Egyptian Journal of Aquatic Biology & Fisheries Zoology Department, Faculty of Science, Ain Shams University, Cairo, Egypt. ISSN 1110–6131 Vol. 26(4): 1305–1318 (2022) www.ejabf.journals.ekb.eg



Evaluation of the immunological status of the common carp (*Cyprinus carpio*) infested with *Lernaea cyprinacea*

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ARTICLE INFO

Article History: Received:May 22, 2022 Accepted:July 27, 2022 Online: Aug. 26, 2022

Keywords:

Lernaea cyprinacea, Common carp, Gene expression, Immunological status, MHC-II, IL-1β

ABSTRACT

This study was initiated to assess the major histocompatibility class II (MHC-II) and Interleukin-1 β (IL-1 β) for the evaluation of the immunological reaction to histopathological reaction of common carp infested by Lernea cyprinacea (L. cyprinacea). Thus, a total of 100 common carp (Cyprinus carpio) fish were collected from Cairo and Giza Governorates in Egypt and investigated for the occurrence of different ectoparasites. Naturally, the infested C. carpio showed hemorrhagic skin with ulcer formation around the copepod; sloughing of the scales. The presence of any external parasites was detected on all fish body. Skin; muscle and gills samples of Cyprinus carpio (C.carpio) were collected for histopathological investigation. The parasitic female L. cyperinacea is elongated with its total length ranging from 10-18 mm ($14 \pm 1.5 \text{ mm}$). In the anterior end; it forms an anchor in which it pierces the skin to insert into the muscle; it has four horns; two dorsal and two ventral. The skin of the infested L. cyprinacea was pale in color; muscles contained multiple millet seed-sized parasitic cysts embedded in C. carpio muscles. Histopathological examination of the skin and muscles of C. carpio revealed parasitic cysts of L. cyprinacea which were attached between the intense degenerated fins with hyperplasia, hypertrophy and loss of its normal lamellar structure. Skin; gills and mucous were dissected from infested fish with L. cyprinacea, all samples were stored aseptically at -20 °C for subsequent investigation. The samples were classified into 4 groups: group 1: samples with 1-5 Lernaea; group 2: samples with > 5-10 Lernaea; group 3: > 10 copepods and group 4: control healthy non-infested fish. The examined genes were elevated according to the degree of infestation levels. In conclusion, infestation with L. cyprinacea in C. carpiois was accompanied by an upregulation of gene expression of MHC-II and IL-1β in C. carpio infested gills, skin and mucous.

INTRODUCTION

Indexed in Scopus

In Egypt, parasitic infections contribute to over 80% of diseases affecting freshwater fish (**Nofalet al., 2016**). It mostly affect the health, growth, and survival of the growing fish. *Lernaea* spp. represents one of the most common parasitic diseases. It accounts for 70 parasitic parasites that harm a wide range of aquatic animals (**Mcallister** *et al., 2011*). *Lernaea* spp. is common ectoparasite in freshwater fish, especially family

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Cyprinids and other fishes. It is found all over the world (El-Mansy, 2009). Lernea cyprinacea can be found in all parts of the fish's body, and they adhere to the fish's body with their anchor mouth and fangs. The parasite feeds on the host's blood and mucus. Lernea cyprinacea has the potential to damage skin tissue in addition to causing fish mortality, especially at the juvenile stage. Lernea cyprinacea will provide access for bacterial pathogens, viruses and fungi to proliferate (Salinas et al., 2020). Moreover, it can produce hemorrhagic and ulcerated lesions as well as severe localized damage to the affected tissue (Abbas et al., 2014). Mechanically removing the parasitic female on a wide scale is difficult since the removal process is often partial, leaving the anchor inside the fish tissue (Rohlenová et al., 2021).

Several studies have shown that fish can produce an efficient immune response to parasite infection and eliminate the parasites that are infecting them (**Zhou** *et al.*, **2018**). Epidemiological evidence suggests that fish can develop acquired immunity to parasites, and that inducing infection with parasites might reduce parasite burden and provide some protection (**Cable** *et al.*,**2007**). The initiation of host immune responses is dependent on gene expression analysis of immune-related genes that can increase pro-inflammatory cytokines (e.g. interleukin (IL)-1) (**Zhou** *et al.*, **2018**). The expression of TNF- α 1, iNOS and COX-2 were significantly higher during the infestation with *Lernaea cyperinacea* **Lindenstrom** *et al.*, (**2004**).

The present study was initiated to analyse the immunological status of the infested common carp (*Cyprinus carpio*) fish with the external parasites *Lernaea cyperinacea*, using gene expression analysis of different cytokines as interleukin and major histocompatibility class II.

