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Cytokine gene expression profiles in goldfish (*Carassius auratus*) during *Gyrodactylus kobayashii* infection

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ABSTRACT

Monogeneans of the genus *Gyrodactylus* are well-known pathogens causing huge mortalities in wild and cultured fish. Cytokine expression is one of most important host defense mechanisms against parasite infections. In this study, the expression pattern of the key pro-inflammatory (IL-1β, IL-8, IFN-γ, TNF-α, IL-12 and iNOS) and anti-inflammatory cytokine genes (IL-10, TGFβ and IL-4) of *Gyrodactylus kobayashii* infected goldfish (*Carassius auratus*) were determined by real-time quantitative PCR analysis. Our results showed that *G. kobayashii* infection caused increased expression of the pro-inflammatory cytokines including IFN-γ, TNF-α and iNOS in all detected tissues throughout the infection period. Among these genes, iNOS has the highest transcript level accompanied with increased nitric oxide (NO) concentration in the serum of all infected goldfish. The mRNA level of IL-1β in the liver, spleen and head kidney was significantly up-regulated during the early stage of infection (days 2–8). While high expression level of IL-8 and IL-12 was observed during the elimination phase of infection (days 10–14). As for anti-inflammatory cytokines, the expression profiles of IL-10 were distinct from those of TGF-β and IL-4. Specifically, the mRNA level of IL-10 did not increase in the spleen and head kidney during the early stage of infection, while increased expression of TGF-β and IL-4 were likewise seen. Besides, all infected fish had significantly higher complement C3 but lower IgM levels than the non-infected fish. The results provide insights into the interaction between gyrodactylids and the fish host, and indicate that systemic cytokine responses are critical for controlling parasite infection in fish.

1. Introduction

Species of *Gyrodactylus* are common ectoparasites found worldwide on freshwater, brackish water and marine fish [1–3]. These helminths are renowned for short generation times with adults giving birth to fully grown pregnant offspring [2]. *Gyrodactylus* infect fish by attaching to the external surfaces where they can cause significant tissue damage [4–6]. Sequentially, the wounds on epidermis facilitate potential mixed infection by other pathogens, resulting in high rates of host mortality and considerable economic losses in aquaculture [2,7,8]. Following the devastating impact that *Gyrodactylus salaris* has had on wild Atlantic salmon, there has been growing interest in controlling *Gyrodactylus* infection. Previous studies have shown that fish rise effective immune response against the infection of *Gyrodactylus* and finally eliminate the parasites [2]. However, the host factors that contribute to the induction of protective immunity remain elusive.

Researches have indicated that complement factors from fish serum and mucus have lethal effects on gyrodactylids [2,9,10], and lectins may be also involved in the immune responses against *Gyrodactylus* infection [11]. In addition, the role of cytokines in controlling the infection of *Gyrodactylus* has been intensively studied. For instance, IL-1β has been shown to be capable of mounting an appropriate response against *Gyrodactylus derjavini* infection [12]. Similar results were reported in Nile tilapia (*Oreochromis niloticus*), where IL-1β and IL-8 expression increased in the skin in response to *Gyrodactylus cichlidarum* infection [13]. Recently, Zhou et al. [14] observed a significant increase of IL-1β expression in goldfish (*Carassius auratus*) after *Gyrodactylus kobayashii* infection, accompanied by a similar response pattern of the immune response mediating TNFα1, TNFα2 and TGF-β in the skin. However, these studies were focused on the role of cytokines on the parasitic site skin. Other tissues particularly in immune organs and the role of anti-inflammatory cytokines during *Gyrodactylus* infection has been underestimated. The balance of pro-inflammatory and anti-inflammatory cytokines is a factor that determines the characteristics of infection and is decisive to reach a necessary balance between parasites and their host [15,16]. There is little information regarding pro- and anti-inflammatory cytokines in fish and their relation to *Gyrodactylus* infection.

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Goldfish (*Carassius auratus*), a freshwater fish in the family Cyprinidae, is one of the most frequently cultured ornamental fish species worldwide [17–19]. However, it is highly susceptible to gyrodactylids infection, particularly to *G. kobayashii* infection, about 80% mortality has been documented [20]. While little knowledge is available on the cytokine network and the various regulatory pathways involved in the immunity of goldfish during *G. kobayashii* infection. In this study, we investigated the expression pattern of a series of pro-inflammatory genes encoding IL-1β, IL-8, IFN-γ, TNF-α, IL-12 and iNOS as well as anti-inflammatory genes encoding IL-10, TGF-β and IL-4 in goldfish liver, spleen and head kidney during *G. kobayashii* infection. Besides, critical host immune factors including nitric oxide (NO), complement C3 and IgM in serum were also examined.

2. Materials and methods

2.1. Fish and parasites

Goldfish were purchased from a local fish farm in Xi'an city, China and kept in indoor aerated tanks with circulating water. After acclimation under laboratory conditions for two weeks, all fish were treated with consecutive baths using praziquantel at a concentration of 2.5 mg/L for 12 h to eliminate possible ectoparasites [6]. Goldfish-*G. kobayashii* model was established following the method described by our previous study [6]. All goldfish were maintained in conformity with the General Recommendation of Chinese Experimental Animals Administration Legislation.

2.2. Experimental infection and tissue sampling

One hundred healthy goldfish (weight, 5.65 ± 1.19 g) were transferred into a 300-L aquarium. After an acclimation for 7 days, all fish were cohabitated with the goldfish single-infested with *G. kobayashii* in a ratio of 1:2. Those infected goldfish (13.56 ± 3.24 g) were maintained in our laboratory and the size was lager to differentiate the healthy ones. During *G. kobayashii* infection, the fish were kept at 20 °C with 200 L water on a simulated natural photoperiod and fed to satiation daily with commercial goldfish diet. Three fish from the aquarium were chosen at random and euthanized with 0.02% MS222 (Sigma) every other day post infection (p.i.) with *G. kobayashii*. The number of gyrodactylids on both sides of the caudal fin was counted under a stereomicroscope for parasitological analysis [14]. Blood was collected from the caudal vein of fish and allowed to clot at room temperature for 1–2 h and then at 4 °C overnight. Serum was collected after centrifugation at 750*g* for 10 min, divided into several aliquots and stored at −20 °C until use [21,22]. Afterwards, the liver, spleen and head kidney were removed from each fish, immediately immersed in TRIzol reagent and stored in −80 °C until RNA extraction [19]. Meanwhile, selected goldfish was checked to verify pathogen status according to the methods described by Noga [23], and the examination results revealed no other pathogens were found excepted *G. kobayashii*. Three uninfected fish at 0 day were processed in the same way as a control.

2.3. Total RNA extraction and cDNA synthesis

Organs stored in TRIzol reagent were homogenized with a motor-driven tissue grinder (Sangon Biotech. Co., Ltd., Shanghai, China). Total RNA was extracted using standard chloroform phase separation and isopropanol precipitation according to the manufacturer's protocol. RNA concentrations were determined by a spectrophotometer at 260 nm and the purity of RNA was assessed by measuring OD260 nm/OD280 nm ratio (range 1.90–2.08). The RNA samples were DNase treated and then reverse transcribed into cDNA using the reverse transcriptase kit from Takara following the instructions of manufacturer. All the RNA and cDNAs templates were stored at −80 °C until used.

Table 1
List of primers sequences used in real time RT-PCR.

