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Short Communication

Dietary emamectin benzoate alters the gill and spleen histoarchitecture of monosex *Oreochromis niloticus* fry

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Abstract
Oral administration of the antiparasitic veterinary drug emamectin benzoate is recommended to control infectious ectoparasites, whose biosafety and effect on vital organs are unknown in several fish species. This study evaluated the influence of dietary emamectin benzoate on the histopathology of hitherto understudied organs, such as gills and spleen of all-male *Oreochromis niloticus* fry at the recommended dose of 50 µg and an overdose of 500 µg kg body weight^{-1} day^{-1} for 7 days. The fry recorded dose-dependent discrepancies in feed intake, survival and biomass. Histologically, noticeable pathological changes in the gill and spleen tissues were documented in both groups. The pathological alterations in the fry fed the recommended dose and increased severity in the overdosed groups indicated the toxic nature of emamectin benzoate and signified exercising caution during its application in tropical aquaculture.

Keywords: all-male tilapia; antiparasitic veterinary drugs; ectoparasites; histopathology; tropical aquaculture

1 | INTRODUCTION

The use of diverse antimicrobial veterinary medicinal products in Indian aquaculture has been systematically documented, with reports detailing the utilisation of different products to reduce disease occurrences (Singha *et al.* 2021; Patil *et al.* 2022). Emamectin benzoate is a novel semi-synthetic biopesticide isolated from the fermentation of soil-dwelling *Streptomyces avermitilis* and a derivative of abamectin (MSD Animal Health 2012). It is a mixture of 90% avermectin B1a benzoate salts and is structurally similar to the abamectin and ivermectin groups. The emamectin benzoate, as an antiparasitic veterinary drug, got its first registration for application in temperate aquaculture in the United Kingdom in 2000 (FAO 2013). Presently, it is commonly used as an in-feed medication for controlling sea lice infestations in fish at a recommended dose of 50 µg kg body weight (BW)$^{-1}$ day^{-1} for 7 days (Anandaraja *et al.* 2020, 2022; St-Hilaire *et al.* 2022).

In tropical aquaculture, the scarcity of data on emamectin benzoate usage and scientific advisories can create uncertainties regarding the safety concerns of farmed fish to humans, and the impact of discharged water on the receiving environment. Our earlier studies focused mostly on the biosafety of emamectin benzoate in *O. niloticus* juveniles and fry (Julinta *et al.* 2020a, 2020b; Singha *et al.* 2022) and haematological and haematomorphological parameters (Das *et al.* 2022, 2023). This study aimed at determining the influence of dietary emamectin benzoate on the histopathological alterations in hitherto understudied organs such as the gills and spleen of monosex (all male) Nile tilapia *Oreochromis niloticus* fry.

2 | METHODOLOGY

This research was conducted as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India. The framework of this study fulfilled the ethical guidelines, including adherence to the legal requirements of India (CPCSEA 2021). Active monosex *O. niloticus* fry (2.92 ± 0.51 cm; 0.46 ± 0.05 g) were obtained from a commercial hatchery (22°49′35″N 88°51′20″E), West Bengal, India, packed in oxygen-filled double-layered plastic bags and brought to the laboratory. The fry, on reaching the laboratory, were disinfected by dipping in 2 ppm potassium permanganate solution for 3 min, transferred to 500-L circular stock tanks at 400 tank^{-1} and acclimatised for 15 days with continuous aeration. The water quality parameters, namely temperature (28.70 ± 1.22°C), pH (7.79 – 8.05) were recorded daily, and dissolved oxygen (5.72 ± 0.21 mg L^{-1}), nitrate (0.04 ± 0.01 mg L^{-1}), nitrite (0.33 ± 0.02 mg L^{-1}), and ammonia (0.04 ± 0.01 mg L^{-1}), were assessed every three days (APHA/AWWA/WEF 1992). During the experimental period, the average daylight duration was 12 hours and 45 minutes. A commercial floating pelleted feed of 0.8 mm diameter with 42% protein (Aquaxcel, Cargill, India) was used thrice daily at 6% BW. Healthy fry of size 0.63 ± 0.03 g, free from external anomalies and infections, were randomly selected and moved into 9 circular tanks at 220 tank^{-1} containing 400-L water and fed as above. About 150 L of water was exchanged on alternate days to remove uneaten feed and faeces.

