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PARASITOLOGICAL ASPECTS AND BIOCHEMICAL CHANGES OF INFECTED CULTURED TILAPIA (*OREOCHROMIS* HYBRID)

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Background. Fish farming has been developed due to decline of traditional fishing where tilapia culture is one of the principal sources for fish production. This study was aimed to investigate the prevalence and impact of helminth infection on the health of cultured hybrid tilapia, *Oreochromis* sp.

Material and methods. Prevalence and intensity of helminth infection and histopathological response of infected organs were studied. Changes of biochemical composition, protein electrophoritic pattern, and heavy metals levels of liver and muscle tissues were determined.

Results. Helminth infection was restricted to the liver of 60% of the examined fish. Of this number 33% were infected by undifferentiated nematode larvae and heterophyid metacercariae while 67% showed the metacercarial infection only. Infection caused a noticeable alternation in histological architecture of the liver that was accompanied by depletion in hepatic glycogen, total lipids, and total protein. Moreover, biochemical components of the muscle tissues were significantly decreased along with intensity of infection. Protein fractions of the liver and muscle tissues were highly variable. Intensity of the parasitic infection was directly correlated with Fe and Mn levels in both liver and muscle.

Conclusions. Cultured fish also suffered from helminth infection that significantly impaired the health and condition of fish as shown by histopathological, biochemical, and protein fractions changes recorded in this study. In additions, intensity of helminth infection might increase the capacity of infected organs in accumulation of heavy metals.

Key words: fish farm, hybrid tilapia, helminthes, histopathology, biochemistry, heavy metals.

INTRODUCTION

Fish farming has been developed due to decline of traditional fishing because of overfishing, pollution, and consequently the increasing demand for high-quality seafood for population growth and health-related considerations. In Egypt, the culture of tilapia and their hybrids is one of the principal sources for fish production.

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Tilapia has been classified as “disease resistant”, and with a minimal presence of pathogens (Popma and Masser 1999). Man-made pollutants and / or intensification of fish culture resulted in an increase of environmental changes, which may be stressful to fish (Lio-Po and Lim 2002). These conditions can result in decreased resistance by the fish, causing spread of disease and parasite infection (Rottmann et al. 1992). Among fish parasites, the helminthes constitute the major threat to the fish health. It has been suggested that some species of parasite are able to accumulate higher concentrations of trace metals than their host (Sures et al. 1997, Zaghloul et al. 2001).

Publication and reports are available on disease of feral and cultured fish in warm fresh water (Kabata 1985, Lim 1990, 1991, Paperna 1991, 1996, Arthur and Lumanlan-Mayo 1997, Fijan 1999) but few were dealing specifically with pathological response to parasitic disease in aquaculture fish and on the possibility of heavy metals bioaccumulation and their impact on fish health. The influence of parasites on host condition is a central problem in evolutionary biology and parasitology (Esch and Fernandez 1993). In this regard, little is known about normal physiology of cultured tilapia and their response to disease (Hrubec et al. 2000). Although, in Egypt, there has been a number of records of parasitic disease of cultured fish, they were mainly descriptive (Zaghloul et al. 2001).

Therefore, it is necessary from a public health viewpoint to estimate difference source of stress on cultured fish, hence, this work was aimed at investigating the prevalence of natural helminth infection and its impact on histopathological and biochemical response of cultured *Oreochromis* sp. In addition, heavy metal levels were considered.

MATERIAL AND METHODS

One hundred and fifty individuals of hybrid tilapia, *Oreochromis* sp., were collected from a farm in Ismailia, Egypt during spring of 2003. The animals were packed in ice and brought to the laboratory on the same day. Total length and weight of the animals were measured. There was no significant difference regarding the length and weight (10.9 ± 2.53 cm and 182 ± 19.8 g, respectively).

