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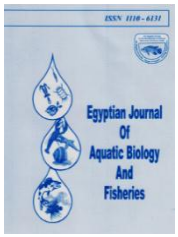

Prevalence, Intensity and Histopathological Alterations Caused by Different Ectoparasites Infesting *Oreochromis niloticus*

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ABSTRACT

This study aimed to investigate the relationship between the ectoparasite infestation of *O. niloticus* with gene expression analysis and its histopathological alteration. Thus, from two localities in Egypt, a total of 423 *O. niloticus* fish were collected and subjected to an investigation during the period from October 2022 to July 2023. Fish were surveyed for ectoparasites. Samples from gills were collected and fixed in 10% neutral buffered formalin. The total RNA was extracted from both the skin, gills, Cpy1a1 and TNF-α were analyzed. The examined fish showed skin hemorrhage, ulcers, sloughed scales, and corneal opacity. Fish were found to have *Clinostomum* spp. on the skin and gills and *Lamproglena* parasitic crustacea were attached to gills. From 423 examined *O. niloticus* fish, 73 fish specimens from various locations in Egypt were examined for their rate of ectoparasites occurrence. Infestation in fishes of the River Nile branch (68.5%) was higher than in cultured fish (57.1%). The rate of infestation was 200 fish with monogenean; 50 fish with protozoa, and 10 fish by crustacean, while the mixed infection was detected in 10 fish individuals with crustacean and monogenean; 3 fish with crustacean and protozoa; 50 fish with monogenean and protozoa, and 5 fish with three types of these ectoparasites. Two protozoan parasites, *Trichodina heterodentata (T.heterodentata)*, and *Myxobolus tilapiae (M. tilapiae*) were among the various ectoparasites found in the examined fish as well as *Centrocestus formosanus (C. formosanus); Cichlidogyrus tilapiae (C. tilapiae*) and *Lamproglena monodi (L. monodi)*. Histopathological examination of gills revealed the presence of parasitic infestation between gill filaments; some cases showed heavy infestation with multiple parasitic worms. Gene expression analysis showed up-regulation of the Cpy1a1, and TNF-α in the M and L groups compared to the negative control group in both skin and gills. Treatment of both infested groups recorded down-regulation of the tested genes in the skin. However, the L-treated group showed a non-significant decrease in the expression level relative to the L group in the gills.

INTRODUCTION

The global tilapia sector has substantially expanded in recent years, providing a cheap source of protein for many underdeveloped nations. Tilapia is now the second most farmed fish in the world **(Garcia *et al.*, 2013; Abdelsalam *et al.*, 2015)**. There are several tilapia species that are commercially raised for food. Egypt is the third-largest producer of the Nile tilapia (*Oreochromis niloticus*) worldwide, following China and Indonesia **(Abdelsalam *et al.*, 2015)**.The Nile tilapia is a popular fish among farmers due to its unique characteristics. It grows quickly, has a high protein content, is easily marketable, has good disease resistance, and can tolerate different stresses in aquatic environments **(El-Sayed, 2019)**. To maximize their profits, most tilapia farmers nowadays use intensive culture methods that involve increasing stocking densities exponentially (**Thomas *et al.*, 2014)**.

Parasites can have a negative impact on the survival of fish by reducing their immunity, altering their behavior, and making them more susceptible to other infections. This can result in significant financial losses in fish farming due to increased mortality and tissue damage **(El Asely *et al.*, 2015)**. Ectoparasites on contaminated angles are more often than not distinguished by scratches, ulcerations of the body, haemorrhagic spots on the skin, and battered blades (**Mahmoud *et al.*, 2011)**. Ectoparasites are the most significant pathogens affecting fish health (**Ebrahimi *et al.*, 2018)**. Myxosporidia are tiny parasites that have significant economic , and they infect a wide range of commercially important fish including tilapias **(El Asely *et al.*, 2015)**. Several species of Myxosporean have been identified in both wild and cultivated cichlids, such as *Myxobolus brachysporus*, *Myxobolus israelensis* **(Eissa *et al.*, 2010)**, and *Myxobolus tilapiae* **(El Asely *et al.*, 2015)**. These parasites have been linked to decreased respiratory capacity **(Székely & Molanr, 1999)**, ovarian disturbance **(Evans *et al.*, 2007)**, and postmortem myoliquefaction of the host **(El Asely *et al.*, 2015; Eissa *et al.*, 2020)**. *M. tilapiae* causes the formation of external injuries in cichlids, including corneal opacity, frontal skin ulcers, and head blisters, which may lead to the development of gaps in the head-like injuries (**Eissa *et al.*, 2006)**.

The metacercariae of *C. formosanus* inhabits the gills of certain farmed fish species, leading to serious neurological changes in the structure of the gills. This can cause respiratory problems, decreased performance, and in some cases, death among young fish **(Bannak *et al.*, 2018)**. Zoonotic cases pertinent to *C. formosanus* have been recorded around the world including the Egyptian Nile Delta, European and Asian nations as a result of eating crude or undercooked angle containing metacercariae **(FAO, 2020)**.