Genes	Primer sequences (from 5′ to 3′)	Accession no.
IL-1β	Fwd: GATGCGCTGCTCAGCTTCT Rev: AGTGGGTGCTACATTAACCATACG	AJ249137
IL-8	Fwd: CTGAGAGTCGACGCATTGGAA Rev: TGGTGTCTTTACAGTGTGAGTTTGG	KC184490
IFN-γ	Fwd: GAAACCCTATGGGCGATCAA Rev: GTAGACACGCTTCAGCTCAAACA	EU909368
TNF-α	Fwd: CATTCCTACGGATGGCATTTACTT Rev: CCTCAGGAATGTCAGTCTTGCAT	EU069817
IL-12	Fwd: CTTCAGAAGCAGCTTTGTTGTTG Rev: CAGTTTTTGAGAGCTCACCAATATC	LN592213.1
iNOS	Fwd: TTGGTACATGGGCACTGAGATT Rev: CCAACCCGCTCAAGAACATT	AY904362
IL-10	Fwd: CAAGGAGCTCCGTTCTGCAT Rev: TCGAGTAATGGTGCCAAGTCATCA	HQ259106
TGF-β	Fwd: GTACACTACGGCGGAGGATTG Rev: CGCTTCGATTCGCTTTCTCT	EU086521
IL-4	Fwd: CGATTGTAGCCGTTACTGGGT Rev: TGGCAAATGTGTTCCTCCG	KX574595
β-actin	Fwd: GATGCGGAAACTGGAAAGGG Rev: ATGAGGGCAGAGTGGTAGACG	AB039726

2.4. Real-time qPCR

Gene-specific primers were designed with Primer Premier 5 software and listed in Table 1, β-actin was used as the internal reference. The real-time PCR reactions were carried out in a CFX96 Real-Time PCR Detection System (Bio-Rad, USA). Each reaction was done in 15 μL final volume containing 7.5 μL SYBR Premix Ex Taq™ (TaKaRa), 1.0 μL of cDNA template, 0.5 μM of each primer. Each individual sample was run in triplicate. The PCR cycling reactions were initially denatured at 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s. The melting curve analysis of 5 s per step from 65 to 95 °C after amplification was conducted to assess primer specificity.

2.5. Assays of complement C3, IgM and NO

At the specific observation time, three fish were sampled to determine the content changes of C3, IgM and nitric oxide in serum during *G. kobayashii* infection. The concentrations of complement C3 and IgM were determined by using the commercially available ELISA kits (Xinlebio, China) according to the manufacturer's instructions. Total NO production in the serum was determined by measuring the concentration of nitrate and nitrite (a stable metabolite of NO) by modified Griess reaction method [24,25]. Total Nitric Oxide Assay Kit (Beyotime, China) was used. The optical densities at 540 nm wavelength were recorded using a Micro-plate Reader (Thermo MutliscanMK3; MA, USA) and total nitrite/nitrate concentration was calculated by using standard of sodium nitrate.

2.6. Statistical analysis

The mRNA expression level of the target cytokines relative to the reference gene were analyzed using the $2^{-\Delta\Delta Ct}$ method [26]. The serum concentration of complement C3, IgM and NO in the infected and control groups was calculated according to instructions of commercial kits. All data were expressed as mean ± SD and the significant difference between the control and infected groups was assessed by one-way ANOVA followed by Dunnett test after normalization. All statistical tests in this study were performed by SPSS 24.0 for Windows and the differences with a p-value of less than 0.05 regarded as significant.

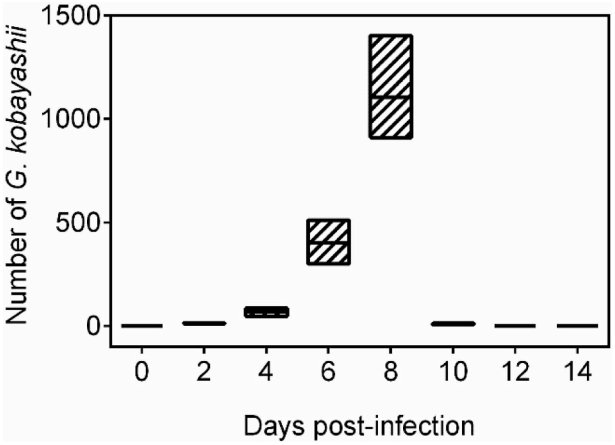

Fig. 1. Abundances of *Gyrodactylus kobayashii* on goldfish (*Carassius auratus*) at various days post infection. Three individuals were sampled every other day post infection for two weeks, and the number of parasites on both sides of the caudal fin was counted under a stereomicroscope. Values are average, floating bars (min to max).

3. Results

3.1. Parasite infection dynamics

Abundances of infection with *G. kobayashii* on goldfish were shown in Fig. 1. During the infection, infecting *G. kobayashii* numbers found on goldfish increased rapidly during the first 8 days post exposure, with a peak at day 8 p.i. (abundance, 1103; SD, 212). Subsequently, the numbers of *G. kobayashii* decreased sharply at day 10 p.i. (abundance, 10; SD, 2.16) and finally the parasite disappeared in the next two samplings. Furthermore, scanning electron micrograph (SEM) observations of a heavy *G. kobayashii* infection and the wounds on the tail fin of goldfish caused by opisthaptor were displayed in Fig. 2.

3.2. Cytokine gene expression in liver of goldfish

To understand the immune response elicited during *G. kobayashii* infection of goldfish, we measured the expression of genes encoding pro-inflammatory cytokines IL-1β, IL-8, IFN-γ, TNF-α, IL-12 and iNOS, and anti-inflammatory cytokines IL-10, TGF-β and IL-4 using quantitative PCR. We compared the cytokines expression in the liver, spleen and head kidney in infected and non-infected goldfish. The mRNA expression levels of pro- and anti-inflammatory cytokines in liver were seen in Fig. 3.

During this experiment, a significant increase in expression levels of the pro-inflammatory cytokine IL-1β was seen during the early stage of infection (days 2–6, Fig. 3A), with a peak occurring at day 2 p.i. (16.75-fold). Correspondingly, significant increases in transcription levels of IL-8 (1.48–3.86 fold) could be observed in all tested points (Fig. 3B). This also applied for IFN-γ and TNF-α, though these apparent increases were not statistically significant (Fig. 3C and D). Besides, lower mRNA levels of another pro-inflammatory cytokine IL-12 were detected in first 8 days after infection, but the transcription levels were significantly upregulated during the elimination phase of infection (days 10–12; Fig. 3E). Curiously, the mRNA levels of iNOS were significantly elevated and attained their peak 220-fold at day 2 p.i. (Fig. 3F). Concerning anti-inflammatory cytokines, the expression of IL-10, TGF-β and IL-4 exhibited the similar patterns with low transcript levels at the early stage of infection but high transcript levels at the late stage of infection (Fig. 3G–I).

3.3. Cytokine gene expression in spleen of goldfish

The expression pattern of the spleen was generally different from what was observed in the liver. An upregulation of IL-1β could be detected in first 8 days p.i. and its transcript level showed no apparent changes at days 10–14 (Fig. 4A). Significant decreases in IL-8 transcription levels were seen from 2 until 10 days p.i., with a tendency to upregulation at days 12 and 14 p.i. Similar expression patterns were detected for IL-12 (Fig. 4E). Obvious increases in the mRNA levels of IFN-γ, TNF-α and iNOS were observed at days 2, 8 and 14 p.i. Similar

