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Research Article
Nerocila bivittata Massive Infestations in *Tilapia zillii* with Emphasis on Hematological and Histopathological Changes

[1]Mamdouh Yousif Elgendy, [2]Azza Morsi Hassan, [1]Mostafa Fawzy Abdel Zaher, [1]Hossam Hassan Abbas, [1]Waleed Salah El-Din Soliman and [1,3]Elsayed Mahmoud Bayoumy

[1]Department of Hydrobiology, Veterinary Research Division, National Research Centre, 33 El-Bohouth St. Dokki, P.O. Box 12622, Giza, Egypt
[2]Department of Pathology, Faculty of Veterinary Medicine, Cairo University, 12211 Giza, Egypt
[3]Department of Biology, Girls Science College, University of Dammam, Dammam, Saudi Arabia

Abstract

Background and Objective: Cymothoid isopods are extremely serious fish parasitic infestations that adversely impact the health of aquatic animals with considerable economic losses. The present study aimed to investigate the massive infestation by the cymothoid isopod, *Nerocila bivittata* (*N. bivittata*) affecting *Tilapia zillii* (*T. zillii*) fish within Lake Qarun Egypt as an attempt to understand the effects of the parasites on the haematological parameters and histopathological alterations in the infested fish. **Materials and Methods:** A total of 150 *T. zillii* were collected alive during June, 2016. Fish were thoroughly investigated visually and microscopically for presence of external parasites. Analysis of blood and serum samples from parasitized and un-parasitized fish was carried out. **Results:** Ninety-six fish (64%) were found to be infected with isopods. All retrieved isopods were further identified as *N. bivittata*. The isopod was found settled in different parts of fish body but gills were the most predilection site. Parasitized fish had lowered erythrocyte counts, haemoglobin and haematocrit values. Leucocyte counts, total protein, albumen and globulin also decreased in infested fish. Severe histopathological alterations were recorded in the skin, muscles and gills of infested fish. Analysis of water samples collected from the Lake revealed unfavorable values for water quality measures and levels of some heavy metals were higher than the recommended values. **Conclusion:** The results of this study indicate that *N. bivittata* are serious parasites in aquaculture and can infect wild fish populations. These parasites feed on the blood of infest fish and put their lives at risk.

Key words: *Nerocila bivittata*, isopods, *Tilapia zillii*, hematology, histopathological alterations

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Corresponding Author: Mamdouh Y. Elgendy, Fish Diseases and Management Researcher, Department of Hydrobiology, Veterinary Research Division, National Research Centre, 33 El-Bohouth St. Dokki, P.O. Box 12622, Giza, Egypt Tel: 00201116893637

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Fish parasitic infestations have critical impacts on the global finfish and shellfish industry, affecting sustainability, production efficiency and economic viability. Infections are commonly detected in both wild and farmed species, however, parasites pose a greater dilemma to fish maintained in captivity[1].

Isopods are extremely serious fish parasitic infestations impacting the health of aquatic animals in fresh, estuarine and marine water habitats. They cause considerable economic losses to fisheries and aquaculture either by causing detrimental injures, stunting growth, reduced fecundity or direct killing of their hosts[2]. These parasites are mainly blood-feeding and settle on the fish outer body surfaces including fins, buccal cavity, gill chambers, nostrils, or occasionally within the muscles of their hosts[3].

Nerocila represent the major genus of Cymothoid isopods infesting numerous commercial wild and cultured fish species. About 65 *Nerocila* species have been reported from different parts of the world. They are obligate parasites feed fundamentally on the host blood, mucus, epithelial and subcutaneous tissues devastating the physiological, behavioral and morphological competences of their hosts[4].

Tilapia zillii is a highly competitive fish species that has been intentionally introduced in several countries for many purposes including; aquaculture, commercial aquarium trade, weed control or as a recreational fishery[5]. *Tilapia zillii* can survive in a wide range of aquatic habitats as well as tolerate highly saline water[6]. *Tilapia zillii* are omnivorous with juveniles being more carnivorous. Adults are especially voracious herbivore with high capacities to alter both the composition and the densities of native aquatic plants. Accordingly, they pose harmful outcomes on the organisms which depend on such plants[7]. *Tilapia zillii* can easily switch their food sources and compete with other cohabitant fish species for food, habitat and spawning sites[8]. *Tilapia zillii* also has a great ability to alter native benthic communities through the elimination of macrophytes as well as it affects the fish communities in its habitats a result of the aggressive behavior towards other cohabitant fish species[6].

