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THE IMPACT OF NATURAL CO-INFECTION OF *Dactylogyrus* spp. AND *Aeromonas hydrophila* ON BEHAVIOURAL, CLINICAL, AND HISTOPATHOLOGICAL CHANGES OF STRIPED CATFISH, *Pangasianodon hypophthalmus* (SAUVAGE, 1878): A CASE STUDY

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Abstract: The present case study reported the effects of *Dactytlogyrus* spp. infection on cultured striped catfish, *Pangasianodon hypophthalmus*. The clinical signs, gross, and histopathological changes inflicted by the parasite on the gills, liver, spleen, and kidney were examined. The fish were sampled from Aquaculture Research Station of Universiti Putra Malaysia, Puchong, Selangor. *P. hypophthalmus* infected with *Dactylogyrus* spp. exhibited several clinical signs, including lethargy, unilateral swimming and sluggish movement on the water surface. Post-mortem examination revealed the congestion of the swim bladder and haemorrhages of the external organs. Examination on the gill indicated hypertrophy and hyperplasia, proliferation of epithelial cells and fusion of the secondary lamellas. The liver sections exhibited severe haemorrhages, vacuolation and congestion of the hepatic vein. Haemorrhages were observed in the kidneys; other lesions were rupture of renal tubules and aggregation of lymphocytes in almost all of the organs examined. Bacteriological examination of the moribund fish revealed secondary infection with bacteria, *Aeromonas hydrophila*. The findings revealed that the damage to the gills could be related to the respiratory impairment caused by *Dactylogyrus* spp., while the external injuries may serve as the point of entry for bacteria, making the fish vulnerable to *A. hydrophila* infection that subsequently resulted to mortality.

Keywords: *Pangasianodon hypophthalmus, Dactylogyrus* spp., clinical signs, histopathology.

Introduction

Disease is one of the factors limiting the growth of the aquaculture sectors (Aly & Albutti, 2014), and parasites are known to be one of the disease causative agents wreaking havoc in the fish farming industry (Scholz, 1999). Parasitic infestations are commonly associated to economic losses in intensive farming, mainly due to the high mortality rate, reduced fecundity, weight loss, and exorbitant cost of therapy (Mohammadi, 2012). *Dactylogyrus* spp. are among the most prevalent monogenean parasites (Shamsi *et al*., 2009); they show strict host and site specificity (Özer & Öztürk, 2005). Its abundance is influenced by many factors, such as seasonal and temperature changes (Özer, 2002; Öztürk & Altunel, 2006), as well as other abiotic and biotic factors (Gonzales-Lanza & Alvarez-Pellitero, 1982). The interaction between host and pathogen is occasionally one-to-one in the aquaculture system (Xu *et al*., 2007). Nevertheless, parasites-bacteria interactions showed evidence of co-interaction, which contributed to the severity of diseases (Bush *et al*., 2003). Numerous studies demonstrated the synergistic interaction of parasites and bacteria resulting in co-infection (Busch *et al*., 2003; Pylkkö *et al*., 2006; Bandilla *et al*., 2006).

The interaction could lead to greater damage in the host tissue, which eventually result in superinfection due to secondary bacterial superimposition (Jalali, 1998; Jiang *et al.*, 2014).

There are reports of infections in gills of pond-cultured striped *P. hypophthalmus* in Southern Vietnam with parasitic monogeneans, *Thaparocleidus siamensis* and *T. caecus* (Thuy & Buchmann, 2008). However, there is still a dearth of information regarding the outbreak of *Dactylogyrus* spp. in *P. hypophthalmus* cultured in a closed system. Considering its economic profitability, it is crucial to observe the clinical signs, behavioural changes, histopathological changes of the gills, liver, and kidney and how it could relate to mortality.