For parasitological examination, the muscles, liver, kidney, and the intestine of fish were taken then left in saline solution for few minutes, cut into small pieces, compressed between glass plates, and examined under a binocular dissecting microscope. Diameters of the metacercariae found in the compressed slides were measured using a micrometer eyepiece and recorded as the mean value of two measurements.

For histopathological examination, infected organs were immediately removed out from fish, fixed in 10% buffered formalin, embedded in paraffin, cut at 5-µm sections, and stained with haematoxylin and eosin. Detection and visual evaluation of hepatic glycogen were carried out by using Periodic acid Schiff’s (PAS) technique. The intensity of parasitic infection was determined by counting the parasites in an area equal to 1 mm^2 in stained paraffin sections.

The total protein content was determined in wet homogenate liver and muscles tissues using Diamond diagnostics kit (BioMerieux, France). Total lipid content was extracted from tissue using a chloroform-methanol mixture (2 : 1) and were determined according to Smedes and Thomasen (1996) using Diamond diagnostics kit. Glycogen content was measured by anthrone reagent as described by Carroll et al. (1956). Results were expressed in mg · g^{-1} w.w. (wet weight).

For electrophoresis analysis, 0.5 g of the raw fish liver and muscle were homogenized with 4 ml of 2% SDS extraction solution (2% sodium dodocyle sulphate, 5% mercaptoethanol, and 60 mM Tris-HCl pH 8.0) (Etienne et al. 1999). The homogenates were centrifuged (20 000 rpm, 15 min, 20°C) and the supernatants were collected. Solutions of samples and the molecular weight marker proteins were applied to the gel according to the protocol provided by Mackie et al. (2000). Analytical SDS--PAGE separations were performed on gel containing 7% polyacrylamide. The protein bands were visualized by staining the gel with Coomassie® brilliant blue (CBB), then rinsed in destaining solution (ethanol : acetic acid : water = 50 : 16 : 143) until the background become clear except the blue protein bands. Finally, the gel was preserved in 7% acetic acid and photographed. Standard protein marker consisted of myosin H-chain (209 KDa), phosphorylase b (112 KDa), bovine serum albumin (83.1 KDa), gultamic dehydrogenase (55.5 KDa), ovalalbumin (49.7 KDa), carbonic anhydrase (31.7 KDa), trypsin inhibitor (21.5 KDa), lysozyme (17.5 KDa) and they were purchased from Merck, Darmstadt, Germany. The acquired images of the gels were analysed with the Whole Band Analyser software of Bio-Image, version 3.3.

H_2SO_4-HNO_3 (Merck) acidic mixture (2 : 3) was used for the digestion of liver and muscles tissues for Co, Cu, Ni, Fe, and Mn determinations (Hamza-Chaffai et al. 1999). Metal concentrations in the samples were measured using a Perkin Elmer AS 2380 flame atomic absorption spectrophotometer and results were expressed in μg · g^{-1} w.w. Detection limits found with this method for the analysis of tissues were: 0.2 μg · g^{-1} for Fe, 0.1 μg · g^{-1} for Mn, 0.04 μg · g^{-1} for Ni, and 0.05 μg · g^{-1} for Cu. A comparison between the amount of heavy metals when added either before or after digestion revealed that there was no loss of the metals during the digestion process with a recovery value of 100–102% for all the heavy metals. All reagents were from Merck, Darmstadt (Germany).

All processing of data was conducted with the software packages Microsoft Excel XP (for data storage) and SPSS version 10.0, for statistical evaluation. Kruskall–Wallis one way ANOVA was used to compare data. Spearman's rank correlation coefficient was used to test for associations between heavy metals concentration and intensity of parasite infection in the fish tissue.

RESULTS

The helminth infection was restricted to the liver of 60% of the examined fish (with no parasites in the other examined tissues). Parasites were mainly heterophyid

metacercariae and undifferentiated nematode larvae. The infected fish showed mixed infection in 33% of cases while 67% were infected only with metacercariae (single infection).