For oral application, the recommended emamectin benzoate dose is 50 µg kg BW^{-1} day^{-1} (1×) for a week (MSD Animal Health 2012). The fry were assigned into 3 groups, viz., group 1: 0× control, group 2: 1× (50 µg) and group 3: 10× (500 µg), in triplicate. The control and medicated feeds with or without the required amount of emamectin benzoate (Sigma-Aldrich, India) were prepared by top-coating as described earlier (Julinta *et al.* 2020a; Singha *et al.* 2022). In brief, the stock solution was prepared by mixing 50 mg of emamectin benzoate powder in 5 mL of ethyl acetate and 50 µL of Tween 80 in a vortex mixer for 5 min. The emulsions containing required volume of emamectin benzoate solution (83 µL and 833 µL) in 5 mL vegetable oil, as a binder, were then mixed with a kilogram of commercial pellet feed separately and admixed vigorously to top-coat and to achieve a dose of 50 µg (1×) and 500 µg (10×) of emamectin benzoate, respectively, when fed the fry at 6% BW. The top-coated emamectin benzoate feeds were thoroughly mixed, air-dried for a day and packed in airtight containers. Before dosing, the fry were reacclimatized in the circular tanks for 7 days and fed the control feed (days 0 – 7: predosing period). On day 8, all fish groups were starved (starvation period) to improve the nutrient and drug absorption (Singha *et al.* 2022). During the experimental tenure, groups 2 and 3 were offered the respective emamectin benzoate feeds for 7 days (days 9 – 15: emamectin benzoate-dosing (ED) period). Subsequently, all the groups were fed the control feed for 42 days (days 16-57: post-emamectin benzoate-dosing (PED) period). Thrice daily at 4-hour intervals starting from 8.00 am, all fry were fed at 6.0% BW in equal shares. The mortality, feed intake and feeding behaviour were observed daily at each feeding time. After 75 minutes of each feeding, the uneaten feeds were collected, air-dried and quantified. The biomass of 10 fry from each tank was measured weekly to adjust the quantity of feed. Two fish tank-1 were arbitrarily netted on days 0 and 7 of ED and day 42 PED, euthanised using clove oil (20 µL L^{-1} water), and carefully dissected to remove the gills and spleen tissues. The tissues were fixed in Bouin's solution for 24 hours. The processing, embedding and sectioning (5 µm) of tissues, as well as Hematoxylin and Eosin staining, were as per Roberts (2012). The sections were observed using a 20× lens in an Olympus trinocular microscope (Model: BX51) fitted with a 16 MP SCO-LUX camera. The capture and processing of images were done by ToupTek ToupView (Version x64, 4.11) software. The analysis of data on mortality and feed intake by one-way ANOVA was executed by Statistical Package for Social Sciences (IBM-SPSS; IBM Corp., Armonk, NY, USA) version 22.0 at a probability level of $p < 0.05$.

3 | RESULTS AND DISCUSSION

Active and aggressive feeding of the ration (100%) was observed in the control group throughout the experimental period, with no signs of abnormal behaviour. Active feeding was rather lacking in the 1× group and absent in the 10× group. The feed intake was reduced significantly ($p < 0.05$) by 10.75% in the 1× and 28.50% in the 10× groups during the ED period. During the PED period, the feed intake was increased close to normal in the 1× group, while in the 10× group, it was significantly low. The fry exhibited no behavioural changes when fed at the recommended dose (1×). On the other hand, the overdosed group showed abnormal behaviour such as loss of equilibrium, lethargy, dark colouration, lack of interest in feeding and gasping for air. Internally, changes such as hepatomegaly, pale and swollen kidneys, splenomegaly and discoloured intestine were observed. Several earlier studies also documented abnormal behaviours in different EB-fed fish species (Roy *et al.* 2000; Stone *et al.* 2002; Anandaraja *et al.* 2020; Julinta *et al.* 2020a). The survival was 97.70% and 96.82% in the 1× and 10× groups during the ED period. Post-dosing recorded a further increase in mortalities, and survival was reduced significantly to 94.77% in the 10× group ($p < 0.05$). The dose-dependent decrease in feeding and survival signified that emamectin benzoate may curtail aggressive feeding and feed intake, which is the first sign of intoxication. The observed morphological changes in the internal organs probably indi-

cated the adaptive changes and tissue alterations in fry exposed to an overdose of emamectin benzoate.