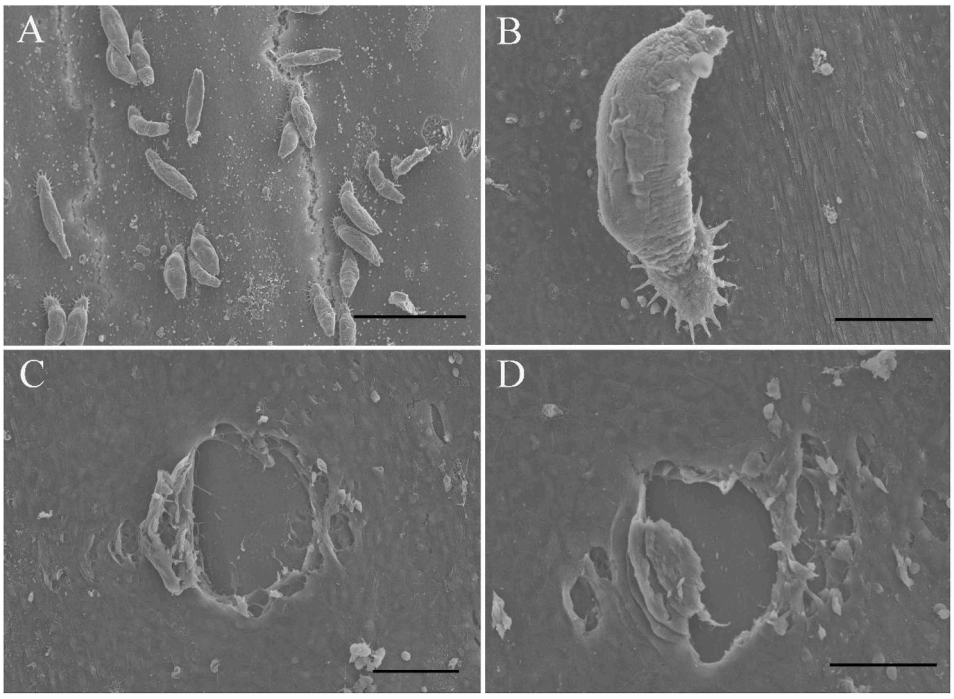

Fig. 2. Scanning electron micrograph of a heavy *Gyrodactylus kobayashii* infection and the wounds on the tail fin of goldfish caused by opisthaptor. Scale bars: A = 300 μm; B-D = 50 μm.

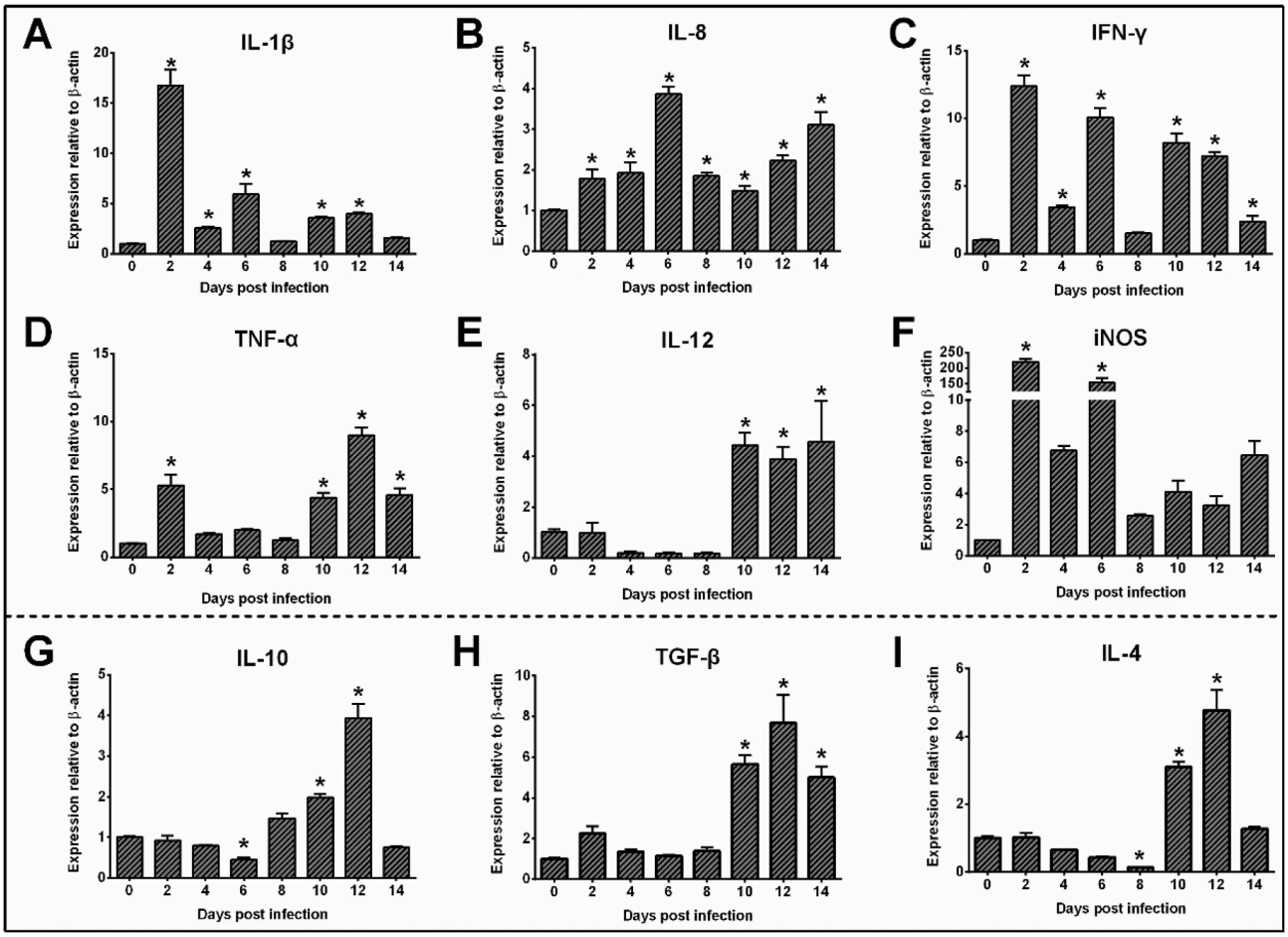

Fig. 3. Transcription levels of IL-1β (A), IL-8 (B), TFN-γ (C), TNF-α (D), IL-12 (E), iNOS (F), IL-10 (G), TGF-β (H) and IL-4 (I) in the liver of goldfish (*Carassius auratus*) during *Gyrodactylus kobayashii* infection. Bars indicate mean value ± SD of three samples at different infection days. Differences between uninfected and infected groups were analyzed by one-way ANOVA, where asterisks indicate a significant difference ($P < 0.05$).

with the findings in liver of *G. kobayashii* infected goldfish, transcription of the pro-inflammatory cytokine iNOS was distinctly upregulated in the acute phase of infection (days 2–8), reaching a peak at day 2 p.i. (250-fold). In the case of anti-inflammatory cytokines, the expression of IL-10 was significantly inhibited in the first 8 days p.i. and then induced at the remaining three time points (Fig. 4G). In addition, the mRNA level of TGF-β was higher during elimination phase of the infection (days 10–14), while transcription of IL-4 was upregulated at almost all sample times with the exception of day 4 p.i. (Fig. 4H and I).

3.4. Cytokine gene expression in head kidney of goldfish

In the head kidney, we observed up-regulation of IL-1β during the acute phase of infection, at days 2, 6 and 8 p.i. (Fig. 5A). This early expression was followed by a depression at days 10 and 12. A late and significant increase in the expression of IL-1β was however seen at day 14 (1.26-fold). Besides, the mRNA levels of IL-8, IFN-γ, TNF-α and iNOS were much higher in infected fish at all time points tested (Fig. 5). With respect to anti-inflammatory cytokines, *G. kobayashii* induced a decrease of IL-10 (0.42-fold) at day 2 p.i., which was followed by up-regulation of this transcript at days 10 and 14 p.i. (Fig. 5G). The mRNA levels of TGF-β were up-regulated at all time points tested apart from day 4 p.i. (Fig. 5H), and similar expression patterns were seen for IL-4.

