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

（オクロコニス症に対するカサゴの年齢に依存した感染感受性に関する免疫学的研究）

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CHAPTER 1

INTRODUCTION

1.1 Background

Infectious diseases are the most crucial factor that caused the huge worldwide economic impact in aquaculture, and the total global loss is estimated to be more than US 6 billion per year (Vijayan et al., 2017). The pathogens such as viruses, bacteria, fungi, and parasites have been known as the cause of these infectious diseases in marine culture fish (Sharma et al., 2012). Fungal infections are one of the problems that affected natural and cultured fish (Ramaiah, 2006). In recent years, a fungal infection caused by genera *Ochroconis* in the Deuteromyctes has been reported in the early age of Japanese marine cultured fish and rarely reported in adult fish (Wada et al., 1995; Wada et al., 2005; Munchan et al., 2006). Evidence from these cases in Japan may indicate that the occurrence of *Ochoconis* infection is age-dependent.

All living organisms have defence mechanisms against infectious diseases. Like mammals, teleost has both innate (non-specific) and adaptive (specific) immune responses (Magnadottir, 2006). The functional development of the immune system is different in each fish species and is important for host protection (Ellis, 2005). Studies on the fish immune system are essential for understanding the host-pathogen relationships in aquacultured fish because the production is affected when there is an occurrence of infections in cultured fish (Leung & Bates, 2013).

The outbreak of infection depends on age, sex, seasonal changes, water quality and water salinity. These factors can affect the immune responses in fish (Noguera et al., 2015). Host defence mechanisms against fungi are various and range from innate (first-line defence) to adaptive immunity (Romani, 2004; Blanco & Garcia, 2008). In general, cell-mediated immunity (CMI) has consistently been shown to mediate protection against various pathogenic fungi, and the formation of granulomatous inflammation is often observed in the tissues infected with the pathogenic fungi (Polonelli et al., 2000).

1.2 *Ochroconis* infection in fish

The genus *Ochroconis* was described by de Hoog and von Arx (1973) for melanised fungi with sympodial conidiogenesis with septate and ellipsoidal conidia. This genus was differentiated from the genus *Scolecobasidium* which was initially described by Abbott (1927). The genus *Ochroconis* (Sympoventuriaeceae, Venturiales) is revised and currently contains 13 species which has been determined using multilocus DNA sequencing (Samerpitak et al., 2013). Up to the present time, three species of the genus: *O. humicola*, *O. tshawytschae* and *O. globalis* have been reported as a pathogen in fish. (Doty & Slater, 1946; Hatai & Kubota, 1989; Wada et al., 1995; Wada et al., 2005; Munchan et al., 2006; Samerpitak et al., 2019).

The first case of *Ochroconis* was in kidney mycosis Chinook salmon (*Oncorhynchus tshawytschae*) caused by *O. tshawytschae* (Doty & Slater, 1946). Later, another *Ochroconis* sp. closely related to *O. tshawytschae* was reported in masu salmon (*Oncorhynchus masou*) (Hatai & Kubota, 1989). *O. humicola* has been reported as an etiologic agent in fish. Ross & Yasutake (1973) firstly described a mycotic disease in Coho salmon (*Oncorhynchus kisutch*) caused by *Scolecobasidum humicola*, later was described as *O. humicola*. Another case was a muscular black spot disease in Atlantic salmon (*Salmo salar*) (Schaumann & Priebe 1994). A cutaneous infection caused by *O. humicola* was reported in Barramundi cod (*Cromileptes altivelis*) (Bowater et al., 2003)

In Japan, *O. humicola* infection has been reported from devil stinger, *Inimicus japonicus* (Wada et al., 1995) with some ulcerations on the body surface. Later, this infection was reported in red sea bream (*Pagrus major*), marbled rockfish, *Sebastiscus marmoratus* (Wada et al., 2005) and striped jack, *Pseudocaranx dentex* (Munchan et al., 2006). Besides fish, *O. humicola* was reported in the frog (Elkan & Philpot, 1973) and a tortoise (*Terrapine carolina var. carolina*) (Weitzman et al., 1985). The clinical data of these cases are summarized in Table 1.

The genus *Ochroconis* can be identified by morphological characteristics, especially with the mode of conidium ontogeny and conidia shape (Hatai, 2012). *Ochroconis* exhibits asexually reproductive mode showing a conidiophore sympodially producing conidia which are two-celled and ellipsoidal to cylindrical shaped (Samerpitak et al., 2013). The fungal

colonies of *O. humicola* showed slow growth, slightly domed, velvety to floccose and pale brown colour (Hatai, 2012).

1.3 Marbled rockfish

The Marbled rockfish, *Sebastiscus marmoratus* (Cuvier, 1829) is important commercially food for its high nutritional values and delicacies (Zhu et al., 2011). It is known as "kasago" in Japanese. Marbled rockfish is widely distributed in the coastal areas of the Northern Pacific Ocean, especially in China, Korea and Japan (Higuchi & Kato 2002; Jin, 2006; Nakabo, 2013). *Sebastes* is a genus of the family Scorpaenidae, and *Sebastiscus* has been called a subgenus of *Sebastes* by Barsukov & Chen (1978) (Boehlert & Yamada, 1991). In general, most of the rockfishes are ovoviviparous, which exhibit a unique pattern of internal fertilisation (Kendall, 2000). A parasitic infection (*Lecithochirium trtraorchis*) was reported in *S. marmoratus* in Taiwan (Shih et al., 2004). Some studies about *Cryptocaryon irritans* infection was reported in *S. marmoratus* (Yin et al., 2014; Yin et al., 2018).

1.4 Immunology of teleosts

Like mammals, teleost fishes have both innate (non-specific) and adaptive (specific) immunity (Magnadottir, 2006). Thymus, kidney and spleen are the major lymphoid organs in teleost (Zapata et al., 2006). Compared to mammals, teleost generally lacks the lymph nodes and bone marrow. The

head kidney in teleost has functional similarity with the bone marrow and lymph nodes in mammals (Press & Evensen, 1999).

1.4.1 Innate immunity

Generally, there are three parameters involved in fish innate immunity. The first category is physical parameters at which fish scales, the mucous surface of skin and gills act as the first barrier of defence (Ingram, 1980; Shephard, 1994; Ellis, 2001). When the pathogen succeeds to pass the first barrier, cells of the innate immune system and the humoral factors are activated (Magnadottir, 2006). Phagocytic cells such as granulocytes, monocytes and macrophages are the key cells involved in the innate immunity (Frøystad et at., 1998; Neumann et al., 2001).

Toll-like receptors (TLRs) are one of the pattern recognition receptors (PRRs) which recognize pathogen-associated molecular patterns (PAMPs) (Li et al., 2017). The epithelial and dendritic cells also participate in the innate defence in fish (Press et al., 1994; Ganassin & Blos, 1996). The humoral immune defence mechanism consists of soluble components such as lysozyme and complement in body fluid, mucous and serum (Magnadottir, 2006; Subramanian et al., 2007).

1.4.2 Adaptive immunity

An acquired immune system is initiated when pathogens surpass the innate defence mechanism and persist beyond the first several days of infection. Activation of the acquired immune responses is relatively slow because of involving cellular differentiation, cellular proliferation and protein synthesis, but the response is long-lasting (Magnadottir, 2010). In contrast to innate immune components, effector cells (lymphocytes) and immunoglobulin function in adaptive immunity which are highly specific to the antigen of the invading pathogens (Kum & Sekkin, 2011). There are two distinct lymphocytes populations: T and B lymphocytes in teleosts (Nakanish et al., 2015). T lymphocytes responsible for the cell-mediated immunity (CMI) and can be further subdivided into CD4 positive (helper T cells) and CD8 positive (cytotoxic T cells) (Scapigliati et al., 2018).

1.4.3 T lymphocytes

T lymphocytes play a central role in regulating the immune responses. Activation of T-cell response requires an interaction involving T cell receptor (TcR) on T lymphocytes and major histocompatibility complex (MHC) molecules on antigen-presenting cells (APCs) (Kumar et al., 2018). In general, CD4 is a co-receptor of helper T cells (Th) that interacts with antigen presented by MHC class II molecules expressed on APCs. On the other hand, CD8 is a co-receptor cytotoxic T cells (CTL) that interacts with MHC class I

molecules expressed on APCs (Konig & Zhou, 2004; Andersen et al., 2006).

1.4.4 Lymphoid organs in fish

The kidney, thymus and spleen are important lymphoid organs in teleost. The maturation of lymphoid organs differs in marine and freshwater teleost (Zapata et al., 2006). The first lymphoid organ develops in freshwater teleost is the thymus, followed by the kidney and the spleen (Hansen & Zapata, 1998; Zapata et al., 2006). However, the order of development in lymphoid organs of marine teleost is different from that of freshwater teleost. In marine teleost, the kidney and the spleen develop firstly, followed by the thymus (Nakanishi, 1986; Pulsford et al., 1994; Zapata et al., 2006). Thymus is an essential organ for development of T lymphocytes from early thymocyte progenitors to functional mature T cells (Nakanishi et al., 2015).

1.5 Aims and outline of thesis

Because *O. humicola* infection in marbled rockfish is reported from juveniles of fish, young age fish seem to be a target of the infection. The correlation between marble rockfish age and *O. humicola* infection has remained unknown. The onset of immune maturation is different in each fish species (Ellis, 2015). Thus, the pattern of the immune response may differ in between younger and older marbled rockfish to *Ochroconis* infection. The purpose of this thesis was to study the effect of age on the susceptibility and pathogenesis of the infectious disease, and to investigate the difference of immune response in marbled rockfish of different age categories.

The aims of this thesis were:

1. To determine effect of age on the susceptibility of marbled rockfish to *O. humicola* infection.

2. To investigate the histopathological features in marbled rockfish of different age categories following experimental challenge.

3. To establish the molecular analysis using immune relevant genes: T cell markers and cytokines in immunological study of marbled rockfish.

4. To study the pattern of the immune response to *O. humicola* in marbled rockfish of different age categories.

Table 1. Cases reported in fish and other cold-blooded animals infected by *Ochroconis* species.

Ochroconis species	Infected animals (species name)	Infected organs	References
Heterosporium tshawytschae (*O. tshawytschae*)	Chinook salmon (*Oncorhynchus tswaytchae*)	kidney	Doty & Slater, 1946 Kirilenko & All Achemed, 1977
O. humicola	Coho salmon (*Oncorhynchus kitsutch*)	kidney	Ross & Yasutake, 1973
O. humicola	Frog	visceral organs	Elkan & Philpot, 1973
O. humicola	Rainbow trout (*Salmo gardneiri*)	kidney	Ajello et al., 1977
O. humicola	Tortoise *(Terrapene carolina carolina*)	Feet, tail ulceration	Weitzman et al., 1985
Ochroconis sp.	Masu salmon (*Oncorhynchus masou*)	kidney	Hatai & Kubota, 1989
O. humicola	Atlantic salmon (*Salmo salar*)	muscle	Schaumann & Priebe, 1994
O. humicola	Devil stinger (*Inimicus japonicus*)	deep skin, open ulcer	Wada et al., 1995
O. humicola	Barramudi cod (Cromileptes altivelis)	liver, swim bladder, kidney	Bowater et al., 2003
O. humicola	Red sea bream (*Pagrus major*), Marbled rockfish (*Sebastiscus marmoratus*)	skin, kidney, brain	Wada et al., 2005
O. humicola	Striped jack (*Pseudocaranx dentex*)	kidney	Munchan et al., 2006
O. globalis	Atlantic salmon (*Salmo salar*)	Kidney and liver	Samerpitak et al., 2019

CHAPTER 2

Effect of age on susceptibility of marbled rockfish, *Sebastiscus marmoratus* to *Ochroconis humicola* following experimental challenge

This chapter has been published in Journal of Fish Diseases.

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2.1 INTRODUCTION

Infection by the fungus *Ochroconis humicola* causes mortality in juveniles of marine fish species including the marbled rockfish, *Sebastiscus marmoratus*, which is cultured in Japan (Wada et al., 1995; Wada et al., 2005; Munchan et al., 2006). Erosive or ulcerative features on the body surface, especially skin lesions on the head, have been reported as the major clinical signs of *O. humicola* infection in Japanese marine cultured fish (Wada et al., 1995; Wada et al., 2005).

These case reports suggest that *O. humicola* consistently infects younger fish. For example, Wada et al. (2005) recorded infections in juvenile marbled rockfish weighing about 1 g. However, there have not yet been any studies investigating the occurrence of *O. humicola* infection in relation to fish body size. Therefore, the objective was to examine the association between susceptibility to infection and age of marbled rockfish.

2.2 MATERIALS AND METHODS

2.2.1 Fish

Juvenile marbled rockfish of three different age categories from the same batch were obtained in August, October, and December from a commercial fish hatchery. Each fish in August, October, and December showed different body length: small (29 ± 2 mm), medium (55 ± 3 mm), and

large (74 ± 6 mm), respectively. The respective mean weights were as follows for the small, medium, and large fish: 0.7 ± 0.2 g, 4.2 ± 0.8 g, and 8.8 ± 2.2 g. The fish were held in 40 L of artificial seawater (Instant Ocean, Aquarium System, Roubaix, France) in a closed system with aeration and a biofilter, and maintained within a temperature range of 22–24°C.

2.2.2 Conidia preparation

Ochroconis humicola NJM1503 isolated from a diseased marbled rockfish in 2015 was used in this study. A conidia suspension of *O. humicola* NJM 1503 was prepared from a fungal colony cultured on PYGS agar (0.125% peptone, 0.125% yeast extract, 3% glucose, and 1.2% agar in artificial seawater). The conidia were suspended with phosphate buffer saline (PBS) and adjusted to a concentration of 1×10^5 conidia per 50 µL of PBS.

2.2.3 Experimental challenge

Fish in each size category (n = 10) were anaesthetized using FA100 (10% solution of eugenol, Tanabe Co. Ltd, Tokyo, Japan). The skin surface on the cranium which is located at a caudal part of the area between eyes was then artificially injured by gently scratching it with nylon-twisted yarn, a material used for fishing nets. Next, 50 µL of the conidia suspension was dropped directly onto the injury site where it was allowed to remain for 15 seconds. Control fish for each size category (n = 10) were infected the same way as described previously, but the conidia suspension was replaced with

PBS. The cumulative mortality was calculated to 60 days post-inoculation (d.p.i.).

2.2.4 Histopathological analysis

For histopathology, the heads of any dead fish and fish surviving to 60 d.p.i (but sacrificed by overexposure to FA100) were fixed in 10% buffered formalin. This was followed by decalcification with 27 mM EDTA, 7.4 mM sodium acetate, and 4% formalin adjusted to pH 7.4 using sodium hydroxide. The heads were then embedded in paraffin. Paraffin-embedded tissue sections (5 μm in thickness) were stained with haematoxylin-eosin (H&E), periodic acid-Schiff (PAS), and Schmorl's method. The Cox proportional hazards model was used to test for age effect on risk of death after the experimental infection.

2.2.5 Ethics Statement

All animal experiments were performed in accordance with the guidelines of the Animal Experiments Committee at Nippon Veterinary and Life Science University, and all experimental protocols were endorsed by the committee according to authorization 30K-32.

2.3 RESULTS

2.3.1 Gross sign and mortality

The control fish in each age category did not show obvious clinical signs. All dead fish had severe ulceration in the head area (Fig. 2.1), except for the small fish died 1 d.p.i. The cumulative mortalities for the small, medium, and large fish were 100%, 20%, and 0%, respectively (Fig. 2.2). The first death in the small fish group was observed at 1 d.p.i., and all fish were dead after 18 d.p.i. The deaths of medium fish were recorded only at 12 and 26 d.p.i. The fish after the experimental infection tended to die at the category of small fish meaning a younger age category ($p < 0.01$).

Fig. 2.1. Gross signs in marbled rockfish infected with *O. humicola* by skin inoculation with artificial injury. Extensive and severe skin ulcers (arrow) in the fish died 17 d.p.i.

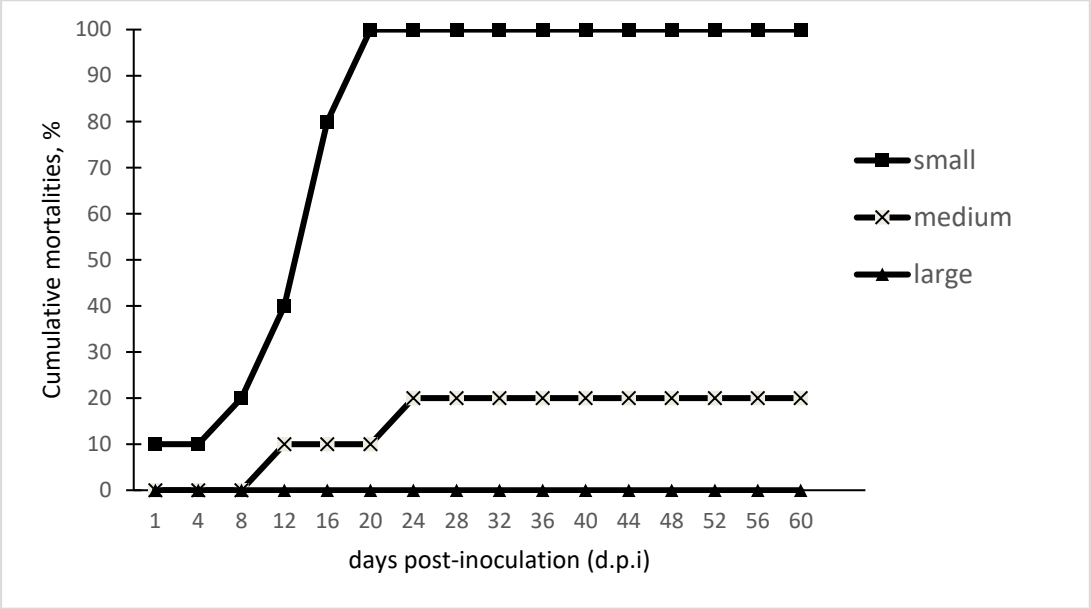

Fig. 2.2. Cumulative mortalities for small, medium, and large fish infected by skin inoculation with artificial injury. The cumulative mortalities for small, medium, and large fish were 100%, 20%, and 0%, respectively.

2.3.2 Histopathological observation

In the histopathological observations, all control fish showed normal tissue structure (Fig. 2.3). No hyphal penetration and inflammatory response was observed in the brain of surviving fish and the small fish that died 1 d.p.i. Tissue damage in the head area was observed in all other dead small and medium fish. The histopathological features observed were destruction of the epidermis and dermis at the skin surface (Fig. 2.4), partial loss of cranial bones (Fig. 2.4), severe degeneration of inflammatory cells infiltrating the adipose tissue below the cranial bone (Fig. 2.5a and 2.5b), and massive invasion of adipose tissue and the brain by fungal hyphae (Fig. 2.6a). The hyphae observed in the tissue were septate (Fig. 2.6b) and stained positively using

Schmorl's method (Fig. 2.6a), suggesting that the presence of melanin in the hyphal walls which characterizes it as dematiaceous fungi. These characteristics indicated that the hyphae originated from the isolate inoculated by experimental infection, because *O. humicola* is a dematiaceous fungus belonging to the Deuteromycetes.