The histopathological changes observed on day 7 emamectin benzoate were lamellar hyperplasia, epithelial lifting, shortening of secondary lamellae, epithelial necrosis, disruption of the cartilaginous core, and swelled secondary lamellar tips in the 1× group (Figures 1A, 1B). While in the 10× group, the changes were more prominent. In addition, the desquamation of epithelial cells and curling of secondary lamellae were noted (Figure 1C). Most of the gill tissue changes were reduced during the PED in the 1× (Figure 1D), but mildly in the 10× groups (Figure 1E). The histopathological alterations in the fry fed the recommended dose and increased vehemence in the overdosed group indicated the toxic nature of emamectin benzoate. The epithelial lifting and lamellar hyperplasia are indications of defence responses. While epithelial necrosis, curling of secondary lamellae and desquamation of epithelial cells are the direct responses to the action of emamectin benzoate that might have damaged the cellular membrane because of depletion of oxygen across the gill filaments alike (Das *et al.* 2022). The epithelial lifting may also increase the gap through which the toxicant can reach the bloodstream. The lamellar hyperplasia possibly acted as a defensive mechanism, leading to a decrease in the respiratory surface and an increase in the toxicant-blood diffusion distance (Cengiz 2006). Therefore, emamectin benzoate overdose can disrupt the gill architecture due to stress, leading to respiratory distress and mortalities. The respiration disturbances may also lead to hyperglycemia and fry growth impairment during the ED and PED periods. The epithelial necrosis may lead to a negative ionic balance, osmoregulatory imbalance, changes in haematocrit and haemoglobin and severe respiratory distress as was observed earlier (Das *et al.* 2022). These results corroborated the observations of pesticide exposure studies with *Cirrhinus mrigala* (Ghayyur *et al.* 2021) and *Anabas testudineus* (Velmurugan *et al.* 2018). The disruption in the perfusion of the gill epithelium probably impacted the membrane permeability of an ionic transport mechanism in the *O. niloticus* fry. The results, thus, suggested that the ED and the possible presence of toxic emamectin benzoate residues in circulation have a role in impacting the fry gill architecture. The tissue alterations were, however, minimal on day 42 PED, implying a sturdy improvement in the gill tissue architecture. Nevertheless, both groups exhibited mild lamellar hyperplasia, indicating the sensitivity of fry to emamectin benzoate even after 6 weeks of PED, or the development of a defence mechanism to prevent the negative effects of emamectin benzoate.