Lamproglena monodi Capart, 1944 (Cyclopoida: Lernaeidae), a shellfish copepod within the family Lernaeidae Cobbold, 1879, is ectoparasite on the gills of primarily cichlid. *Trichodina centrostrigeata, T. acuta, T. kalimbeza, T. linyanta, T. pediculus, T. velasquezae, T. nigra, T. minuta, T. heterodentata, T. compacta, T. fultoni, T. salmincola,*

T. canton, T. magna, and others have all been detected to infect the Nile tilapia. The most important trichodinid are *Tripartiella orthodens, T. migala*, *Trichodinella tilapiae*, and *Paratrichodina africana* **(Aly *et al.*, 2020)**. The flatworm group's monogeneans, which are found on fish gills and skin, are the most significant ectoparasites of fish (**Mono, 2015)**.

Common monogenic ectoparasites in the Nile tilapia are *Dactylogyrus extensus* and *Cichlidogyrus tilapiae*. Therefore, this work aimed to assess the reaction of the fish body against external parasites before and after treatment using gene expression analysis and histopathological alteration.

MATERIALS AND METHODS

Collection of samples

Between October 2022 and July 2023, a total of 423 *O. niloticus* fish, with a length of 10-15cm, were collected from three different localities in Egypt. Out of these, 350 fish were from Kafrel Sheikh fish farms, while 73 fish were collected from the Nile River (Al Bahr Al Aazam). The purpose of this collection was to survey the fish for ectoparasites. The fish were transported alive to the laboratory for further investigations, including parasitological, histopathological, and biochemical analysis. Fish specimens were kept in separate, aerated, covered glass aquaria.

Clinical examination of fish

According to **Amlacher (1970)**, any clinical abnormalities were examined in collected fish.

Parasitological examination of fish

Each part of fish was carefully examined under a light microscope; OLYMPUS; CX41. Smears were prepared with methanol and stained with Giemsa from the mucous surrounding the skin, gills, and fins (**Attia *et al.*, 2021)**.

Histopathological examination

Tissue specimens from gills were collected, fixed in neutral buffered formalin 10%, washed, dehydrated, cleared, and embedded in paraffin. The paraffin-embedded blocks were sectioned at 5 micron thickness and stained with Hematoxylin and Eosin (**Bancroft *et al.*, 2012)** for histopathological examination via a light microscope (Olympus BX50, Japan).

Treatment trials

Natural treatment trials using ginger plant extract with a dose of 2.2mg/ L of water (**Pramita *et al.*, 2023)** were conducted on *O.niloticus* infested with *monogenean* and *Trichodina*; chemical control was performed using metrifonate (organophosphorus compound) according to **Untergasser (1989)**.

Metrifonate was handled at 1g/ L of water as stock and used immediately as 100ml stock to 100L of water aquarium at 25°C and pH 6.7 for three days to *O.niloticus* infested with *monogenean* and *Trichodina* after which samples from skin, gills, and fins were taken for gene expression analysis.

Gene expression

Total RNA extraction and cDNA synthesis

The total RNA was extracted from both the skin and gills samples using the QIAmp RNA mini kit (Qiagen, Hilden, Germany) according to the protocol provided. The first strand of cDNA was then synthesized using M-MuLV reverse transcriptase (Fermentas, EU).

Real-time PCR (qPCR)

The PCR reactions were prepared using the iQ SYBR GREEN PERMIX (BIO-RAD 170–880, USA) in the BIO-RAD Cycler thermal cycler and the MyiQ real-time PCR detection system **(Ibrahim & Ibrahim, 2014)**. The primer used to amplify the target genes was designed based on the sequence published in the Gene Bank of *O. niloticus* (Table 1). The program used was as follows: pre-incubation for 10min at 95^{o}C, then 40 cycles of denaturation for 20s at 95^{o}C, annealing at 60^{o}C for 20s, and extension at 72^{o}C for 30s **(Ahmed *et al.*, 2021)**. Each assay was performed twice and included a no-template negative control **(Ibrahim *et al.*, 2020; Younis *et al.*, 2020)**. The GAPDH is used as an internal control to normalize expression data **(Ko *et al.*, 2009)**. Gene expression data were calculated according to $2^{\Delta\Delta}$CT.

Table 1. Primers used in transcription levels of the examined genes

Gene	**Formard primer**	**Reverse primer**	**Accession number**	**Amplicon (bp)**
Cyp1a1	TAAACTGCAGAGCGAGAGCA	CTTTCGACCCCAGATAACCA	XM_019365993.2	190
TNF-a	GCCTCACAATTCTCAGCCAC	AAACACGCCAAAGAAGGTCC	AY428948.1	248
GAPDH	GCTGTACATGCACTCCAAGG	ACTCAAACACACTGCTGCTG	NM_001279552.1	182

RESULTS

1.Clinical signs

The examined fish showed a variety of non-specific health issues, including skin hemorrhaging, scale sloughing with ulcers, and corneal opacity. *Clinostomum* spp., a yellow grape-like parasite, was found on the gills of the fish, causing what is known as

yellow grape disease. Additionally, *L. monodi* parasitic crustaceans were discovered attached to the gills (Fig.1).