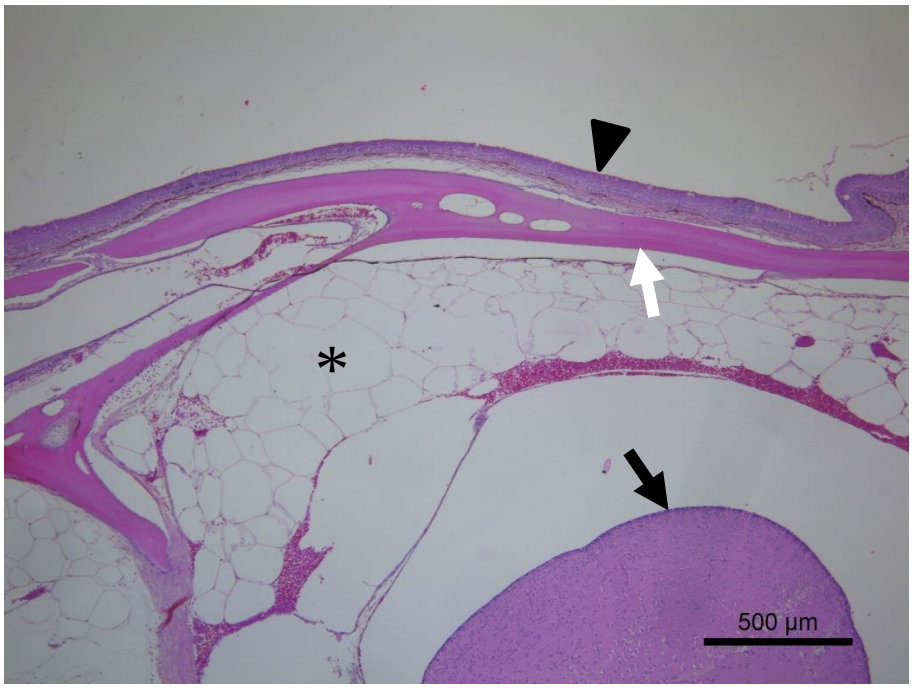

Fig. 2.3. Non-damaged tissue including normal skin (arrow head), adipose tissue (*), a cranial bone (white arrow), and brain (black arrow) in a surviving fish. H&E staining. Scale bar: 500 µm.

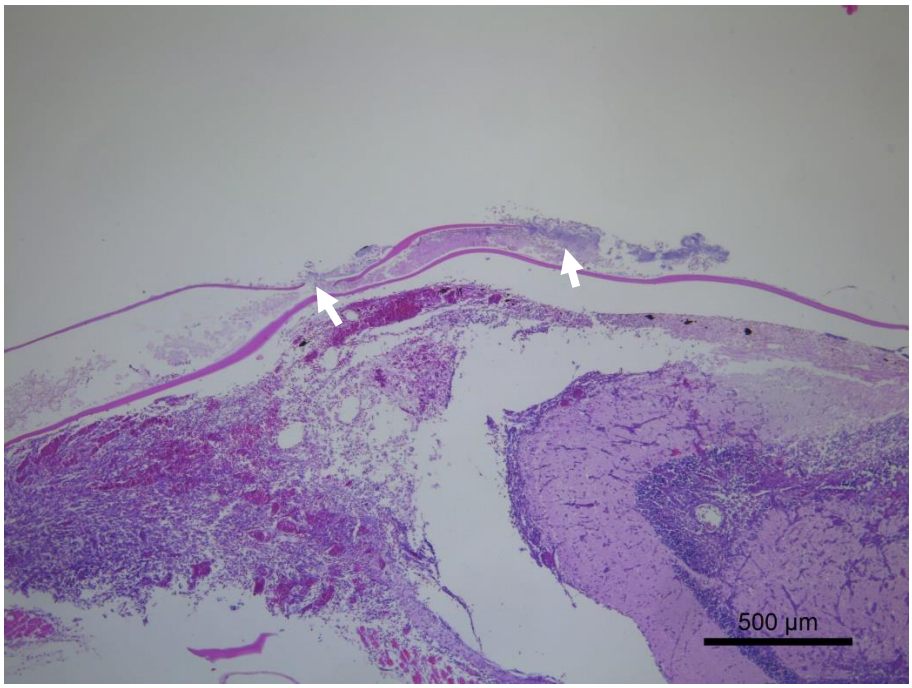

Fig. 2.4. Damaged tissue including destruction of the epidermis and dermis and partial loss of cranial bones (white arrows) in a small fish died 13 d.p.i. H&E staining. Scale bar: 500 µm.

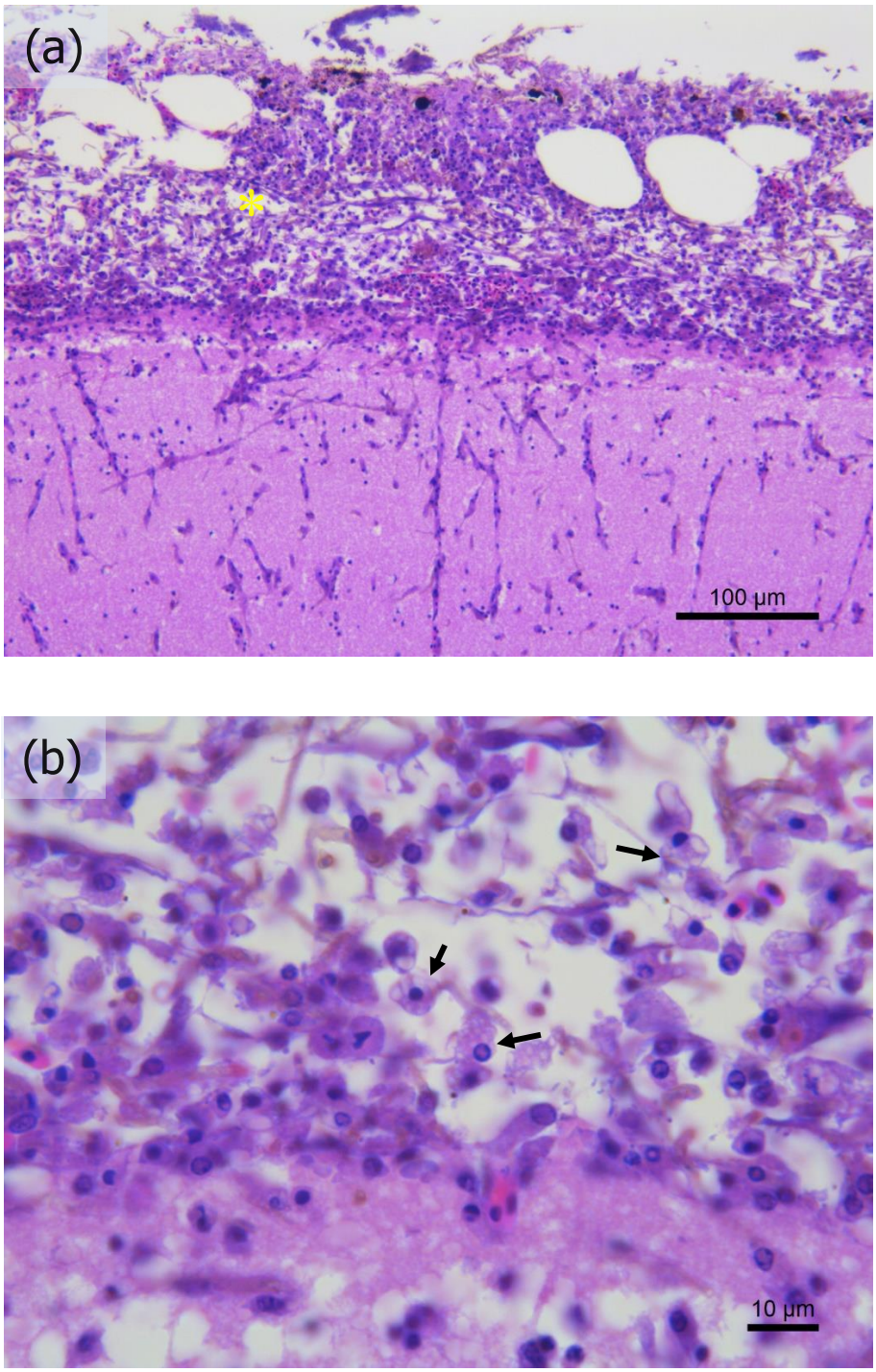

Fig. 2.5. **(a)** Inflammatory cells infiltrating the adipose tissue (*) below the cranial bone of a small fish died 13 d.p.i. H&E staining. Scale bar: 100 µm. **(b)** Severe degeneration of inflammatory cells observed in small fish died 17 d.p.i. These cells showed nuclear condensation and were swollen in shape (arrows). H&E staining. Scale bar: 10 µm.

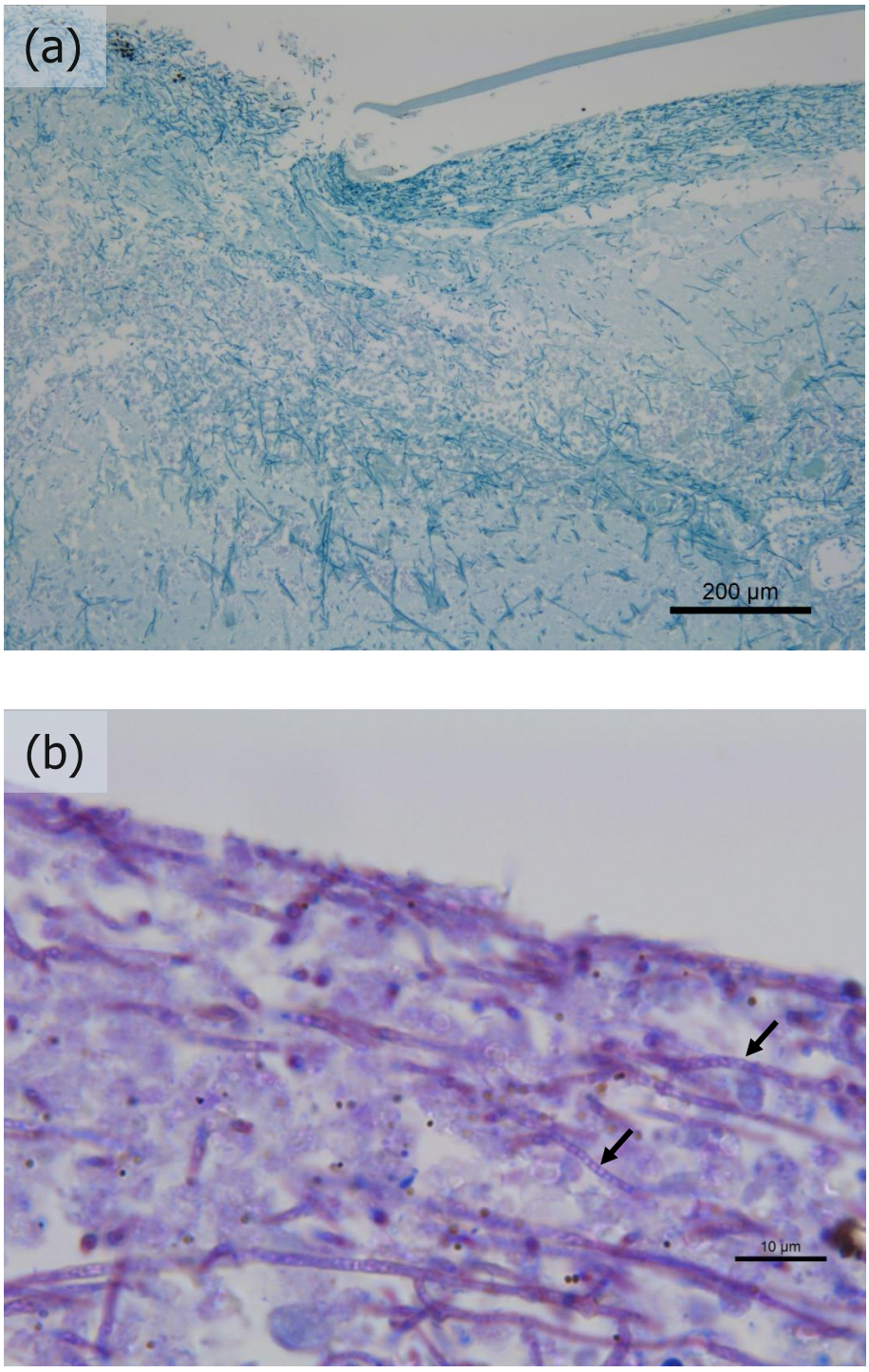

Fig. 2.6. **(a)** Massive invasion by fungal hyphae (blue colour) in the adipose tissue and brain of a small fish died 13 d.p.i. Schmorl staining. Scale bar: 200 µm. **(b)** Septate hyphae (arrows) of the infected fungi in the adipose tissue. PAS staining. Scale bar: 10 µm

2.4 DISCUSSION

This study demonstrated that the younger fish among juveniles were more sensitive to *O. humicola* infection. We have also obtained the same finding by preliminary trials using juvenile marbled rockfish. This finding is consistent with the observation that the outbreaks on marbled rockfish aquaculture farms have only occurred among small juveniles. The gross and histopathological features found in the fish experimentally infected with *O. humicola* were similar to those found in naturally infected fish (Wada et al., 2005), indicating that we succeeded in reproducing the signs of *O. humicola* infection in marbled rockfish.

The experimental challenge without artificial injury failed to produce any pathological lesions (data not shown). This suggests that natural infection is more likely to occur when a fish is injured. Schaumann and Priebe (1994) described *O. humicola* as an opportunistic fungal pathogen that might also be highly potent under suitable conditions. Skin damage of fish may occur when individuals bite one another in the rearing environment and when they are handled by aquaculturists. As the damage would enable the pathogen to infect the fish, care must be taken to avoid injuring fish in hatcheries.

2.5 SUMMARY

The present study demonstrated that younger fish among juveniles were more susceptible to *O. humicola* infection. It was suggested that *O. humicola* infection was age-dependent in marbled rockfish. The pathological features caused by the experimental challenge was characterized by ulceration in the head area and encephalitis associated with severe inflammation and numerous fungal hyphae in younger fish, while no inflammatory responses and tissue damage was characterized in older fish.

CHAPTER 3

Histopathological study of inflammatory response induced by experimental infection with *Ochroconis humicola* in different age categories of Marbled rockfish, *Sebastiscus marmoratus*

3.1 INTRODUCTION

In chapter 2, I demonstrated that younger age fish were more susceptible to *O. humicola* infection when the fish were experimentally challenged by a direct contact method to the skin surface on the cranium. The younger marbled rockfish showed destruction of the epidermis and dermis at the skin surface, partial loss of the cranial bone, severe degeneration of inflammatory cells infiltrating the adipose tissue below the cranial bone and massive invasion of fungal hyphae in the adipose tissue and the brain (Lee et al., 2020).

On the other hand, the older age fish did not show any pathological features described above, even in the same experimental situation (Lee et al., 2020). Therefore, I suppose that marbled rockfish possesses different immune reactivity against the pathogen by their ages. In this chapter, the inflammatory response and disease development in younger and older marbled rockfish were characterized by histopathological observation after intraperitoneal injection (i.p.) with *O. humicola*. Intraperitoneal injection challenge method was chosen to induce systemic immune responses in marbled rockfish.

3.2 MATERIALS AND METHODS

3.2.1 Fish

The fish obtained from different months was categorized as follows: younger fish (52 ± 1.2 mm, 3.5 ± 0.2 g) and older fish (76 ± 3.9 mm, 12.6 ± 1.7 g). The fish were held in 40 L of artificial seawater (Instant Ocean, Aquarium System, Roubaix, France) and aerated with biofilters system. The fish maintained within temperature range 22 -24ºC and fed a commercial diet once a day

3.2.2 Preparation of conidia suspension

O. humicola NJM 1503 was cultured on PYGS agar (0.125 % peptone, 0.125 % yeast extract, 3 % glucose, and 1.2 % agar in artificial seawater) at 25ºC for three weeks. Conidia were suspended from the fungal colony with phosphate buffer saline (PBS) by gently scratching using at 1.0 µL inoculation loop (Sarstedt AG & CO., Nümbrecht, Germany). The collected conidia were counted using a haemocytometer (Erma, Tokyo, Japan) and adjusted to a concentration of 1×10^5 conidia per 50 µL of PBS.

3.2.3 Experimental challenge

3.2.3.1 Experiment 1

The histopathological features in moribund or dead fish caused by intraperitoneal injection with *O. humicola* were studied. Fish in each age category (n = 10) were anaesthetized using FA100 (10% eugenol solution, Tanabe Co. Ltd, Tokyo, Japan) and intraperitoneally (i.p.) injected with 50 µL of the conidia suspension. As a control, fish in each age category were similarly injected i.p. with 50 µL of PBS instead of conidia suspension. The injected and control fish were separately reared in 40 L of artificial seawater within temperature range 22-24ºC.

During the experiment period, the moribund or dead fish were sampled for histopathological observation. The experiment was finished after 30 days post-injection (d.p.i.), and the survived fish was sacrificed by overexposure to FA 100. The kidney, spleen, liver and adipose tissues of all experimental fish were fixed in 10% buffered formalin and embedded in paraffin. Paraffin-embedded tissues were sectioned to 5 µm and the sections were stained with hematoxylin and eosin (H & E), Schmorl method and Elastica van Gieson (EV).

3.2.3.2 Experiment 2

The histopathological development in the fish i.p. injected with *O. humicola* was studied. A total of 15 fish in each age category were injected i.p. with the same amount of the conidia suspension described in Experiment 1. Three fish from each age category were sampled at 3, 5, 7, 10 and 13 d.p.i., and sacrificed with overexposure to FA100. Internal organs (kidney, spleen, liver and intraperitoneal adipose tissue) were fixed in 10 % buffered formalin, embedded in paraffin and sectioned to 5 μm. The sections were stained with hematoxylin and eosin (H&E) and Schmorl method.

3.2.4 Ethics statement

All animal experiments were performed under the guidelines of the Animal Experiments Committee at Nippon Veterinary and Life Science University, and all experimental protocols were endorsed by the committee according to authorization 2019K-39.

3.3 RESULTS

3.3.1 Experiment 1

3.3.1.1 Mortality and gross sign

Mortality of younger marbled rockfish started at day 15 after the injection, and the cumulative mortality reached 100 % at the end of the experiment. By contrast, older marbled rockfish and PBS-injected fish did not die for the whole experiment period. Dead fish showed gross signs such as external ulcerative lesions in the injected site. Accumulation of pale red ascites in the peritoneal cavity and hepatic haemorrhages were also observed (Fig. 3.1). The survived fish showed no clinical signs.

3.3.1.2 Histopathological observation

Younger fish

Hyphal penetration was observed in intraperitoneal adipose tissues (Fig. 3.2a), liver (Fig. 3.2b), spleen (Fig. 3.3a) and kidney (Fig. 3.3b) of all injected fish except for one fish died on day 15 showing hyphae only in intraperitoneal adipose tissue and liver. The intraperitoneal adipose tissues were almost occupied with necrotic inflammatory cells (Fig. 3.4a). In the liver, hepatitis, necrosis and invasion of the hyphae were commonly seen. The infiltration of mononuclear cells were obviously found around the hyphae and most of them necrotized (Fig. 3.4b). Tissue degeneration was also shown in the spleen (Fig.