Despite the high competitively and tolerability of *T. zillii*, the existence of this species in Lake Qarun an enclosed in landsaline waterbody in Egypt, was threatened by a heavy isopod infestations in 2015 and extended to 2017 with enormous economic losses. Hence the present study aimed to investigate the effects of *Nerocila* sp. infestations on *T. zillii* within the Lake Qarun and their outcomes on the hematological parameters of the host fish. Further, the study aimed to investigate the histopathological alterations associated with these parasitic infestations in their host fish.

MATERIALS AND METHODS

Area of study and fish sampling: Lake Qarun is located in Fayoum governorate South West of Cairo. The Lake extends about 40 km from East to the West and its maximum breadth ranging about 9.25 km in the Western part. The mean depth of the lake is about 4.2 m. The water level of the lake fluctuated between 43-45 m below mean sea level. The main source of the lake water is the agricultural drainage water. The salinity of the lake constantly increases approaching up to 40% in some parts.

A total of 150 *Tilapia zillii* were collected during June, 2016. Fish body weights ranged about 75±5 g. Fish were transferred alive within tanks filled with water from the lake and supplied with continuous oxygen source to the Department of Hydrobiology, National Research Centre. Fish were maintained in the lab in glass aquaria supplied with water from the lake.

Clinical and parasitological examination: Fish were visually investigated thoroughly by naked eye for the presence of any external parasites in external body surfaces, the gills and the buccal cavity. Isopods when detected were removed and immediately preserved in 70% ethanol. Smears were freshly prepared from fish skin, gills and fins. Then fixed with methanol, stained with 10% Giemsa stain and examined dissecting microscope[9]. The number of parasites, life stages and their predilection sites on fish were recorded. Accordingly, fish were divided into two groups, isopods infested fish and isopods free fish. The presence of blood parasites in all fish indicated for hematological examination was also investigated. Blood films were prepared, air dried, fixed and stained with diluted Giemsa stain then examined[10].

Physical and chemical water analysis: Temperature, dissolved oxygen (DO), pH and salinity were measured on spot by digital apparatus. The other water quality parameters including; un-ionized ammonia (NH_3), nitrites and nitrates were determined in the laboratory according to methods adopted from APHA[11]. Furthermore, heavy metals in water samples were detected by atomic absorption (Thermo Electron Corporation S series AA Spectrometer,USA). The samples were prepared and analyzed for iron (Fe), copper (Cu), zinc (Zn), cobalt (Co), cadmium (Cd) and lead (Pb).

Hematological examination: Blood samples were collected from 10 randomly selected *T. zillii* fish confirmed via both visual inspection as well as microscopic examination to be parasitized only with isopods. Furthermore, samples were taken also from fish found to be free from any parasitic infestations (10 randomly collected fish). Fish were anesthetized with benzocaine solution (50 mg L^{-1}); blood was withdrawn from the caudal vein of fish using 1.0 mL, sterile disposable plastic syringes rinsed with a drop of 10% EDTA. Blood smears were stained with Giemsa/May Grunwald for differential counting of leucocytes[12]. Hematocrit (Htc) was determined according to Goldenfarb *et al.*[13]. Haemoglobin (Hb) content was detected using the hemoglobin assay kit (Sigma-Aldrich Co. Ltd, Dorset, UK). Total red blood cells (RBCs) were counted in a haemocytometer. White blood cells (WBCs) and thrombocytes were counted in blood extension by the indirect method[14]. Serum was also collected for assaying the total protein and albumen, blood was drawn via syringe without anticoagulant, then tubes were kept in slanting position for about 2 h and thereafter centrifuged at 1600 rpm for 25 min at 4°C, followed by collection of straw colored serum with micropipette and stored at -20°C for further analysis. Different sera were analyzed for total protein and albumin content following the methods adopted from Doumas *et al.*[15] and Lowry *et al.*[16], respectively. Furthermore, globulin content was calculated by subtracting albumin from the total protein then albumin: Globulin ratio was determined. The mean values of all parameters were calculated.

Histopathological examination: Tissue samples from skin, muscle and gills at the parasite attachment sites were fixed in 10% neutral buffered formalin, routinely processed and embedded in paraffin. Tissue sections of 5 µm thickness were stained with H and E and PAS stains for routine histopathological examination to demonstrate the mucous secreting cells[17].