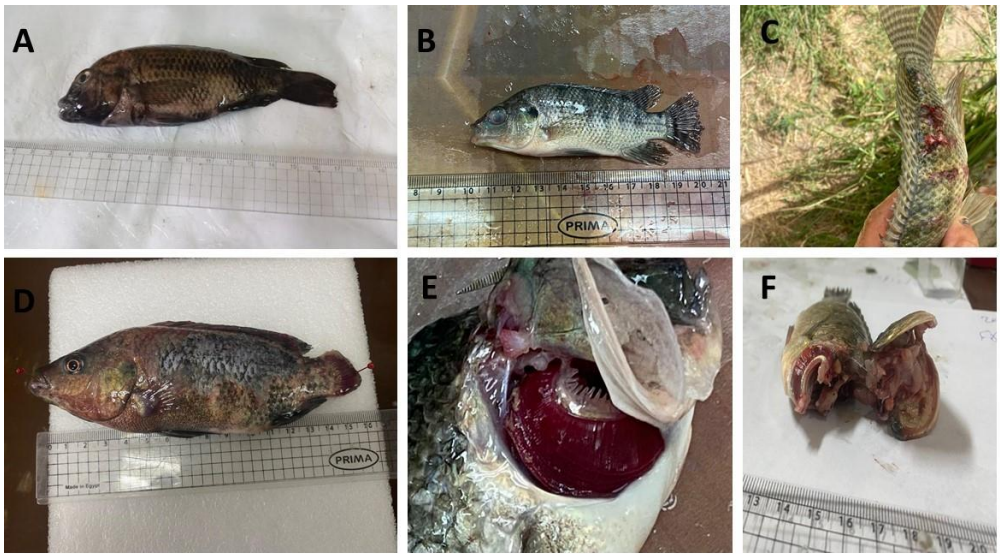


Fig. 1. Photos showing: **A)** Stressed fish; **B)** Fish with corneal opacity; **C)** Fish showing ulceration; **D)** Fish showing sloughing of scales; **E)** *L. monodi* attached to fish gill, and **F)** EMC of *Clinostomum* spp. attached to fish gill.

2. Prevalence of ectoparasites in *O.niloticus*

Out of a total of 423 *O. niloticus*fish species examined from various locations in Egypt between October 2022 and July 2023, 73 were taken from the River Nile and 350 were taken from fish farms. The infestation rate among fish from the River Nile (68.5%) was higher than that of cultured fish (57.1%).

3. Seasonal variation of ectoparasites

From 423 examined *O.niloticus* fish species;73 were infected with ectoparasites in winter, 200 in spring, 55 in summer, and 50 in autumn.The prevalence of examined fishes in winter showed the highest record (78%), while in spring it was 25%, for summer, it was 9%, and during autumn, a value of 50% was recorded (Table 2).

Table (2):Seasonal variation of examined *O.niloticus* in different seasons

O.niloticus	**Total number**	**Positive infected fish**	**Prevalence (%)**
Winter	73	57	78%
Spring	200	50	25%
Summer	55	5	9%
Autumn	50	25	50%
Total	378	137	36.2%

4.Mixed infection with ectoparasites

The rate of infestation was 200 fishwith monogenean,50 fish with protozoa, and 10 fish with crustacean, while the mixed infection was 10 fish with crustacean and monogenean,3 fish with crustacean and protozoa,50 fish with monogenean and protozoa, finally 5 fish with three types of these ectoparasites; (**Table 3)**.

Table (3):Mixed infection of examined *O.niloticus*with different ecto-parasites

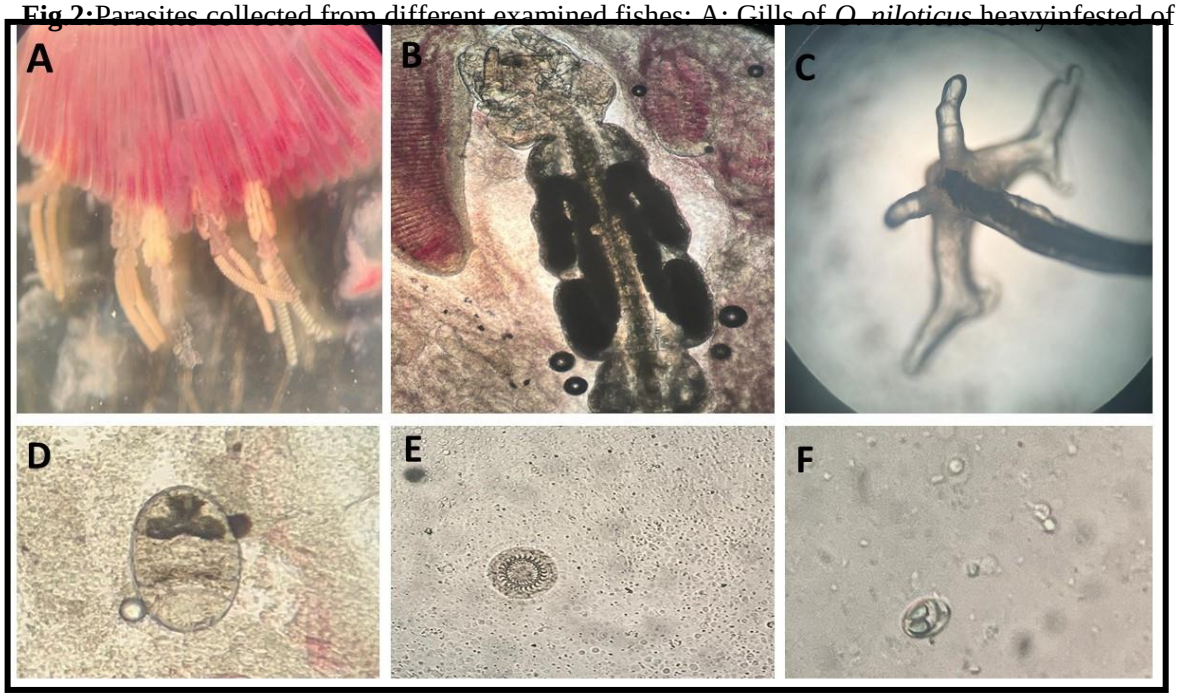
Species / ***Fish***	**Total number**	**C**	**M**	**P**	**(C+M)**	**(C+P)**	**(M+P)**	**Three mixed**
O.niloticus	423	10	200	50	10	3	50	5