3.5. The ratios of pro-inflammatory to anti-inflammatory cytokines

To better understand the temporal trends in gene expression of pro- and anti-inflammatory cytokines in the liver, spleen and head kidney post infection, the ratios of pro-inflammatory to anti-inflammatory cytokines were evaluated using the pairwise comparison method. A significantly increased ratios of IL-1β/IL-10 in all the tissues were observed at days 2 and 6 p.i., and followed by decrease and remained low levels at late stage of infection (Fig. 6A). Similar temporal trends were also suitable for the ratios of IL-1β/TGF-β and IL-1β/IL-4 only in the liver, their ratios remained low throughout the infection in head kidney (Fig. 6 D, G). The IFN-γ/IL-10 and IFN-γ/TGF-β ratios were the highest at day 2 p.i. in the spleen and head kidney. While the peak of the ratio of IFN-γ/IL-4 was seen at day 6 p.i. in liver of the infected goldfish (Fig. 6 H). When compared to the noninfected group, the infected groups presented higher ratios of iNOS/IL-10, iNOS/TGF-β and iNOS/IL-4 only during the acute stage of infection (d 2-8 p.i., Fig. 6 C, F, I). In regard to the ratios of another three pro-inflammatory cytokines (IL-8, TNF-α and IL-12) to anti-inflammatory cytokines (IL-10, TGF-β and IL-4), their results were irregular and displayed in Fig. 1S.

3.6. Contents of nitric oxide complement C3 and IgM

NO production is regulated by iNOS expression, which was significant up-regulated during *G. kobayashii* infection in this study, hence, we further examined the NO contents in serum of goldfish during *G. kobayashii* infection. As shown in Fig. 7, significant increases of serum NO concentrations could be observed at all time points tested, with the peak occurring at day 8 p.i. with *G. kobayashii.* What's more, as it was shown that host complement has a devastating effect on monogeneans

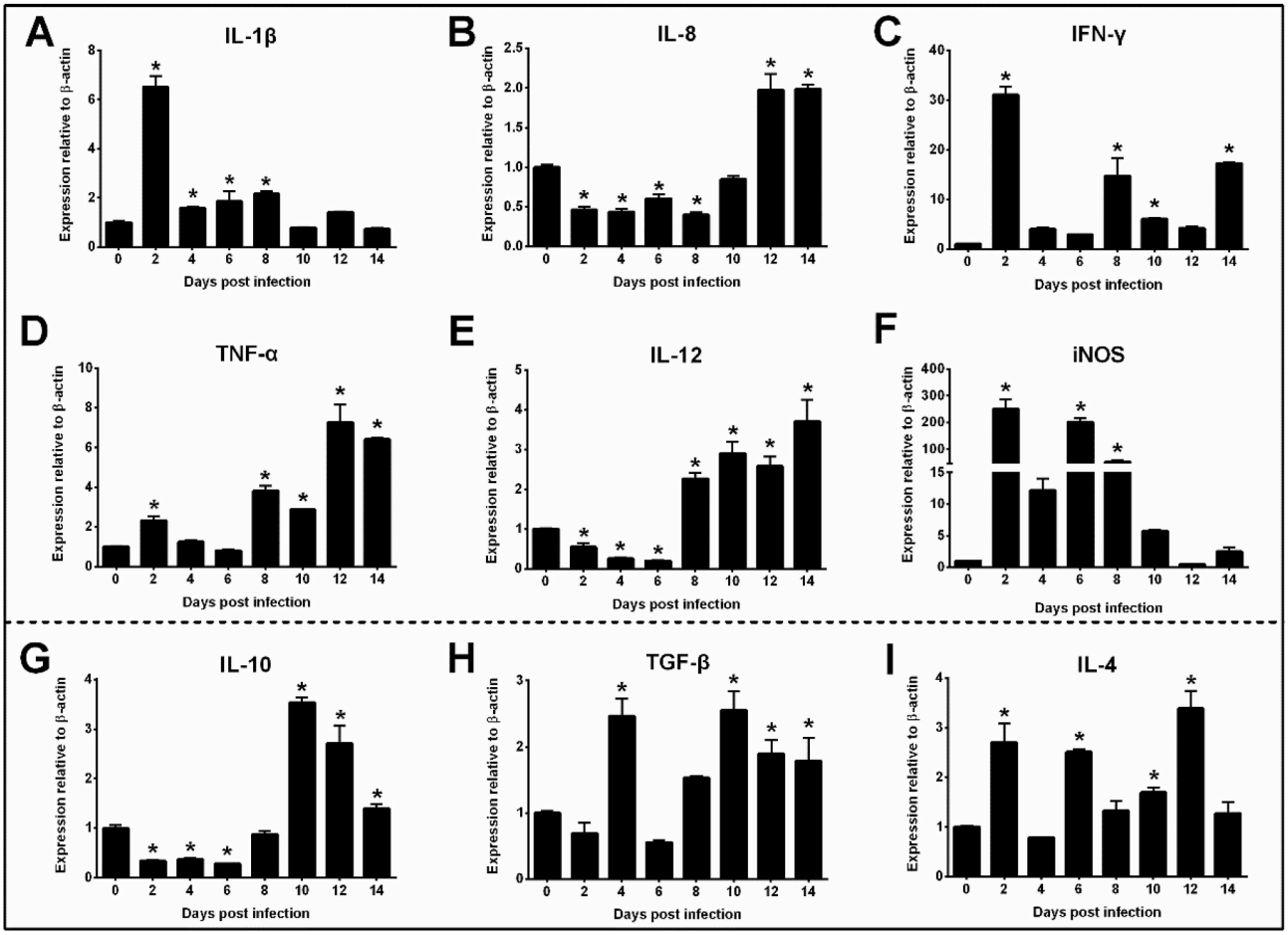

Fig. 4. Transcription levels of IL-1β (A), IL-8 (B), TFN-γ (C), TNF-α (D), IL-12 (E), iNOS (F), IL-10 (G), TGF-β (H) and IL-4 (I) in the spleen of goldfish (*Carassius auratus*) during *Gyrodactylus kobayashii* infection. Bars indicate mean value ± SD of three samples at different infection days. Differences between uninfected and infected groups were analyzed by one-way ANOVA, where asterisks indicate a significant difference ($P < 0.05$).

[9,10], we also examined the contents of complement C3 in serum of goldfish during *G. kobayashii* infection. The results were seen in Fig. 8, we found a delayed (day 6 and day 8) but a significant increase of complement C3 contents in the serum of infected fish. Moreover, there was no change observed in complement C3 content in the early stage of infection (days 2 and 4 p.i.). Serum IgM contents were decrease after infection with *G. kobayashii*, except for a slight increase but it was not statistically significant at day 12 p.i. (Fig. 9).

4. Discussion

Gyrodactylus spp. are monogenean ectoparasites of fish that cause severe disease and high mortality in aquaculture [2]. In this work, we have described the parasite load using the goldfish-*G. kobayashii* model during a two-week period. Besides, the gene expression of pro-inflammatory cytokines such as IL-1β, IL-8, IFN-γ, TNF-α, IL-12 and iNOS, anti-inflammatory cytokines including IL-10, TGF-β and IL-4 in the liver, spleen and head kidney of goldfish after infection with *G. kobayashii* were also evaluated.

Gyrodactylids are renowned for short generation times with adults giving birth to fully grown pregnant offspring [6,27]. In the present study, an explosive growth of *G. kobayashii* on goldfish was observed, it took only eight days for parasite population to reach a significantly high level (1103 parasites/fish). Subsequently, the abundance rapidly declined and finally disappeared at late stage of infection. Almost all gyrodactylid-vertebrate interactions which have been examined show evidence of parasite population limitation and decline [2]. And the profiles of average load of *G. kobayashii* were in accordance with those previous reports [13,14,28,29], while the speed of population growth here was much faster. This difference may be attributed to the high stocking density and higher parasite populations on the donor fish, which will reduce the fish resistance to pathogenic infections.