Immunohistochemical investigations: The proliferating epidermal and lamellar epithelial cells were demonstrated by the proliferating cell nuclear antigen (PCNA) while apoptotic cells were detected via Caspase-3 immune stain, according to the methods of Hegazy *et al.*[18] and Ibrahim *et al.*[19]. Tissue sections were de-paraffinized and incubated in 3% H_2O_2. For blocking of the non-specific immune reaction, sections were incubated with normal goat serum at 37°C. Rabbit polyclonal anti- PCNA and anti-caspase-3 (Abcam, Ltd., USA) were used as biotinylated primary antibodies. Visualization of the immune reaction was performed using the chromogen diaminobenzidine (DAB). Cells with dark brown nuclei were considered positive for PCNA while cells with dark brown cytoplasm and/or nuclei were considered positive for caspase-3.

RESULTS

Clinical and postmortem examination: Macroscopic parasitic isopods were noticed evidently on the external body surfaces on the head region, ventral body surface nearby the pectoral fin, inside the gill chamber and in the buccal cavity. Haemorrhages, loss of scales and extensive skin erosions spread widely on the external body surfaces sometimes ulcers were also existed. Petechiae were extensive, especially around the lesions. Skin damage in the head area was also noticed. Gills were pale and anemic with excessive mucus secretions. Complete absence of the gill cover was noticed in some cases. Furthermore, thickening of the gill arch and gill rakers were characteristic. Some cases showed lack of gill rakers. In cases where the parasite was detected in the buccal cavity, tongue was found to be destroyed. Internally, paleness of liver was detected in the majority of investigated specimens. In some other cases liver was congested and haemorrhagic (Fig.1a-d).

Parasitological examination: Ninety-six fish (64%) were found to be infected with isopods. All retrieved isopods (131) were further identified as *Nerocila bivittata* (Fig. 2a-c). The maximum number of parasites exist on the same fish was 4 parasites. Gills were the most predilection site for existence of *Nerocila bivittata*, 46.56%. It sometimes contained more than one parasite at once. Isopods were either emerged completely inside the gill chamber or with its posterior parte protrude outside it. On the other hand, isopods frequently settled on the lateral body surfaces nearby the pectoral fin 16.03% as well as the ventral body surface just below the gills, 25.95%. No infestations were recorded on the peduncle region. Moreover, the buccal cavity has been noticed also as an organ of isopod attachment, 11.45%. Interestingly, no other parasitic infestations were recorded in all investigated specimens.

Water analysis: Unfavorable values were recorded for some water quality measures in lake Water. The un-ionized ammonia (NH_3) was slightly higher. Additionally, levels of some detected heavy metals were greater than the marine high reliability trigger value recommended for saltwater fish (Table 1).

Hematological examination: Hematological parameters are illustrated in (Table 2). The total erythrocyte count

Fig. 1(a-d): (a, c) *Tilapia zillii* infested with *N. bivitata* settling inside the gill chamber with hemorrhages on the external body surfaces, (b) *T. zillii* infested with *N. bivitata* settling inside the buccal cavity and (d) *T. zillii* showing pale anemic gills, damage and pressure atrophy of the gill filaments a result of *N. bivitata* infestations