C: Crustacae**;** M: Monogenea**;** P: Protozoa

5.Parasitological examination

Two protozoan parasites, *T.heterodentata*, and *M.tilapiae*, were among the various ectoparasites found in the fish under examination. as well as *C.formosanus and C.tilapiae as well as L. monodi*; **Fig. 2;3**

Fig 2: Parasites collected from different examined fishes; A: Gills of *O. niloticus* heavyinfested of



L. monodi; B: Light microscopic micrograph of *L. monodi* isolated from gills; C: *Lernaea cyprinacea* isolated from goldfishes; D: *C.formosanus* isolated from *O. niloticus* infested gills; E: *T.heterodentata* from *O. niloticus*; F: *M. tilapiae* from gills of infested *O. niloticus*.

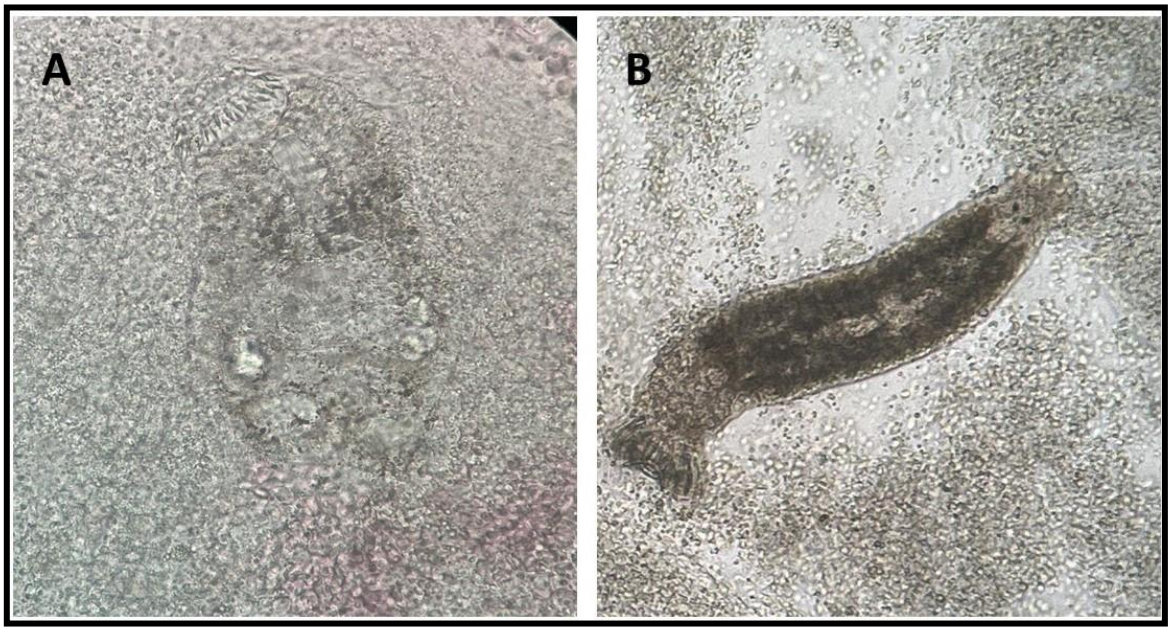


Fig. 3: Gills of *O. niloticus* infested with *C. formosanus*(A) and*C. tilapiae*(B).

6.Histopathological examination of O. niloticus infested with ectoparasites

Histopathological examination of gills revealed presence of parasitic infestation between gill filaments (Fig. 4a), some cases showed heavy infestation with multiple parasitic worms (Fig. 4 b) penetrating secondary gill lamellae (Figs. 4 c, d, e & f), with various degrees of gill lamellae destruction and necrosis (Fig.4 g), some sections revealed presence of multiple EMC in gill arch (Fig. 4 h), gill arch connective tissue infiltrated with eosinophilic granule cells surrounding the parasitic cysts (Fig. 4 i). Parasitic infestation in gills revealed multiple alterations in tissue as it induced activation of gill mucus cells (Fig. 5 a), also there was hyperplasia of primary gill lamellae cartilage (Fig. 5 b), secondary gill lamellae showed hyperplasia and fusion (Fig. 5 c), the connective tissue of gill arch showed a severe inflammatory reaction, edema, hemorrhage and vascular congestion (Fig. 5 d), there was also congestion of primary gill lamellae blood vessels (Fig. 5 e) and telangiectasis of secondary gill lamellae capillaries (Fig. 5 f), the base of gill filament showed heavy infiltration of eosinophilic granule cells (Fig. 5 g).