3.5a) and kidney (Fig. 3.5b). In the kidney, hematopoietic cells and renal tubes necrotized (Fig. 3.5b).

Fig. 3.1. Gross lesions in the younger fish at 20 d.p.i. with *O. humicola.* Ulceration at the injected area (an inserted picture) and hepatic haemorrhages (arrow) with ascites accumulation were observed

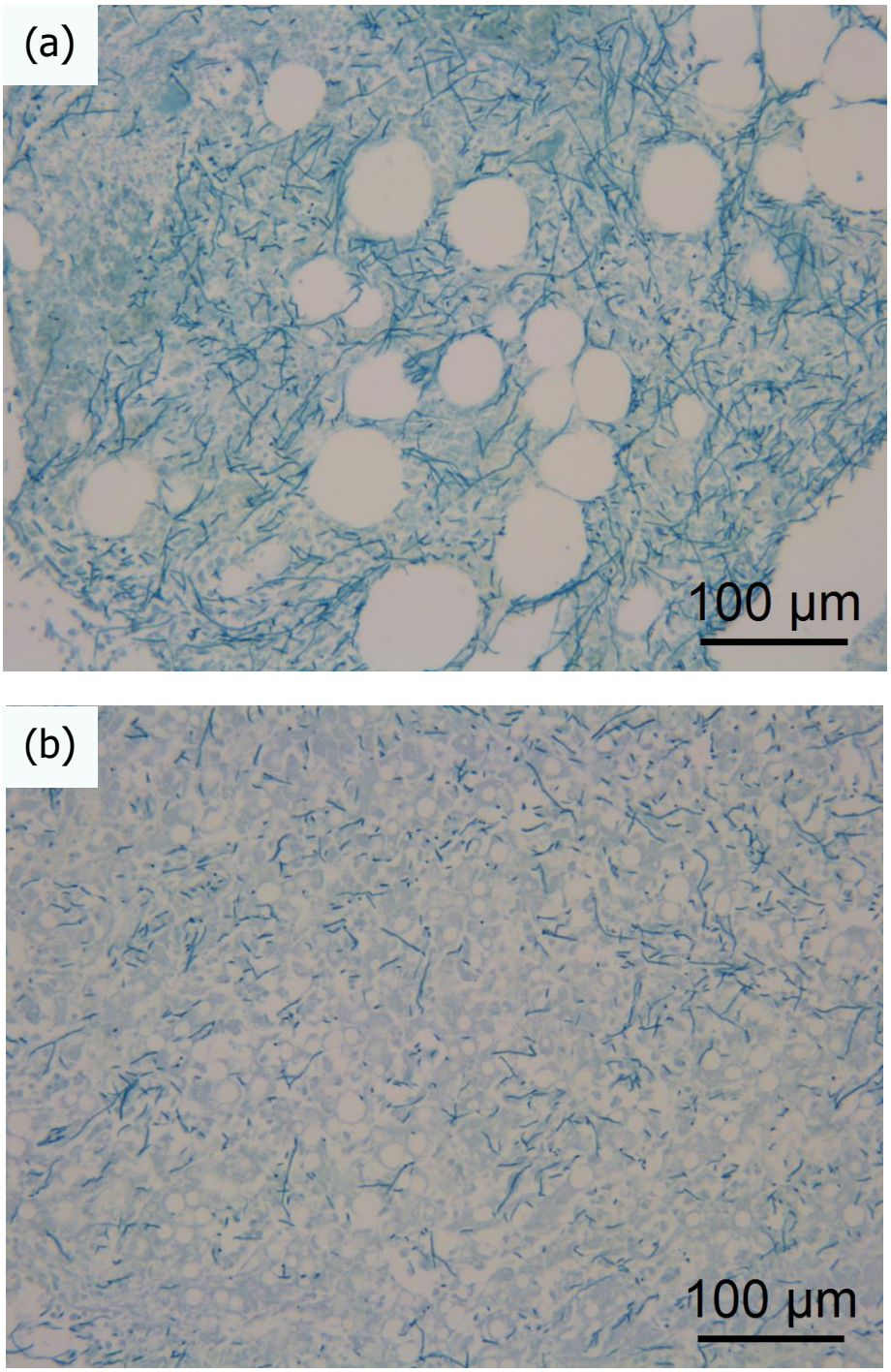

Fig. 3.2. Hyphal penetration in the intraperitoneal adipose tissues (a) and liver (b) of the younger fish died 15 d.p.i. The fungal hyphae were Schmorl positive (blue colour). Scale bar: 100 µm.

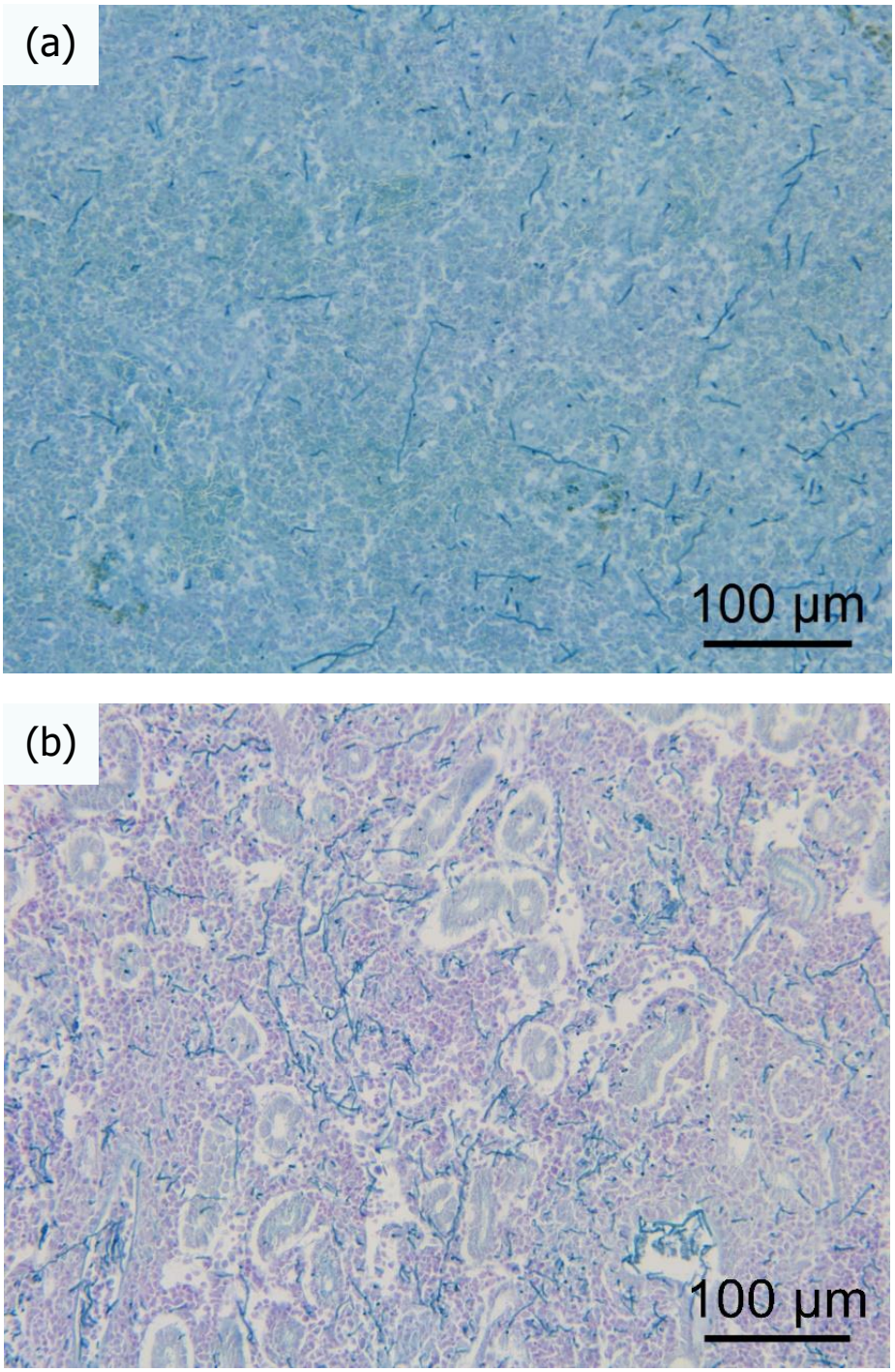

Fig. 3.3. Hyphal penetration in the spleen (a) and the kidney parenchyma (b) of the younger fish at died 24 d.p.i. The fungal hyphae were Schmorl positive (blue colour). Scale bar: 100 µm.

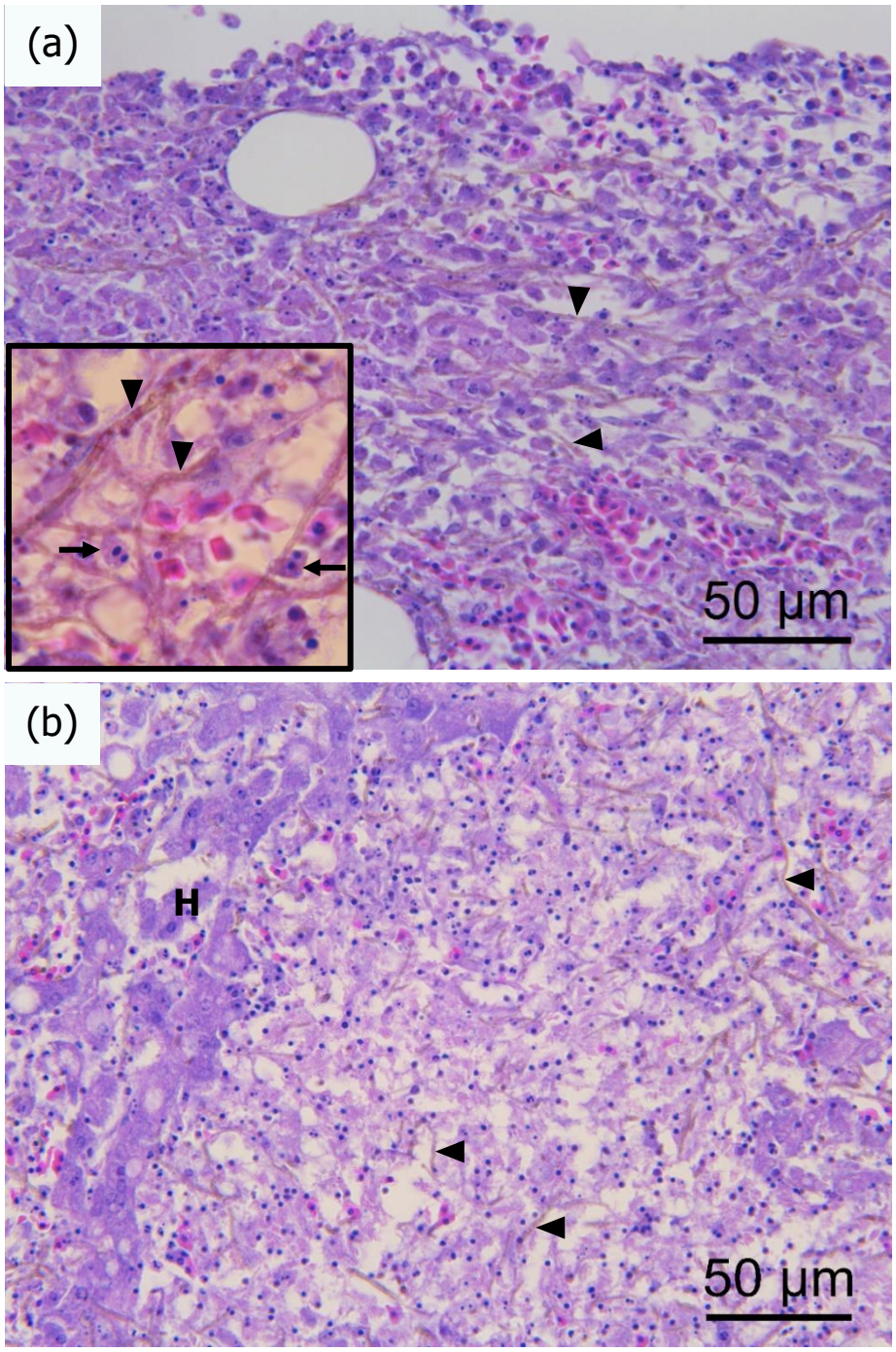

Fig. 3.4. Necrosis and inflammation were observed in the adipose tissues (a) and liver (b) of the younger fish died 21 d.p.i. (a) Inflammatory cells were infiltrated in the adipose tissues and associated with fungal hyphae (arrowheads). The insert picture showed inflammatory cell degeneration with nuclear fragmentation and pyknosis (arrows). H & E staining. Scale bar: 50 µm. (b) Hepatocytes (H) were replaced by numerous mononuclear cells and associated with fungal hyphae (arrowheads). H & E staining. Scale bar: 50 µm.

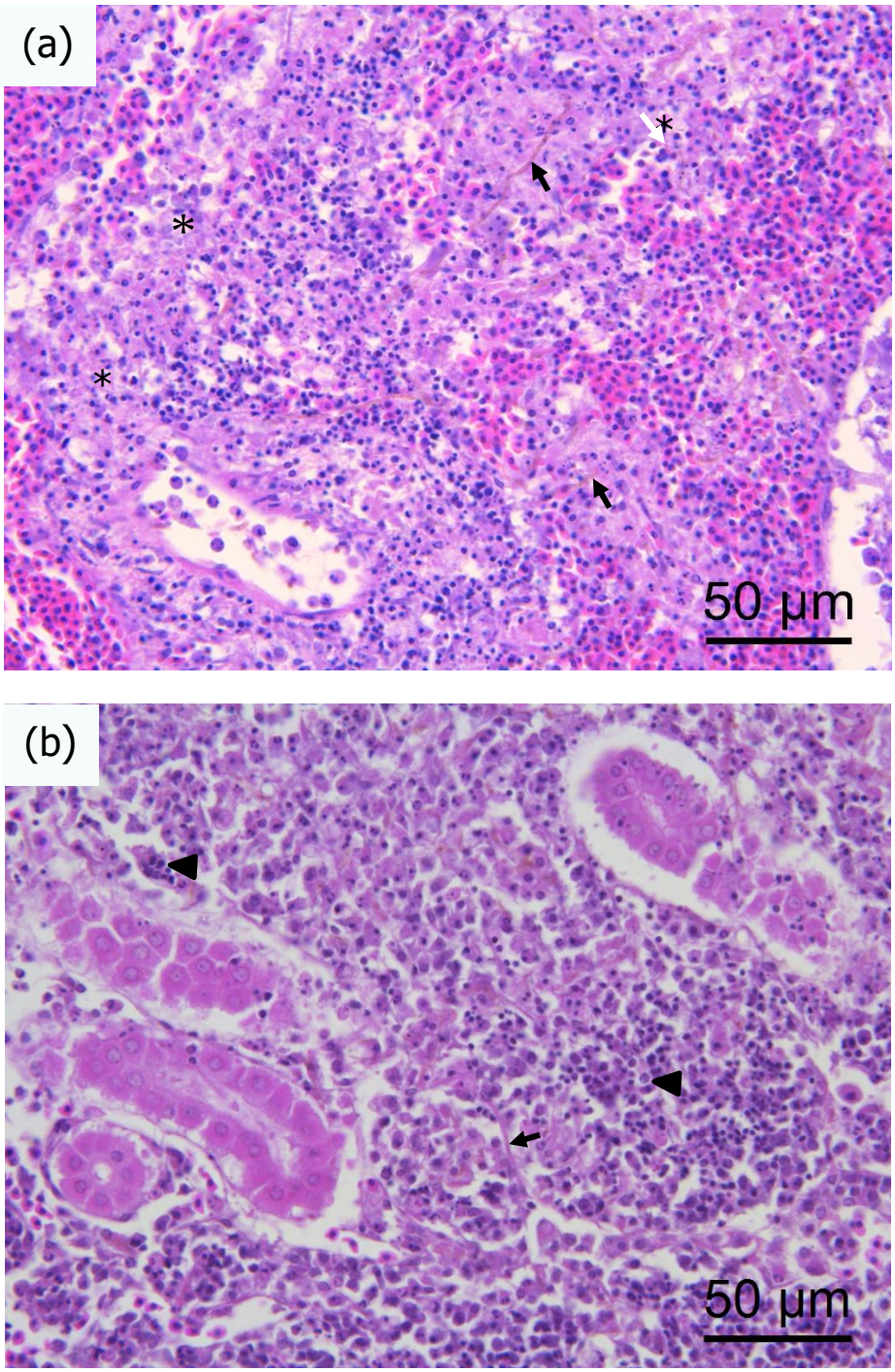

Fig. 3.5. Necrosis and inflammation were observed in the spleen (a) and kidney (b) of the younger fish died 24 d.p.i. and 21 d.p.i., respectively. (a) Splenititis (*) and the fungal hyphae (arrows) were observed in the parenchyma. H & E staining. Scale bar: 50 μm. (b) Necrotic degeneration of hematopoietic cells (arrowheads) associated with fungal hyphae (arrow). H & E staining. Scale bar: 50 μm.

Older fish

In contrast to the younger fish, the older fish showed neither inflammatory response nor hyphal invasion in the liver, the spleen and the kidney. However, apparent granulomatous inflammation was observed in only the peritoneal adipose tissues of 4 fish among 10 injected fish (Fig. 3.6). Formation of collagen fibres characterized the epithelioid cell granuloma in the adipose tissues (Fig. 3.7a). Also, invasion of the fungal hyphae was limited in the granulomatous inflammatory area (Fig. 3.7b).

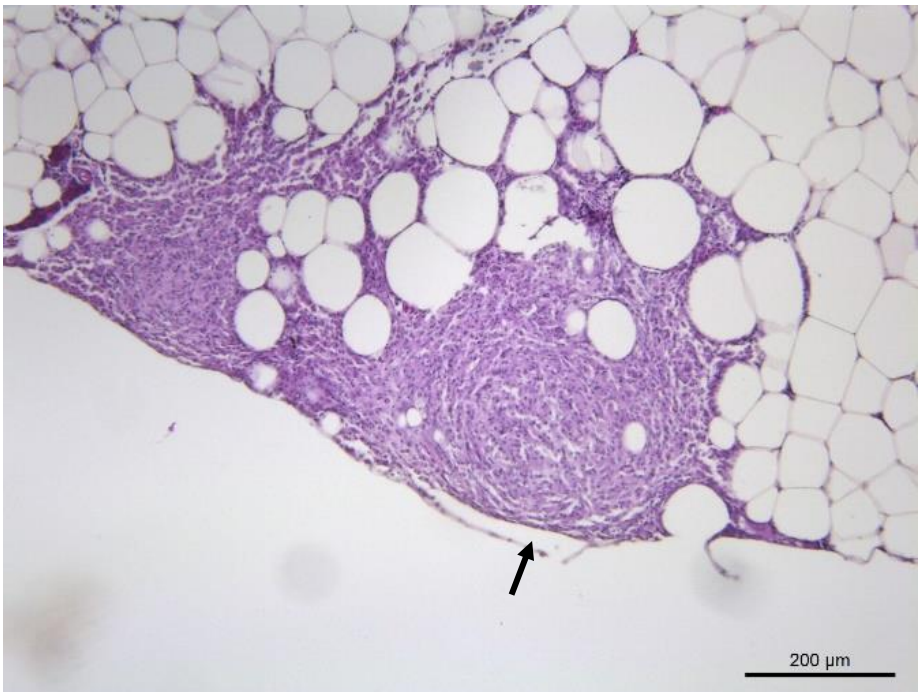

Fig. 3.6. The epithelioid cell granuloma (arrow) was observed in the adipose tissues of the older fish at 30 d.p.i. H&E staining. Scale bar: 200 µm.