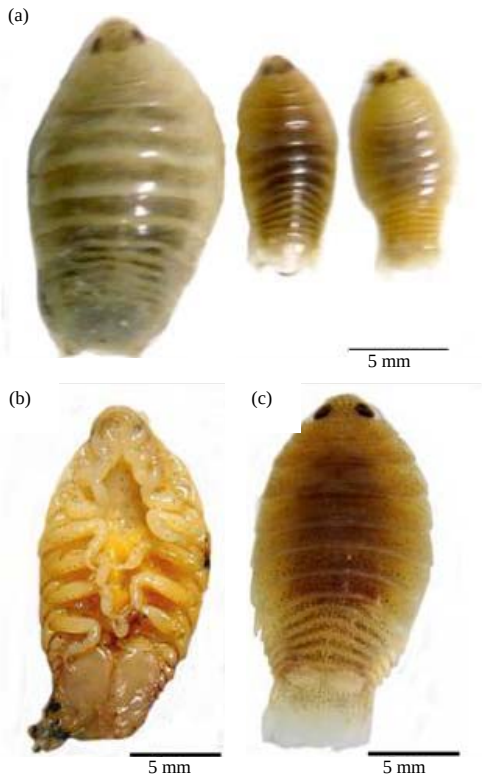

Fig. 2(a-c): *Nerocila bivitata* retrieved from *Tilapia zillii*

Table 1: Water analysis of water samples collected from Lake Qarun

Parameters	Value	Parameters (mg L^{-1})	Value
Temperature (°C)	30.13	Cl^-	341.000
Salinity (%)	40	SO_4	92.000
EC (dS m^{-1})	39.1	Ca^{2+}	23.800
TDS (mg L^{-1})	1759.67	Mg^{2+}	84.300
TSS (mg L^{-1})	76.33	Na^+	324.300
pH	8.62	K^+	4.800
DO (mg L^{-1})	6.6	Fe	0.532
BOD (mg L^{-1})	6.87	Zn	0.048
COD (mg L^{-1})	8.13	Mn	0.095
NH_4 (mg L^{-1})	1.96	Cu	0.074
NH_3 (mg L^{-1})	0.83	Co	0.606
NO_2^- (mg L^{-1})	0.014	Pb	0.310
NO_3^- (mg L^{-1})	0.078	Ni	o.437
HCO^{-3} (mg L^{-1})	4.2	Cd	0.169

(RBCs count), hemoglobin (Hb) and hematocrit (Ht) were lower in infested fish than non-infested accompanied with an increase in the mean corpuscular hemoglobin concentration (MCHC) as well as the size of erythrocytes (MCV).

WBCs severely decreased in infested fish a result of lymphopenia and were concomitantly accompanied by an enhanced percentage of monocytes and neutrophils. On the other hand, the total protein, albumen and globulin values reduced in infested fish in comparison with non-infested fish. On contrast, the albumin/globulin (A/G) ratio was lower in parasitized individuals than non-parasitized fish (Table 2).