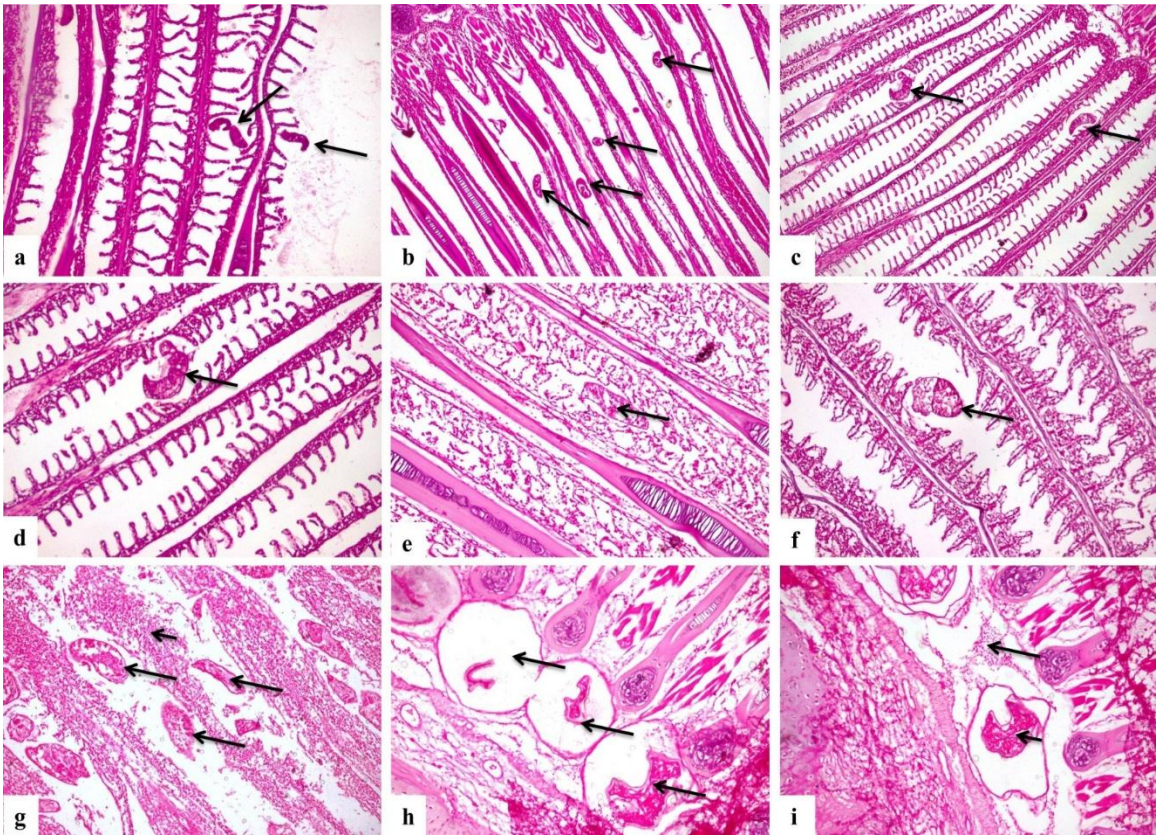


Fig.4:Photomicrograph of gills showing. **(a)** Sections of parasites between gill filaments (arrows) (H&EX100). **(b)** heavy infestation with multiple parasitic worms (arrows) (H&EX100). **(c)** parasitic worms penetrating secondary gill lamellae (arrows) (H&EX100). **(d)** higher magnification of the previous photo illustrating the destruction of secondary gill lamellae by parasitic worm (arrow) (H&EX200). **(e)** and **(f)** parasitic infestation between gill filaments with minimal tissue reaction (arrow) (H&EX200). **(g)** gill lamellar destruction and necrosis (short arrow) by heavy parasitic infestation (long arrows) (H&EX200). **(h)** multiple EMC in gill arch (arrows) (H&EX200). **(i)** gill arch connective tissue infiltrated with esinophilic granule cells (long arrow) surrounding the parasitic cysts (short arrow) (H&EX200).

