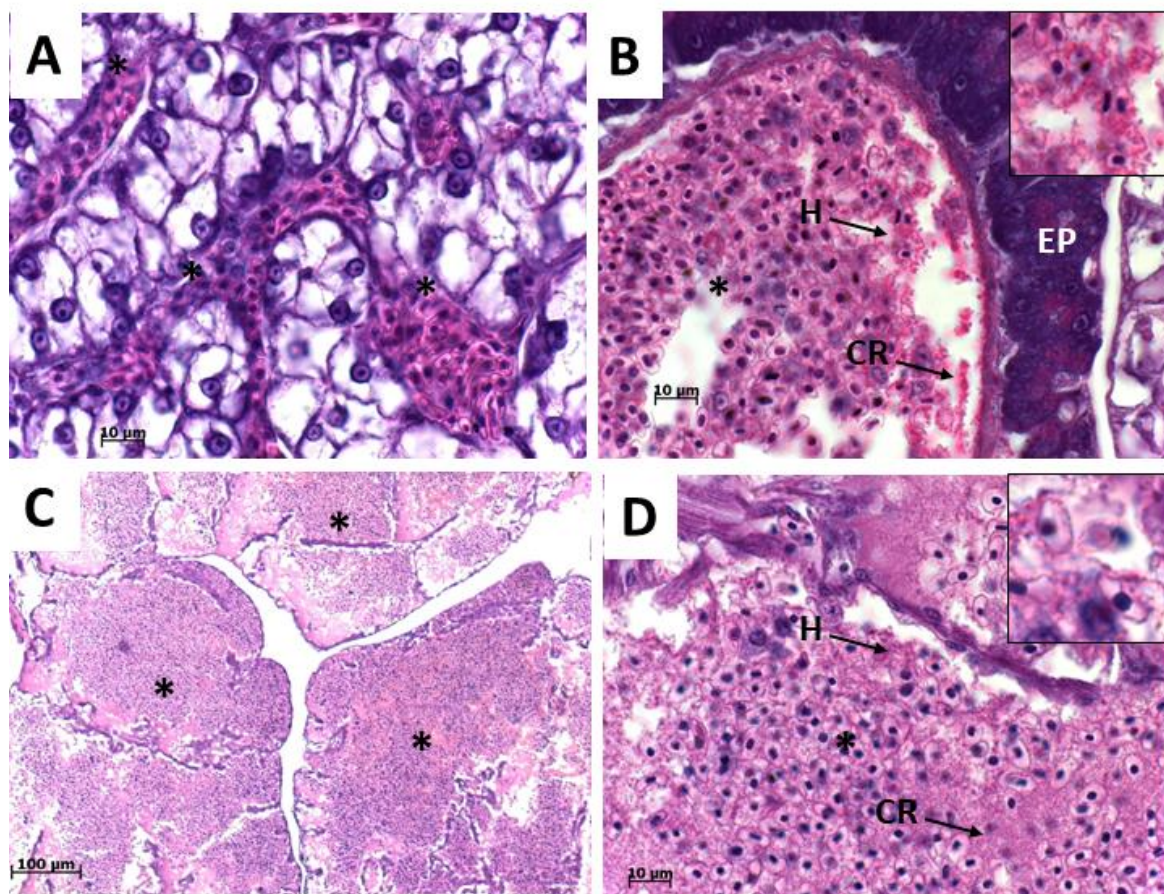


Figure 4. *Totoaba macdonaldi*. Histopathological findings in the liver and heart's atrium. A & B. Hepatopancreas. C & D. Heart's atrium. Abbreviations: CR, cell remnants; EP, exocrine pancreas; H, hemolysis. Asterisk marks the abundant erythrocytes in all the organs. Hematoxylin-eosin.

DISCUSSION

The mortality event described in this study was strongly related to the increase in the culture tanks' temperature, which varied from 16 to 25.5°C in all of them, due to unusual warm summer season. The normal temperature fluctuation during summer in the region is from 16 to 21°C. This condition has been related to a predisposing factor for vibriosis, which has been called a “summer disease” (Noga, 2010; Patarnello and Vendramin, 2017).