Damage of fish epidermis is quite obvious in *Gyrodactylus* infection owing to the penetration of marginal hooklets [12]. SEM observations corroborated earlier histological studies have demonstrated that *Gyrodactylus* infection induce a marked inflammation of the epidermis [2]. In mammals, it is known that nearly all inflammatory reactions are followed by the production of IL-1 and TNF [30,31]. In this study, an early and significantly elevated expression of IL-1β was detected in all the tested tissues. IL-1β is central for the initiation of host reactions and the involvement of this cytokine in anti-gyrodactylid responses has also been reported [12]. Furthermore, IL-1β has been reported to activate neutrophils and macrophages, which in turn induce the release of reactive oxygen metabolites [32] and complement factors with known lethal effects on *Gyrodactylus* spp [9,33].

Similar to IL-1β, TNF-α is an important component of early inflammatory events. In the present study, changes in the TNF-α transcript levels in liver and spleen of infected fish were different from those observed in the head kidney. The differences observed may be owing to the fact that these lymphoid organs have different type of cells also associated to differential functions. In head kidney, there are a remarkable number of macrophages able to produce large amounts of TNF-α [34]. Production of pro-inflammatory cytokines has been studied previously in goldfish infected with *G. kobayashii*. Likewise, an increased expression of IL-1β and TNF-α was detected in the skin of goldfish [14]. Besides, it has been reported that infections with other

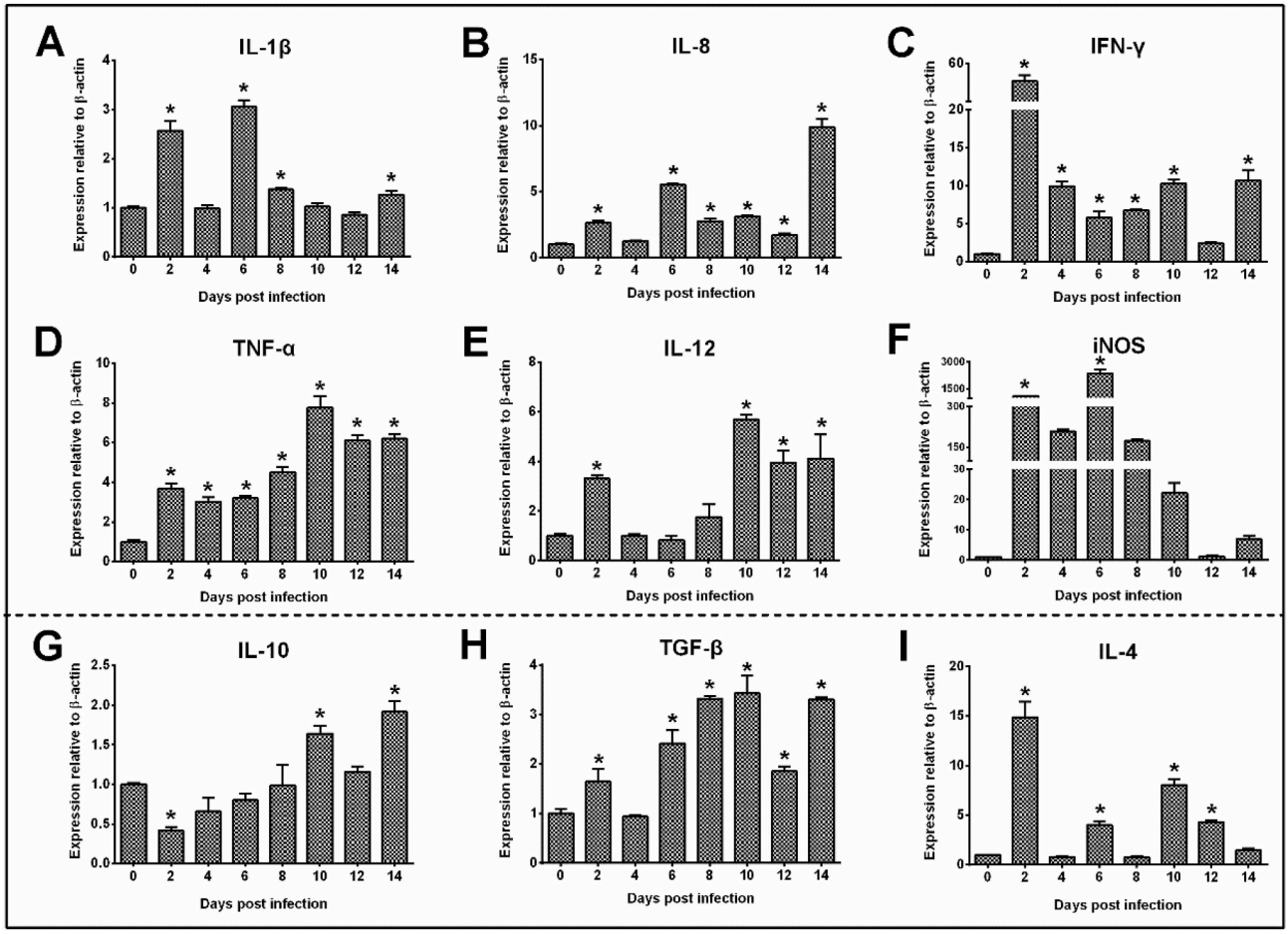

Fig. 5. Transcription levels of IL-1β (A), IL-8 (B), TFN-γ (C), TNF-α (D), IL-12 (E), iNOS (F), IL-10 (G), TGF-β (H) and IL-4 (I) in head kidney of goldfish (*Carassius auratus*) during *Gyrodactylus kobayashii* infection. Bars indicate mean value ± SD of three samples at different infection days. Differences between uninfected and infected groups were analyzed by one-way ANOVA, where asterisks indicate a significant difference ($P < 0.05$).

species of *Gyrodactylus* also induced higher expression of these two cytokines [12,35]. These results point to an important role for both IL-1β and TNF-α in the regulation of *Gyrodactylus* infection.

IFN-γ is one of the most pivotal cytokines that plays a vital role in macrophage activation resulting in the inhibition of parasite replication [36]. An increased IFN-γ transcription in all detected tissues was observed throughout the infection period, and the peak of the expression levels was reached at day 2 p.i. Similar results have been reported for fish infected with parasitic ciliate *Ichthyophthirius multifiliis* and the protozoan pathogen *Trypanosoma cruzi* [37,38]. IFN-γ is the major cytokines promoting Th1 differentiation, which in turn activates cell-mediated immunity that is important in parasites clearance. Unfortunately, there is only one work where an attempt was made to detect the IFN-γ in fish challenged with *Gyrodactylus* [14]. Hence, the specific role of the important pro-inflammatory cytokine IFN-γ against monogenean infection needs to be further studied.

Surprisingly, our results showed that *G. kobayashii* infections elicited strong iNOS expression in all tested tissues. Additionally, significant increases in the contents of NO in goldfish serum were detected at all time points. In mammals, pro-inflammatory cytokines such as IL-1β, TNF-α and IFN-γ have been implicated in the upregulation of iNOS expression via NF-B activation and induction of NO [39,40]. Numerous evidences have indicated that NO plays a vital role against invading parasites, through either direct killing or by limiting growth [40]. Due to low IgM levels in the serum of infected goldfish, we speculated that the decline in parasite abundances might be mediated by NO production. Meanwhile, unregulated NO production may lead to a variety of damaging effects [40]. Thus, future studies on the possible adverse effects of NO production on *Gyrodactylus* parasites need to be performed to establish the mechanism of NO production against fish monogenean.

