Histological and immune response in the fish Centropomus viridis elicited by the parasite Rhabdosynochus viridisi

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ABSTRACT

Objective. To analyze histological and immunological changes in the Pacific white snook (PWS) Centropomus viridis during primary infection and re-infection with the monogenean Rhabdosynochus viridisi. Materials and methods. Samplings were performed at three timepoints (0, 1, and 2). Histological alterations in gills were evaluated by the severity degree and the degree of tissue change. RT-qPCR assays were developed to investigate the expression of il1B, il8, il10, il12, il17, igM, igT, ifnγ, tnfa, tbet, hsp70, foxp3a, stat4, and cmip in gills and head kidney. Results. The prevalence of infection was 100% in challenged fish. During the primary infection, the mean intensity was 152 parasites per fish at Time 1 and 94 at Time 2, while in the re-infection, it was 367 parasites at Time 1 and 129 at Time 2. Histological analysis of gills showed fusion of the secondary lamellae, hyperplasia, infiltration of mononuclear inflammatory cells, and increase of chloride cells in both primary infections and re-infections. Only the expression of cmip in gills at Time 1 was significantly higher in re-infections than in primary infections, and the expression of il12β showed a fold-change value >100 in head kidney at Time 2 in primary infections. Conclusions. The monogenean R. viridisi may cause histological alteration in its fish host. As showed by the decrease of the intensity of infection from Time 1 to Time 2, it seems that the PWS is able to combat R. viridisi; however, our immunological analysis did not reveal strong evidence of a possible mechanism.

Keywords: Aquaculture; Immunology; Platyhelminthes; Monogenea; Ectoparasite; Centropomidae (Source: CAB).

RESUMEN

Objetivo. Analizar los cambios histológicos e inmunológicos en el robalo blanco del Pacífico Centropomus viridis infectados por primera vez y reinfectados con el monogeneo Rhabdosynochus viridisi. Materiales y métodos. Se realizaron muestreos en tres tiempos (0, 1 y 2). Las alteraciones histológicas en las branquias se midieron con el grado de severidad y grado de cambio de tejido. Se


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midió la expresión de il1B, il8, il10, il12, il17, igM, igT, ifny, tnfa, tbet, hsp70, foxp3a, stat4 y cmip en branquias y riñón cefálico mediante RT-qPCR. **Resultados.** La prevalencia de infección fue 100% en los peces desafiados. Durante la primera infección, la intensidad promedio fue de 152 parásitos por pez en el Tiempo 1 y 94 en el Tiempo 2. Durante la reinfección, fue de 367 en el Tiempo 1 y 129 en el Tiempo 2. Las branquias infectadas mostraron fusión de lamelas secundarias, hiperplasia, infiltración de células inflamatorias e incremento de células de cloro. Sólo la expresión de cmip en branquias en el Tiempo 1 fue significativamente mayor durante la reinfección que durante la primera infección, y la expresión de il12β aumentó más de 100 veces en riñón cefálico en el Tiempo 2 en infecciones primarias. **Conclusiones.** El monogeneo R. viridisi puede causar daños histológicos en los peces. Se observó una reducción de la infección del Tiempo 1 al Tiempo 2, lo cual sugiere que los peces pueden combatir a R. viridisi; sin embargo, los análisis inmunológicos no proveen evidencia concreta de los posibles mecanismos.

**Palabras clave:** Acuicultura; Inmunología; Platyhelminthes; Monogenea; Ectoparásito; Centropomidae (Fuente: CAB).

**INTRODUCTION**

Marine fish commonly known as snooks or robalos (Centropomus) are highly appreciated for human consumption and have the potential for mariculture (1). In northwestern Mexico, aquaculture of the Pacific white snook (PWS), *Centropomus viridis*, has been investigated for large-scale commercial production (2). However, infectious diseases represent a significant threat to finfish aquaculture, causing important economic deficits such as lost feed days, expenses for treatments against pathogens, and direct losses associated with fish mortality. Particularly, infections by the monogenean *Rhabdosynochus viridisi* (Diplectanidae family) represent a threat to the culture of PWS (3,4). Nevertheless, little is known regarding disease pathogenesis and the host-parasite interaction at immunological level. To better understand the response to infection and re-infection in PWS exposed to *R. viridisi*, the present study aimed to analyze histological changes in gills and the expression level of different immune related genes in both gills and head kidney.