MATERIALS AND METHODS

1. Fish samples

A total of 100 *Cyprinus carpio* (*C. carpio*) fish were collected and investigated for the occurrence of ectoparasites (*L. cyperinacea*). The fish were collected from several aquaria in Cairo and Giza Governorates in Egypt. Then, the collected fish were transferred to the laboratory in isothermal boxes with aerators for further analysis, using parasitological and genetic characterization (Abdelsalam *et al.*, 2020; Attia *et al.*, 2021a).

2. Ethical procedure

This research followed the ethical procedure in sampling and was approved by the Institutional Animal Care and Use Committee, Faculty of Veterinary Medicine, Cairo University, Egypt.

3. Parasitological examination

External parasites were examined out in all fish samples. All the examined samples (copepoda) were measured with minimum-maximum (mean \pm standard deviation), measuring 50 specimens from each examined copepoda. The identification was based on the studies of **Eissaet al. (2021)**, **Abu-Elala et al. (2021)** and **Attia et al. (2021b)**.

4. Assessment of major histocompatibility class II (MHC-II) and Interleukin-1 β (IL-1 $\beta).$

Skin, gills and mucous were collected from the infested fish with *L. cyprinacea*; all samples were aseptically stored at -20°C for subsequent research work. The samples were

classified into 4 groups: group 1: samples with 1-5 *Lernaea*; group 2: samples with > 5-10 *Lernaea*; group 3: > 10 copepoda, and group 4: control healthy non infested fish.

5. Extraction of RNA

Total mRNA kit (Ambion, Applied Biosystems) was used to extract mRNA from 100 mg of infested skin according to the manufacturer's instructions.

By using the FastPrep-24 homogenizer (MP Biomedicals, 2 cycles of 30 s at 6 m/s), the skin and gills of fish were homogenized and placed in Lysing Matrix D tubes (MP; Biomedicals). The quantity and purity of mRNA were determined using Nano drop (Thermo Scientific). According to the manufacturer's recommendations, 500 ng of mRNA were obtained using DNaseI amplification grade (Invitrogen). The reverse transcription of treated RNA was achieved using the following method of **Tu** *et al.* (2019) and **Younis** *et al.* (2020), the High-Capacity cDNA Archive Kit (Applied Biosystems) was used.

Table (1) lists the qRT-PCR primer specially for IL-1 β and MHC-II specifically for *C. carpio* based on the sequences deposited in the GenBank; samples were obtained from 1cm of skin and gills infested with *L. cyperinacea*. The procedure described in **Tu** *et al.* (2019), Youniset al. (2020) and Attia *et al.* (2021a, b, c) was followed for the extraction and synthesis of the analysed mRNA.

Genes	Sequence	Accession no.
IL-1β	F-GAA GCC GCTTAATGCGAC ACA G	AJ401030
	R:GTG ACGCTGAGTGCAGGA ACC ATT	
MHC-II	F: ACAGCTCCCGTGATTTCAGT	EU860997
	R:CTCTGCGTTATATATACTCCAAGTGC]
β-actin	F: GATGCGGAAACTGGAAAGGG	AB039726
	R:ATGAGGGCAGAGTGGTAGACG	

Table 1: Primers of the gene expression analysis of C. carpio

6. Real-Time PCR

The tests were carried out on a Step One TM Real-Time PCR System (Applied Biosystems, USA). 10 microliters of (SYBR[®] Premix Ex TaqTM (TliRNase H Plus), ROX plus (TaKaRa, Japan)), 1µL of mDNA, and 0.5µL of the produced primer (100 nM) were mixed with an ultra-pure water to 20µL. The cycling conditions used were those of *et al.* (2021a); the ^{Δ}CT value was calculated by the subtraction of the controlled gene (β-actin) from the result of the examined gene; Where, ^{Δ} CT acts as an internal control.

Conditions for PCR cycling

Condition of 40-cycle amplification, denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 45 seconds were used. A real-time PCR technique was developed by **Tu** *et al.* (2019). Samples were taken three times.

7. Histopathological examination

Cyprinus carpio skin and muscle samples were taken and evaluated for any gross abnormalities before being fixed in 10% neutral buffered formalin. The fixed samples were cleaned, dehydrated, clarified, and paraffin embedded. The paraffin blocks were sectioned at a 5-micron thickness. Hematoxylin and eosin were used to stain the sections according to **Bancroft and Layton (2012)**, and then they were examined under a light microscope (Olympus BX50, Japan).