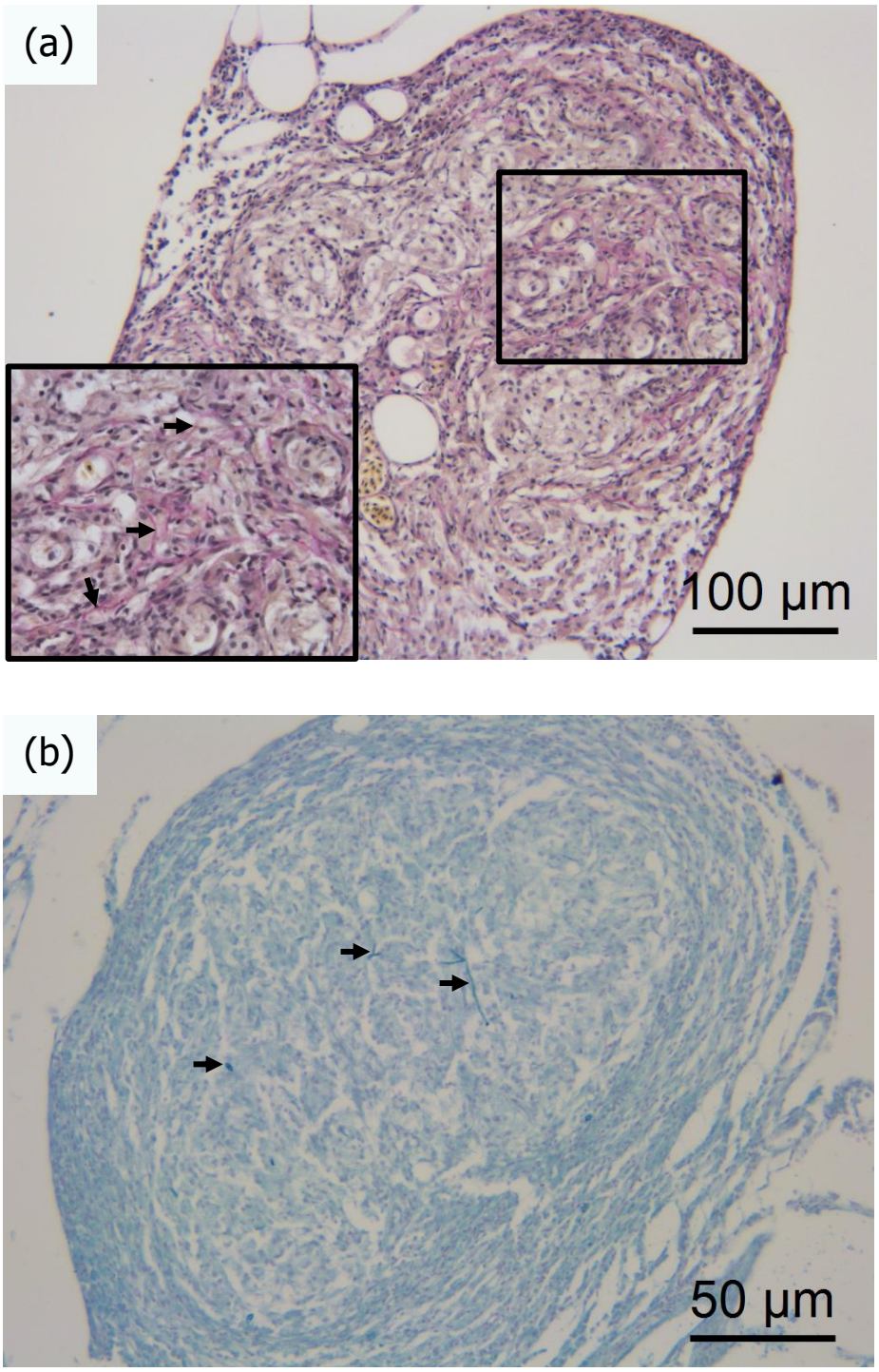

Fig. 3.7. (a) The outer layer of epitheliod cell granuloma containing collagen fibres (arrows) showing EV positive (red colour). Scale bar: 50 µm. (b) Fungal hyphae showing Schmorl-positive (arrows) were encapsulated inside the granuloma. Scale bar: 100 µm.

3.3.2 Experiment 2

The prominent pathological changes were observed in intraperitoneal adipose tissues and the liver of younger marbled rockfish. No obvious pathological changes were observed in the spleen and the kidney of both younger and older marbled rockfish in this experiment.

3.3.2.1 Intraperitoneal adipose tissues

In the younger fish, histopathological features appeared in intraperitoneal adipose tissues at 3 d.p.i., and the inflammation diffused over time. By day 3, infiltration of mononuclear cells was occasionally observed in 2 out of 3 experimental fish, and the cells surrounded the fungal elements (Fig. 3.7a). On day 5, the infiltration of inflammatory cells became more evident in all 3 experimental fish. A small number of fungal elements were observed together with round- or flat-shaped mononuclear cells (Fig. 3.7b).

The all experimental fish developed the granulomatous inflammation from day 7 to day 10 (Fig. 3.8a), and the number of fungal hyphae was increased. At day 13, severe inflammation associated with a high number of fungal hyphae were observed in all 3 experimental fish (Fig. 3.8b). Mononuclear cells were highly infiltrated, and some of them showed cell degeneration with nuclear fragmentation and pyknosis (Fig. 3.9). No obvious histopathological features were found in the adipose tissues of the older fish in the whole experimental period.

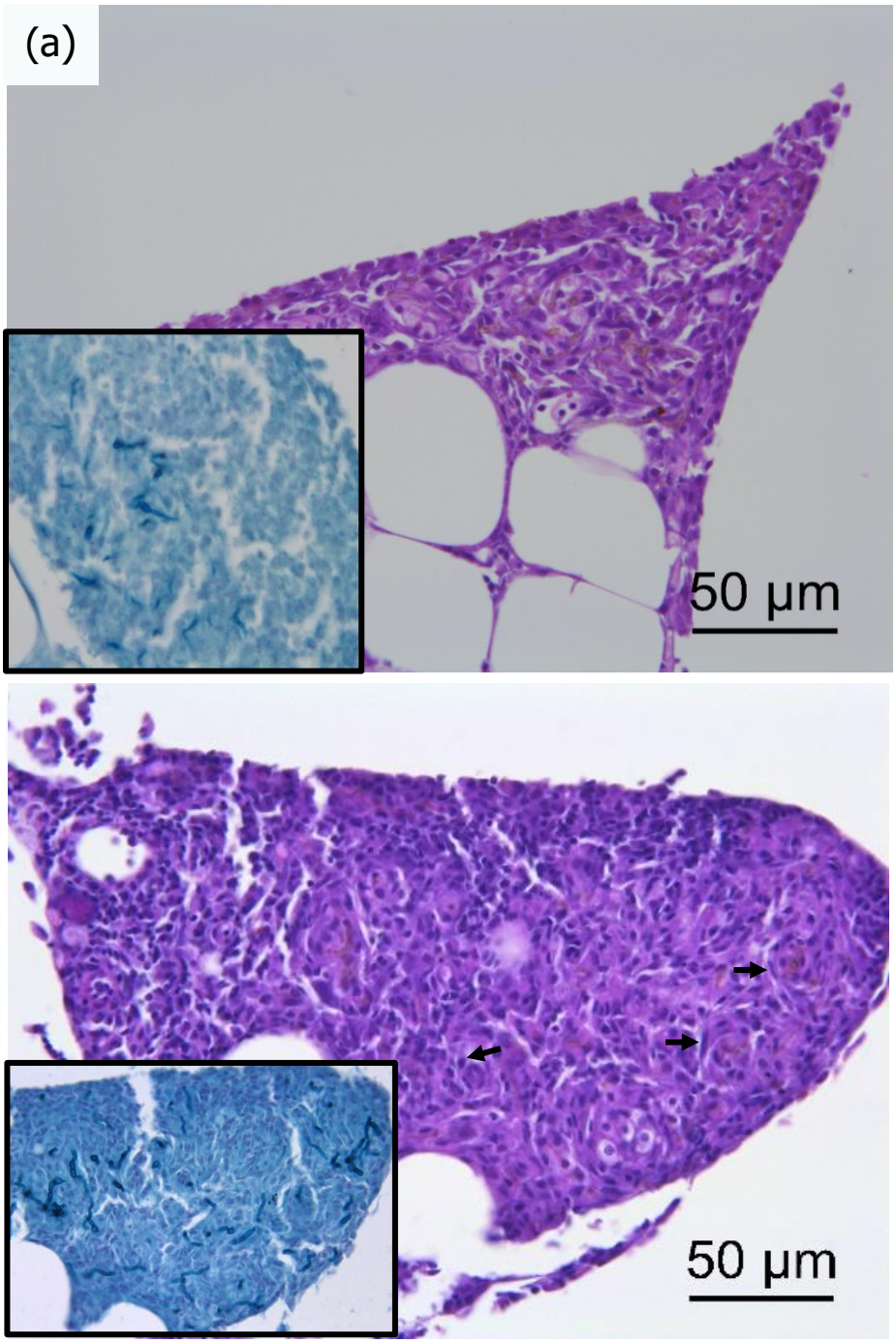

Fig. 3.7. Histopathological observation of the intraperitoneal adipose tissue of the younger fish. (a) Weak inflammation associated mononuclear cell infiltration and fungal elements (insert) were with Schmorl positive (blue colour) on 3 d.p.i. H & E staining. Scale bar: 50 μm. (b) At day 5, infiltration of round- or flat-shaped mononuclear cells (arrows) and fungal elements (insert) were with Schmorl positive (blue colour). H & E staining. Scale bar: 50 μm.

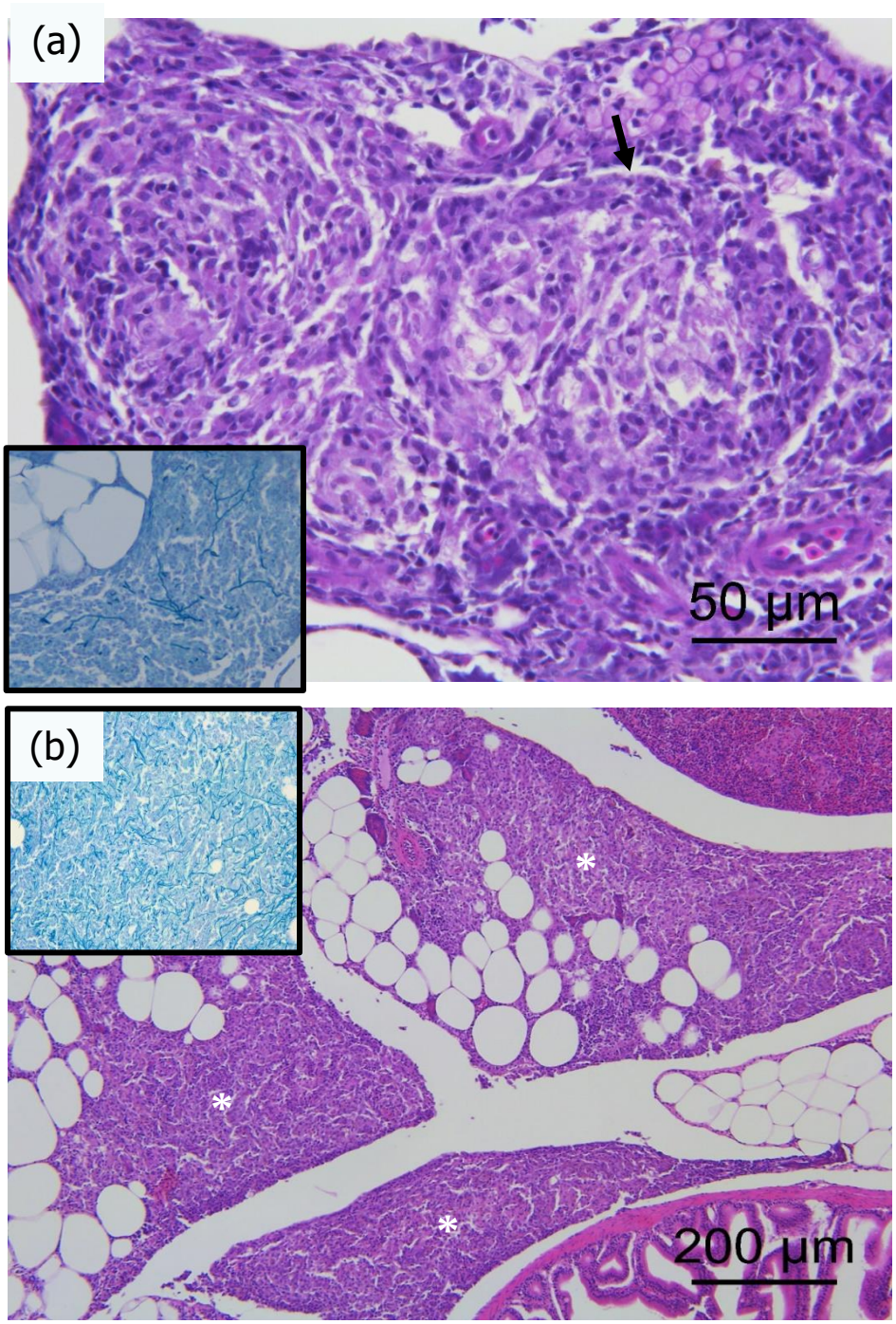

Fig. 3.8. Intraperitoneal adipose tissues observed in the younger fish at 10 d.p.i. (a) and 13 d.p.i. (b). (a) At day 10, more prominent granulomatous inflammation (arrow) was observed. H & E staining. Scale bar: 50 µm. (b) At day 13, granulomatous inflammation (*) highly observed in the adipose tissues. H & E staining. Scale bar: 200 µm. Both inserts showed the fungal hyphae with Schmorl positive (blue colour).

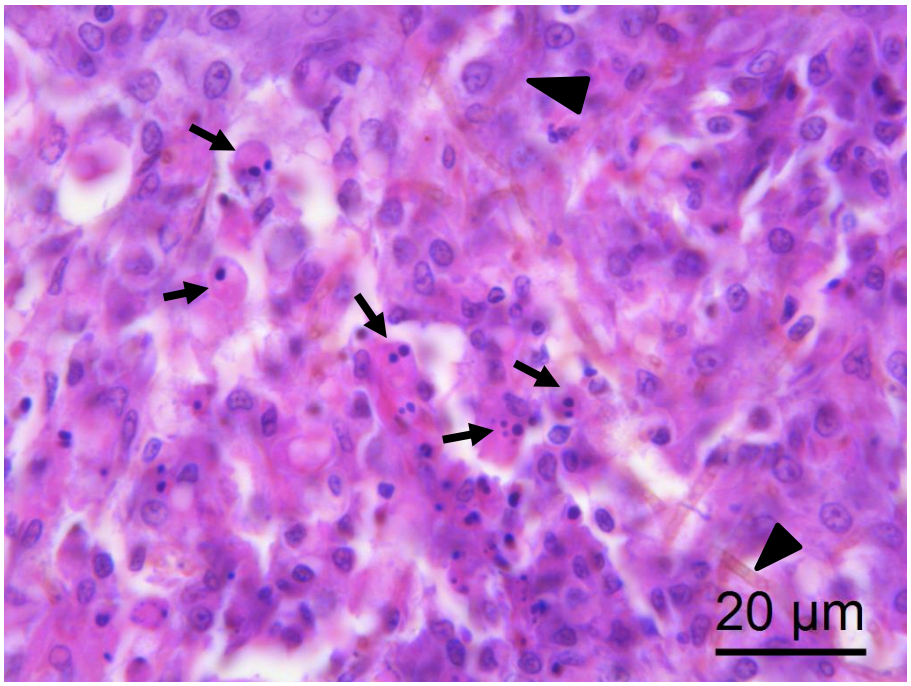

Fig. 3.9. A high number of mononuclear cells were infiltrated in response to fungal hyphae (arrowheads), and some were necrosis (arrows) in adipose tissues of younger fish at 13 d.p.i. H&E staining. Scale bar: 20 µm.

3.3.2.2 Liver

At 3 and 5 d.p.i, inflammation was not observed in the liver of all younger experimental fish, but a small number of fungal hyphae were occasionally found in the liver parenchyma (Fig. 3.10a). Aggregation of mononuclear cells around blood vessels appeared (Fig. 3.10b) in all 3 experimental fish at 7 d.p.i. and associated with a small number of fungal hyphae (Fig. 3.10b). Multifocal hepatitis was observed in the liver of all experimental fish at 10 d.p.i (Fig. 3.11a), and the mononuclear cells were diffused around the invading hyphae (Fig. 3.11b). Prominent granulomatous inflammation was observed at 13 d.p.i. (Fig. 3.12a) and the fungal hyphae were associated with the granuloma (Fig. 3.12b). However, all older experimental fish sampled each time showed normal structure without inflammation and hyphal invasion.