Materials and Methods

Clinical, Parasitology, and Histopathological Examinations

The fish (*P. hypophthalmus*) were collected from the Puchong hatchery of Universiti Putra Malaysia during infestation. The fish (body weight: 1.61±0.58 kg; total length: 57.63±5.98 cm) were reared in a 40-tonne square concrete tank (3 m × 3.3 m × 4 m) with a stocking density of 2 tails L^{-1}. The fish were sampled twice: first (i) during early infestation, the fish were sampled (n=10); and, second (ii) two weeks later, moribund fish (n=4) with external necrotic lesions. During sampling, clinical examination of the sampled fish was employed to observe any clinical signs. The fish were subjected to parasitological investigation as described previously by Lucky (1977). The gills, liver, and kidney were taken and immediately fixed in a 10% neutral buffer formalin, processed using a standard fish histological procedure by sectioning at 5 µm, stained with hematoxylin and eosin (H&E).

Bacteriological Examination

Blood sample and swab from the posterior kidney were taken for bacterial isolation and identification. Prior to euthanasia and dissection, blood was drawn (approximately 0.3 mL) from the caudal peduncle, and after euthanasia, and dissection swabs were obtained from the posterior kidney; both were inoculated onto the Trypton Soy Agar (TSA) (Difco) agar and incubated at 37°C for 24 h. A single colony from each plate was sub-cultured onto another TSA agar and conventional biochemical analysis was conducted and subsequently identified using commercial kit API 20E® (Biomėrieux,USA).

Results

Clinical signs

During early infestation, the clinical signs observed on *P. hypophthalmus* were inappetence, lethargy, slightly darker skin pigmentation, unilateral swimming, sluggishness, swimming close to the water surface, aggregating near the water inlet and hurtling on the tank walls. The infestation had increased the stress threshold of the fish, evident with exhibition of aggressive swimming behaviour and erratic movements, which eventually led to injuries on the frontal part of the head and mouth due to crashing against the tank walls. Fish that showed the aforementioned behaviour eventually died due to suffocation, hence termed as traumatised-mortality. Meanwhile, fish that were hurtling against the tank walls survived, but were swimming sideways and/or upside-down. After two weeks of the first sampling, those moribund fish showed skin discolouration (pale) with necrotic lesions on the mouth and head parts (Figure 3.A).

Infected gill of P. hypophthalmus

Wet mounts revealed that the gills of the affected fish were congested and covered with excessive mucous (Figure 1.A), and intense infestation with *Dactylogyrus* spp., as evident with the presence of parasites attached to the gill filaments (Figure 1.B). Histological examination revealed the proliferation of epithelial cells (Figure 2.A.1), hyperplasia and hypertrophy of the gills (Figure 2.B.2). Besides, hyperplasia that developed from active proliferation of the epithelial cells

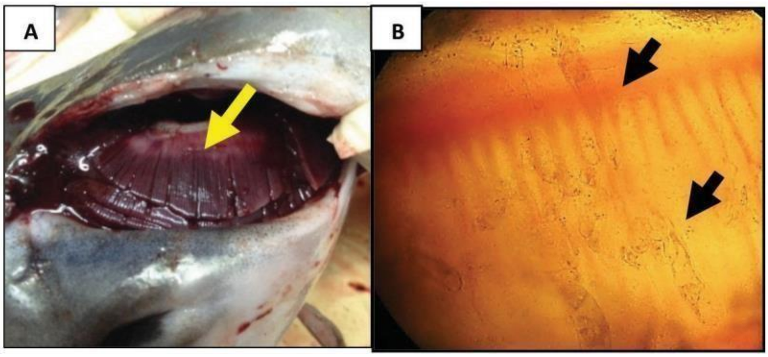

Figure 1: (A) Congested gills of an infected fish with whitish discolouration at the base due to excessive mucous secretion. (B) Wetmount showing *Dactylogyrus* spp. attached to the filaments of the gills (magnification ×100)