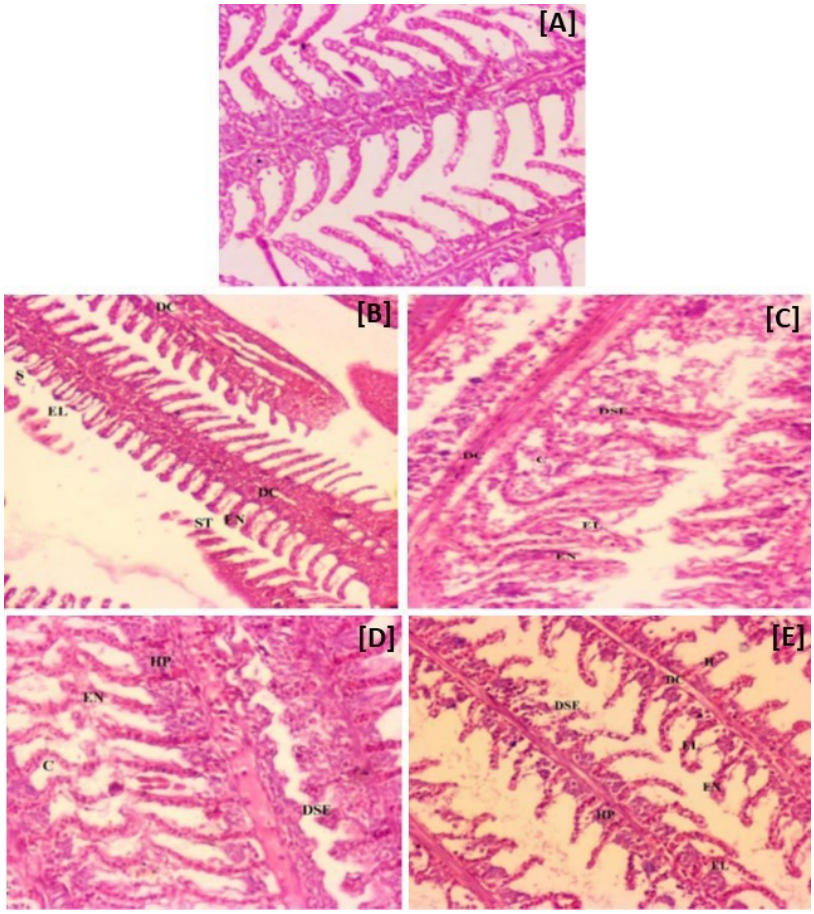

FIGURE 1 Histopathological changes in the gill tissues of the [A] control and emamectin benzoate-dosed *Oreochromis niloticus* fry for 7 consecutive days at 50 µg kg body weight (BW)$^{-1}$ day^{-1} (1× group) [B] on day 7 emamectin benzoate-dosing (ED), ×200; [C] day 42 post-emamectin benzoate-dosing (PED), ×200; and at 500 µg kg BW^{-1} day^{-1} (10× group) [D] on day 7 ED, ×200; [E] day 42 PED, ×200 showing lamellar hyperplasia (HP), swelled secondary lamellar tip (ST), epithelial lifting (EL), shortening of secondary lamellae (S), epithelial necrosis (EN) curling of secondary lamellae (C), disruption of cartilaginous core (DC), desquamation of epithelial cells (DSE), and lamellar epithelial hypertrophy (H), H&E staining.

The splenic necrosis, increased sinusoidal space, a slight lipid infiltration, and increased eosinophilic bodies were the noticeable changes in the spleen of both groups (Figures 2A, 2B, 2C). The spleen architectural alterations became nearly normal on day 42 PED with the regeneration of white and red pulps in the 1× group, while these changes, though reduced, were still persistent in the 10× group (Figures 2D, 2E). The results indicated the splenic toxic effect of emamectin benzoate, possibly due to the accumulation of residues in the spleen. However, the changes were only minimal. One of the major functions of the spleen is to remove or filter the damaged erythrocytes, and the accumulation of emamectin benzoate in the splenic tissue may disrupt the sinusoidal space and lead to the depletion of oxygen. Similarly, in an exposure study, splenic necrosis, lipid infiltration and increased sinusoidal space were noted in the spleen of *Oncorhynchus mykiss* (Capkin *et al.* 2010). The unusual behaviour during the overdosing period indicated the fish to be anaemic, plausibly due to the continuous release of erythrocytes from the spleen. Regeneration of red and white pulps with slight lipid infiltration was noted on day 42 PED, indicating a steady recovery of the spleen parenchyma at the recommended dose. While the changes were still visible in the spleen tissues of the overdosed group, showing the long-term effect of emamectin benzoate toxicity if abused. The results of the present study revealed that oral administration of emamectin benzoate, even at the recommended dose, can reduce the survival, feed intake and biomass and alter the gill and spleen histoarchitecture of *O. niloticus* fry and ultimately disrupt their physiology. Nevertheless, the alterations were reversible with dose discontinuation.