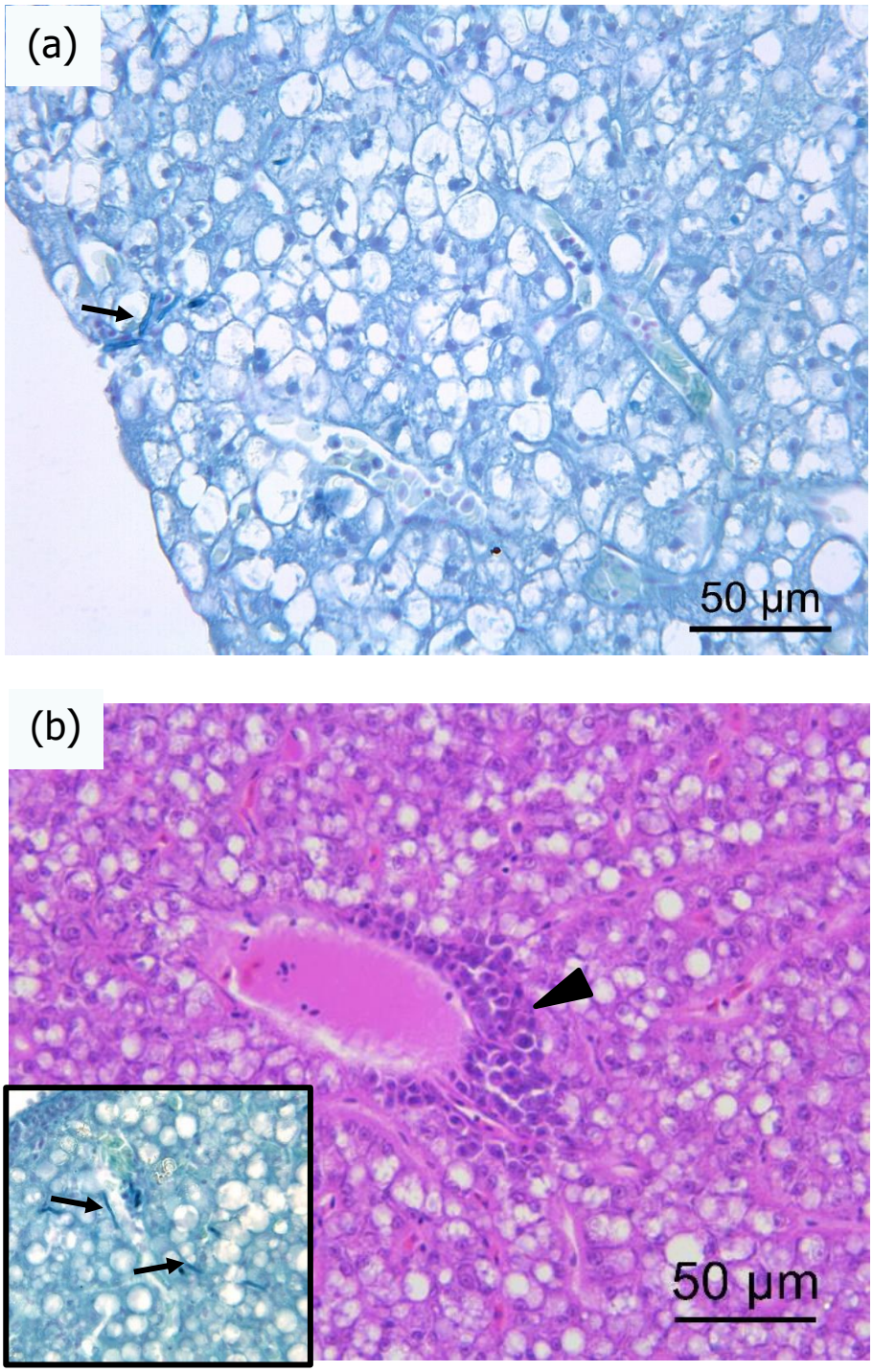

Fig. 3.10. (a) A small number of fungal hyphae (arrow) in the liver parenchyma of the younger fish at 5 d.p.i. Schmorl staining. Scale bar: 50µm. (b) Mononuclear cells (arrowhead) aggregated around the blood vessels in the liver of the younger fish at 7 d.p.i. H & E staining. Scale bar: 50 µm. Insert showed fungal hyphae with Schmorl positive (arrows) were scattered in the parenchyma.

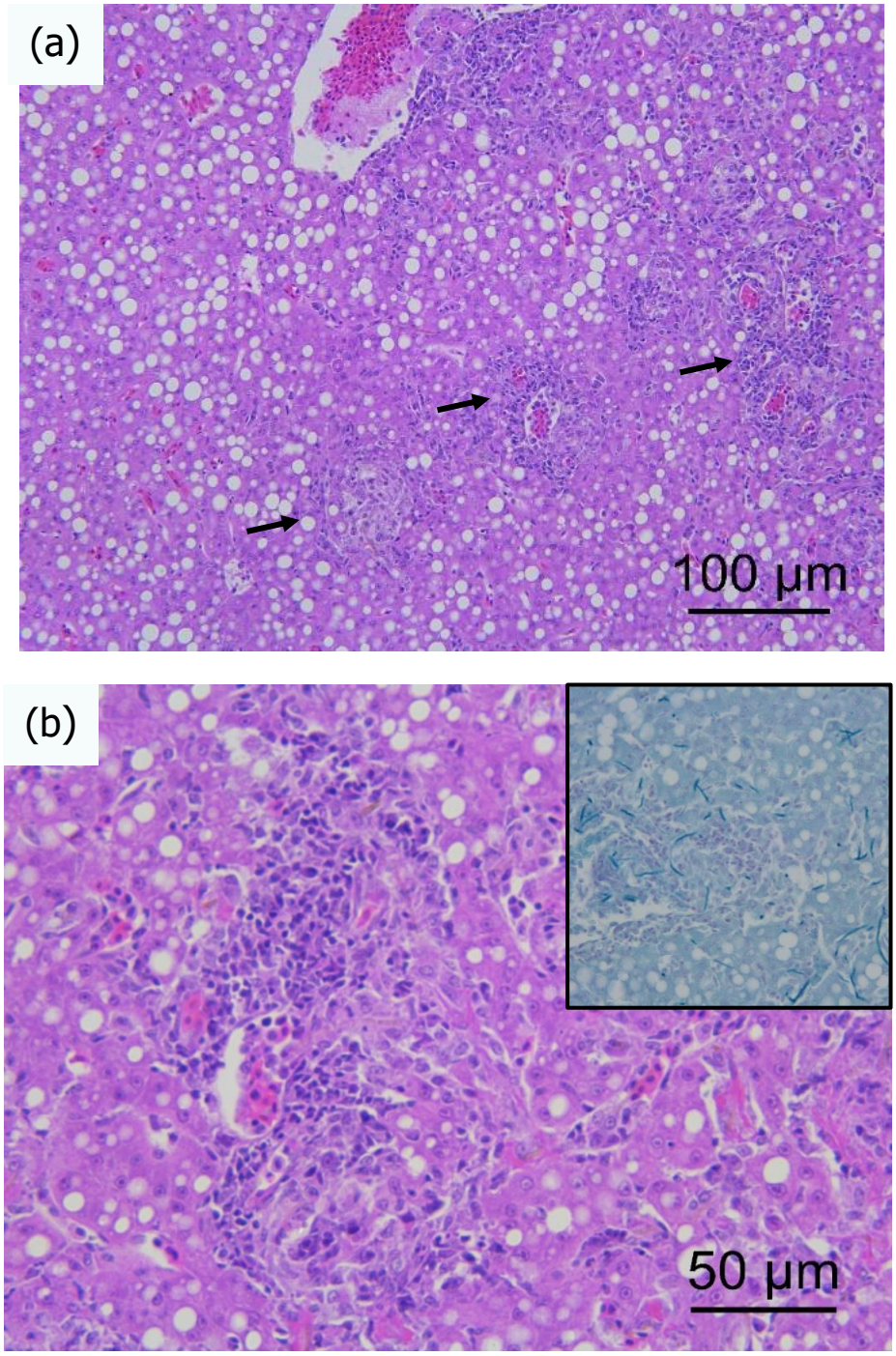

Fig. 3.11. (a) Multifocal hepatitis (arrows) were observed in the liver of the younger fish at 10 d.p.i. H & E staining. Scale bar: 100 µm. (b) Infiltration of mononuclear cells around the fungal hyphae in the liver parenchyma of the younger fish at 10 d.p.i. Insert picture showed the fungal hyphae with Schmorl positive (blue colour). H & E staining. Scale bar: 50µm

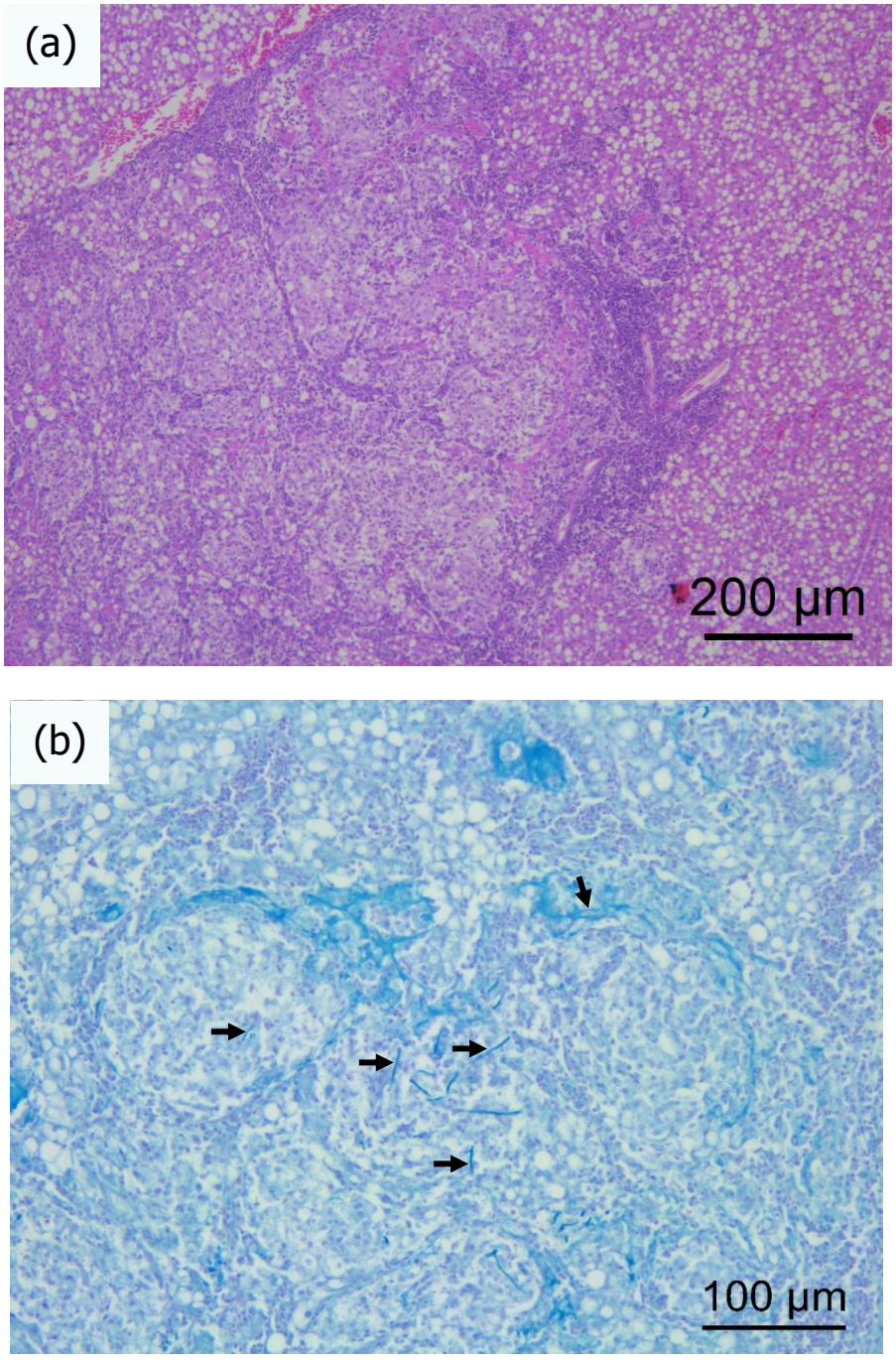

Fig. 3.12. (a) Prominent granulomatous inflammation was observed in the liver of the younger fish at 13 d.p.i. H & E staining. Scale bar: 200 µm. (b) The fungal hyphae were Schmorl-positive (arrows) and associated with the granuloma. Scale bar: 100 µm.

3.4 DISCUSSION

In the case of younger marbled rockfish, fungal hyphae were scattered around in the intraperitoneal adipose tissues, the liver, the spleen and the kidney. Severe inflammation involving the infiltration of mononuclear cells and tissues necrosis was also observed. By contrast, older marbled rockfish fish did not show prominent inflammation and hyphal invasion in the organs, except for the appearance of granuloma in the adipose tissues of some experimental fish. From these observations, I demonstrated that the younger fish developed histopathological features such as inflammation and hyphal invasion than the older fish in the experimental challenge by intraperitoneal injection with *O. humicola*.

The first experiment demonstrated that injection of *O. humicola* caused mortality and histopathological changes in younger marbled rockfish. All examined younger fish were moribund or dead. Almost all the fish showed infiltration of a lot of mononuclear cells and invasion of numerous hyphae in the examined organs. Severe necrosis of the examined organs was also characterized in the younger fish. The necrotic cells were observed in not only inflammatory cells but also parenchymal cells.

The second experiment showed the development of inflammation in the organs of the younger marbled rockfish after intraperitoneal injection with *O. humicola*. The younger fish showed a quick response in the histopathological changes. At 3 d.p.i., appearance of mononuclear cells was firstly observed in the adipose tissues. In the adipose tissue, the infiltration of

mononuclear cells progressed over time. Inflammatory cell infiltration was also observed in the liver followed by the changes in the adipose tissues.

Both younger and older marbled fish developed the formation of inflammation in the adipose tissues, but the inflammation in the older fish was limited in the small area. Furthermore, the younger fish showed a quick inflammatory response after the injection with *O. humicola* in the adipose tissues. Pignatelli et al. (2014) reported that teleost adipose tissue is capable of collecting antigens from the peritoneal cavity. The adipose tissues of rainbow trout (*Oncorhynchus mykiss*) act as an active immune site capable of playing a role in immune responses (Veenstra et al., 2019). The findings in this study which a quick inflammatory response in the adipose tissues may be related to the immune function of fish adipose tissues.

3.5 SUMMARY

The results clearly showed that histopathological changes in younger marbled rockfish was characterized by intense inflammatory response, while low or no inflammatory response was characterised in older marbled rockfish. Even though the younger fish well developed the inflammatory response against the hyphal invasion in several organs, eventually the inflammation could not prevent the hyphal invasion and the fish were mortal. 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 defence against the pathogen *O. humicola.*

CHAPTER 4

Molecular cloning and characterization of immune relevant genes of Marbled rockfish, *Sebastiscus marmoratus*

4.1 INTRODUCTION

In chapter 2 and 3, the disease development of *O.humicola* infection in marbled rockfish was demonstrated. The older fish were believed to be disease resistant to this infection. Both younger and older fish showed different inflammatory response to *O. humicola* injection. It is reasonable to say that the resistance or susceptibility to *O. humicola* injection may be caused by the host immune system. However, the immune mechanism involved in the resistance or susceptibility development remains unknown.

The functional development of the cell-mediated immunity (CMI) in fish has been investigated in some fish and the onset of maturation of the immune response is important for host protection (Ellis, 2005). The functional maturation of T cells in common carp, and rainbow trout was observed 16 days post-hatching (d.p.h.) (Botham & Manning, 1981) and 14 d.p.h. (Tatner & Manning, 1983), respectively. The late appearance of lymphocytes were observed in the lymphoid organs of marbled rockfish. The morphologically matured lymphocytes appeared in the thymus at three weeks post-birth, followed by the pronephros at four weeks and the spleen at six weeks (Nakanishi, 1991). Nakanishi (1986) suggested that immunological maturation is apparently depended on the age and the cell-mediated immunity in marbled rockfish was not sufficiently matured for 1 to 2 months after post-hatching.

Inflammation plays an important role in the elimination of any infiltrating pathogens (Nathan & Ding, 2010). Cytokines are one of the important soluble mediators to regulate the onset, progression and resolution

of the inflammatory response (Grayfer & Belosevic, 2012). Cytokines such as interleukin-1 beta (IL-1β) and interferon gamma (IFNγ) enhance antimicrobial functions of immune cells and help in pathogen clearance (Zou & Secombes, 2016).

IL-1β protein has been known to be important in the mammalian immune system, which is an essential cytokine to trigger inflammatory and immune response (Dinarello, 1997). The biological activity of recombinant IL-1β has been studied in several fish species, indicating that fish IL-1β is involved in the regulation of immune relevant genes, lymphocyte activation, migration of leukocytes and phagocytosis (Hong et al., 2001; Peddie et al., 2001; Buonocore et al., 2005; Reyes-Cerpa et al., 2012). The bioactivity of teleost IFN-γ has been studied about activation of macrophages in several fish species (Zou et al., 2005; Jung et al., 2012; Yang et al., 2017).

The study of the immune system in marbled rockfish helps to reveal the difference between the younger and the older fish in immunological response to *Ochroconis* infection. However, understanding of immunity in marbled rockfish is still largely unexplored. Recently, the development of immunological study is achieved by molecular analysis of immune relevant genes. Therefore, identification of immune genes such as T cell markers and cytokines in marbled rockfish is necessary for the study of interaction between host immune response and infection. The purpose of this chapter was to identify and characterize marbled rockfish immune relevant genes such as T cell markers (CD4, CD8β, CD3ε, CD28), and cytokines (IL-1β and IFN-γ).

4.2 MATERIALS AND METHODS

4.2.1 RNA sequencing

To get RNA sample, a marbled rockfish (6.4 g, 7.5 cm) was stimulated by intraperitoneal injection with *Mycobacterium* sp. (NJB 1604). The isolate was incubated on Middlebrook 7H10 agar supplemented with 10% OADC enrichment. The bacterial suspension was prepared by suspending a bacterial colony with 1mL of phosphate buffered saline (PBS). The concentration of bacteria was adjusted to 1 x 10^6 CFU/mL. Marbled rockfish were anaesthetised using FA100 and intraperitoneally injected with 50 µL of the bacterial suspension. Fish was sacrificed after one week, and the kidney tissues were removed for RNA extraction. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad CA, USA) following the manufacturer's instruction. RNA sequencing was performed using the RNA sample by Macrogen Japan Co.

4.2.2 Primer designs

Partial sequences of several immune relevant genes were found from the RNA sequencing data. Gene-specific primers were designed based on the partial sequences for determination of the full-length gene sequences by a Rapid amplification of cDNA ends (RACE) method (Table 4.1).

4.2.3 Cloning of the full-length immune relevant gene cDNA

First-strand cDNA was synthesized with 1μg of total RNA using ReverTra Ace RT Master Mix (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. The full lengths of marbled rockfish immune relevant gene cDNA were determined by 3′ – and 5′- RACE methods using FirstChoice RLM-RACE kit (Invitrogen) according to the manufacturer's instruction.

PCR products obtained by RACE methods were purified by a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and ligated into pGEM T easy vector (Promega, WI, USA) and then transfected into competent cells of JM109 *Escherichia coli* (Promega, WI, USA). The positive clones were screened by ampicillin selection and colony PCR. The plasmids inserted with target genes were purified by NucleoSpin®Plasmid EasyPure (Takara, Shiga, Japan) according to the manufacturer's instruction. The DNA sequencing was performed by FASMAC Co. Ltd.

4.2.4 Structural and phylogenetic analyses

The nucleotide sequences were edited and translated into amino acid sequences using GENETYX ver 11.0 (GENETYX, Tokyo, Japan) and the similarities were compared using BLASTX of National Centre for Biotechnology Information (NCBI). Signal peptide, the transmembrane domains, N-linked glycosylation sites and O- glycosylation sites of the proteins were predicted using the following internet tools, www.cbs.dtu.dk/services/SignalP.

www.cbs.dtu.dk/services/TMHMM, www.cbs.dtu.dk/services/NetNGlyc, http://www.cbs.dtu.dk/services/NetOGlyc/, respectively. Immunoglobulin-like domains of the proteins were predicted by SMART online tool http://smart.embl-heidelberg.de/. Multiple sequence alignments were generated using Clustal Omega program www.ebi.ac.uk/Tools/msa/clustalo/. Phylogenetic trees were constructed by the neighbour-joining method using MEGA version 7.0 software (Kumar et al., 2008) and validated with 1000 bootstrap replications.