Metacercariae were rounded, yellowish-brown with black pigmentations and different in sizes that related to different developmental stages; no organs were found (Fig. 1).

Mean diameters of metacercariae recovered from single- and mixed-infected fish were summarized in Table 1. Diameters of metacercariae of mixed-infected fish were significantly higher as compared to those of single infection ($P < 0.05$, $F = 7.9$). Intensity of metacercariae per mm^2 of liver tissue was higher in case of mixed infection ($P < 0.01$, $F = 10.2$) where more than 60% of fish had more than three metacercariae per 1 mm^2. The intensity of infection with undifferentiated nematode larvae was 0.9 ± 0.04 spec. per mm^3 of the liver (Table 1).

Table 1

Summary statistic of sizes of heterophyid metacercariae and intensity of helminth infection in liver of hybrid *Oreochromis* sp.

Type of infection		Parameter	$\bar{x} \pm s$	Range
Single infection	Heterophyid metacercariae	Diameter µm	48.7 ± 4.49	31.75–68.75
		No./mm^2	2.2 ± 0.3	1–4
Mixed Infection	Heterophyid metacercariae	Diameter µm	82.6 ± 11.2	37.5–125
		No./ mm^2	3.3 ± 0.12	2.67–4
	Nematode larvae	No. larvae/ mm^2	0.9 ± 0.04	0.67–1

Histological examination of mixed-infected liver showed invasion of undifferentiated nematode larvae and heterophyid metacercariae to liver parenchyma (Figs. 2, 3) with a remarkable degeneration to the surrounding area.

The histopathological response to infection was represented by a cytoplasmic vacuolation, fatty degeneration, eosinophilic and lymphocytic infiltration, and hepatocellular focal necrosis (Figs. 3–5). In addition to the above, a noticeable damage to pancreatic cells was found (Fig. 5). In a single-infected fish, the severity of histopathological changes in liver was limited as compared to mixed infection (Figs. 6, 7). However, in all cases, there was no inflammatory response to the parasites observed in the tissues.

A high depletion of polysaccharides material was observed in the hepatocytes of mixed infected fish as compared to single one (Figs. 8, 9). Proteins analysis of liver and muscle of examined fish was shown in Table 2.

In a single-infected fish, protein content was decreased significantly to 113.4 and 195.1 mg · g^{-1} w.w. in both liver and muscles tissues, respectively, as compared to non-infected tissues. In mixed-infected fish, protein content decreased significantly by 37.25 and 9.02 percentage points, respectively as compared to non-infected and single-infected fish.

Table 2

Protein contents (mg · g^{-1}) of liver and muscles of both non-infected and infected hybrid *Oreochromis* sp.

Fish state	Examined organ			
	Liver		Muscles	
	$\bar{x} \pm s$	Range	$\bar{x} \pm s$	Range
Non-infected	140.3 ± 3.2	120.4–146.2	282.9 ± 2.9	278.8–288.6
Single infection	113.4 ± 2.1[a]	109.8–117.2	195.1 ± 3.4 [a]	188.7–200.4
Mixed infection	88.3 ± 2.9 [a, b]	83.0–93.3	177.5 ± 6.1 [a, b]	167.7–188.7

[a] Significantly different as compared with normal control.

[b] Significantly different as compared with corresponding single infection.

As shown in Table 3, lipid content of muscle decreased significantly in both single- and mixed infection ($P < 0.01$, $F = 20.11$; $P < 0.002$, $F = 162.94$, respectively) as compared to non-infected muscle. Liver lipid content decreased significantly in both infection levels as compared to non-infected tissue ($P < 0.01$ and $2.4 \cdot 10^{-5}$; $F = 18.23$ and 496.3, respectively).

Table 3

Lipid content (mg · g^{-1} wet weight) of liver and muscles of both non-infected and infected hybrid *Oreochromis* sp.

