Immunological study on age-dependent susceptibility to *Ochroconis* infection in marbled rockfish, *Sebastiscus marmoratus*

Summary of Doctoral Thesis

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Marbled rockfish, *Sebastiscus marmoratus* (Cuvier, 1829) is an important marine fish species in Japanese fishery. Parasitic infection such as *Lecithochirium trtraorchis* and *Cryptocaryon irritans* has been reported in marbled rockfish. In recent, fungal infection caused by *O. humicola* was reported in marbled rockfish (Wada et al., 2005). This infection was consistently reported from juvenile fish cultured in Japan, including devil stinger (Wada et al., 1995) seabream (Wada et al., 2005), striped jack (Munchan et al., 2006). With these case reports, *O. humicola* infection more likely targets juvenile fish and seems to be an age-dependent in these fish species.

The immune system of fish share many similarities with mammalian counterparts. The fundamental immune molecules: T lymphocytes, B lymphocytes, macrophages and cytokines are similar between fish and mammals. In recent, the fish immune system has been extensively investigated in several fish species due to its aquaculture importance (Zou et al., 1995; Wang & Secombes, 2013). The immune responses in fish can be affected by these factors: age, sex, seasonal changes, water quality and water salinity (Noguera et al., 2015). Host defence mechanism against pathogenic fungi are various and ranges from innate immunity to adaptive immunity (Romani, 2004; Blanco & Garcia, 2008). In general, cell-mediated immunity has shown to mediate host protection against various pathogenic fungi. (Polonelli et al., 2000).

The focus of this thesis has been to study the host immune response of marbled rockfish to *O. humicola* with fish age. *O. humicola* (NJM 1503) was used as a pathogen to study histopathological features, survival and susceptibility changes in different age category. Additionally, immune relevant genes of marbled rockfish were cloned and characterized. In-vitro studies using kidney leukocytes stimulated with LPS and poly I:C demonstrated the functionality of these cloned genes. The host immune response to *O. humicola* in marbled rockfish of different age categories was investigated by analyzing the gene expression of T cell markers and cytokines in the spleen.

1. Effect of age on susceptibility to *O. humicola*

In chapter 2, effect of age on susceptibility of marbled rockfish to *O. humicola* was studied by experimental challenge. Juvenile marbled rockfish of three age categories were obtained from a commercial fish hatchery. Each age category showed different body length: small (29 ± 2 mm), medium (55 ± 3 mm), and large (74 ± 6 mm). The fish were experimentally challenged with 1×10^5 conidia/50 μL of *O. humicola* NJM 1503. The skin surface on the cranium was artificially injured by nylon-twisted yarn and conidia suspension was drop directly on the injury site, and then all fish were kept for 60 days. The cumulative mortalities for small, medium and large fish were 100 %, 20 % and 0 %, respectively.

All dead fish had severe ulceration in the head area. Severe encephalitis associated with tissues damage were also observed in all dead fish. Histopathological symptoms in dead fish were characterised by partial loss of cranial bones, severe degeneration of infiltrated inflammatory cells and massive invasion of fungal hyphae in the infected area. In contrast, no hyphal penetration and inflammatory response were observed in the brain of surviving fish. These results demonstrated that the younger fish among juveniles were more sensitive to *O. humicola*.

2. Histopathological study of the inflammatory response induced by *O. humicola*

The previous chapter has been demonstrated that younger marbled rockfish were more susceptible to *O. humicola* injection. In chapter 3, the inflammatory response and the disease development in younger and older marble rockfish were investigated by histopathological observation. Younger fish (52 ± 1.2 mm, 3.5 ± 0.2 g) and older fish (76 ± 3.9mm, 12.6 ± 1.7g) were intraperitoneally injected with 1×10^5 conidia/50 μL of *O. humicola.* Two sampling method was performed. For the first experiment, all fish were kept for 30 days and moribund or dead fish were sampled during the experimental period. For the second experiment, fish in both ages was sampled at day 3, 5, 7, 10, and 13 after injected with *O. humicola.*

In the first experiment, all the younger fish died and failed to control the fungal invasion. The histopathological symptoms in the dead fish were characterized by

severe inflammation associated with a high number of hyphae and necrosis (mononuclear cells and parenchymal cells) in the infected tissues. In contrast, the older fish survived and some of them showed epitheliod cell granulomas in the intraperitoneal adipose tissues.

From the second experiment, the infiltration of mononuclear cells progressed over time and followed by increasing of hyphae number was observed in the adipose tissues and the liver of younger fish. Cell degeneration of the inflammatory cells was observed at 13 d.p.i. This results clearly showed that even though younger fish well developed the inflammatory response against hyphal invasion, eventually the inflammation could not prevent hyphal invasion leading the fish died. Low or no inflammatory response was characterized in the older fish. Taken together these observations, the quick and intense inflammation observed in the younger fish seems to be the cause of tissue damage in the organs of infected fish rather than play the immune diffense against the pathogen *O. humicola.*

3. Molecular cloning and characterisation of immune relevant genes

In the recent, immunological study is developed by molecular analysis of immune relevant genes. Characterisation of immune genes such as T cell markers (CD4, CD8β, CD3ε and CD28) and cytokine (IL-1β and IFN-γ) was conducted in chapter 4. The full length of cDNA of genes was determined by RACE PCR method.

The primary structures for each gene are highly conserved among the teleost based on the multiple alignments of amino acid sequences and phylogenetic analysis. T cell markers and IFN γ in marbled rockfish showed high homology with that in orange-spotted grouper. By contrast, SebIL-1β showed the highest amino acid identity with that of large yellow croaker. These genes were useful for studying the immune responses to *Ochroconis* infection in marbled rockfish.

4. Analysis of Immune Relevant Genes Expression

Chapter 5 was divided into three parts: (i) expression analysis of the genes (CD4, CD8β, CD3ε and CD28) in several organs of healthy marbled rockfish, (ii) in-vitro functional analysis of the genes using immune stimulants, (iii) expression

analysis of the genes in a lymphoid organ of the younger and the older fish after the injection with *O. humicola*

The highest level of gene expression of SebCD4, SebCD8β, SebCD3ε were found in the thymus These data suggest that these genes can be identified as T cell markers in marbled rockfish. In contrast, SebCD28 showed highest expression in the liver and with moderate expression in other lymphoid organs. With the different expression pattern of SebCD28, it has remained unknown whether CD28 is truly expressed in T cells of marbled rockfish.

The gene expression of SebCD4, SebCD8β, SebIL-1β and SebIFN-γ was upregulated in the kidney leukocytes after stimulation with LPS and poly I:C. Therefore, these genes have a similar role in immune response as observed in mammals.

The difference of gene expression between younger and older marbled rockfish was observed under the stimulation with the pathogen *O humicola*. The gene expression showed a unique profile that the younger fish characterized quick and intense response, but the older fish characterized slow and moderate response. The quick and intense response of SebCD4 and SebIFN-γ gene expression in the younger fish probably initiates the inflammatory reaction in the adipose tissues and the liver described in chapter 3. The expression of these genes could be crucial for the recruitment for inflammatory cells to eliminate the fungal hyphae but this response in younger fish failed in elimination of the pathogen. The younger marbled rockfish could be characterized by operation of ineffective immune response which provokes intense inflammation resulting in tissue damage. It may be associated with the susceptibility of marbled rockfish against *O. humicola* infection.

A future study needs to find immune relevant genes associated with resistance capacity of the older marbled rockfish by transcriptome analysis which is able to access a large number of expression genes.