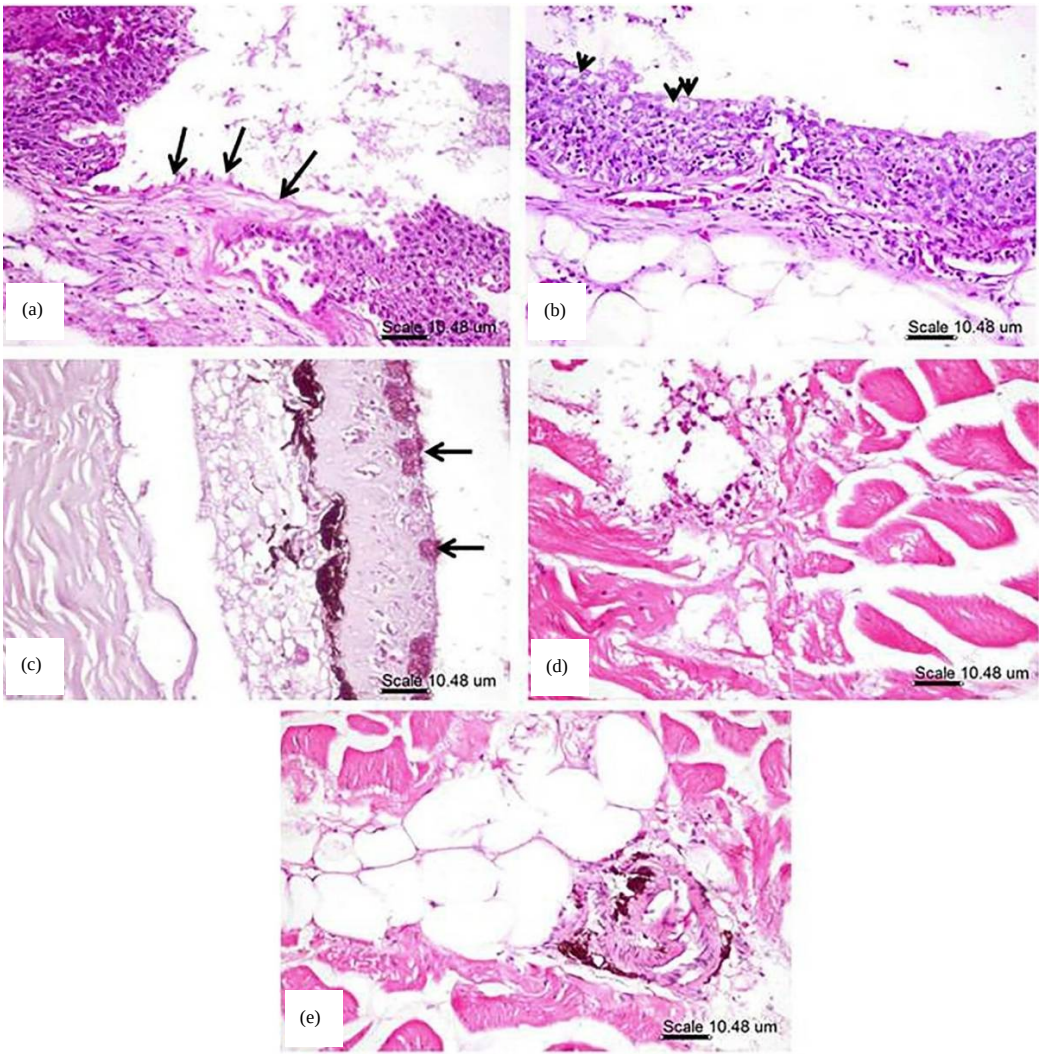

Fig. 3(a-d): (a) Skin of *T. zillii* showing focal erosive lesion (arrows) with complete detachment of the superficial epidermal cells, (b) Skin of *T. zillii* showing infiltration of epidermal and dermal cell layers with macrophages and lymphocytes associated with activation of mucous secreting cells (arrow heads), (c) Skin of *T. zillii* showing mucous secreting cells that appeared in magenta (arrows), (d) Muscle of *T. zillii* showing necrosis of muscle fibers associated with infiltration of mononuclear cells and (e) Muscle of *T. zillii* showing presence of fat cells associated with aggregation of melanomacrophage cells, eosinophilic granular cells and lymphocytes
(H and E for a, b, d and e; PAS stain for c, scale bar,10.48 µm)

Histopathology: Variable and severe histopathological alterations were demonstrated in the skin, muscle and gills of infested *T. zillii*. Skin lesions varied from focal erosive lesion with complete detachment of the superficial epidermal cells associated with dermal and hypodermal edema (Fig. 3a) to extensive necrosis and sloughing of all its constituent malpighian cells. The edge of the focal erosive lesions was surrounded by hyperplastic malpighian cells which were confirmed in PCNA immunohistochemically stained sections. The epidermal and dermal cell layers were infiltrated with inflammatory cells mostly macrophages and lymphocytes with activation of mucous secreting cells (Fig. 3b) that appeared in magenta with PAS-stained sections (Fig. 3c).

Muscles revealed necrosis of muscle fibers with presence of large fat cells and aggregations of melanomacrophage cells, eosinophilic granular cells and lymphocytes (Fig. 3d, e). Gills revealed diverse histopathological alterations ranging from extensive necrosis and desquamation of lamellar epithelium (Fig. 4a) to diffuse hyperplasia and fusion of secondary gill lamellae with activation of mucous secreting cells that were