Fish state	Examined organ			
	Liver		Muscles	
	$\bar{x} \pm s$	Range	$\bar{x} \pm s$	Range
Non-infected	354.88 ± 12.3	340.68–379.4	81.80 ± 4.6	74.15–89.97
Single infection	286.48 ± 10.2 [a]	267.13–301.97	56.72 ± 3.2 [a]	52.03–62.87
Mixed infection	50.85 ± 2.9 [a,b]	45.08–54.7	13.25 ± 1.1 [a,b]	11.54–15.38

[a] Significantly different as compared with normal control.

[b] Significantly different as compared with corresponding single infection.

Table 4

Glycogen content (mg · g^{-1} wet weight) of liver and muscles of both non-infected and infected hybrid *Oreochromis* sp.

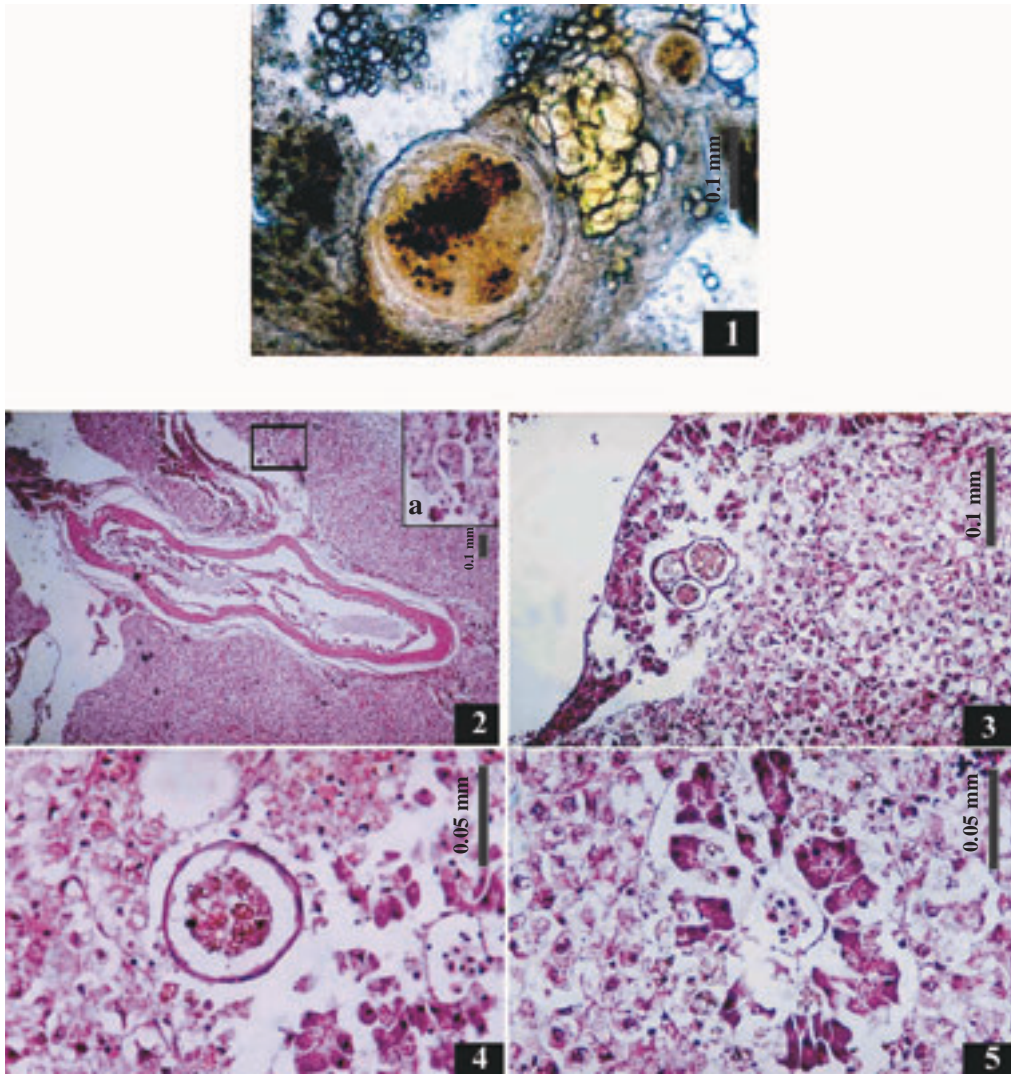
Fish state	Examined organ			
	Liver		Muscles	
	$\bar{x} \pm s$	Range	$\bar{x} \pm s$	Range
Non-infected	8.61 ± 0.7	7.83–9.30	3.20 ± 0.30	2.70–3.6
Single infection	5.20 ± 0.4 [a]	4.80–5.50	2.38 ± 0.10 [a]	2.25–2.6
Mixed infection	3.86 ± 0.1[a,b]	3.76–4.03	2.01± 0.04 [a , b]	1.92–2.1