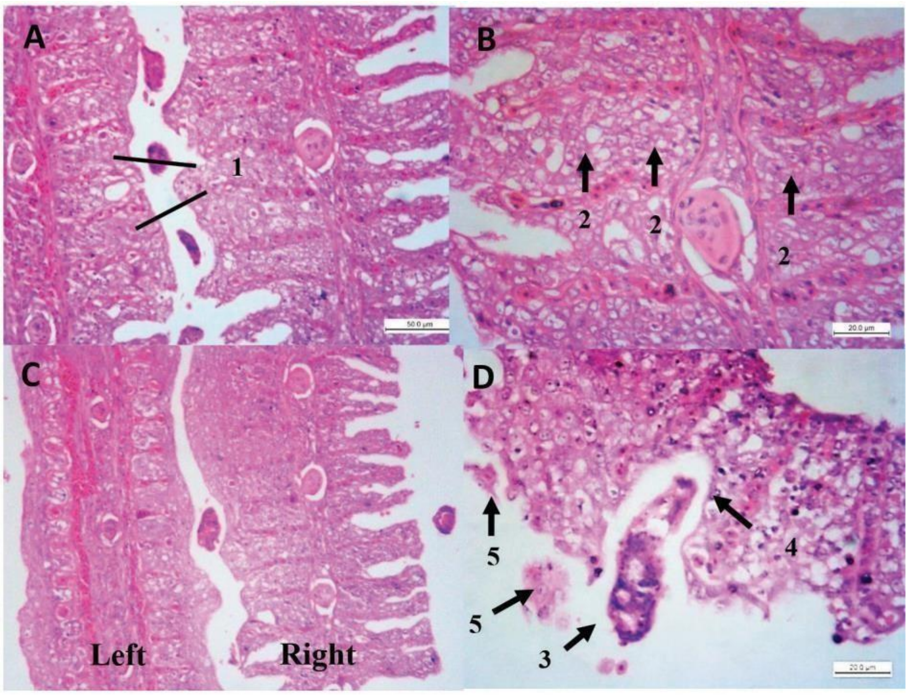

Figure 2: Changes on gills of infected *P. hypophthalmus*. (A.1) Proliferation of epithelial cells; (B.2) hyperplasia and hypertrophy of the epithelial cells; (C) complete (left) and partial (right) fusion of the adjacent secondary lamellae. (D.3) Photomicrograph showing the *Dactylogyrus* spp. in-between of the successive secondarylamellae; (D.4) premature cells death and (D.5) lifting of the epithelial cells. H&E (magnification A, C ×100; B, D ×400)

consequently caused partial and complete fusion of the adjacent secondary lamellas, respectively (Figure 2.C. left and right). *Dactylogyrus* spp. were observed in between the secondary lamellas (Figure 2.D.3) with certain level of necrosis on the gills and premature cells death due to the attachment of the parasites (Figure 2.D.4); epithelial lifting was also observed on the gills of affected fish (Figure 2.D.5).

Effects on Liver, Spleen, and Kidney of P. hypophthalmus

Post-mortem on the fish that died due to traumatised-mortality during the first sampling showed congestion of blood vessels on the surface of the air bladder (Figure 3.B). Meanwhile, on the second sampling, there were necrotic lesions on the liver (Figure 3.C) with excessive intra-peritoneal fluid (Figure 3.D) in the moribund fish sampled. During the first sampling, the *Dactylogyrus* spp. were found prevalent in approximately 50%, with nearly 70% of cumulative mortality recorded during the outbreak. Bacteriological examination showed an absence of bacterial isolates (*A. hydrophila*) from the fish that were sampled earlier on the first sampling (traumatised-mortality). However, the moribund fish (sampled two weeks after the first sampling) tested positive for *A. hydrophila*. The bacterial isolates from the fish were identified as *A. hydrophila*. The API 20E profile showed positive for b-galactosidase, arginine dihydrolase, lysine decarboxylase, indole production, acetoin production (Voges Proskauer), gelatinase, glucose, mannitol, sucrose, and amygdalin.

Histopathological examinations on liver tissues showed severe haemorrhages (Figure 4.A.1), vacuolations (Figure 4.A.2), and congestions of the hepatic veins (Figure 4.A.3). The congestions can be seen as the aggregation of red blood cells within the blood vessels. There were presence of melano-macrophage centres (MMCs) in the spleen and kidney tissues, with higher concentration of melanophore melanin pigments observed in the spleen (4.B.4) and kidney tissues than the liver (Figure 4.C.4). In the kidney tissues, there were degeneration of excretory tubules and glomeruli (Figure 4.D.5), aggregation of lymphocytes (Figure 4.D.6), detachment of kidney tubules (Figure 4.D.7) and congestion of the renal tissues with increased number of the red blood cells in those areas (Figure 4.D.8).