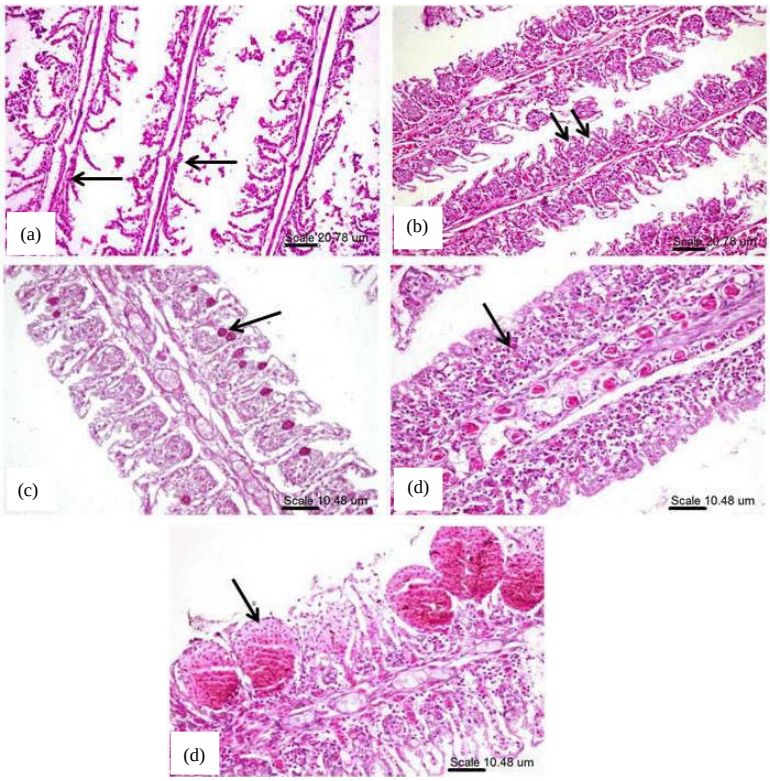

Fig. 4(a-e): (a)Extensive necrosis and desquamation of lamellar epithelium (arrows), (b) Diffuse hyperplasia and fusion of secondary gill lamellae with activation of mucous secreting cell (arrows), (c) Mucous secreting cells appeared in magenta (arrow), (d) Congestion of venous sinuses with intense diffuse infiltration of gill lamellae with inflammatory cells mostly lymphocytes and EGCs (arrow) and (e) Lamellar telangiectasis
(H and E for a, b, d and e, PAS stain for c, scale bar, 20.78 um for a and b; 10.48 µm for c, d and e)

Table 2: Blood analysis of Parasitized and non Parasitized *T. zillii* fish Mean value±SD

Aspect	Parasitized *T. zillii* No. (10)	Non parasitized *T. zillii* No. (10)
Hematocrit (%)	17.30±0.65	20.07±0. 86
Haemoglobin (g dL^{-1})	6.10±0.25	10.50±0.41
Erythrocytes ($\times 10^6$ μL^{-1})	1.42±0.15	2.32±0.24
Thrombocytes ($\times 10^3$ μL^{-1})	25.34±0.35	44.12±0.28
Total leukocytes ($\times 10^3$ μL^{-1})	35.50±0.71	69.21±0.49
MCHC (g dL^{-1})	52.31±0.21	35.26±0.18
MCV (fL)	121.83±1.42	86.50±0.58
Lymphocyte (%)	71.00±0.66	89.00±0.85
Monocytes (%)	2.30±0.48	1.40±0.29
Neutrophils (%)	26.70±0.51	9.60±0.16
Total protein (g dL^{-1})	1.98±0.12	4.81±0.23
Albumen (g dL^{-1})	0.60±0.05	1.90±0.12
Globulin	1.38±0.14	2.91±0.20
Albumin/Globulin (A/G) ratio	0.43	0.65

distended with mucin which appeared as faint bluish materials in H and E stained sections (Fig. 4b) and in magenta in PAS stained one (Fig. 4c). The respiratory epithelium appeared large, hypertrophied and intensely infiltrated with EGCs and lymphocytes concomitantly with presence of apoptotic changes that were confirmed in caspase-3 immunohistochemically stained sections. Branchitis was evident and characterized by congestion of venous sinuses associated with intense infiltration of gill lamellae with inflammatory cells mostly macrophages and EGCs (Fig. 4d) in addition to focal lamellar hemorrhages and telangiectasis (Fig. 4e).