[a] Significantly different as compared with normal control.

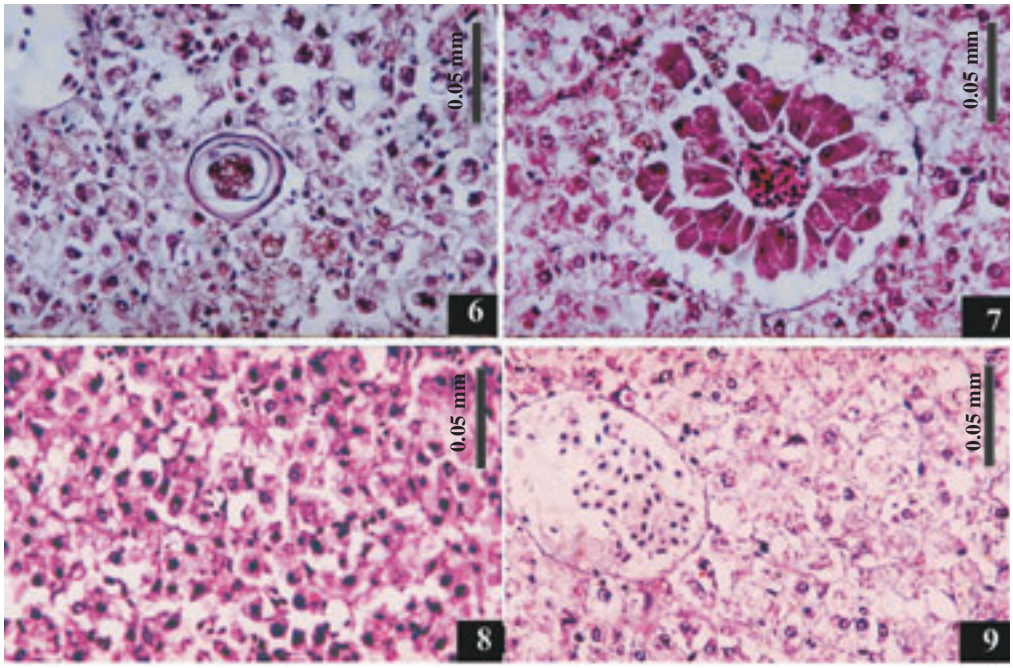
[b] Significantly different as compared with corresponding single infection.