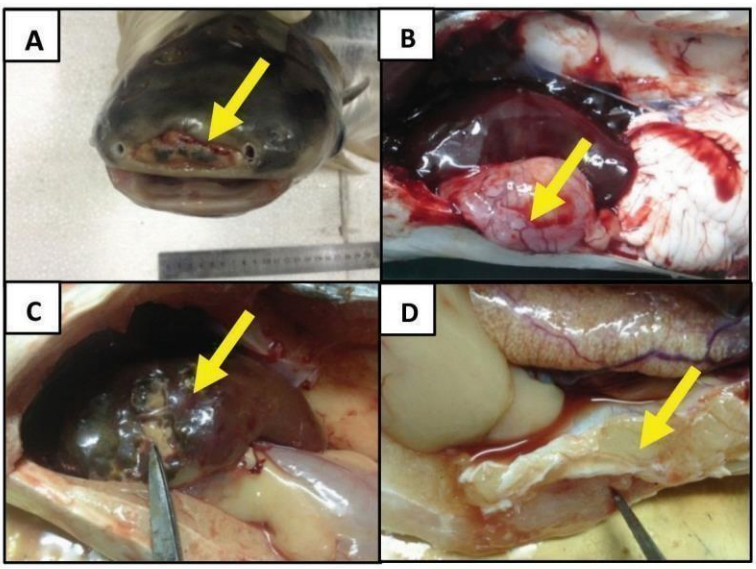

Figure 3: Gross lesions in the infected moribund *P. hypophthalmus* (A) gross necrotic lesions on the upper jaw; (B) congested swim bladder following traumatized-mortality; (C) gross necrotic lesion on the liver and (D) excess intra-peritoneal fluid