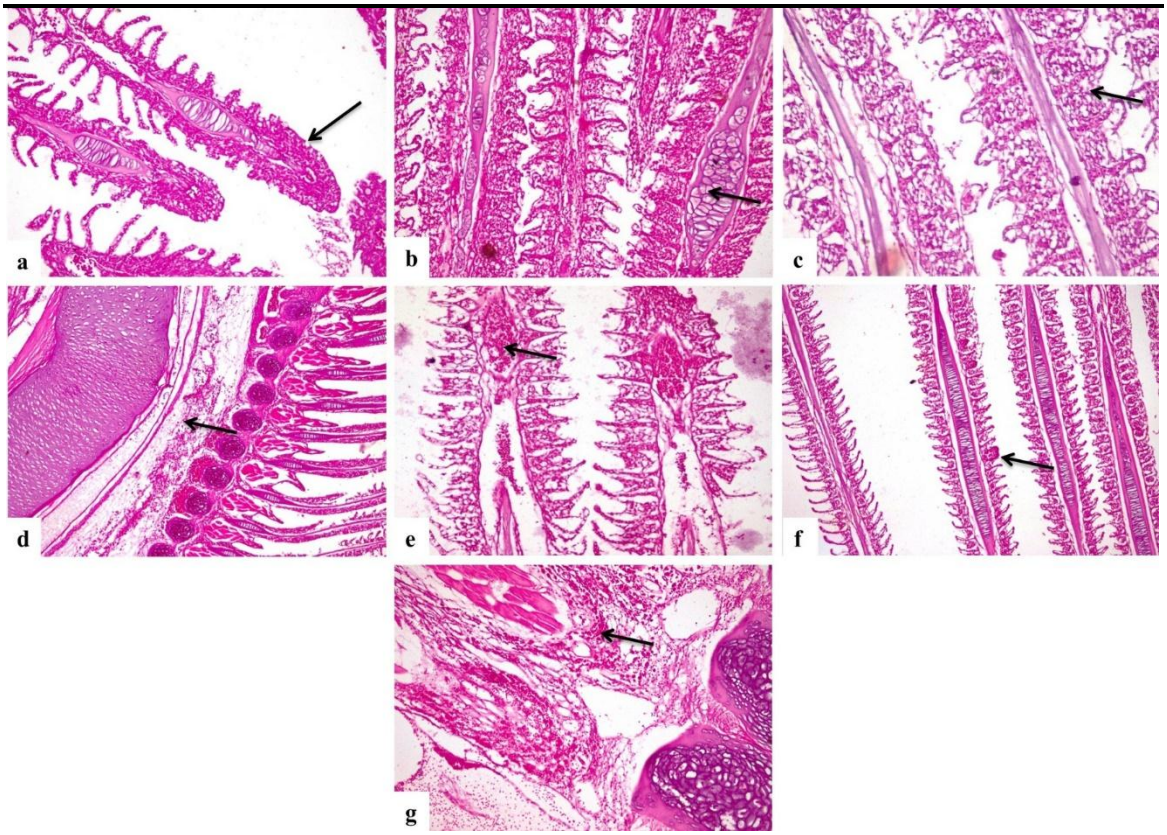


Fig. 5:Photomicrograph of gills showing. **(a)** hyperactivation of gill mucus cells (arrow) (H&EX100). **(b)** hyperplasia of primary gill lamellae cartilage (arrow) (H&EX200). **(c)** hyperplasia and fusion of secondary gill lamellae (arrow) (H&EX200). **(d)** inflammatory reaction, edema, hemorrhage and vascular congestion of gill arch connective tissue (arrow) (H&EX100). **(e)** congestion of primary gill lamellae blood vessels (arrow) (H&EX200). **(f)** telangiectasis of secondary lamellae capillaries (arrow) (H&EX100). **(g)** infiltration of esinophilic granule cells at the base of gill filaments (arrow) (H&EX200).

7.Gene expression analysis

Up-regulation of the Cpy1a1 and TNF-α was seen in the M and L groups compared to the negative control group in both the skin and gills. Treatment of both infested groups recorded downregulation of the tested genes in the skin. However, the L-treated group showed a non-significant decrease in the expression level relative to the L group in the gills (**Figs.6,7)**