8. Statistical analysis

One-way ANOVA was used to compare the means of the groups. Statistical significance was defined as a value of P<0.05. Predictive Analytics Software (PASW) Statistics, Version 18.0, was used for all statistical studies (SPSS Inc., Chicago, IL, USA).

RESULTS

1.Morphological analysis

Naturally infested *C. carpio* showed hemorrhagic skin with ulcer formation around the copepoda; sloughing of the scales.

1a.Morphological characterization of L. cyperinacea

The parasitic female of *L. cyperinacea* is elongated with its total length ranging from 10-18mm (14 ± 1.5 mm) it's anterior end has anchor in which it pierces the skin to be inserted into the muscle; it has four horns; two dorsal and two ventral. The dorsal pair is a Y shape while the ventral one is simple. The mouth is located between the two dorsal horns. The body of the female-runs elongated and cylindrical forming a cylindrical neck to reach to a small; short abdomen. The abdomen has two elongated egg sacs which are containing several eggs. Egg sacs are ranged in length from 3.5 - 5.5 mm (4.5 ± 0.5) (**Fig.1**).



Fig.1: The parasitic female *L. cyperinacea*; A: elongated in the anterior end; it forms anchor (a) with appearance of the four horns; the mouth (mo)is located between the two horns. B:The body of the female *L. cyperinacea* which run elongated and cylindrical forming cylindrical neck. C:*L. cyperinacea* abdomen which appear small; short with two elongated egg sacs which are containing several eggs.

2. Gross findings

The skin of the infested *L. cyprinacea* was pale in color; muscles contained multiple millet seed-sized parasitic cysts embedded in *C. carpio* muscles.

3. Histopathological findings

Histopathological examination of the skin and muscles of *C. carpio* revealed the presence of parasitic cysts of *L. cyprinacea* attached between the intense degenerated fins with hyperplasia, hypertrophy and loss of its normal lamellar structure; the copepoda embedded and circled with connective tissues. The skin was infiltrated with inflammatory cells; the blood vessels were severely congested. The muscles were hyalinized, fragmented and deteriorated. The muscle had granuloma with melanin pigments in between degenerated muscle fibers (**Fig.2-4**).



Fig.2:Photomicrographs of histopathological changes in *C. carpio* infected with *L. cyprinacea*. (A) Section of parasite (arrow) attached between degenerated fins (arrow head). (B) Section of fins encircled by connective tissue capsule of *L. cyprinacea* (arrow). Notice infilammatory cells infiltration along slide. (C) Section displayed the attachment site of lernaea (arrow) and its surrounding of hyalnized and deteriorated muscle fibers (arrow head). (D) Section highlighted the aggregated inflammatory cells (arrow) and congested blood vessles (arrow head). (H&E staining, 100x magnification, scale bar = 200μ m).



Fig. 3: Photomicrographs of pathological alterations in fins of *C. carpio* infested by *L. cyprinacea*. All sections of (**A**, **B**, **C**) highlighted the intense degree of degenerated fins; hypareplasia, hypertrophic, and loss its normal lammellar structure. Section (**B**) presented also the epithelial desquamation (arrow head) and fragmented muscle fibers (thin arrow). Section (**D**) emphasized the accumulated inflammatory cells (arrow). (H&E staining, A&B (100x magnification, scale bar = 200μ m), C&D (400x magnification, scale bar = 50μ m)).



Fig.4: Photomicrographs of histopathological findings in muscle of *C. carpio* infested with *L. cyprinacea*. (A) Section of hyalinized and degenerated muscle fiber (arrow head). Also aggregated inflammatory cells (arrow) were detected. Sections of (**B** & C) highlighted granuloma inbetween muscle fibers (Granulomatus myoscitis) (arrow). Notice congesed blood vessles (arrow head). (D) Section emphasized the melanin granules (arrow) in between impaired muscle fibers (arrow head). (H&E staining, A&B (100x magnification, scale bar = 200µm), C&D (400x magnification, scale bar = 50µm).

4. Gene expression Assessment of Major histocompatibility class II (MHC-II) and Interleukin- 1β (IL- 1β) in *C. carpio*-infested gills; skin and mucous.

Skin, gills and mucous were collected from infested fish with *L. cyprinacea*. All samples were stored aseptically at -20 °C for subsequent research work. The samples were classified into 4 groups: group 1: samples with 1-5 *Lernaea*; group 2: samples with > 5-10 *Lernaea*; group 3: > 10 copepoda; group 4: control healthy non infested fish.