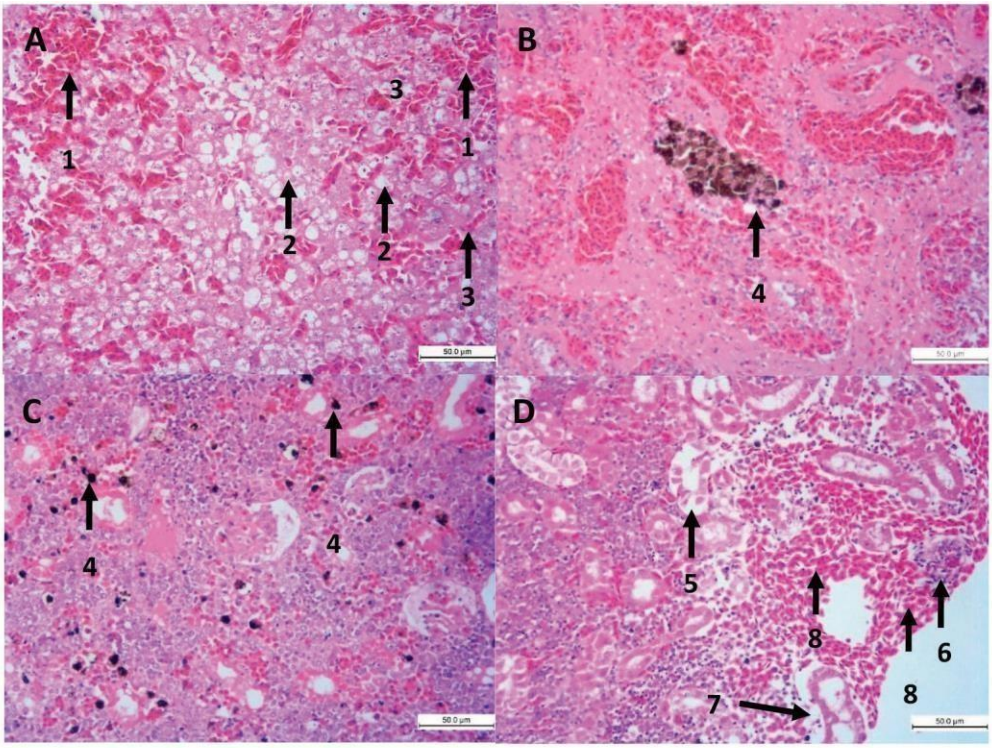

Figure 4: Photomicrographs of the liver, spleen, and kidney tissues. (A.1) Liver with multiple haemorrhagic points; (A.2) multiple areas of vacuolation in the liver; (A.3) congestion of the hepatic veins. (B.4) (C.4) Presence of melano-macrophages centres aggregates on both spleen and kidney, respectively. (D.5) Degeneration of the kidney tubules; (D.6) lymphocytic aggregation; (D.7) detachment of kidney tubules; (D.8) congestion of the renal tissues H&E (magnification ×100)

Discussion

Generally, the gill is a very sensitive organ that plays important roles in gaseous exchange, osmoregulation, excretion, circulation, and hormone production (Hassaninezhad *et al*., 2014). The gill filaments and lamellas that are covered with epithelial cells, mucous, and blood vessels are primarily facilitating in providing the extensive surface contact with the environment (Mir & Channa, 2009). In the present study, the attachment of *Dactylogyrus* spp. on the gills of *P. hypophthalmus* had caused severe irritation, triggered the proliferation of the epithelial cells, hyperplasia of secondary lamellae, fusion of adjacent secondary lamellae, epithelial lifting, and leukocytic infiltration. Similarly, these lesions were found in various fish species with parasitic infestation, such as the American eel and European eel (Abdelmonem, 2010). The oscar, *Astronotus ocellatus,* discus, *Symphysodon discus,* (Mohammadi *et al*., 2012), Persian sturgeon, *Acipenser persicus,* Stellate sturgeon, *Acipenser stellatus,* are other species that presented and similar lesions at the peak of parasitic infestation (Movahedinia *et al*., 2012).

There were excessive mucous secretions on the gills of *P. hypophthalmus*. According to Kennedy (2001), parasites that feed on the gills often cause excessive mucous secretion, erosions, ulcers discoloration, epithelial hyperplasia, and focal haemorrhages in the fish host. Different studies reported hyperplasia as the most frequent gill lesion as a result of a heavy parasite infestation. In addition, the fusion of adjacent secondary lamellae and proliferation of epithelial cells are part of the common findings (Movahedinia *et al*., 2012). Depledge

(1992) also mentioned that the proliferation and ultimate fusion of lamellae cells occurred due to the suction of the parasites in response to irritation.

In the current study, the damaged gills may contribute to the prompt death or traumatised-mortality of *P. hypophthalmus*. The possible reason might be due to respiratory impairment, as insufficient oxygen intake after the decline in oxygen diffusing capacity of the fish due to gill damage could also serve as a predisposing factor. In this case, the situation was seen to have become worse after the increase in oxygen demand when the fish were exposed to stress conditions during the sampling, especially in the fish that sustained injuries after crashing against the tank walls. There are several factors contributing to the severity of the gill damage that influences the level of respiratory impairment and this includes the intensity of parasitic load attached to the host fish (Raissy & Ansari *et al*., 2011). Besides, the prevalence and abundance of parasites is influenced by the age and size of the host and are often higher in older fish (Özer & Öztürk, 2005) because the body size reflects the surface area of the gills (Koskivaara *et al*., 1992). Therefore, the greater the surface area of the gill, the higher the water flows over it, hence a higher parasitic load (Beamish, 1964). In addition, larger individuals could attract more parasite due to an increase in physical (ventilation volume) and chemical (mucous) stimuli that serve as a source feed to the parasites (Kearn, 1967).