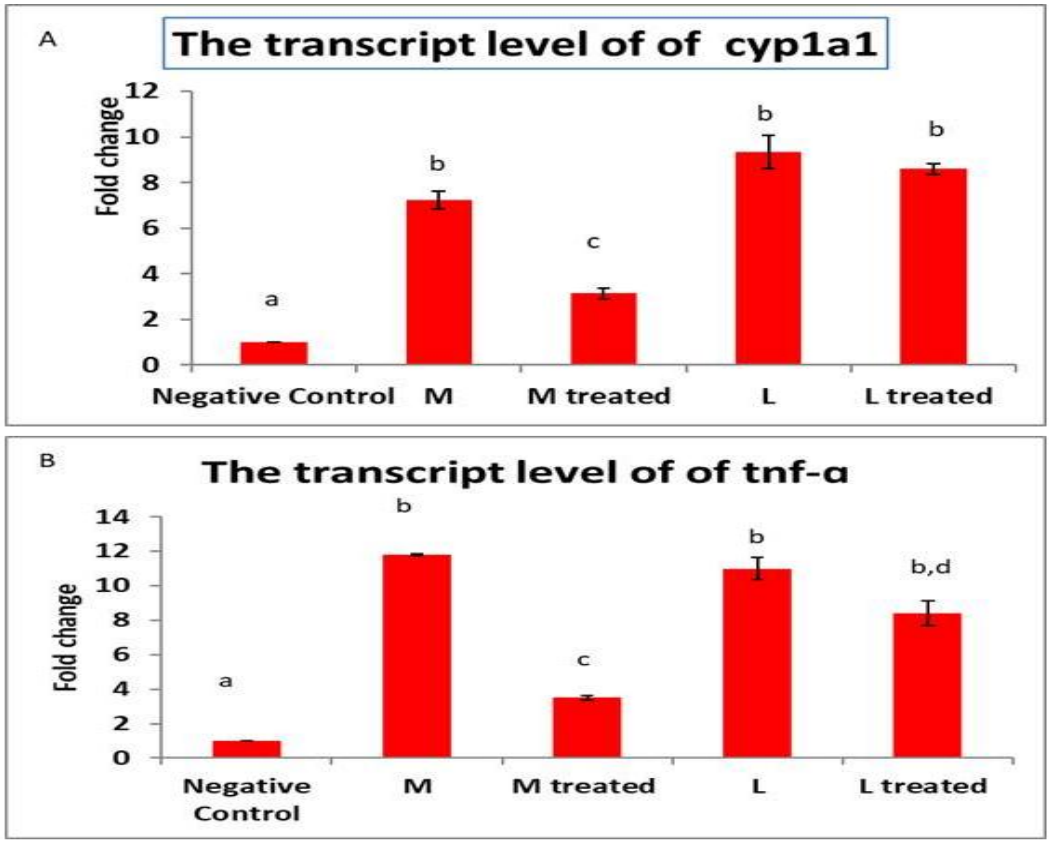


**Fig.6: The bar chart of the transcript level in the skin of A): cyp1a1; B) :tnf-a in the skin. Values are presented as mean± SEM. (n = 5 fish/group). ***indicates a statistically significant difference at $p < 0.05$.

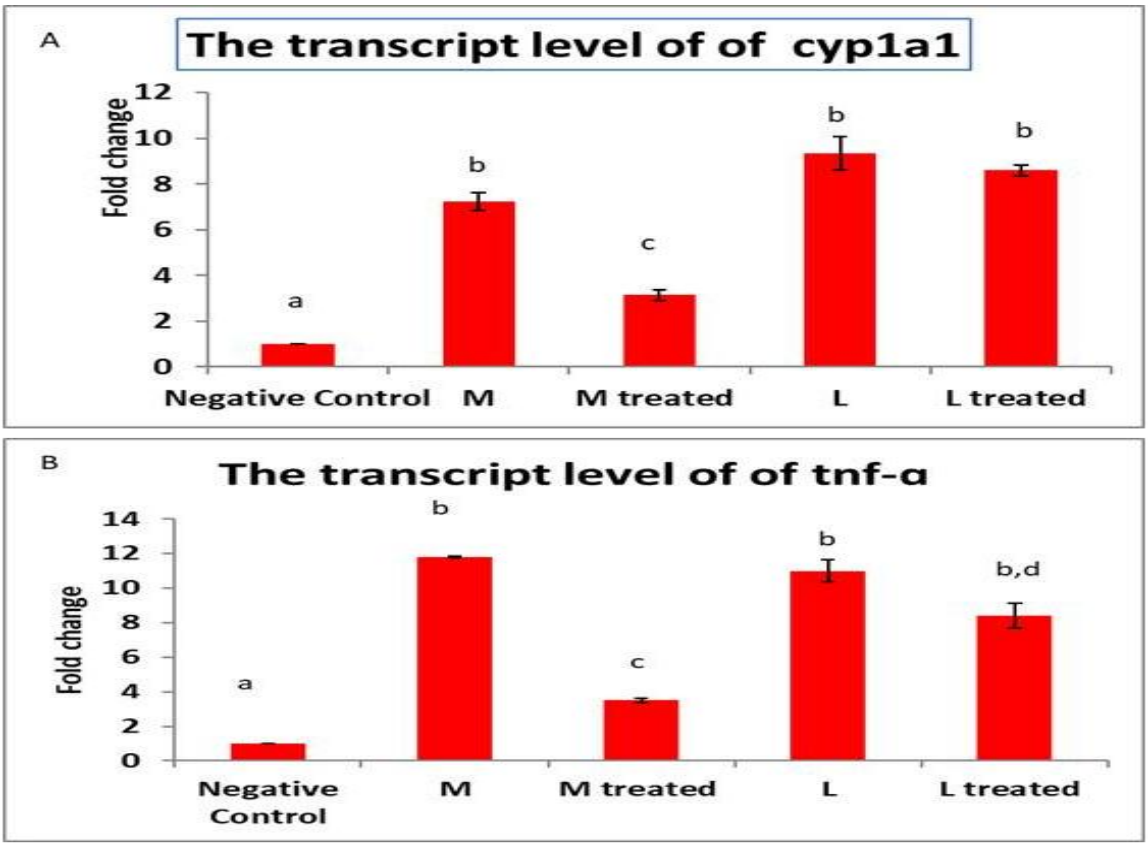


**Fig.7: The bar chart of the transcript level in gills of A): cyp1a1; B) :tnf-a in the in the gills. Values are presented as mean± SEM. (n = 5 fish/group). ***indicates a statistically significant difference at $p < 0.05$.

DISCUSSION

This study focused on the primary ectoparasites (monogenean and trichodinids) on the skin and gills of *O. niloticus*, the Nile tilapia, and how they relate to the seasonality of infection, pond management, and water quality. The impact of seasonality on ectoparasite infestation produced some unexpected findings. On the one hand, our investigation revealed that, in all farms examined, the seasons had no discernible impact on parasite prevalence, intensity, or abundance ($P < 0.05$). Similar findings were reported by **Jerônimo *et al*.(2011)**, who discovered that monogenoidea was prevalent in fish ponds all year round. However, in Saudi Arabia's eastern area, where the prevalence of trichodinawas highest in the spring and winter and lowest in the autumn and summer**,** **Hassan (1999)** discovered some effect of seasonality **(Suliman and Al- Harbi, 2016)**.