**MATERIALS AND METHODS**

**Ethical statement.** The experiment was performed following the American Veterinary Medical Association Guidelines for the Euthanasia of Animals (5), as well as the ethical standards of the Centro the Investigación en Alimentación y Desarrollo (CIAD).

**Experimental challenge.** Hatchery PWS (11 months old; weight: 61.5±16.1 g), from a single spawning batch, were divided into three experimental groups: control (uninfected), primary and re-infection, each containing 45 fish distributed in triplicate 400-L tanks supplied with flow-through-filtered seawater (25±0.5°C, 35‰, dissolved oxygen at 5.2±0.1 mg/L, and 15 fish per tank) (Figure 1). Each tank of the primary infection and reinfection was challenged with ~6000 *R. viridisi* eggs, following methods published elsewhere (3,6). Briefly, wild PWS naturally infected with *R. viridisi*, caught in Mazatlán, Mexico, were transported to the laboratory at CIAD-Mazatlán. Parasite eggs were collected with cotton threads and placed into the tanks to promote experimental infections on hatchery PWS. The parasite species was identified by morphology (4).

**Figure 1.** Schematic diagram of experimental challenge of Pacific white snook *Centropomus viridis* (PWS) infected with the monogenean *Rhabdosynochus viridisi*. 
Hatchery PWS of the reinfection group were obtained as follows. Two months prior to the start of the experiment, 55 hatchery PWS were put into a 400-L tank and infected with *R. viridisi*, as described above. Once the infection was confirmed, by observing the gills of five examined fish and detecting newly laid monogenean eggs, fish were treated with three consecutive baths of formalin (JT Baker) (200 µL/L for 40 min, at 3-day intervals at 24±0.5°C) to remove parasites. Fish were allowed to recover for 25 days and then used for reinfection in the experiment.

Three sampling times were chosen (Figure 1). At Time 0 (one day prior to egg introduction into the tanks) three fish per group were evaluated. At Time 1 (eight days after egg introduction) nine fish per group were sampled. This time point corresponds to the early phase of the infection when parasites are deemed “juveniles”. The final time point (Time 2) occurred 14 days after egg introduction when parasites reached the adult stage. During time point 2, nine fish per group were evaluated. Fish were anesthetized with 125 mg/L MS-222 (Sigma-Aldrich, San Louis, MO, USA) buffered with sodium bicarbonate (1:1), and then euthanized by pithing, following general ethical guidelines (5). Biopsies of the most external gill arch from the right branchial chamber were collected for molecular and histological analyses (Figure 2). The head kidney was also collected for molecular analysis. Samples for molecular analysis and histology were immersed in RNAlater and 10% buffered formalin, respectively. Samples in RNAlater were preserved at −70°C. The remaining gill arches were immersed in RNAlater and 10% buffered formalin, respectively. Samples in RNAlater were preserved at −70°C. The remaining gill arches were examined under a dissecting microscope to detect contaminating genomic DNA. After reverse transcription, the samples were used as templates for qPCR.

**Histology.** Based on conventional histological methods, gills were stained with hematoxylin-eosin/phloxine (HE), and observed under a light microscope. Histological alterations were measured semi-quantitatively by calculating the severity degree (SD) (8) and the degree of tissue change (DTC) (9). Briefly, SD and DTC were used to calculate the histological index (HI) for each fish by mean of the equation: \( HI = SD \times DTC \). Five categories of HI were established: 0-10 functionally normal organ; 11-20 slightly damaged organ; 21-50 moderately damaged organ; 51-100 severely damaged organ; and >100 irreparably damaged organ. All slides were examined by two researchers.

**Gene expression.** Primers were designed using Beacon designer™ software (Premier Biosoft) and NCBI/Primer-BLAST, based on a transcriptome available for PWS (GenBank, PRJNA497139). The sequences of *il1B*, *il8*, *il10, il12, igM, igT, ifnγ, tnfa, hsp70, foxp3α, stat4*, and *cimp* were used to analyze immune responses (Table 1). Primer efficiencies were measured using 10-fold dilution series of cDNA templates. Total RNA was extracted with the RNeasy Plus Mini Kit (Qiagen, Redwood City, CA, USA). RNA concentration and purity, determined by 260/280, were determined using a ThermoFisher Scientific NanoDrop One/OneC Spectrophotometer. Reverse transcription of 1 µg extracted RNA in 20 µL reactions was performed using the QuantiTect Reverse Transcription kit (Qiagen, Redwood City, CA, USA). Controls with omitted reverse transcriptase were performed to detect contaminating genomic DNA. After reverse transcription, the samples were used as templates for qPCR.