The loss of scales and the skin lesions documented in this study were similar to those observed in the vibriosis associated with *V. harveyi* in the sea bass (*Dicentrarchus labrax*) reported by Jale and Gülşen (2008). Similarly, the skin lesions previously mentioned were superficial like the ones described by Hashem and El-Barbary (2013) in the vibriosis event by *V. harveyi* in the Arabian surgeon fish (*Acanthurus sohal*), which showed lesions of necrosis of the epidermis and hypodermis at the histological level. Even with these lesions, the leukocyte infiltration was light and restricted to the hypodermis. According to Ferguson (2006), this is related to the movement of pathogens and the inflammatory processes.

Rod bacteria were identified in scales of the epidermis, dermis, and hypodermis. Mostly, the bacterial morphology agreed with the isolated bacteria in which 4 of the 8 colonies were related to *V. harveyi* by partial sequencing (~700 nt) of the 16S rRNA gene. It is important to underscore that some of the strains isolated from the diseases *T. macdonaldi* showed a high sequence identity to other characterized bacterial strains known to cause diseases in the aquatic organisms. For instance, one *V. harveyi* isolate from this study showed the highest identity to a *V. harveyi* strain (MZ093615.1) that was isolated from diseased Turbot

(*Scophthalmus maximus*). On the other hand, gill's paleness is a sign denoted by Toranzo *et al.* (2005) as a manifestation of serious anemia in moribund fish. In juvenile totoabas, paleness was only noted in the distal segments of gill filaments and could indicate the start of anemia. However, histologically, the gill's lamella showed critical alterations such as dissociation of epithelial cells, shorting of the lamella, and loss of the normal lamella structure. Particularly, the dissociation of epithelial cells is an acute alteration that could diminish the area for exchange between water and blood (Olha *et al.*, 1989; Speare and Ferguson, 2006). This could be related to an effort to obtain more oxygen due to the systemic hemolysis in congested hepatic portal vessels and the heart's atrium, and the relative absence of red pulp which is caused by the lack of oxygen (Noga, 2006).

Ruwandeeepika *et al.* (2012), reviewed the pathogenesis virulence factors and virulence regulation of vibrios that belong to this genus. Hemolysins are among the major exotoxins identified in this clade, and, interestingly, haemolysins have also been shown to have cardiotoxic activities that can be related to the alterations observed in the heart's atrium. The most pathogenic strains generated extracellular factors with a great capacity to hemolyze fish erythrocytes (Zhang and Austin, 2000). Zhang *et al.* (2001) found a duplication of hemolysin genes in a virulent isolate of *V. harveyi*.

Some bacterial diseases are commonly characterized by the induction of anorexic signs related to a chronic process (Noga, 2010; Patarnello and Vendramin, 2017). In the present study, the histopathological results evidenced the acute nature of this mortality event in which the combination of the lesions could have led the fish to a hypoxic and systemic decompensation, probably related to the increase in temperature and the proliferation of *Vibrio* spp. and, possibly, *V. harveyi* strains in particular (Lin *et al.*, 2010; Zhang *et al.*, 2018). However, it is also important to take into account that *V. rotiferianus* and *V. brasiliensis* have been considered pathogenic to rainbow trout (*Oncorhynchus mykiss*), *Artemia nauplii*, and with shrimp (*Penaeus vannamei*) (Thompson *et al.*, 2003; Austin *et al.*, 2005; Li *et al.*, 2021) and that *V. shilonii* has been a cause of bleaching in the coral *Oculina patagonica* (Kushmaro *et al.*, 1996; Rosenberg and Falkovitz 2004; Stratus *et al.*, 2017) and has been involved in the acute hepatopancreatic necrosis disease of the white shrimp (*Litopenaeus vannamei*) (Quang *et al.*, 2020). Corroboration of the identity of isolated *Vibrio* spp. by sequencing of the complete 16S rRNA gene, as well as specific studies based on Koch postulates are needed, to determine if a particular species of these *Vibrio* or if a synergy of them, induced this vibriosis associated with sudden temperature increments.

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