As one of the primary immunosuppressive cytokines, TGF-β is well known to play essential roles in the progress of inflammation, as well as tissue regeneration and wound healing [41,42]. Severe tissue damages were observed in the skin and fins of fish with *Gyrodactylus* parasitizing [2]. In this study, the increased transcription level of TGF-β was observed mainly at the late stage of infection. These results are in line with previous studies on TGF-β gene expression in tissues infected by monogeneans [33,43,44]. In addition, it was suggested that increased production of TGF-β could suppresses production of NO from macrophages [45], limiting extensive tissue damage caused by high NO production [38]. Therefore, the higher TGF-β expression could be explained as an immunoregulatory mechanism that retains *G. kobayashii* as chronic infection at a certain intensity even elimination of infection.

We also tested expression of IL-10, which has been described as a potent anti-inflammatory cytokine that, among other functions, modulates the expression of other cytokines [46–48]. In the present study, the increase of IL-10 transcripts was only observed in all the three tissues at late stage of infection, which coincided with the absence or low levels of induction of pro-inflammatory cytokine IL-1β. Moreover, higher ratios of IL-1β/IL-10 were observed in the first eight days, followed by decrease and remained low levels at late stage of infection. This also applied for the ratios of IFN-γ/IL-10 and iNOS/IL-10. Our results are in accordance with the role of IL-10 during infections, including regulation and inhibition of pro-inflammatory cytokine expression, which contributes to the resolution of infections and reduction of the tissue damage caused by these cytokines [49,50].

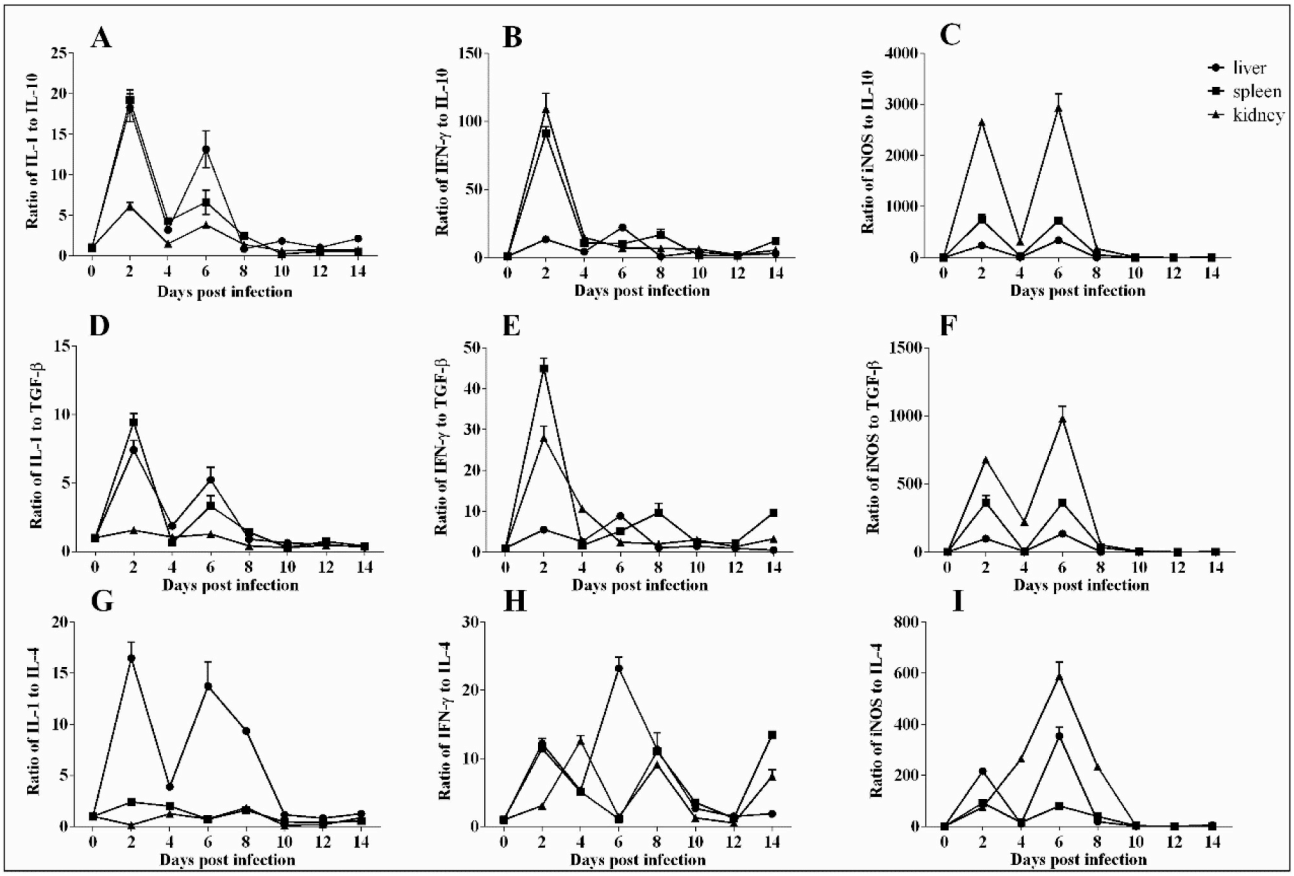

Fig. 6. Ratios of pro-inflammatory (IL-1β, IFN-γ and iNOS) to anti-inflammatory cytokines (IL-10, TGFβ and IL-4) in the different tissues (liver, spleen and head kidney) of goldfish during *Gyrodactylus kobayashii* infection. Error bars indicate the SD.

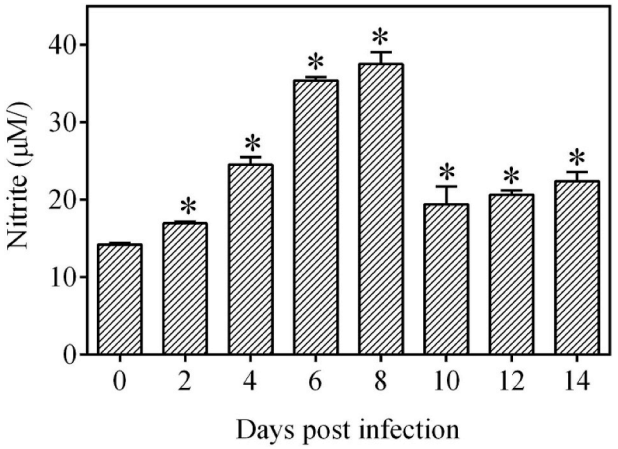

Fig. 7. NO production in the serum of goldfish during *Gyrodactylus kobayashii* infection within a two-week period. At the specific observation time, three fish were sampled to determine the nitric oxide contents by using total nitric oxide assay kit. Values are presented as mean ± SD. Asterisks denote a response that is significantly different from the control (one-way ANOVA, $p < 0.05$).

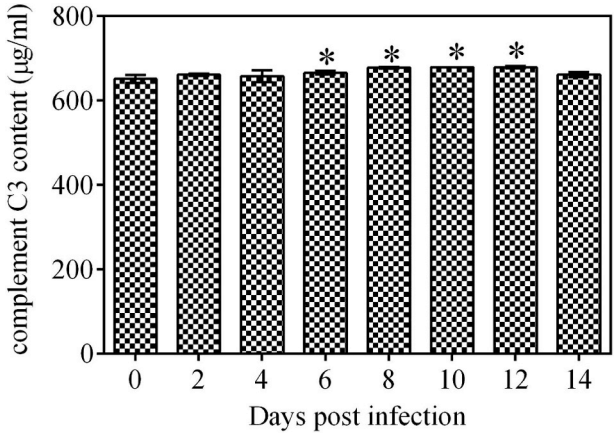

Fig. 8. The contents of complement C3 in serum of goldfish infected with *Gyrodactylus kobayashii* during a two-week period. Values are presented as mean ± SD (n = 3). The asterisk represents a statistically significant difference when compared with the controls (one-way ANOVA, $p < 0.05$).