Relative quantification of cytokines was made using the *ef1α* reference gene for normalization in a QuantStudio 5 Real-Time PCR System (ThermoFisher Scientific, Waltham, MA, USA). PCR-wells contained 5 µL of template cDNA, 1x SYBR Green Master Mix (ThermoFisher Scientific), and 0.2 µM of primers (Invitrogen, Carlsbad, CA, USA). The RT-qPCR was run as follows: 1 cycle at 50°C 2 min, 1 cycle at 95°C 10 min, and 40 cycles of amplification at 95°C for 15 s and annealing at 60°C for 1 min. A melting curve determination was performed at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. Negative controls without sample templates were included for each run. Relative gene expression was calculated using the 2^−ΔΔCT method and normalized against the expression of the average of the reference gene (10). Tissues from control

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**Figure 2.** Schematic diagram of the sample processing of Pacific white snook *Centropomus viridisi* infected with the monogenean *Rhabdosynochus viridisi*. Reprinted with permission from Rev MVZ Córdoba. 2024. May-August; 29(2):e3381. https://doi.org/10.21897/rmvz.3381

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PWS were used as calibrators for all genes. Two-way ANOVA with a posteriori Tukey’s test was used to compare the expression levels between samples, based on log10 transformed data (each sample n=3). The significance level was set at p<0.05. This test was performed using GraphPad Prism (GrahPad Software, San Diego, CA, USA).

**Table 1.** Primer details for immune-related genes of PWS.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5’-3’)</th>
<th>Amplicon size (bp)</th>
</tr>
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| ef1ab  | F: AGGTCATCACATCGAACCACC  
       | R: GAGCAACTTGGGGGTTGCTCAA  | 155                |
| il1β   | F: ACCTGTCATGCCAACAAGGAA  
       | R: ATGTACCCATCACCCCAAGCG  | 178                |
| il8    | F: GTACATGCACCGCCCATAGT  
       | R: TGGCAGGAATCGAGCTCCACC  | 157                |
| il10   | F: CCGATGACTACTCGCTGT    
       | R: AGCACCTGGGTGGGTAGAGA  | 86                 |
| il12β  | F: ACCCAAGTTCCTGGAGACGT  
       | R: TGAAACCATCGGGGTCTTTGT  | 130                |
| ilM    | F: CAGACGAGGCTTCCAGCAA   
       | R: TGGCCTTCTCCCAGTACGT   | 127                |
| ilT    | F: CTGGGCAGGTGTGATGTGCA   
       | R: CATCATCATGCAAGCGATCC   | 176                |
| ifnγ   | F: ACCTCCTCGACAGAAGGTAAG  
       | R: AATACCCCGACCCCTAGT    | 88                 |
| tnfα   | F: CAGCACCAAAAATCGAAGCA   
       | R: CACTCCAGCCTTTCTCCGA   | 81                 |
| tbet   | F: GGACAACAAGAAGAGGAGAGTG  
       | R: AGTGAGGGTGATGACCATAGT  | 128                |
| hsp70  | F: AGACACATCGACACAAACAGAG  
       | R: TCTGGTGATGGAGGTGTAAG   | 145                |
| foxp3a | F: AGCTCCAGGTGGAAGACGC   
       | R: AGAGGGTGCTATGTGTC      | 153                |
| stat4  | F: TGCTTGTCTTTTGTGATCTTG  
       | R: GCTGTGTCTGACCTTCTTGG   | 144                |
| cmip   | F: TGCAAGCCACCGCTTTATG    
       | R: TGCTGTATCCAGCTTCTGC   | 233                |
| il17   | F: GCTGGCTTTACAGGCTTCCA  
       | R: ATAAGGTTTCCCTGTGCCG   | 155                |

**RESULTS**

Control fish remained uninfected, with gills showing a normal appearance (Figure 3a), although a few cases (< 10%) of epitheliocystis were detected at Time 2 (Figure 3b). At Time 0, all fish were parasite free. At Time 1 and Time 2, the prevalence of infection reached 100% in exposed fish.

In the primary infection group, the mean intensity was 152 at Time 1 and decreases but not significantly to 94 at Time 2 (p>0.05); gills showed fusion of the secondary lamellae, hyperplasia, infiltration of mononuclear inflammatory cells (Figure 3c), and increase of chloride cells, which resulted in \( H_{i} = 17 \) at Time 1 and \( H_{i} = 30 \) at Time 2. We did not observe apparent damage at the sites where the parasites were found (Figure 3d), although the parasite hook within the branchial filament was observed in some cases (arrowed in Figure 3e). In the reinfected group, the median intensity was 367 at Time 1 and decreased significantly to 129 at Time 2 (p<0.05); gills showed the same histological alterations observed in the primary infection, although with more severity (Figure 3f), with \( H_{i} = 24 \) at Time 1 and \( H_{i} = 58 \) at Time 2.