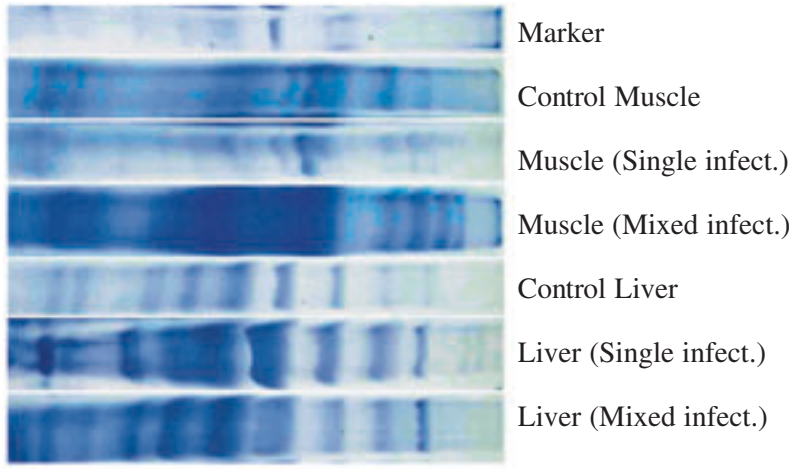
Table 4 showed that the glycogen resources in the liver were depleted significantly in single- and mixed infection to 5.2 and 3.86 mg · g^{-1} w.w. as compared to non-infected tissue (8.6 mg · g^{-1}) ($P < 0.01$, $F = 28.1$; $P < 0.001$, $F = 118.9$, respectively). Significant difference in glycogen content of muscles of both single- and mixed-infected fish was found as compared to non-infected one ($P < 0.01$, $F = 17.4$; $P< 0.001$, $F = 59.5$), respectively).

Table 5 summarized the protein pattern being separated on a SDS-PAGE for liver and muscle of cultured tilapia. There were 23, 23, and 24 bands detected for none-, single-, and mixed-infected fish muscle, respectively (Fig. 10), with appearance and disappearance of some bands. The amount of the enzyme carbonic anhydrase (31.7 KDa) slightly decreased in single infection (7.72 percentage points) and recorded more decrease in mixed infection (2.15 percentage points) as compared to non--infected fish (9.4 percentage points). The amount of enzyme gultamic dehydrogenase (55.5 KDa) increased significantly in both single and mixed-infected fish (by 18.1 and 13.1 percentage points, respectively). Oval albumin bands (49.7 KDa) decreased in all infected groups. Trypsin inhibitors (21.5 KDa) disappeared in muscle of mixed--infected fish.



Figs. 1–5. Tissues of hybrid *Oreochromis* sp. infected with parasites. Fig. 1. Hetrophyid metacercariae in a compressed liver tissue. Fig. 2. Stained section of infected liver showing invasion of undifferentiated nematode larvae and metacercariae (a) to liver parenchyma with a remarkable degeneration to the surrounding area. Fig. 3. Metacercariae in parenchyma with marked degeneration in liver parenchyma. Fig. 4. Section of mixed-infected liver showing cytoplasmic vacuolation, fatty degeneration; leukocytic infiltration, focal necrosis. Fig. 5. Section of liver showing the damage occurred to pancreatic cells





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Figs. 6–10. Histopathological and electrophoretic aspects of the pathology of hybrid *Oreochromis* sp. infected with parasites. Figs. 6–7. Section of metacercariae-infected liver showing that the histopathological alternations were mild as compared to mixed infection. Fig. 8–9. PAS-stained liver sections showing a presence of considerable amount of polysaccharide content and remarkable depletion in this content in single and mixed infection, respectively. Fig. 10. SDS-PAGE pattern of proteins of liver and muscle tissues of non-infected and infected fish

Table 5

SDS-PAGE Proteins binding pattern of liver and muscle tissues of non-infected and infected hybrid *Oreochromis* sp.