Table 4.1. Primer designed for 5'- and 3'-RACE PCR.

Gene	Primer sequence (5' - 3')
SebCD4 F (3ROP)	CACCATTTCCACCCGACTC
SebCD4 RT F (3RIP)	CTCACTGATCATCACAAACATC
SebCD4 R (5ROP)	GTTTTGCATTGGCAGAGTTTAC
SebCD4 R2 (5RIP)	GAGAGGCCCGGATTTAGACGTATAC
SebCD28 F (3ROP)	CTGTGTACCTGCCGGAAAC
SebCD28 F2 (3RIP)	CACACTCTCAGTGCAAAAGATC
SebCD28 R (5ROP)	GACGCGGTATAGGAATTTGAAC
SebCD28 R2 (5RIP)	CTCCAGTGAGCATGAAAGTGTC
SebCD8β F (R) (3ROP)	GTGGACGAAAAACGCATC
SebCD8β RT F (3RIP)	GTGACCAAAGAGGACACAG
SebCD8β RT R (5ROP)	GGTTTGGGCTTTGAGAAAAAG
SebCD8β RT R2 (5RIP)	CACCTGGATAAATAATGTTGTCATC
SebCD3e RT R (3ROP)	CAAACATGTATTCTCATTTACCTC
SebCD3e RT R2 (3RIP)	CAGGTGACTTGTCTCTATCCCATC
SebCD3e RT F (5ROP)	GAAGCCGGGAATTGGTACAAG
SebCD3e RT F2 (5RIP)	CATTATTTTGGACATGTTGTTGAC
SebIL-1β F2 (3ROP)	CTGTCCGATGCTTTAGACAGCTC
SebIL-1β F (3RIP)	GTAGAAGAGGAACCGGTCCATG
SebIL-1β R (5ROP)	CAGACGATGCCTCCATCTAG
SebIL-1β R2 (5RIP)	GGTGACCGAAACCATCGTC
SebIFNg2 RT F (3ROP)	GGAGACGTACGAAAAGCTG
SebIFNg2 RT F2 (3RIP)	GACAGCCGAGAGCAACAAC
SebIFNg2 RT R (5ROP)	CAGAAAAGAAATATGAAAAAGTTGG
SebIFNg2 RT R2 (5RIP)	CAATGCTTTGCTCTGGACAAC

3ROP: outer primer for 3' RACE, 3RIP: inner primer for 3' RACE,
5ROP: outer primer for 5' RACE, 5RIP: inner primer for 5' RACE

4.3 RESULTS AND DISCUSSION

4.3.1 Characterization of *Sebastiscus marmoratus* CD4 (SebCD4)

The full-length cDNA SebCD4 (GenBank accession no. LC565789) was found to be 2320 bp and including an open reading frame that encoded 464 amino acids. SebCD4 contained a signal peptide, four extracellular Ig domains (D1-D4), a transmembrane region (TM) and a cytoplasmic tail with p56 Lck binding (C-X-C) motif that plays an important role for T cell activation (Edholm et al., 2007; Buonocore et al., 2008). An analysis of SebCD4 sequence predicted four potential N-linked glycosylation sites and one O-glycosylation site.

To date, CD4 has been reported from a few fish species, including fugu (Suetake et al., 2004), rainbow trout (Laing et at., 2006), channel catfish (Edhom et al., 2007), ginburna crucian carp (Nonaka et al., 2008), European seabass (Buonocore et al., 2008), Atlantic halibut (Patel et al., 2009), Atlantic salmon (Moore et al., 2009) and large yellow croaker (Mao et al., 2017). Multiple alignment of SebCD4 amino acid sequence with other teleosts, mouse and human CD4 sequences was shown in Fig. 4.1. Phylogenetic tree analysis showed that SebCD4 formed a cluster with CD4-1 molecules in other fish species (Fig. 4.2). SebCD4 shared highest amino acid identity with orange-spotted grouper CD4 (63 %), but the lowest identity 16-25 % with mouse CD4 and human CD4 (Table 4.2).

The SebCD4 has many common characteristics of other teleost species such as the primary structure and conserved amino acid residues. The cysteine residues found in SebCD4 were mostly conserved among other CD4 molecules but not all. When compared with mouse and human CD4 sequences, teleost CD4 including SebCD4 lacks the first cysteine in D1 (Moore et al., 2009). Moreover, SebCD4 lacks 2nd cysteine within the domain which conserved among other teleost CD4 sequences in D1.

The D2 and D4 of SebCD4 has a pair of cysteine residues, like other teleosts, mouse and human CD4. D3 domain of SebCD4 has two conserved cysteine residues that only found in teleost CD4 molecule. These cysteines residues in D3 which could be involved in the formation of an additional disulphide bond which lacking in human and mouse CD4 molecules (Patel et al., 2009). The difference in the number and location of the disulfide bonds in CD4 molecules has been observed. Dog, cat and whale CD4 lack the first cysteine in D2 for forming disulfide bond (Norimine et al., 1992; Milde et al., 1993; Koskinen et al., 1999), and both cysteines were missing in D3 of human and mouse CD4 molecules. The interaction between SebCD4 and the MHC II molecule may be similar to that in other teleost CD4s, as the disulfide bonding pattern in the Ig domains is highly conserved in teleost.

Table 4.2. Amino acid identity of SebCD4 among teleost and mammalian species.

Species (scientific name)	GeneBank accession number	Amino acid identity (%)
Orange spotted grouper CD4 (*Epinephelus coioides)*	AER42417.1	63
Japanese seabass CD4 (*Lateolabrax japonicus)*	AFK73394.1	60
European seabass CD4 (*Dicentrachlus labax)*	CAO98732.1	56
Japanese flounder CD4-1 (*Paralichthys olivaceus*)	BAM7205.1	48
Fugu CD4-1 *(Takifugu rubripes)*	BAD37152.1	51
Common carp CD4 (*Cyprinus carpio)*	ABD58988.1	34
Channel catfish CD4-1 (*Ictalurus punctatus)*	ABD93351.1	34
Rainbow trout CD4-1 (*Oncorhynchus mykiss)*	AAY42070.1	41
Atlantic salmon CD4-1 (*Salmo salar*)	ABZ81916.1	41
Chicken CD4 (*Gallus gallus*)	CAA72740.1	21
Mouse CD4 *(Mus musculus)*	AAC36010.1	16
Human CD4 (*Homo sapiens*)	AAH25782.1	25

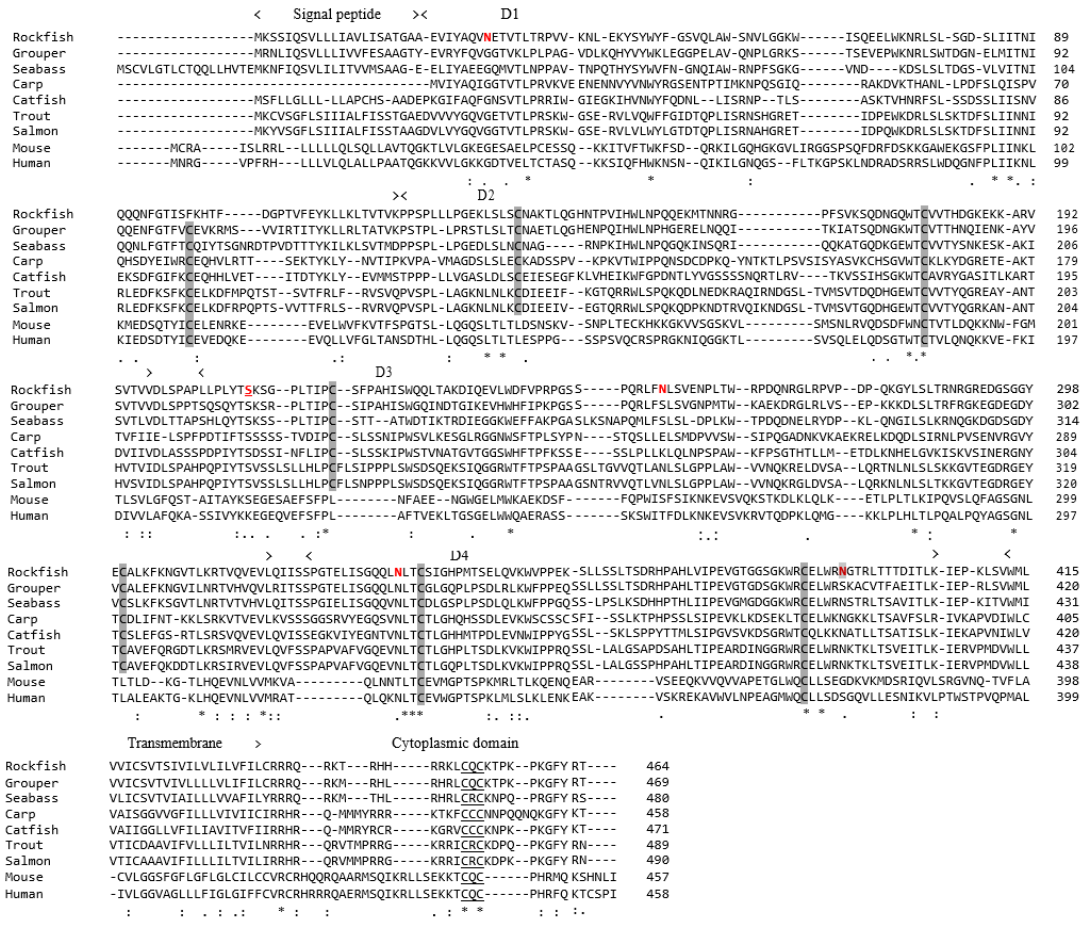

Fig. 4.1. Multiple alignment of teleost and mammalian CD4 amino acid sequences. The predicted signal peptide, four Ig domains (D1-D4), a transmembrane region and a cytoplasmic tail of SebCD4 are labelled above the alignment. The conserved cysteine residues among the species are shaded in grey, and the p56 Lck binding motif is underlined. The predicted N glycosylation sites were bold in red and O-glycosylation site was bold in red and underlined. Conserved residues within all the sequence are marked with asterisks (*), while double (:) and single dots (.) indicated the similarities between the amino acids. The corresponding GeneBank accession number: Orange-spotted Grouper (*Epinephelus coioides*, AER42417), European Seabass (*Dicentrarchus labrax*, CAO98732), Common Carp (*Cyprinus carpio*, ABD58988), Channel Catfish (*Ictalurus punctatus*, ABD93351), Rainbow Trout (*Oncorhynchus mykkiss*, AAY42070), Atlantic Salmon (*Salmo salar*, ABZ81916), Mouse (*Mus musculus*, AAC36010) and Human (*Homa sapiens*, AAH25782)

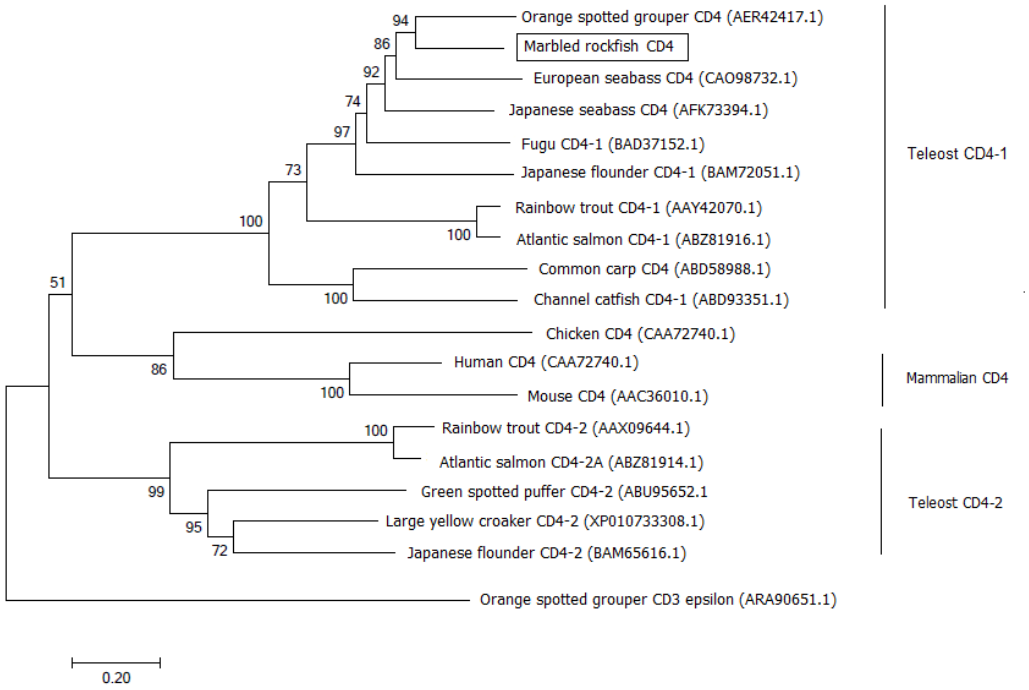

Fig. 4.2. Phylogenetic tree analysis of the Seb CD4 with other known teleost and mammalian CD4 molecules. SebCD4 was clustered into teleost CD4-1 molecules. The sequences were aligned using CLUSTAL W, and the tree was generated using neighbour-joining method in MEGA 7.0. Orange-spotted grouper CD3ε was used as an outgroup. The bootstrap values are shown at the branches, and the branch length scale indicated below the tree represented genetic distance.

4.3.2 Characterization of *Sebastiscus marmoratus* CD8β (SebCD8β)

The full-length cDNA was found to be 1253 bp (GenBank accession no. LC573977) and containing an open reading frame that encoded 210 amino acids. An analysis of SebCD8β sequence revealed the presence of a signal peptide, an extracellular Ig domain, a transmembrane region and a cytoplasmic tail. Two potential of each N-linked glycosylation sites and O-glycosylation sites were predicted.

To date, CD8β gene has been cloned from Atlantic salmon and brown trout (Moore et al., 2005), fugu (Suetake et al., 2007), common carp (Sun et al., 2007), Atlantic halibut (Patel et al., 2008) and orange-spotted grouper (Xu et al., 2011). The current result revealed that the overall structure of SebCD8β molecule was conserved among teleost and mammalian CD8β (Patel et al., 2008). Multiple alignment of SebCD8β amino acid sequence with other teleosts, mouse and human CD8β sequences was shown in Fig. 4.3.

A phylogenetic tree showed clustering the SebCD8β into other teleost species CD8β molecules but not into those CD8α molecules (Fig. 4.4). Among the teleost, SebCD8β shared highest amino acid identity with orange-spotted grouper CD8β (70%), but the lowest identity 26-28% with mouse and human CD8β (Table 4.3).

The Ig domain of SebCD8β contained four conserved cysteine residues. Of these cysteine residues, two were highly conserved in all CD8β to form intra chain disulfide bond in stabilising Ig domain (Suetake et al., 2007). Like in grouper CD8β (Xu et al., 2011), additional cysteine residues were observed

in Ig domain of SebCD8β. These extra cysteine residues conserved in teleost maybe the characteristics of teleost CD8β molecules but the physiological role was remained to be investigated (Sun et al., 2007). The conserved cysteine residues in the hinge region, potentially involved in dimerisation, which form inter chain disulfide bond in CD8αβ heterodimer (Duncan et al., 2009), were also found in SebCD8β

The cytoplasmic region of CD8α teleost lacks p56Lck (C-X-C) binding domain. Instead of C-X-C motif, all teleost CD8α possessed C-X-H motif at the cytoplasmic domain (Xu et al., 2011). The C-X-H motif found in teleost CD8α suggested that this motif may represent a primordial Lck binding site (Quiniou et al., 2011). All teleost CD8β including SebCD8β has conserved C-R-H motif (Patel et al. 2008), and Xu et al. (2011) suggested that this region found in CD8β has a similar function to C-X-H motif in teleost CD8α.

Table 4.3. Amino acid identity of SebCD8β among teleost and mammalian.

Species (scientific name)	GeneBank accession number	Amino acid identity (%)
Orange spotted grouper CD8β *(Epinephelus coioides)*	ACS68186.1	70
European seabass CD8β (*Dicentarchus labrax*)	AKC57344.1	56
Japanese flounder CD8β (*Palichthys olivaceus*)	BAM72050.1	61
Atlantic salmon CD8β (*Salmo salar*)	AAW33877.1	52
Rainbow trout CD8β (*Oncorhynchuss mykiss*)	AAT68458.1	50
Common carp CD8β (*Cyprinus carpio*)	ABC59224.1	37
Channel catfish CD8β (*Ictalurus punctatus*)	ACN96561.2	38
Human CD8β (*Mus musculus*)	AAA35664.1	26
Mouse CD8β (*Homo sapiens)*	NP_033988.1	28

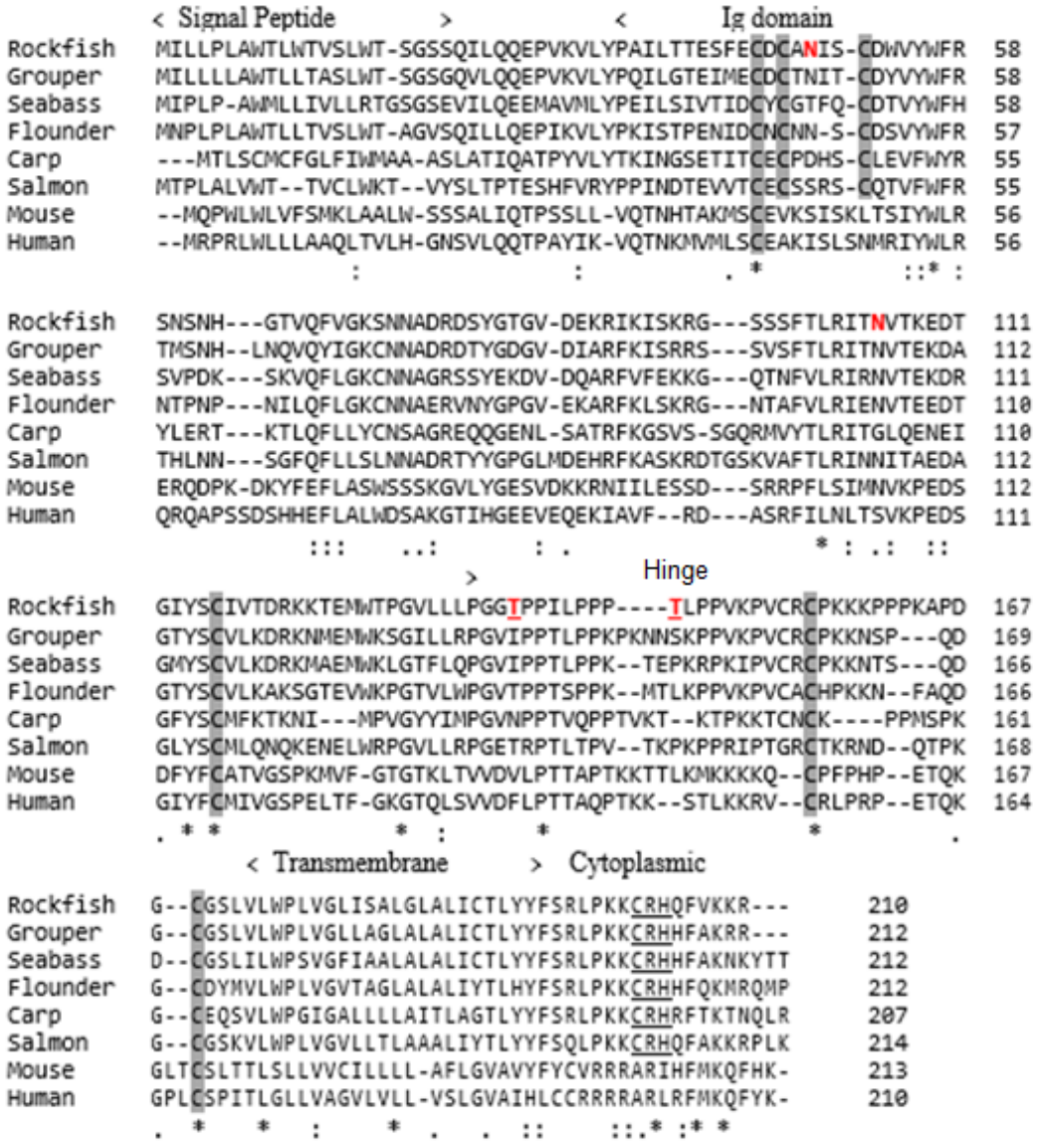

Fig. 4.3. Multiple alignment of teleost and mammalian CD8β amino acid sequences. The predicted signal peptide, Ig domain, transmembrane and cytoplasmic tail of SebCD8β are labelled above the alignment. Conserved cysteine residues are shaded in gray. The conserved CRH motif within the teleosts is underlined. The predicted N-linked glycosylation sites were bold in red and O-glycosylation sites were bold in red and underlined. Conserved residues within all the sequence are marked with asterisks (*), while double (:) and single dots (.) indicated the similarities between the amino acids. The corresponding GeneBank accession numbers: Orange-spotted Grouper (*Epinephelus coioides*, ACS68186), European Seabass (*Dicentrarchus labrax*, AKC57344), Japanese Flounder (*Paralichthys olivaceus*, BAM72050), Common Carp (*Cyprinus carpio*, ABC59224), Atlantic Salmon (*Salmo salar*, AAW33877), Mouse (*Mus musculus*, NP_033988) and Human (*Homo sapiens*, AAA35664).