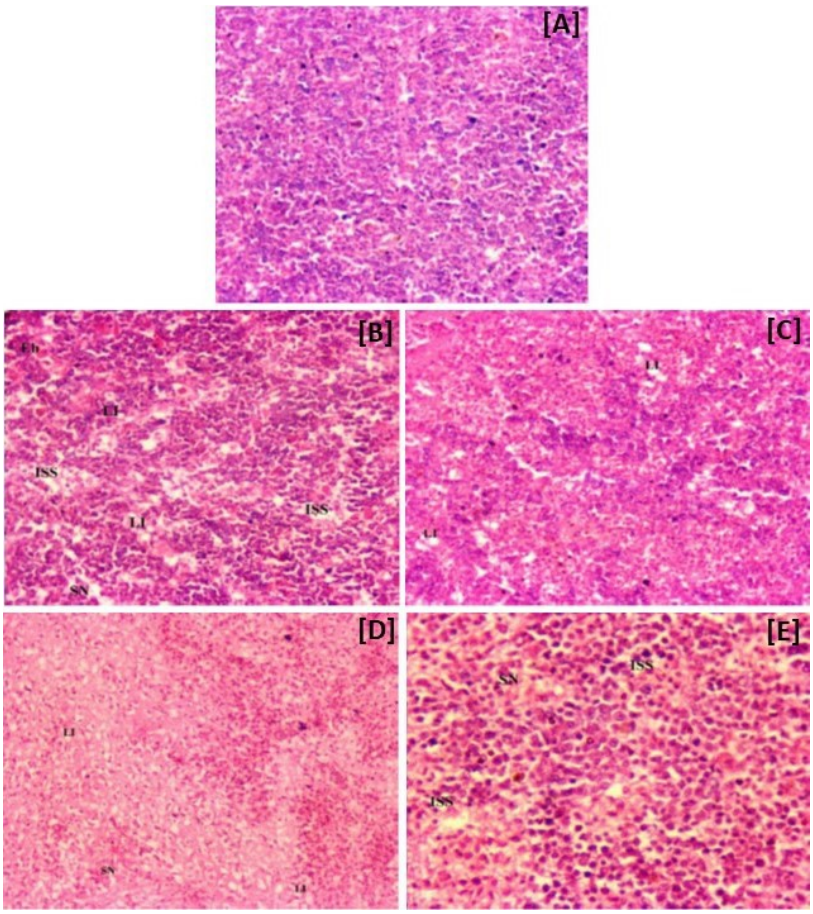

FIGURE 2 Histopathological changes in the spleen tissues of the [A] control and emamectin benzoate-dosed *Oreochromis niloticus* fry for 7 consecutive days at 50 µg kg body weight (BW)$^{-1}$ day^{-1} (1× group) [B] on day 7 emamectin benzoate-dosing (ED), ×200; [C] day 42 post-emamectin benzoate-dosing (PED), ×200; and at 500 µg kg BW^{-1} day^{-1} (10× group) [D] on day 7 ED, ×200; [E] day 42 PED, ×200 showing splenic necrosis (SN), increased sinusoidal space (ISS), eosinophilic bodies (Eb), lipid infiltration (LI) and regeneration of white pulp and red pulp, H&E staining.

4 | CONCLUSIONS

The histopathological examinations of emamectin benzoate-fed fry documented dose-dependent gill and spleen tissue damage. The pathophysiological recovery of the fry was demonstrated by the observed reversible changes following the termination of dosing at the recommended dose. Overall, the results suggested exercising appropriate caution during the application of emamectin benzoate in tropical aquaculture to avoid potential adverse effects on cultured fish health and production. Furthermore, additional research is warranted to develop species-specific emamectin benzoate safety guidelines or withdrawal periods for tropical aquaculture systems to ensure the safety of fish and human consumers.

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ETHICAL STATEMENT

This study was performed in strict accordance with the guidelines for experimentation on fishes, Government of India. The experimental protocols were approved by the Indian Council of Agricultural Research, Government of India, New Delhi, under the All-India Network Project on Fish Health vide F.No. CIBA/AINP-FH/2015-16 dated 16.07.2015.

CONFLICT OF INTEREST

The author declares no conflict of interest.

AUTHORS' CONTRIBUTION

Both authors have equal contributions.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on a reasonable request from the corresponding author.

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