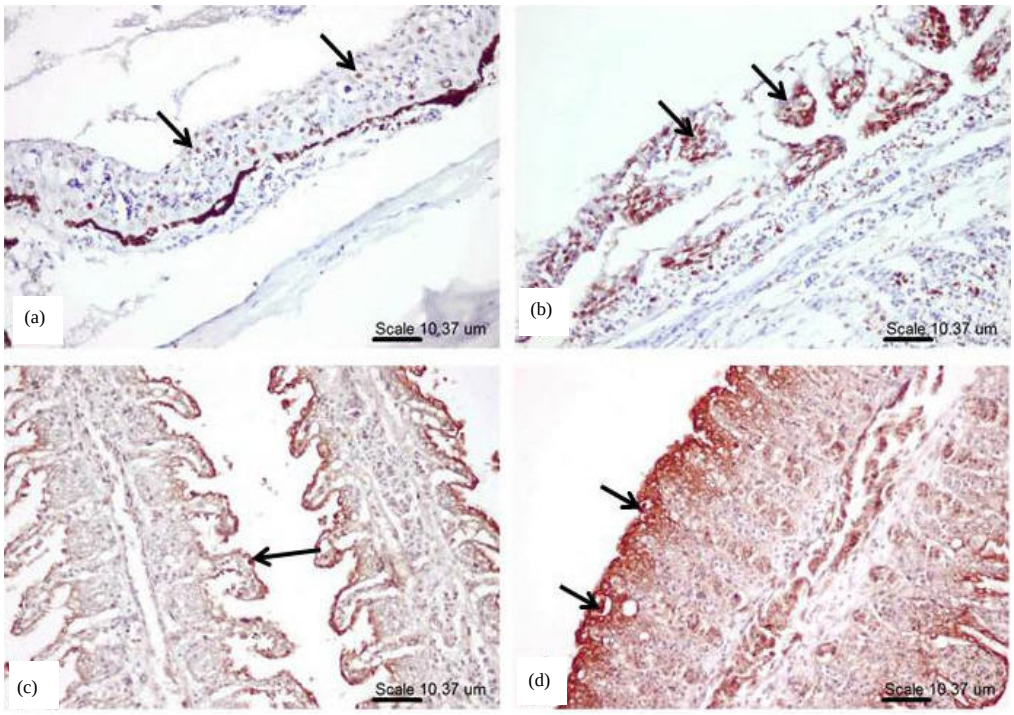

Fig. 5(a-d): (a) Skin of *T. zillii* showing PCNA immune reactive cells (arrows), (b) Gills of *T. zillii* showing PCNA immune reactive cells (arrows), (c) Gills of *T. zillii* showing caspase-3 immune stained apoptotic body (arrow) (d) Gills of *T. zillii* showing caspase-3 immune stained cells (arrows)
(Immunohistochemical staining for PCNA (a and b) and Caspase-3 (c and d), scale bar, 10.48 µm)

Immunohistochemistry: Immunohistochemical analysis of skin and gills revealed presence of PCNA immune reactive cells that exhibited dark brown nuclei (Fig. 5a, b, respectively). Furthermore, caspase-3 immune reactive cells was also demonstrated in the lamellar epithelium. The immune-stained cells exhibited strong dark brown cytoplasm and/or nuclei (Fig. 5c, d).

DISCUSSION

Parasitological examination revealed higher prevalence of *N. bivittata* in *T. zillii* (64%) in comparison to earlier studies in *Lithognathus mormyrus* (10.26%)[20]. Cymothoids infestations affecting natural fish populations are extremely variable[21]. Higher Cymothoids infestations with *Ceratothoa* sp. and *Cymothoa* sp. approaching frequencies up to 73% have been recorded in some reports[22,23]. Cultured fish are also susceptible to isopods infections Rajkumar *et al.*[24,25] with extremely higher prevalence of infections (98%) Sievers *et al.*[26]. In the majority of cases the source of infection is related to infested wild fish coming in close contact with the farmed species[27].

Climatic conditions strongly affect the reproduction of cymothoids[28]. Rainfall, salinity and temperature are critical issues affecting the intensity and prevalence of infestations[29]. The prevalence reached peak values during the summer and post monsoon seasons[30]. Higher temperature and salinity similare to that recorded in Lake Qarun; 30.13°C and 40%, respectively, are supposed to be appropriate for their growth and reproduction[24].

The levels of some metals detected in Lake Qarun water exceeded the safe limits recommended for protection of aquatic species. According to ANZECC and ARMCANZ[31] the marine high reliability trigger value recommended for; copper, iron, lead, zinc, cobalt and cadmium are; 1.3, 300, 4.4, 15, 1 and 5.5 µg L^{-1}, respectively. The surplus discharges from agriculture drainage, sewage as well as some industrial effluents released into Lake Qarun are alleged to be the main sources for such detected metals[32]. Such unfavorable environmental circumstances deteriorate the physiological condition of fish lowering their immune defense mechanisms, therefore, rendering fish susceptible to numerous opportunistic infections[33].

Nerocila bivittata were collected from: Gills, the lateral body surfaces nearby the pectoral fin, the ventral body surface just below gills and inside the buccal cavity indicating that these sites were the most predilections for *N. bivittata.* The preference of attachment site may be relevant to the body movement, morphology and habits of the host fish[34]. The existence of these parasites inside the gill chamber as well as inside the buccal cavity may be related to the high protection provided in these sites. Isopods inhabiting the gill chamber cause severe injuries to the gills through pressure exerted by the parasite as well as via their feeding activities and the extent of damages is proportional to the duration of settlement as well as the size of the isopods[35]. The majority of investigated *T. zillii* specimens demonstrated extensive skin erosions, thickening of gill arch and some cases also showed complete absence of gill rakers, which may be a result of the severe irritations caused by biting as well as the sucking mouth parts of isopods. The pale appearance of gills may be due to either loss of blood or consequence to obstruction of branchial circulation[36]. However, some reports have not shown any serious effects of infection with cymothoids[37].