The clinical signs of lethargy and unilateral swimming observed in the current study were similar to the findings of Baker *et al*. (2007) and Mohammadi *et al*. (2012). According to Barber (2007), the parasites may influence the swimming performance of infected fish by distressing their hydrodynamic properties by increasing the energetic cost of locomotion and exacerbating the intrinsic energy costs of infested fish. Nevertheless, loss of swimming equilibrium (sideways or/and upside-down) observed in present study is likely due to the congestion of the swim bladder, leading to the dysfunction of the organ, which subsequently diminished the fish's ability to swim. Although there are no specific pathognomonic clinical signs in this case, the infection intensity and size of the host fish may influence the behavioural changes significantly as observed by Barber (2007). Interestingly, the aggressiveness of the fish during the first sampling was not reported in other studies, presumably occurred due to the increased level of stress during the infestation, handling could contribute to the sternness.

Evidence of parasite and bacterial co-interaction was observed in the present case study, as there was evidence of bacterial infection, *A. hydrophila,* on *P. hypophthalmus*. The bacterial infection may occur as a result of stress caused by the parasites, reduced the fish natural resistance to infection (Bower *et al*., 2000), and the gross lesions caused by the parasites may provide the bacteria with a route of entry. Holzer *et al.* (2006) stated that there is an increased risk of secondary bacterial diseases in parasitised fish, where the parasites act as transporter to transmit the pathogens. In the current case study, it is observed that the parasite-induced injuries are likely the main entry point for bacterial agents into the body of the fish with superinfection. Subsequently, the bacterial agent could enter the bloodstream through the damaged gills or lesions on the snout of the fish.

There is another possible reason that could lead to thebacterial co-infection of the fish, which is the ability of the parasite to destroy the cells of the gill lamellae, besides its ability to damage the tissues and perforate blood vessels, giving the opportunity to the bacteria to enter the blood stream as observed by Raissy & Ansari, (2011). Sugumar *et al.* (2002) similarly reported that the potentially pathogenic bacteria might enter the body of the fish via the induced gills damage, leading to septicaemia and mortality. In a similar study, the authors also stated *Dactylogyrus* spp. may trigger the production that; mucous and possibly the virulent strains of *Aeromonas* spp. are attracted to the mucous to invade the fish. In a study by Xu *et al*. (2012), the results revealed the enhancement of susceptibility of channel catfish to bacterial infection with *Edwardsiella ictaluri* after parasitism by *Ichthyophthirius multifiliis*. Likewise, mortality was observed in fish co-infected with a ciliate, *Epistylis*, and *A. hydrophila* (Miller & Chapman, 1976), where the *Epistylis* provided the medium for the attachment of the bacteria (Huizinga *et al*., 1979).

Although our hypothesis suggested that the gross lesions on the frontal parts of the upper jaw and head of the fish could have possibly developed due to mechanical injuries, there is another factor, such as potent bacterial proteases (proteolytic enzymes), that could result in gross lesions of that nature (Toranzo *et al*., 2005). Moreover, the synergistic interaction between parasites and bacteria could aid in the escalation of the occurrence (Kotob *et al*., 2016). Since there is no strong evidence to prove the real cause of the necrosis, further work is therefore necessary to clarify the effects of co-infection in an experimental study, as many other factors may contribute to the mortality.

Conclusion

In summary, the findings revealed that the infestation of *Dactylogyrus* spp. has led to the destruction of the gills. This is evident with the severe proliferation of epithelial cells which developed into hyperplasia, ultimately increasing the fusion of lamella, thereby reducing the surface areas of the gills for oxygen exchange, and this may contribute to the traumatised-mortality of *P. hypophthalmus* due to respiratory impairment under stress conditions. The present investigation also suggested that there is a co-interaction between *Dactylogyrus* spp. and *A. hydrophila* and the secondary infection had increased the severity of the outbreak, which eventually lead to mortality. The present study also provides helpful and baseline information on the early detection of *Dactylogyrus* spp. infestation in striped catfish, *P. hypophthalmus.* Since there is co-interaction between *Dactylogyrus* spp. and *A. hydrophila* infection, precaution and proper handling should be given priorities when there is an outbreak to reduce the fish's stress level as it could lead to mortality due to injuries or secondary infection.

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