In the skin infested with group 1; IL-1 β elevated to 8 folds than control noninfested fish (3.5± 0.5); while in group 2; the mRNA genes were 17 folds upregulated than in comparison with control non infested group. In the group 3 infested copepoda the mRNA gene was higher elevated to 28 folds than control healthy fish.

In the collected mucous of infested group 1; IL-1 β elevated to 12 folds than control non-infested fish (3.0± 0.8); while in group 2; the mRNA genes were 17 folds up regulated in comparison with control non infested group. In the group of 3 infested copepoda the mRNA gene was higher elevated to 29 folds than control healthy fish.

While the gills of infested group 1; IL-1 β elevated to 10 folds upregulation than control non-infested fish (3.0 ± 0.4); while in group 2; the mRNA genes were 19 folds up regulated than in comparison with control non infested group. In the group 3 infested copepoda the mRNA gene was higher elevated to 35 folds than control healthy fish (**Fig.5**).



Fig.5.Gene expression assessment of IL-1 β in *C. carpio* in infested gills; skin and mucous.

In the skin infested with group 1; MHC- II elevated to 15 folds than control non infested fish (4.0 ± 0.3) ; while in group 2; the mRNA genes was 26 folds up regulated than in comparison with control non infested group. In the group 3 infested copepoda the mRNA of MHC- II was higher elevated to 35 folds than control healthy fish.

The collected mucous of infested group 1; MHC- II elevated to 12 folds than control non infested fish (3.5 ± 0.3) ; while in group 2; the mRNA genes was 25 folds up regulated than in comparison with control non infested group. In the group 3 infested copepoda the mRNA of MHC- II was higher elevated to 39 folds than control healthy fish.

While the gills of infested group 1; MHC-II elevated to 20 folds than control noninfested fish (3.0 ± 0.4) ; while in group 2; the mRNA genes was 29 folds upregulated than in comparison with control non infested group. In the group 3 infested copepoda the mRNA of MHC-II was higher elevated to 45 folds than control healthy fish(**Fig. 6**).



Fig.6.Gene expression Assessment of MHC-II in *C. carpio* infested gills; skin and mucous.

DISCUSSION

Aquaculture has emerged as the fastest-growing food production sector, as well as a reliable source of high-quality protein production (**Bilal** *et al.*, **2021**). However, it faced several emerging hazards that threatened sustainable aquaculture production (**Iqbal** *et al.*, **2020**).

Lernaea cyprinacea is considered an ectoparasite of C. carpio, it can be found in all parts of the fish's body, and they adhere to the fish body with their anchor (Rohlenová et al., 2021). The parasite will suck blood and mucous from the host. L. cyprinacea parasites have the potential to damage skin tissue as well as cause fish mortality, especially in the juvenile stage. Lernaea will offer access for bacterial pathogens, viruses, and fungi to proliferate (Abu-Elala et al., 2018). Ornamental and stocked fish have spread the disease throughout most of the world. L. cyprinacea was found in the fish farm and common carp (C. carpio) may be the most likely vector (Abu-Elala et al., 2018). Yoshimine et al., 2015 found that the incidence of the disease was linked to the water temperature and the fish's average length, most parasites (90%) were found at the base of the fins. Lerneacyprinaceais still predominantly identified by a combination of morphological characteristics, most notably the shape of the anchors, which is considered the most accurate characteristic for identification. (Hua et al., 2019). The shape of anchors in L. cyprinacea can be affected by the parasite's age, host species, and infestation place (Salinas et al., 2016). When parasitizing on different hosts, L. cyprinacea can have a variety of morphological characteristics. Considering its extensive host range, it's thought that conspecific parasites' host-specific morphology has resulted in various taxonomic artefacts, such as mis-identifications of morphotypes as new species, and that many of the L. cyprinacea described are the same species (Hua et al., 2019).

The expression of immune genes in the skin, a primary tissue site for parasite infection, was studied to understand understand the molecular processes underlying C. carpio's susceptibility to parasite infection (**Zhou** *et al.*, **2018**). Pro-inflammatory genes

like IL-1 β 2, TNF- α 1 and TNF- α 2 were shown to be considerably up-regulated in correlation with parasite load, indicating that the inflammatory immune pathway is activated. At a later stage of infection, no elevated expression of pro-inflammatory genes was found, despite the fact that parasite infection was still present. Complement factor C3, which is known to play a role in microbial responses, was shown to be downregulated. This means parasites could devise techniques to selectively disable the host's antimicrobial defences in order to establish infection (Livak and Schmittgen, 2001).

Interleukin-1 (IL-1) is a pro-inflammatory cytokine increasing the host immune response after infection by beginning and boosting other cytokines, chemokines, and adhesion molecules production (**Dinarello, 1997**).