Hematological analysis demonstrated that blood parameters (cell counts, haemoglobin content and haematocrit) were decreased in parasitized fish than non-parasitized fish. The decline in erythrocyte count and haemoglobin content have long been considered as distinctive features of post-haemorrhagic anemia induced by the blood feeding activities of parasites[38]. Similar findings were also reported in some isopods infestations; *Alitropus typus* in *Channa striatus* fish[39] and *Ceratothoa oestroides* in *Dicentrarchus labrax*. Previous reports on cymothoids infestations affecting wild fish have been found to follow similar patterns[36]. These changes evidently signify impaired oxygen blood carrying capacity a result of sever gill damages.

The size of erythrocytes (MCV) has been increased in parasitized fish than others. This may be alleged as an alternative strategy for enhancing the capacity of oxygen transport [40,41]. Swelling of erythrocytes may be relevant to the effect of synthesized catecholamine in response to parasitic infestations[42]. Additionally, the erythrocyte swelling was concomitantly associated with increasing the (MCHC) which may be a result of haemoglobin synthesis by the circulating erythrocyte[43]. Results were in concordance with that reported by Tavares-Dias *et al.*[44] in hybrid tambacu fish naturally infested with branchiuran fish lice, *Dolops carvalhoi*, fish demonstrated low haematocrit and augmented MCHC values. On the contrary, some studies showed no differences in MCV and MCHC values between fish infected with parasites and other non-infected[36,45]. Additionally, some fish parasitic infestations also have been found to be accompanied by significant decrease in MCHC and increased MCV values[46].

Leukocytic count decreased in fish infected with *N. bivittata* which may be a result of the haematophagy of these parasites reducing the whole blood cell count[45]. A link between impaired immunological responses of fish in concomitant with parasitic infestations has been demonstrated in previous studies[47,48]. Romestand [36] indicated no change in the leucocyte count between parasitized and unparasitized fish. On the contrary some studies detected enhanced leukocytic count in parasitized fish than non-infected individuals[45]. Furthermore, infested fish showed lymphopenia, enhanced monocytes and neutrophils levels. The greater number of monocytes may be discussed as a cell defense mechanism against the invading parasites[49]. The total protein and albumen levels were also decreased in parasitized fish which may be linked to the depletion of energy stores[50,51].

Variable histopathological alterations were demonstrated. The diversity of the lesions is commonly linked to the size of the parasite and the duration of parasitic establishment[34]. The erosive skin, necrotic lesions in fish muscles and the destruction of gill filaments demonstrated in the present study are attributed to heavy pressures exerted by the invading isopods at their attachment sites as well as to the powerful irritations induced by their feeding activities on host tissues[10,52].

The inflammatory reaction demonstrated in the skin and gills associated with mucous cell activation is in concordance with some earlier reports[53,35]. Perfuse infiltration of tissues with lymphocytes and granulocytes is a common feature in isopods infestations affecting fish[52]. Cymothoids infestations are usually coupled with destructive changes in the fish respiratory system. Parasites usually reside for long periods within the gill chamber consequently reduce the respiratory surface area, hinder the normal growth of the gill arches and cause erosion of the gill arch with fusion of gill lamellae[30]. Hyperplastic and hypertrophic activities of both epidermal and lamellar epithelia were recorded in some sections. These alterations are either a response in order to regenerate the sloughed epidermal cells[52] or to protect the underlying tissues[53]. The hyperplastic cells appeared with dark brown nuclei in PCNA immunohistochemical stained sections confirming the proliferation activity.

CONCLUSION

Parasitic isopods are serious parasites that have great ability to threaten the lives of fish in natural fisheries. These parasites cause severe tissue damage at their attachment sites.

They feed on fish blood, therefore, detrimentally affect the physiological status of the host fish and put their lives at risk. High levels of pollutants noticed in Lake Qarun provide an optimum environment for isopod growth and multiplication. The study recommends keen monitoring and restriction of the discharges released into the lake to save the life of its aquatic animals.

SIGNIFICANCE STATEMENTS

This study confirmed that parasitic isopod, *N. bivittata,* is increasingly serious problem in aquaculture. *N. bivittata* can infect wild fish population in natural fisheries causing massive economic loses. These parasites detrimentally affect the physiological status of the host fish and put their lives at risk. This study tackled the role of polluted aquatic environments in providing circumstances optimum for existence of *Nerocila bivittata* which will be helpful for strategies established for their control, therefore, the study recommends restriction of harmful discharges released into aquatic environments to save aquatic animals health and highlight the need to establish methods to control these serious parasites .

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