Lanes	Marker		Non-infected fish muscle		Single-infected fish muscle		Mixed-infected fish muscle		Non-infected fish liver		Single-infected fish liver		Mixed-infected fish liver	
Rows	Mol wt	amount %	Mol. wt	amount %	Mol. wt	amount %	Mol. wt	amount %	Mol. wt	amount %	Mol. wt	amount %	Mol. wt	amount %
r1			495.37	0.7894	490.25	0.474					490.25	0.738	490.25	0.187
r2			398.2	0.5053	398.2	0.402	398.2	2.35			398.2	0.534	398.2	0.162
r3			323.44	0.5031	333.69	0.464	313.51	2.1					347.86	0.0959
r4					279.63	0.404	279.63	2.36			288.49	0.0838	288.49	0.183
r5	209	1.7991	209	1.0895	252.01	1.46	211.18	2.73	209	1.66			209	
r6			173.33	0.8969	209	0.844	178.82	4.62			209	2.09	209	4.8
r7					159.49	3.69	128.21	1.11						
r8	112	1.8379	105.86	6.1825	105.86	2.69	105.86	3.99	105.86	3.23	105.86	5.41	105.86	4.78
r9			98.739	1.9263			98.413	0.682					98.739	
r10			84.77	1.271	84.77	0.245	92.097	0.102	84.77	7.25	84.77	6.4	84.77	4.39
r11	83.1	4.9359	68.975	5.1157	65.836	3.55							68.975	
r12	55.5	11.479	52.394	10.598	52.394	18.1	52.394	13.1					52.394	
r13	49.7	8.1847	45.749	2.7197	45.749	3.09	45.749	2.51	45.749	13.3	45.749	12.9	45.749	5.67
r14			37.634	2.645	36.537	2.1	38.308	0.681					37.634	
r15	31.7	13.575	31.233	9.3991	31.233	7.72	31.233	2.15	31.233	23.3	31.233	14.4	31.233	15.1
r16			29.554	61.197			29.634	7.48					29.554	
r17					28.268	3.64			28.04	9.51	27.965	5.3	27.553	8.99
r18			26.892	0.9646			27.074	2.61					26.892	
r19					26.142	1.58	26.283	1.43	26.177	2.92			25.931	0.273
r20			25.481	0.3251			25.481	0.359	25.481	3.59	25.481	2.19	25.481	
r21											25.038	4.1	25.106	5.18
r22			24.143	0.0384	24.143	0.958	24.143	0.0062	24.143	0.343			24.143	0.0344
r23			22.691	1.9599	21.91		21.91	9.27					21.91	0.8
r24	21.5	5.6303	19.801	8.5298		4.04			21.149	4.95	19.801	2.63	20.718	0.288
r25	17.5	14.352	15.531	11.379	16.863	4.04	15.531	8.27	15.531	6.82	15.531	8.41	15.531	3.21
r26					14.421	10.5	13.897	4.16						
r27			13.613	3.0214	13.118	7.14			13.281	1.32			13.064	4.69
r28			11.982	2.1094	11.982	5.59	11.982	5.05	11.982	1.03	11.982	7.75	11.982	5.36
r29			10.163	0.1755	10.417	2.56	10.417	0.335	10.766	2.43			10.59	3.35
r30							8.763	0.0634						
Sum		61.794		78.342		82.5		77.6		81.7		72.9		67.6
In lane		100		100		100		100		100		100		100

SDS-PAGE revealed that liver proteins of non-, single-, and mixed-infected fish were separated to 14, 14, and 19 bands, respectively, with remarkable changes in protein bands pattern (Fig. 10).

Myosin H-chain band (209 KDa), was found in non-infected fish liver and disappeared in infected one. Oval albumin band (49.7 KDa) decreased in mixed infection (5.67 percentage points) as compared to non-infected fish (13.3 percentage points). While carbonic anhydrase band in the liver protein (31.7 KDa) significantly decreased in both single and mixed infection (14.4 and 15.1 percentage points, respectively) as compared to the non-infected fish (23.3 percentage points).

Results showed that Fe, Mn, Cu, and Ni were detected in liver and muscle of all examined fish while there was no evidence for Co. The level of heavy metals of muscles was summarized in Table 6. Generally, concentrations of Fe and Mn were significantly higher in liver than muscle tissues and those of Cu and Ni were very low as compared to the Fe and Mn. There was a positive significant correlation between concentrations of Fe and Mn and intensity of parasite infection (Table 6).

Table 6

Heavy metals concentration of liver and muscle tissues of non-infected and infected hybrid *Oreochromis* sp.