The IL-1 α and IL-1 β members of the growing IL-1 family of regulatory proteins are well-known (**Kumar** *et al.*, **2000**; **Engelsma***et al.*, **2003**). IL-1 α and IL-1 β are distinguished by the presence of a hydrophobic leader sequence and a b-barrel shape. IL-1 α , IL-1 β , on the other hand, requires proteolytic cleavage in order to mature into a physiologically active mature protein.

The C3 gene was also found to be expressed in the gills and kidney of *Dactylogyrus* intermedius-infested C. carpio (Zhang et al., 2015), and in skin, head kidney and liver of rohu (Labeoro hita) infested with a freshwater louse Argulus siamensis (Karet al., **2015**). There was no significant upregulation of IFN- γ , a cytokine that promotes macrophage antimicrobial responses (Grayferet al., 2009), implying that parasites may suppress the host antimicrobial system to establish infection. This is explained by the fact that immune-suppressive TGF- β was induced in contaminated fish. It is known that TGFβ plays a key role in controlling excessive inflammation, tissue regeneration and wound healing. MHC-II β and TCR-1 β , two marker genes involved in adaptive immunity, were also studied. During infection, no substantial changes in expression were seen, which is consistent with earlier research in rainbow trout infected with G. derjavini(Zhou et al., 2017). Infection of gyrodactylids in other fish species did not change the expression of genes like CD-4, CD-8, TCR- α , or MHC- II, nor did it trigger a particular antibody response (Haddad et al., 2008). The fact that TCR-1^β expression was greatly increased in fish infested with a large number of gyrodactylids shows that cell-mediated immunity is involved. However, further work is required to confirm this.Lower-level infection, according to Faliexet al., (2008), cannot trigger the adaptive immune response. Finally, the current study contributed to the existing of evidence supporting the infection model. proposed by (Zhou et al., 2017). Infection with Gyrodactylids causes inflammatory reactions in fish skin and reduces antimicrobial defences in the early stages of infection. Adaptive immune genes like MHC-IIB and TCR-B1 maintain their expression throughout infection (Zhou et al., 2018).

The lesions generated by L. cyprinacea varied in intensity from discrete to highly prominent haemorrhages, scale destruction, and mucus excess on the body surface (Hanganet al., 2013). Similar cases have been reported in lizaaurata and lizahaematocheila, in addition to the examined species (Hanganet al., 2013). L. cyprinacea is the most common lernaeid parasite in cyprinids. Because this parasite has no host specificity, it can infect any freshwater fish. In still and slow-flowing water, L. cyprinacea is more common than in fast-flowing streams (Iqbal et al., 2012). L. cyprinacea can cause major pathogenic effects on the fish's skin and fins as a result of

attachment. Young fish are the most vulnerable to mortality. Peritonitis and mortality can result from *L. cyprinacea* invading the visceral cavity, including the heart (Ali *et al.*, 2014).

Regarding the ability of the *C. carpio* to control infection with parasites (acute cases), it was observed upregulation in the expression of interferon-gamma (IFN- γ), and tumor necrosis factor alpha-2 (TNF- α 2) in the kidney and spleen but not in gills of the fish. After sub-chronicinfection significant increases in mRNA levels of proinflammatory genes in the gill (IFN- γ , interleukin-1 beta 1 [IL1- β 1], TNF- α 2), kidney (IL1- β 1, TNF- α 2), and spleen (IL1- β 1). Upregulation of immune gene expression in the gill and kidney (IFN- γ , IL1- β 1, and TNF- α 2) and spleen (IL1- β 1, TNF- α 2) was observed.

Upregulation of the interferon-gamma (IFN- γ) and tumour necrosis factor alpha-2 (TNF- α 2) expression in the kidney and spleen, but not in the gills, was detected in the ability of the *C. carpio* to control parasite infection (in acute cases). Significant increases in mRNA levels of proinflammatory genes in the gill (IFN- γ , interleukin-1 beta 1 [IL1- β 1], TNF- α 2), kidney (IL1- β 1, TNF- α 2) and spleen (IL1- β 1) after sub-chronic infection. Immune gene expression was upregulated in the gills and kidneys (IFN- γ , IL1- β 1, and TNF- α 2) as well as the spleen (IL1- β 1, TNF- α 2) (**Hanganet al., 2013**).

CONCLUSION

Infestation with *L. cyprinacea* in *C. carpio* is accompanied by up regulation of gene expression of MHC-II and IL-1 β in *C. carpio* infested gills; skin and mucous.

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