Cytokines are not typically stored proteins and their synthesis is initiated after stimulation by gene transcription, transcript levels can often be interpreted as protein levels [51]. In many cases, their correlation is very strong, for instance, in the case of IL-12 [52] and IL-10 [53]. In the present study, the cytokine expression during *G. kobayashii* infection were only determined in transcript levels by using qRT-PCR analysis. Besides, the fish before infection (day 0) were used as control to compare the dynamic gene expression changes in a time-resolved fashion. The current work represents a good beginning in the comprehension of host defense against infection with *Gyrodactylus*. It is noteworthy that a time matched controls will be better understanding of the roles of cytokine expression during pathogen infection.

In conclusion, this study reports on the expression of genes that encode pro- and anti-inflammatory cytokines during *G. kobayashii* infection in goldfish. Our results indicate that IL-1β, TNF-a, IFN-γ and iNOS are expressed in goldfish during the early phase of an infection with *G. kobayashii* probably initiating the inflammatory reaction. The expression of these genes could be crucial for the recruitment of

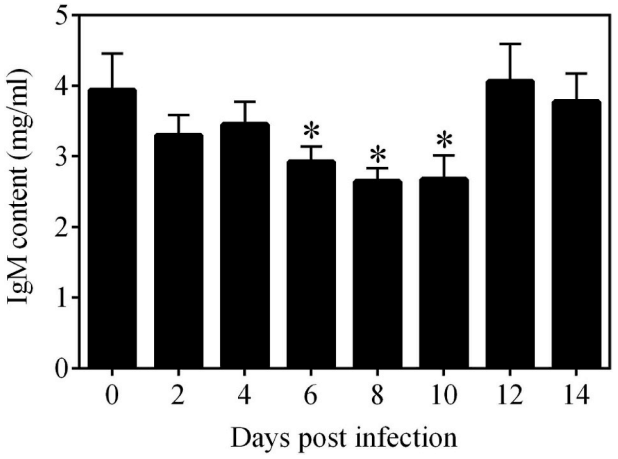

Fig. 9. IgM contents in serum of goldfish during *Gyrodactylus kobayashii* infection. Values are presented as mean ± SD (n = 3). The asterisk represents a statistically significant difference when compared with the controls (one-way ANOVA, $p < 0.05$).

relevant immune cells necessary for the initiation of the immune reactions needed to clear the infection. Besides, the production of anti-inflammatory cytokines IL-10, TGF-β and IL-4 mainly expressed during the late stage of infection. The balance between the pro- and anti-inflammatory cytokines could explain the resolution of the inflammatory response and the survival of infected fish.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.11.035.

References

[1] P.D. Harris, A.P. Shinn, J. Cable, T.A. Bakke, Nominal species of the genus *Gyrodactylus* von Nordmann 1832 (Monogenea: Gyrodactylidae), with a list of principal host species, Syst. Parasitol. 59 (2004) 1–27.
[2] T.A. Bakke, J. Cable, P.D. Harris, The biology of gyrodactylid monogeneans: the "Russian-doll killers", Adv. Parasitol. 64 (2007) 161–376.
[3] B.I. Kuperman, V.E. Matey, S.H. Hurlbert, Parasites of fish from the Salton sea, California, U.S.A, Hydrobiologia 466 (2001) 195–208.
[4] G. Levy, D. Zilberg, G. Paladini, S. Fridman, Efficacy of ginger-based treatments against infection with *Gyrodactylus turnbulli* in the guppy (*Poecilia reticulata* (Peters)), Vet. Parasitol. 209 (2015) 235–241.
[5] C.A. Harms, Treatments for parasitic diseases of aquarium and ornamental fish, Semin, Avian Exotic Pet Med, 5 1996, pp. 54–63.
[6] X. Tu, A. Huang, Y. Hu, F. Ling, G. Wang, Arctigenin: an emerging candidate against infections of *Gyrodactylus*, Aquaculture 495 (2018) 983–991.
[7] A. Akmirza, Monogeneans of fishes near Gökçeada, in Turkey, Turk. J. Zool. 37 (2013) 441–448.
[8] S. Zhou, W.X. Li, Y.Q. Wang, H. Zou, S.G. Wu, G.T. Wang, Anthelmintic efficacies of three common disinfectants and extracts of four traditional Chinese medicinal plants against *Gyrodactylus kobayashii* (Monogenea) in goldfish (*Carassius auratus*), Aquaculture 466 (2017) 72–77.
[9] P.D. Harris, A. Soleng, T.A. Bakke, Killing of *Gyrodactylus salaris* (Platyhelminthes, Monogenea) mediated by host complement, Parasitology 117 (1998) 137–143.
[10] K. Buchmann, Binding and lethal effect of complement from *Oncorhynchus* mykiss on *Gyrodactylus derjavini* (Platyhelminthes: Monogenea), Dis. Aquat. Org. 32 (1998) 195–200.
[11] K. Buchmann, Lectins in fish skin: do they play a role in host-monogenean interactions? J. Helminthol. 75 (2001) 227–231.
[12] T. Lindenstrøm, K. Buchmann, C.J. Secombes, *Gyrodactylus derjavini* infection elicits IL-1beta expression in rainbow trout skin, Fish Shellfish Immunol. 15 (2003) 107–115.
[13] T. Zhi, X. Xu, J. Chen, Y. Zheng, S. Zhang, J. Peng, C.L. Brown, T. Yang, Expression of immune-related genes of Nile tilapia *Oreochromis niloticus* after *Gyrodactylus cichlidarum* and *Cichlidogyrus sclerosus* infections demonstrating immunosupression in coinfection, Fish Shellfish Immunol. 80 (2018) 397–404.
[14] S. Zhou, W.X. Li, H. Zou, J. Zhang, S.G. Wu, M. Li, G.T. Wang, Expression analysis of immune genes in goldfish (*Carassius auratus*) infected with the monogenean parasite *Gyrodactylus kobayashii*, Fish Shellfish Immunol. 77 (2018) 40–45.
[15] S. Reyes-Cerpa, F.E. Reyes-López, D. Toro-Ascuy, J. Ibañez, K. Maisey, A.M. Sandino, M. Imarai, IPNV modulation of pro and anti-inflammatory cytokine expression in Atlantic salmon might help the establishment of infection and persistence, Fish Shellfish Immunol. 32 (2012) 291–300.
[16] D. Maubon, D. Ajzenberg, M.P. Brenierpinchart, M.L. Dardé, H. Pelloux, What are the respective host and parasite contributions to toxoplasmosis? Trends Parasitol. 24 (2008) 299–303.
[17] X. Tu, F. Ling, A. Huang, G. Wang, The first report of *Dactylogyrus formosus* Kulwiec, 1927 (Monogenea: Dactylogyridae) from goldfish (*Carassius auratus*) in central China, Parasitol. Res. 114 (2015) 2689–2696.
[18] S.Y. Wang, L. Jing, R.W. Murphy, S.F. Wu, C.L. Zhu, G. Yun, Y.P. Zhang, Origin of Chinese goldfish and sequential loss of genetic diversityaccompanies new breeds, PLoS One 8 (2013) e59571.
[19] X. Tu, L. Liu, X.Z. Qi, W.C. Chen, G. Wang, F. Ling, Characterization of Toll-like receptor gene expression in goldfish (*Carassius auratus*) during *Dactylogyrus intermedius* infection, Dev. Comp. Immunol. 63 (2016) 78–83.
[20] X. Tu, F. Ling, A. Huang, G. Wang, An infection of *Gyrodactylus kobayashii* Hukuda, 1940 (Monogenea) associated with the mortality of goldfish (*Carassius auratus*) from central China, Parasitol. Res. 114 (2014) 737–745.
[21] K. Hao, X.H. Chen, X.Z. Qi, B. Zhu, G.X. Wang, F. Ling, Display of GCRV vp7 protein on the surface of *Escherichia coli* and its immunoprotective effects in grass carp (*Ctenopharyngodon idella*), Fish Shellfish Immunol. 72 (2018) 199–209.
[22] D. Yang, Q. Liu, C. Ni, S. Li, H. Wu, Q. Wang, J. Xiao, Y. Zhang, Gene expression profiling in live attenuated *Edwardsiella tarda* vaccine immunized and challenged zebrafish: insights into the basic mechanisms of protection seen in immunized fish, Dev. Comp. Immunol. 40 (2013) 132–141.
[23] E.J. Noga, Fish Disease: Diagnosis and Treatment, Wiley-Blackwell, 2010.
[24] Y.L. Peng, Y.N. Liu, L. Liu, X. Wang, C.L. Jiang, Y.X. Wang, Inducible nitric oxide synthase is involved in the modulation of depressive behaviors induced by unpredictable chronic mild stress, J. Neuroinflammation 9 (2012) 2012 75.
[25] S.W. He, G.H. Wang, B. Yue, S. Zhou, M. Zhang, TO17, A teleost antimicrobial peptide that induces degradation of bacterial nucleic acids and inhibits bacterial infection in red drum, *Sciaenops ocellatus*, Fish Shellfish Immunol. 72 (2018) 639–645.
[26] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (2001) 402–408.
[27] S.J. Denholm, R.A. Norman, A.S. Hoyle, A.P. Shinn, N.G. Taylor, Reproductive trade-offs may moderate the impact of *Gyrodactylus salaris* in warmer climates, PLoS One 8 (10) (2013) e78909.
[28] A. Soleng, T.A. Bakke, The susceptibility of grayling (*Thymallus thymallus*) to experimental infections with the monogenean *Gyrodactylus salaris*, Int. J. Parasitol. 31 (2001) 793–797.
[29] J. Cable, O.C. Van, The role of innate and acquired resistance in two natural populations of guppies (*Poecilia reticulata*) infected with the ectoparasite *Gyrodactylus turnbulli*, Biol. J. Linn. Soc. 90 (2010) 647–655.
[30] R.G. Titus, B. Sherry, A. Cerami, The involvement of TNF, IL-1 and IL-6 in the immune response to protozoan parasites, Immunol. Today 12 (1991) A13–A16.
[31] J. Sigh, T. Lindenstrøm, K. Buchmann, Expression of pro-inflammatory cytokines in rainbow trout (*Oncorhynchus mykiss*) during an infection with *Ichthyophthirius multifiliis*, Fish Shellfish Immunol. 17 (2004) 75–86.
[32] K. Buchmann, J. Bresciani, Rainbow trout leucocyte activity: influence on the ectoparasitic monogenean *Gyrodactylus derjavini*, Dis. Aquat. Org. 35 (1999) 13.
[33] K. Buchmann, Binding and lethal effect of complement from *Oncorhynchus mykiss* on *Gyrodactylus derjavini* (Platyhelminthes: Monogenea), Dis. Aquat. Org. 32 (1998) 195–200.
[34] P. Scapini, J.A. Lapinet-Vera, S. Gasperini, F. Calzetti, F. Bazzoni, M.A. Cassatella, The neutrophil as a cellular source of chemokines, Immunol. Rev. 177 (2000) 195–203.
[35] T. Lindenstrøm, C.J. Secombes, K. Buchmann, Expression of immune response genes in rainbow trout skin induced by *Gyrodactylus derjavini* infections, Vet. Immunol. Immunopathol. 97 (2004) 137–148.
[36] R.T. Gazzinelli, I.P. Oswald, S. Hieny, S.L. James, A. Sher, The microbicidal activity of interferon-γ treated macrophages against *Trypanosoma cruzi* involves an L-arginine-dependent, nitrogen oxide-mediated mechanism inhibitable by IL-10 and transforming growth factor-β, Eur. J. Immunol. 22 (1992) 2501–2506.
[37] A. Oladiran, D. Beauparlant, M. Belosevic, The expression analysis of inflammatory and antimicrobial genes in the goldfish (*Carassius auratus* L.) infected with *Trypanosoma carassii*, Fish Shellfish Immunol. 31 (2011) 606–613.
[38] I.A. Abrahamsohn, R.L. Coffman, *Trypanosoma cruzi*: IL-10, TNF, IFN-γ, and IL-12 regulate innate and acquired immunity to infection, Exp. Parasitol. 84 (1996) 231–244.
[39] F. Aktan, iNOS-mediated nitric oxide production and its regulation, Life Sci. 75 (2004) 639–653.
[40] L.R. Brunet, Nitric oxide in parasitic infections, Int. Immunopharm. 1 (2001) 1457–1467.
[41] T. Hanada, A. Yoshimura, Regulation of cytokine signaling and inflammation, Cytokine Growth Factor Rev. 13 (2002) 413–421.
[42] P.L. Beck, I.M. Rosenberg, R.J. Xavier, T. Koh, J.F. Wong, D.K. Podolsky, Transforming growth factor-β mediates intestinal healing and susceptibility to injury *in vitro* and *in vivo* through epithelial cells, Am. J. Pathol. 162 (2003) 597–608.