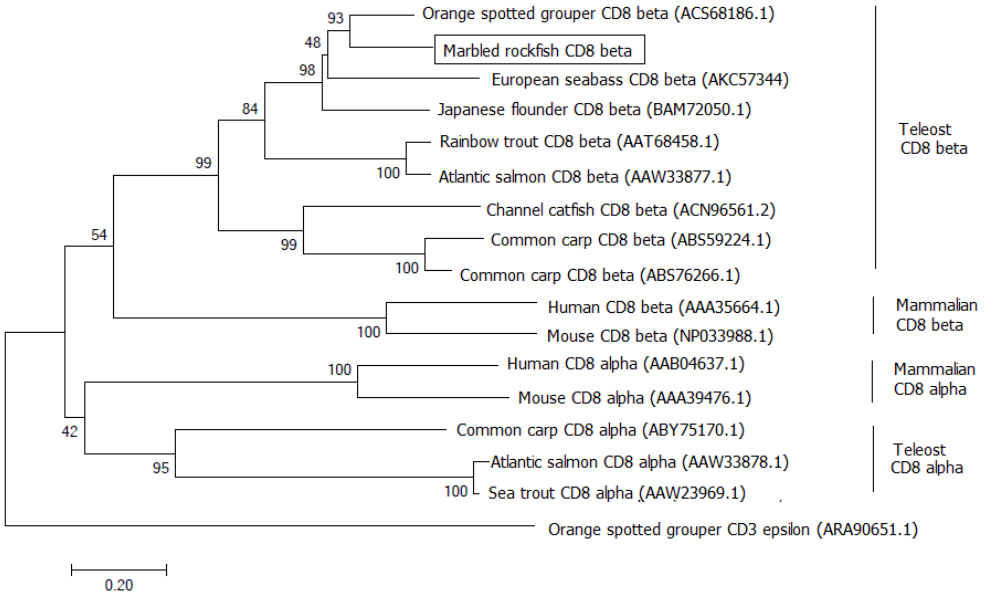

Fig. 4.4. Phylogenetic tree analysis of the Seb CD8β with other known teleost and mammalian CD8β molecules. The SebCD8β was cluster into teleost CD8β molecules. Orange-spotted grouper CD3ε was used as an outgroup. The sequences were aligned using CLUSTAL W, and the tree was generated using neighbour-joining method in MEGA 7.0. The bootstrap values are shown at the branches, and the branch length scale indicated below the tree represented genetic distance.

4.3.3 Characterization of *Sebastiscus marmoratus* CD3ε (SebCD3ε)

The full-length cDNA SebCD3ε (GenBank accession no. LC573976) was found to be 1500 bp and containing an open reading frame that encoded 183 amino acids. An analysis of SebCD3ε sequence revealed the presence of a signal peptide, an extracellular Ig domain, a transmembrane and a cytoplasmic tail. Seven O-glycosylation sites and none of N-linked glycosylation sites were predicted in SebCD3ε.

Fish CD3 genes have been cloned and characterized in Japanese flounder (Park et al., 2001), sterlet (Alabyev et al., 2001), fugu (Araki et al., 2005), Atlantic salmon (Liu et al., 2008), common carp (Shang et al., 2008) and Atlantic halibut (Øvergård et al., 2009). The multiple amino acid alignment of SebCD3ε with other teleosts, mouse and human CD3ε sequences was shown in Fig. 4.5. Phylogenetic analysis showed that SebCD3ε formed a cluster with other teleost CD3ε (Fig. 4.6). Among the teleost, SebCD3ε shared highest amino acid identity with orange-spotted grouper CD3ε (58 %), but the lowest identity 24-28 % with mouse and human CD3ε (Table 4.6).

The cysteine residues found in the extracellular domain were conserved among other teleost and mammalian CD3ε. These cysteine residues are important for Ig fold stabilization by forming a disulfide bond (Gold et al., 1987; Øvergård et al., 2009). The CXXC motif is also conserved in extracellular Ig domain of SebCD3ε. The CD3 subunits comprise heterodimers of CD3εγ and CD3εδ, and a homodimer of CD3ζζ (Punt et al., 1994). This motif may function in the dimerization of CD3ε as heterodimer or homodimer (Liu et al.,

2008; Wang et al., 2009).

Also, the CXXC motif of CD3ε is critical for T cell development and TCR signalling. Mutation of the cysteine residues in the CXXC motif showed a significant effect on pre TCR and TCRαβ development and signalling (Wang et al., 2009; Brazin et al., 2014).

The cytoplasmic tail of SebCD3ε possessed an immunoreceptor tyrosine-based activation motif (ITAM). The first ITAM module and second ITAM module in the cytoplasmic tail of SebCD3ε are YEPL- and YSVV-, respectively (Fig. 4.5). The spacing between the two ITAM modules in SebCD3ε was 9 amino acids. This characteristic is similar to CD3ε in fugu, Atlantic salmon and Japanese flounder, in contrast to the spacing of 7 amino acids found in mammalian CD3ε. The difference space between the ITAM modules indicates that the mechanisms of signal transduction through phosphorylation of ITAMs in teleost could be slightly different from that in mammals (Øvergård et al., 2009).

Table 4.4. Amino acid identity of SebCD3ε among teleost and mammalian species.

Species	GeneBank accession number	Amino acid identity (%)
Orange spotted grouper CD3ε (*Epinephelus coioides)*	ARA90651.1	58
Japanese seabass CD3ε (*Lateolabrax japonicus*)	AND78199.1	57
Japanese flounder CD3ε (*Paralichtyhs olivaceus*)	BAC87847.1	45
Atlantic halibut CD3ε (*Hippoglossus hippoglossus*)	ACY54760.1	51
Fugu CD3ε (*Takifugu rubripes*)	BAD93376.1	43
Atlantic salmon CD3ε (*Salmo salar*)	ABO10202.1	36
Human CD3ε (*Homo sapiens*)	AAH49847.1	28
Mouse CD3ε (*Mus musculus*)	AAA40296.1	24

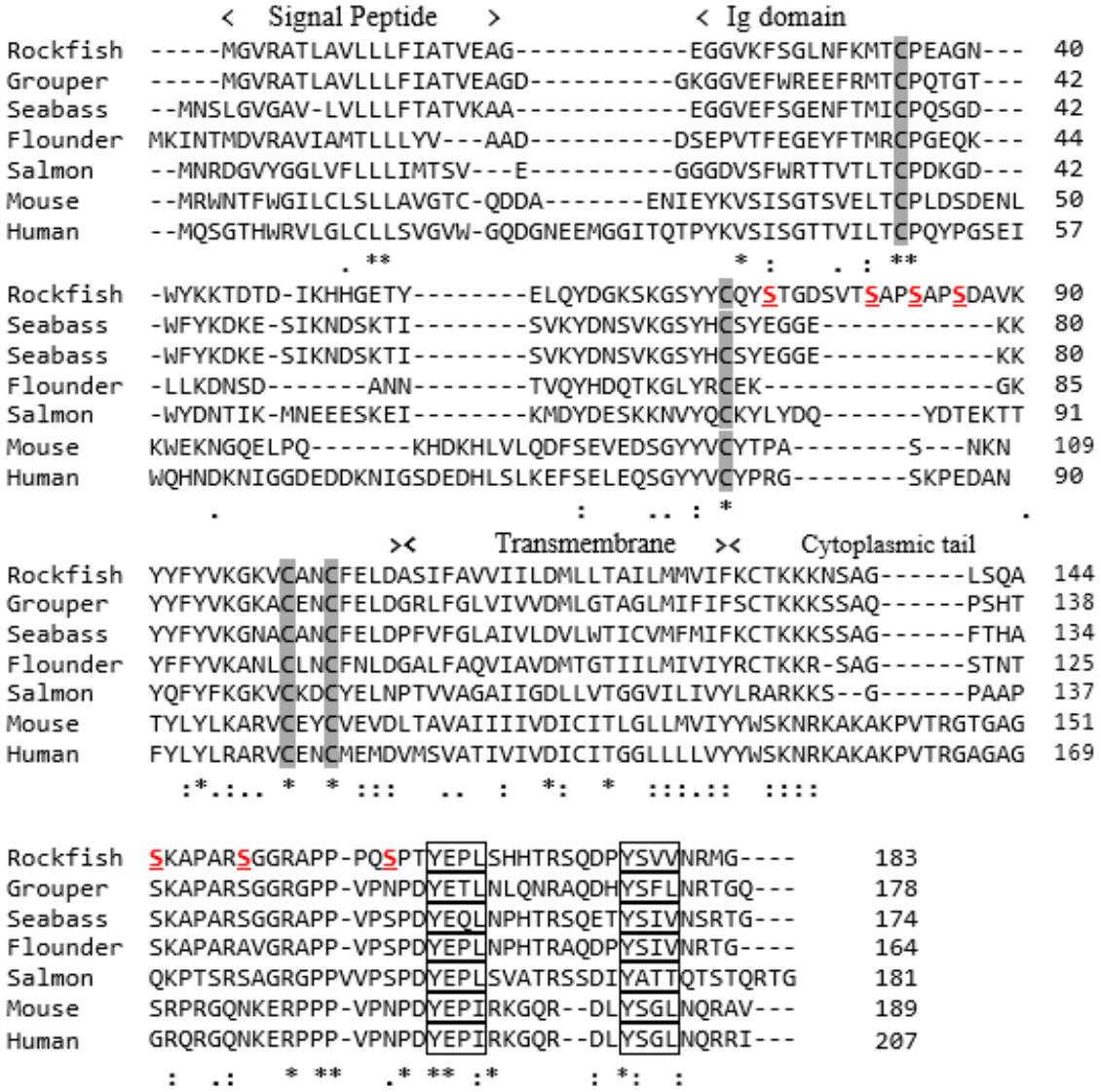

Fig. 4.5. Multiple alignment of teleost and mammalian CD3ε amino acid sequences. The predicted signal peptide, Ig domain, transmembrane and cytoplasmic tail of marbled rockfish CD3ε are labelled above the alignment. Conserved cysteine residues are shaded in grey. Conserved residues within all the sequence are marked with asterisks (*), while double (:) and single dots (.) indicated the similarities between the amino acids, conserved ITAM modules were boxed. The predicted O-glycosylation sites were bold in red and underlined. The corresponding GeneBank accession numbers: Orange-spotted Grouper (*Epinephelus coioides*, ARA90651.1), Japanese Seabass (*Lateolabrax japonicus*, AND78199.1), Japanese Flounder (*Paralichthys olivaceus*, BAC87847.1), Atlantic Salmon (*Salmo salar*, ABO10202.1), Mouse (*Mus musculus*, AAA40296.1) and Human (*Homo sapiens*, AAH49847.1).

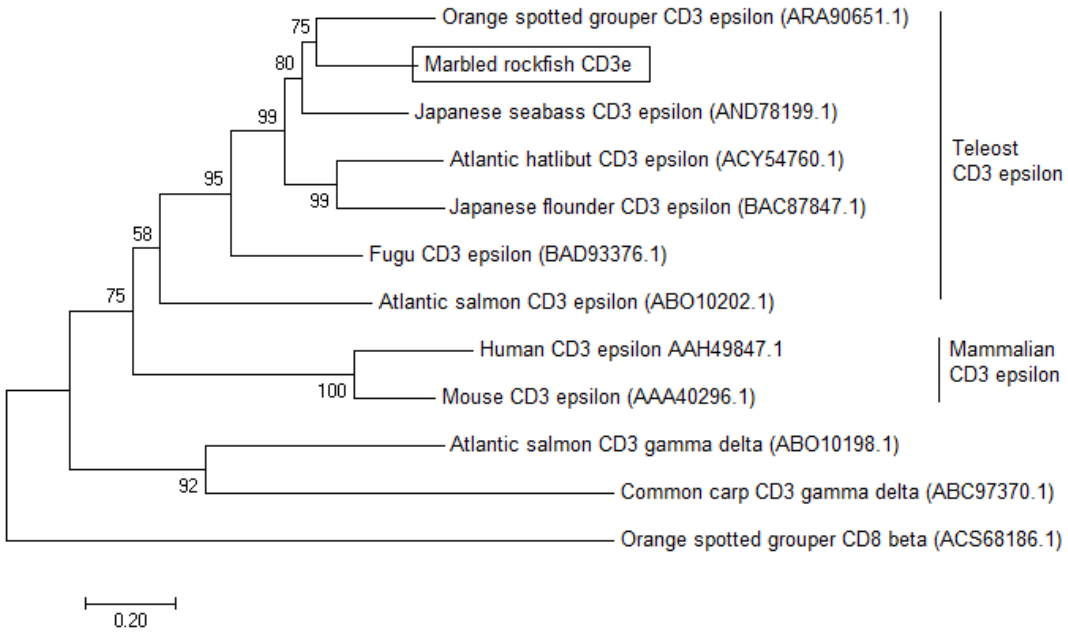

Fig. 4.6. Phylogenetic tree analysis of the SebCD3ε with other known teleost and mammalian CD3ε molecules. The SebCD3ε was cluster into teleost CD3ε molecules. Orange-spotted grouper CD8β was used as outgroup. The sequences were aligned using CLUSTAL W and the tree was generated using neighbour-joining method in MEGA 7.0. The bootstrap values are shown at the branches, and the branch length scale indicated below the tree represented genetic distance.

4.3.4 Characterization of *Sebastiscus marmoratus* CD28 (SebCD28)

The full-length cDNA SebCD28 (GenBank accession no. LC573975) was found to be 1168 bp and containing an open reading frame that encoded 222 amino acids. An analysis of SebCD28 sequence revealed the presence of a signal peptide, an extracellular Ig domain, a transmembrane region and a cytoplasmic tail. Two potential of each N-linked glycosylation sites and O-glycosylation sites were predicted in SebCD28. To date, CD28 gene was reported from rainbow trout (Bernard et al., 2006), half smooth tounge sole (Hu et al., 2012), rock bream (Jeswin et al., 2017) and Nile Tilapia (Huang et al., 2018).

Multiple alignment of SebCD28 amino acid sequence with other teleosts, mouse and human CD28 sequences was shown in Fig. 4.7. Among the teleost, SebCD28 shared highest amino acid identity with orange-spotted grouper CD28 (56 %), but the lowest identity 23-27 % with mouse and human CD28 (Table 4.5). Phylogenetic analysis showed that SebCD28 formed a cluster with teleost CD28 molecules (Fig. 4.8).

SebCD28 possessed three conserved cysteine residues found in other teleost and mammalian CD28. These conserved cysteine residues are known to be involved in disulphide bond and dimerisations (Hu et al., 2012). The MYPPPY motif, which is conserved from mammals to fish, was included in the extracellular domain of SebCD28. CD28 binds to B7 molecules such as CD80/86 using this motif and the interaction initiates the co-stimulatory signal transduction cascade (Freeman et al., 1989; Balzano et al., 1992; Azuma et

al., 1993).

In the SebCD28, the YMNT motif in the cytoplasmic has highly conserved between teleost fish such as orange-spotted grouper and European seabass. The YMNT motif likely corresponds to the mammalian YMNM binding motif. In human and mouse, this motif is responsible as a binding domain for phosphatidylinositol-3 kinase (PI3-kinase), and mediating proliferation and IL-2 secretion (Harada et al., 2001; Boomer et al., 2014).

Table 4.5. Amino acid identity of SebCD28 among teleost and mammalian species.