Organs	Heavy metals concentration ($\mu g \cdot g^{-1}$ w.w.)			
	Element	Non-infected fish	Single-infected fish	Mixed-infected fish
Liver	Fe	159.3 ± 5.2	265.0 ± 2.2 [a] r = 0.78	388.6 ± 3.5 [a,b] r = 0.76
	Mn	48.6 ± 0.7	91.0 ± 1.3 [a] r = 0.52	110.9 ± 0.3 [a,b] r = 0.93
	Ni	0.075 ± 0.01	0.12 ± 0.01	0.11± 0.03
	Cu	0.47 ± 0.1	0.41± 0.001	0.39 ± 0.02
Muscle	Fe	55.1 ± 2.7	72.1 ± 0.9 [a] r = 0.92	106.6 ± 1.5 [a,b] r = 0.99
	Mn	79.90 ± 0.2 [a]	79.90 ± 0.2 [a] r = 0.88	96.68 ± 8.9 r = 0.95
	Ni	0.063 ± 0.01	0.05 ± 0.01	0.05 ± 0.02
	Cu	0.21 ± 0.1	0.34 ± 0.01	0.27 ± 0.01

[a] Significantly different as compared with normal control.

[b] Significantly different as compared with corresponding single infection.

r, Spearman correlation coefficients, showing principal interactions in explaining data variation between heavy metals concentration and intensity of helminth infection.

DISCUSSION

Fish diseases and histopathology, with a broad range of causes, are increasingly being used as indicators of environmental stress since they provide a definite biological end-point of historical exposure (Stentiford et al. 2003). Wild fish have greeter parasite diversity but with lower population abundance and the reverse is true for cultured fish (Lio-Po and Lim 2002). Consequently, the present study revealed that only undifferentiated nematode larvae and/or heterophyid metacercariae parasitized the liver of a high percentage of examined cultured tilapia. The infections resulted in changes in histological architecture of the liver and depletion in hepatic glycogen reserves, total lipids, and protein. Muscle biochemical complements were also affected. In this regard, histopathological alterations due to infection of fresh water fishes with helminthes parasites were variable and almost potentially pathogenic in

heavy infection (Taraschewski 2000) and this was in accordance with our results. In addition, Zaghloul et al. (2001) pointed out that alterations of the metabolic pathways of fish can be by a variety of biological and physiological factors.

Dramatic changes of muscle and liver composition (protein and enzyme expression) due to helminth infection were in three main ways; the disappearance of some protein bands, fading away of some existing bands or appearance of new bands, it also affected the relative quantities of protein fraction either by being increase or decrease. However, this fluctuation in protein bands might be considered as a reflection of stressor impact on ribosome and RNA levels and consequently on protein synthesis (Guyton 1961, de Bruin 1976).

Results showed that metal concentrations (Fe and Mn) were higher in the liver than in the muscle as the target tissues of heavy metals are the tissues of high metabolic activity (Canli et al. 1998). Sorensen (1991) reported that concentration of heavy metal does not depend only on the structure of the organ but also on the interaction between the particular metal and the target organ. Consequently, results highlighted on a possible positive correlation between the level of heavy metals and intensity of infection taking in consideration a fixed size class of examined fish, since size may be an important factor in biochemical content and heavy metals concentration of aquatic animals (Sures et al. 1997, Sures 2001). Low concentrations of Ni and absence of Co in the muscle and liver of fish was not surprising because aquaculture is a system organized to constitute an optimal environment, in contrast to wild and feral fish that generally face human impact (Watson and Yanong 2002). The heavy metal levels recorded in this study were lower than the permissible limits for human consumption (Anonymous 1981, Ellen et al. 1990).

In conclusion, the helminth infection had an impact on condition and health of cultured tilapia. However, the histopathology, biochemical changes, and the protein fractions recorded in this study were taken into account in elevating the response of fish to stressors where parasitism could be adversely affect metabolism of fish.

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