[43] C. Lu, F. Ling, J. Ji, Y.J. Kang, G.X. Wang, Expression of immune-related genes in goldfish gills induced by *Dactylogyrus intermedius* infections, Fish Shellfish Immunol. 34 (2013) 372–377.
[44] E. Faliex, S.C. Da, G. Simon, P. Sasal, Dynamic expression of immune response genes in the sea bass, *Dicentrarchus labrax*, experimentally infected with the monogenean *Diplectanum aequans*, Fish Shellfish Immunol. 24 (2008) 759–767.
[45] F.M. Omer, J.A. Kurtzhals, E.M. Riley, Maintaining the immunological balance in parasitic infections: a role for TGF-beta? Parasitol. Today 16 (2000) 18–23.
[46] K.W. Moore, A. O'Garra, W.M.R. De, P. Vieira, T.R. Mosmann, Interleukin-10, Annu. Rev. Immunol. 11 (1993) 165–190.
[47] K.W. Moore, R.D.W. Malefyt, R.L. Coffman, A. O'Garra, Interleukin-10 and the interleukin-10 receptor, Annu. Rev. Immunol. 19 (2001) 683–765.
[48] W. Ouyang, S. Rutz, N.K. Crellin, P.A. Valdez, S.G. Hymowitz, Regulation and functions of IL-10 family cytokines in inflammation and diseases, Annu. Rev. Immunol. 29 (2010) 71–109.
[49] W. Ouyang, S. Rutz, N.K. Crellin, P.A. Valdez, S.G. Hymowitz, Regulation and functions of the IL-10 family of cytokines in inflammation and disease, Annu. Rev. Immunol. 29 (2011) 71.
[50] J.L. Mege, S. Meghari, A. Honstettre, C. Capo, D. Raoult, The two faces of interleukin 10 in human infectious diseases, Lancet Infect. Dis. 6 (2006) 557–569.
[51] S. Reyescerpa, F. Reyeslópez, D. Toroascuy, R. Montero, K. Maisey, C. Acuñacastillo, J.O. Sunyer, D. Parra, A.M. Sandino, M. Imarai, Induction of anti-inflammatory cytokine expression by IPNV in persistent infection, Fish Shellfish Immunol. 41 (2014) 172–182.
[52] J. Liu, S.L. Cao, X. Ma, Differential regulation of interleukin (IL)-12 p35 and p40 gene expression and interferon (IFN)-gamma-primed IL-12 production by IFN regulatory factor 1, J. Exp. Med. 198 (2003) 1265–1276.
[53] K.W. Moore, R. de Waal Malefyt, R.L. Coffman, A. O'Garra, Interleukin-10 and the interleukin-10 receptor, Annu. Rev. Immunol. 19 (2001) 683–765.