Pavanelli *et al.*(2008) state that a number of stressors, including nutritional status, handling, and transportation, as well as the water quality and organic load of the production units, affect how susceptible fish are to parasites and diseases. Low temperatures can weaken the immune system, decrease appetite and growth, and make fish more vulnerable to infestation (**Kubitza, 2000)**.

The rates of parasitism in fish were higher in the intermediate and final periods compared to the initial phase. The component most significantly connected with the number of parasite species present is host body size, according to **Zuben (1997).** Larger hosts might harbor more species and provide more room for parasites. As such, a wider range of niches are open to habitation, permitting the coexistence of many parasite species (**Poulin, 1995).** Furthermore, significant hosts discharge copious amounts of nitrogen compounds, which build up in fish cages and have the potential to escalate the parasitism rates of specific parasite species, like Trichodina spp. Few studies compare the frequencies of parasitism in *O. niloticus* throughout growth phases; those that do exist often focus on the presence of parasitism in a single fish growth phase.

The intensity of monogenic species, according to **Eiras (2006)**, exhibited clearly defined yearly patterns of infection, with a rise in parasites at higher temperatures (i.e., the rainy season) and a fall at lower temperatures (i.e., the dry season).

The seasonality results of this study support those of **Jerônimo *et al*.(2011)**, who found that in three regions of Santa Catarina State, Brazil, *O. niloticus* reared at higher rates of infestation by protozoans during lower temperature months (fall and winter) and at higher rates of monogenic parasitism during higher temperature months (spring and summer) (**Zago *et al*., 2014)**.

All fish species, across all locations and ecosystems, share copepods as a common component of their ectoparasite assemblages (**Boxshall and Halsey, 2004**). Copepods are the third-largest group of parasites in freshwater hosts and the second-largest group in marine fish in the Neotropics (**Luque and Tavares, 2007**). Copepods are important components of pond ecosystems, acting as intermediate hosts for fish parasites, food for small fish, fish parasites, micro predators of fish and other creatures, and hosts and vectors of human diseases (**Piasecki *et al*., 2004**).

Since most members of the genus *Lamproglen*a are gill-dwellers (**Eissa, 2002**), they may result in fish mortality in aquaculture. There are around 40 nominal species in the genus *Lamproglena;* **Piasecki (1993**). They are found in Asia (**Kuang and Qian, 1985; Kumari *et al*.1989; Yambot and Lopez, 1997),** Europe **(Caki´c *et al*.1998** and **Galli *et al*.2001**), and Africa (**Marx and Avenant-Oldewage, 1996, Ibraheem and Izawa,2000**). Only the mature females of both the genus *Lamproglena* and the species *Lernaea* are fish gill parasites (**Eissa, 2002, Lester and Hayward, 2006**). The parasite is present in 36.7%

of the *O. niloticus*. 20% of *O. niloticus*, 16% of *S. galilaeus*, and 20% of *T. zillii*were found to be infested in a prior study conducted by **(Ibraheem and Izawa,2000)** at El-Minya in the Nile River system. Therefore, our research showed a prevalence rate that was larger than previously noted in the same species, which may call attention to the infestation's expanding geographic range over time. But **Ghirardelli *et al*.(2006)** found that Lamproglena sp. was more common (90%) than previously thought.

The current research's overall monogentic infestation rate was 31%, greater than the 1.77% reported in Fayom, Egypt (**Al-Bassel, 2003)**.Our current findings also surpass a number of earlier international studies, such as those conducted in Ethiopia and Thailand, which found*Dactylogyrus*spp. in 4% and 15% of the fish studied, respectively (**Tesfaye *et al*.,2017**;**Vargas, 2000)**, and external protozoa infection rates of 6% and 4% for *Trichodina* and *Icthyophthiriusmultifiliis*, respectively (**Abd-ELrahman *et al*., 2023)**.

Parasitic infestations can cause significant stress in fish, leading to a series of reactions including the activation of signaling pathways and the modulation of gene expression. These events can impact the overall health of the fish as well as their immune response **(Durrani *et al*.2012; Gosh *et al*.2019)**.

The upregulation of CYP1A1 in tilapia infested with monogeneans and trichodinids suggests the activation of detoxification mechanisms to counteract the injurious effects of these ectoparasitic infections as the CYP1A1 is a crucial member of the CYP-450 family (**Goldstone *et al*., 2010)**.

TNF is a pro-inflammatory cytokine that plays a significant role in the immune response to infections and the regulation of inflammation (**Zhi *et al*., 2018)**. The upregulation of TNF in tilapia infested with monogeneans and trichodinids indicates the activation of the immune system and the initiation of an inflammatory response including the recruitment and activation of immune cells

CONCLUSION

The upregulation of CYP1A1 and TNF in tilapia infested by monogeneans and trichodinids reflects the activation of detoxification mechanisms and the immune response. This coordinated response contributes to the fish's ability to fight the ectoparasites and maintain health .

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