The expression of all genes did not differ significantly between control and experimentally infected fish (Figures 4 and 5). The expression of cmip in gills at Time 1 was significantly higher in reinfections than in primary infections (p<0.05). Despite the absence of significant difference, the expression level of il12β showed a fold-change value > 100 in head kidney at Time 2 in primary infections, whereas all other genes showed low fold-change values (typically < 2) (Figures 4 and 5).
Figure 3. Photomicrographs of gills of PWS infected with *Rhabdosynochus viridisi*. a. Normal appearance in control (uninfected) fish; b. Cystic of epitheliocystis between the secondary lamella (arrow); c. Infiltration of mononuclear inflammatory cells (arrows) and hyperplasia (arrowhead) in primary infection; d. Parasite attached to the primary lamella (arrows); e. Parasite hook within the lamella (arrow); f. Severe infiltration of mononuclear inflammatory cells in reinfected fish. H&E stain.

Figure 4. Expression of immune-related genes in head kidney of Pacific white snook *Centropomus viridis* during *Rhabdosynochus viridisi* primary infection and reinfection at times 0, 1 and 2.
**Figure 5.** Expression of immune-related genes in gills of Pacific white snook *Centropomus viridis* during *Rhabdosynochus viridisi* primary infection and reinfection at times 1 and 2.
DISCUSSION

The histological alterations in gills of PWS caused by the monogenean diplectanid *R. viridisi* are similar to those caused by another diplectanid (*Diplectanum sciaenae*) in meagre in Mediterranean aquaculture (11). In the present study, the attachment site where parasites were detected seemed unaltered, possibly because parasites migrate from an affected site of the gill filament toward an unaltered gill section, where the immune response has not yet reached or where it is not strong enough to kill them (12).

The parasite load reduction observed in reinfected PWS at Time 2 suggest an enhanced response for a faster expulsion of monogeneans, possibly due to acquired immunity (13, 14). Recently, an analysis *in silico* indicated that 63% of the predicted excretory/secretory proteins of *R. viridisi* are potential antigens and, therefore, could be presented to T cell receptors and recognized by host antibodies (15). So, it could be possible that fish reacts specifically towards those antigens and brings immunological memory into play, which is an important step to reaching an adaptive response (16).

This is the first study about the immune response of PWS infected with the monogenean *R. viridisi*. We evaluated the local (gills) and systemic (head kidney) response of immune-related genes in PWS infected with *R. viridisi*. However, we found that the expression of all genes did not differ significantly between control and experimentally infected fish. Only the expression of *cmip* in gills at Time 1 was significantly higher in reinfections than in primary infections. It could be indicating the effort of fish to activate adaptive immunity and could be related to the decrease of parasites in reinfections at Time 2. *cmip* is not typically measured in fish-parasite studies. One of the few studies reported no differences in expression levels of *cmip* in three-spined stickleback (*Gasterosteus aculeatus*) uninfected and infected with *Gyrodatylus* (17).

The high expression level of *il12β* in head kidney at Time 2, in both primary infections and reinfections, is in agreement with Pérez-Cordón et al. (18) who observed, although in a myxozoan-fish model, that among 19 IL-related genes analyzed *il12β* reached the highest expression levels. Likewise, in fish infected with bacteria, Zhang et al. (19) showed that its expression occurs in multiple tissues but is highest in the kidney. In a study similar to ours, Tu et al. (20) observed that the expression of *il12* in the spleen, liver, and head kidney of goldfish was low at the beginning of the monogenean infection, but increased significantly later, during the elimination phase of infection. These findings suggest that *il12*, a pro-inflammatory cytokine well known to be critical against intracellular pathogens, could also has a pivotal role against monogeneans.

In summary, the present study shows that the monogenean *R. viridisi* may cause changes in the gill tissue of its fish host. In addition, the decrease of the intensity of infection from Time 1 to Time 2, suggests that the fish is able to combat *R. viridisi*; however, our immunological analysis did not reveal strong evidence of a possible mechanism.

Conflicts of interest

The authors have no conflict of interest to declare in regard to this publication.

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REFERENCES