Species (scientific name)	GeneBank accession number	Amino acid identity (%)
Orange-spotted grouper CD28 (*Epinephelus coicodes*)	DR711304.1	56
Rock bream CD28 (*Oplegnathkus fasciatus*)	ARP51378.1	50
European seabass CD28 (*Dicentarchus labrax*)	AIK66542.1	43
Fugu CD28 (*Takifuge rubripes*)	DAA05843.1	43
Rainbow trout CD28 (*Oncorhynchuss mykiss*)	AAW78853.1	32
Human CD28 (*Homo sapiens*)	CAD57003.1	27
Mouse CD28 (*Mus musculus*)	NP_031668.3	23

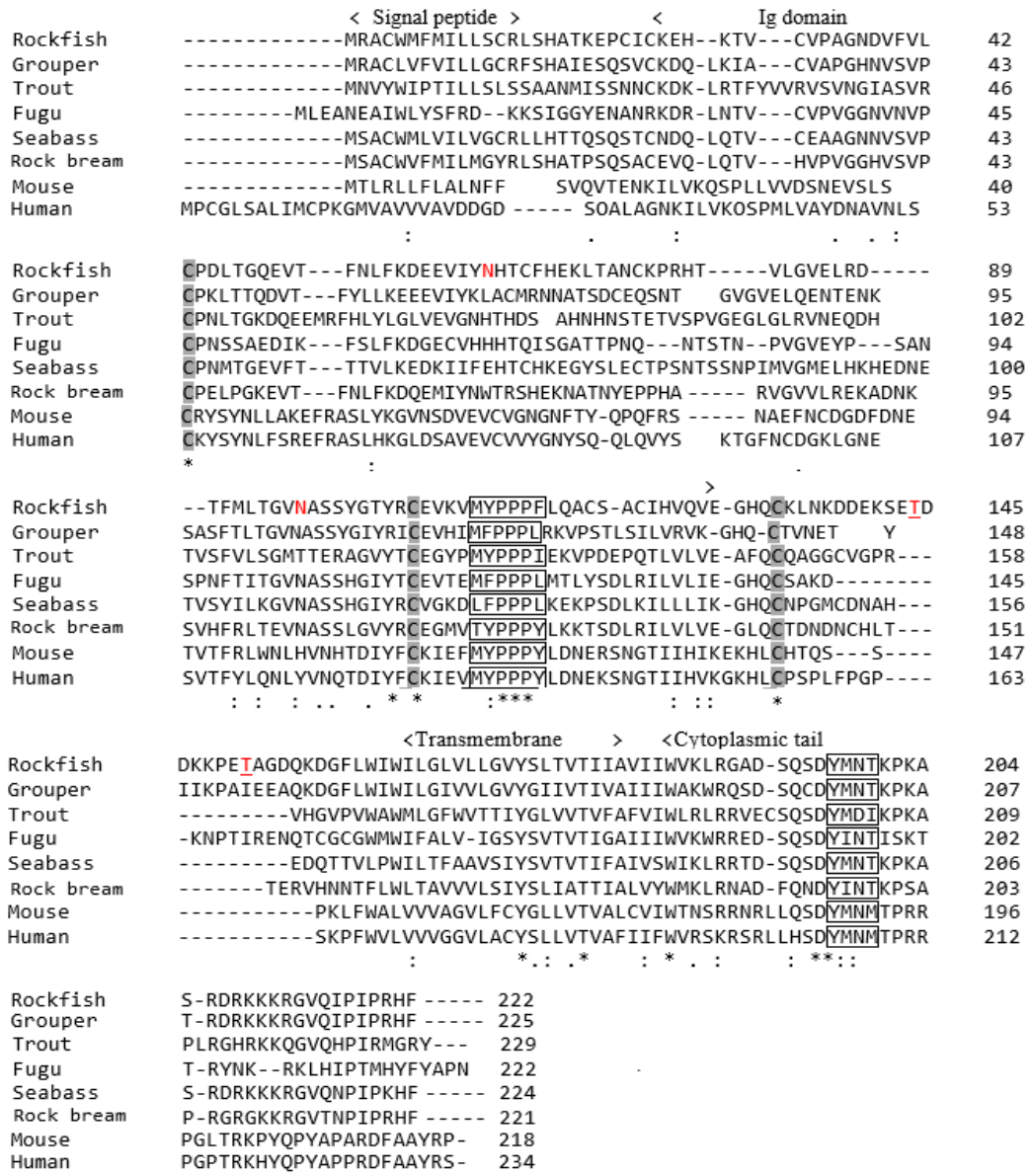

Fig. 4.7. Multiple alignment of teleost and mammal CD28 amino acid sequences. The predicted signal peptide, Ig domain and transmembrane of marbled rockfish CD28 are labelled above the alignment. Conserved cysteine residues are shaded in grey. Conserved residues within all the sequence are marked with asterisks (*), while double (:) and single dots (.) indicated the similarities between the amino acids. The conserved MYPPPY motif and YMNT motif were boxed. The predicted N-glycosylation site were bold in red and O-glycosylation were bold in red and underlined. The corresponding GeneBank accession numbers: Orange-spotted Grouper (*Epinephelus coioides*, DR711304.1), Rainbow Trout (*Oncorhynchus mykiss*, NP_001118004.1), Fugu (*Takifugu rubripes*, DAA05843.1), European Seabass (*Dicentrarchus labrax*, AIK66542.1), Rock bream (*Oplegnathus fasciatus*, ARP51378.1), Mouse (*Mus musculus*, NP_031668.3) and Human (*Homo sapiens*, CAD57003.1).

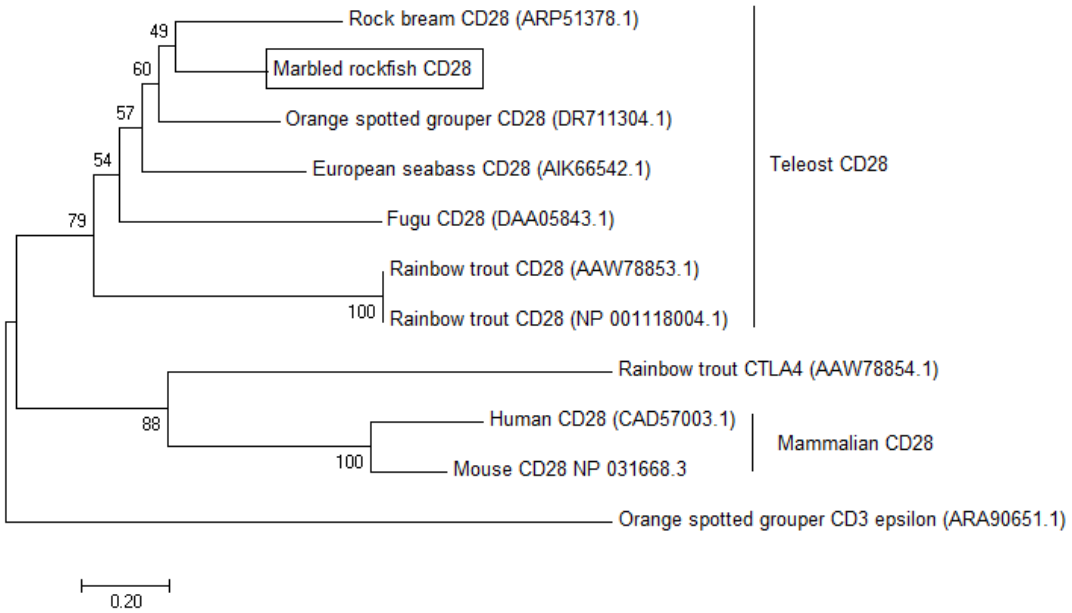

Fig. 4.8. Phylogenetic tree analysis of the SebCD28 with other known teleost and mammalian CD28 molecules. The SebCD28 was cluster into teleost CD28 molecules. Orange-spotted grouper CD3ε was used as an outgroup. The sequences were aligned using CLUSTAL W and the tree was generated using neighbour-joining method in MEGA 7.0. The bootstrap values are shown at the branches, and the branch length scale indicated below the tree represented genetic distance.

4.3.5 Characterization of *Sebastiscus marmoratus* IL-1β (SebIL-1β)

The full-length cDNA SebIL-1β (GenBank accession no. LC573978) was found to be 1330 bp and containing an open reading frame that encoded 264 amino acids. The IL-1 family signature was well conserved in the teleost and was found between 227 and 247 amino acids in SebIL-1β. An analysis of SebIL-1β sequence revealed the one N-linked and six O-linked glycosylation sites. Like other teleost IL-1β, SebIL-1β lacked a signal peptide. To date, IL-1β has been identified from rainbow trout (Zou et al., 1999), common carp (Fujiki et al., 2000), European seabass (Scapigliati et al., 2001), channel catfish (Wang et al., 2006), yellowfin seabream (Jiang et al., 2008), orange-spotted grouper (Lu et al., 2008), ayu (Lu et al., 2013), Atlantic bluefin tuna (Lepen Pleic et al., 2014), and large yellow croaker (Wu et al., 2015).

Multiple alignment of SebIL-1β amino acid sequence with other teleosts, mouse and human IL-1β sequences was shown in Fig. 4.9. Among the teleost, SebIL-1β shared highest amino acid identity with large yellow croaker (57 %), but the low identity 23-26 % with mouse and human IL-1β (Table 4.6). Phylogenetic analysis showed that SebIL-1β formed a cluster with teleost IL-1β (Fig. 4.10).

It has been reported that IL-1β gene of rainbow trout lacks the classical IL-1β-converting enzyme (ICE) clevage site which is necessary for production of IL-1β protein from the precursor molecule in mammalian IL-1β (Black et al., 1989; Kostura et al., 1989; Howard et al., 1991; Zou et al., 1999).

This feature has been observed in other teleost IL-1β including SebIL-1β. Non-mammalian vertebrates processing IL-1β with no cleavage site and release active IL-1β protein though specific enzyme actions (Irmler et al., 1995). Several studies have shown that recombinant IL-1β protein enhanced inflammatory response to bacterial or parasitic infection in fish (Watzke et al., 2007; Morrison et al., 2012). Vojtech et al. (2012) demonstrated the processing and secretion of zebrafish IL-1β related with caspase orthologs in response to a pathogen *Francisella noatunensis.* Teleost fish probably has different mechanisms to produce active mature IL-1β from that in mammals, but the functions in the immune system may be similar to that in mammalian IL-1β.

Table 4.6. Amino acid identity of IL-1β among teleost and mammalian species.

Species (scientific name)	GeneBank accession number	Amino acid identity (%)
Large Yellow croaker IL-1β (*Larimichthys crocea)*	KAE8285371.1	57
Turbot 1β (*Scophthalmus maximus*)	CAC33867.2	34
Nile Tilapia 1β (*Oreochronis niloticus*)	XP_019221386.1	34
Orange-spotted grouper IL-1β (*Epinephelus coioides*)	ABV02593.1	33
Atlantic salmon IL-1β (*Salmo salar*)	AAT36642.1	33
Zebrafish IL-1β (*Danio rerio*)	AAQ16563.1	32
Common carp IL-1β (*Cyprinus carpio*)	BAA24538.1	32
Japanese flounder IL-1β (*Paralichthys olivaceus*)	BAM66988.1	30
Gilthead seabream IL-1β (*Sparus aurata*)	CAC81783.2	30
Human IL-1β (*Homo sapiens*)	AAA36106.1	26
Mouse IL-1β (*Mus musculus*)	NP_032387.1	23

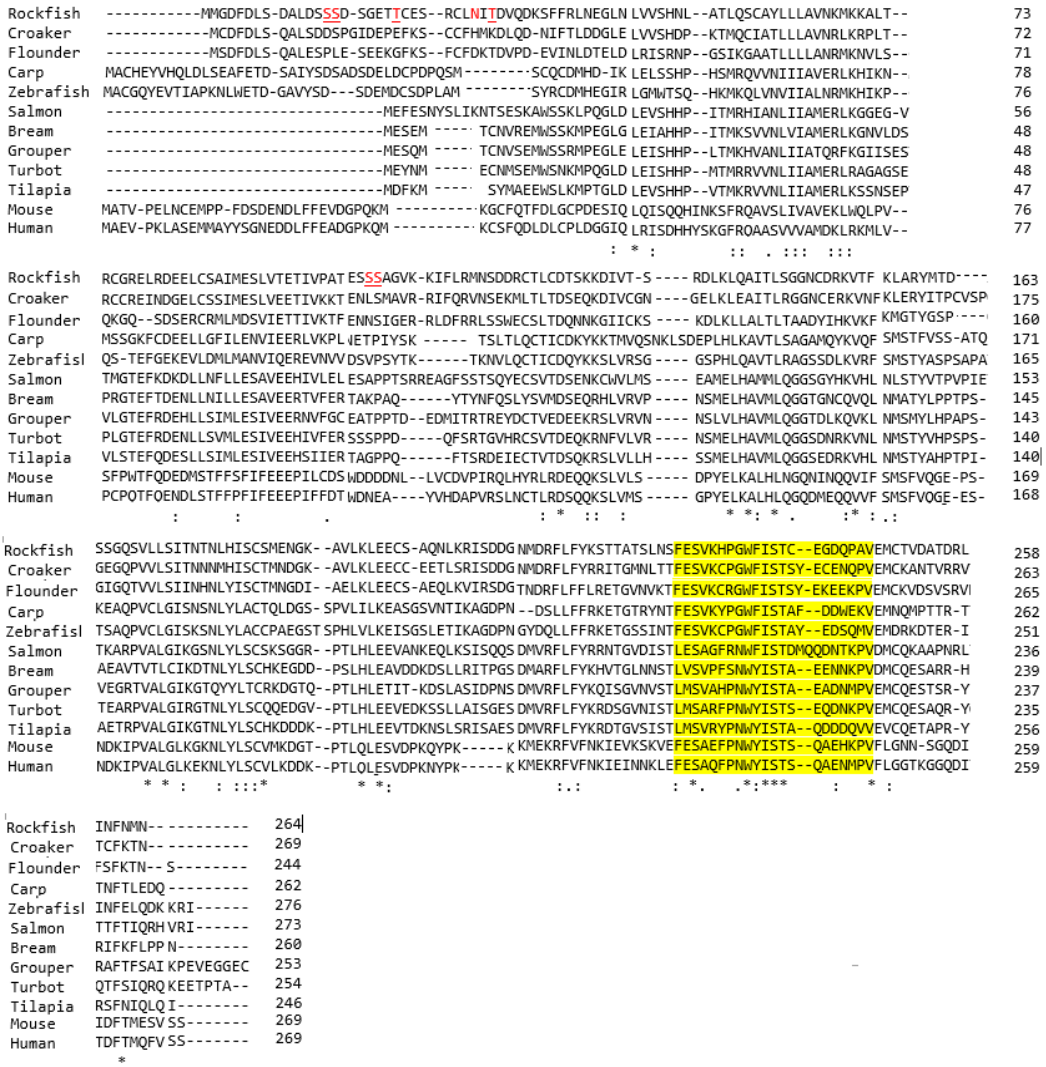

Fig. 4.9. Multiple sequence alignment of teleost and mammal IL-1β amino sequences. Conserved residues within all the sequence are marked with asterisks (*), while double (:) and single dots (.) indicated the similarities between the amino acids. The conserved motif of IL-1 signature are highlighted in yellow. The predicted N-link glycosylation sited were bold in red and O-glycosylation sites were bold in red and underlined. The corresponding GeneBank accession numbers: Large Yellow Croaker (*Larimichthys crocea*, KAE8285371.1), Japanese Flounder (*Paralichthys olivaceus*, BAM66988.1), Common Carp (*Cyprinus carpio,* BAA24538.1), Zebrafish (*Danio rerio*, AAQ16563.1), Atlantic Salmon (*Salmo salar*, AAT36642.1), Gilthead seabream (*Sparus aurata*, CAC81783.2), Orange-spotted Grouper (*Epinephelus coioides*, ABV02593.1), Turbot (*Scopahthalmus maximus*, CAC33867.2), Nile Tilapia (*Oreochromis niloticus*, XP_019221386.1), Mouse (*Mus musculus*, CAA28637.1) and Human (*Homo sapiens*, AAA36106.1)

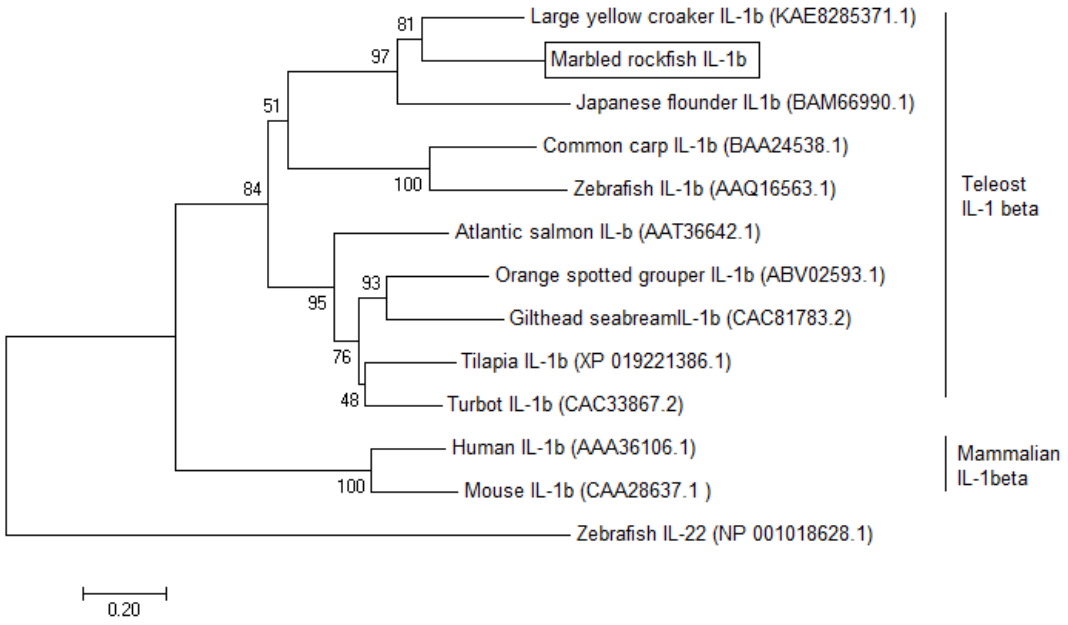

Fig. 4.10. Phylogenetic tree analysis of the SebIL-1β with other known teleost IL-1β molecules. The SebIL-1β was cluster into teleost IL-1β molecules. Zebrafish IL-22 was used as an outgroup. The sequences were aligned using CLUSTAL W and the tree was generated using neighbour-joining method in MEGA 7.0. The bootstrap values are shown at the branches, and the branch length scale indicated below the tree represented genetic distance.

4.3.6 Characterization of *Sebastiscus marmoratus* IFN-γ (SebIFN-γ)

The full-length cDNA SebIFN-γ (GenBank accession no. LC573979) was found to be 1187 and containing an open reading frame that encoded 206 amino acids. An analysis of SebIFN-γ sequence revealed the presence of a signal peptide. One N-linked and ten O-glycosylation sites were predicted in SebIFN-γ. To date, IFN-γ has been reported from fugu (Zou et al., 2004), rainbow trout (Zou et al., 2005), green spotted pufferfish (Igawa et al., 2006), Atlantic salmon (Robersten, 2006), channel catfish (Milev-Milovanovic et al., 2006), common carp (Stolte et al., 2008), goldfish (Grayfer & Belosevic, 2009), Atlantic cod (Furnes et al., 2009) and turbot (Pereiro et al., 2016).

Multiple alignment of SebIFN-γ amino acid sequence with other teleosts, mouse and human IFN-γ sequences was shown in Fig. 4.11. Phylogenetic analysis showed that SebIFN-γ formed a cluster with other teleosts IFN-γ (Fig. 4.12). Among the teleost, SebIFN-γ shared highest amino acid identity to orange-spotted grouper (70 %), but the low identity 21-28 % with mouse and human IFN-γ (Table 4.7).

C-terminal region of SebIFN-γ showed an IFN-γ signature sequence (I/V-Q-X-K/Q-A-X2-E-L/F-X2-I/V) and NLS motif that was conserved in other IFN-γ molecules (Peng et al., 2018). The nuclear localisation sequence (NLS) in mammals is essential for the biological activities and translocation of STAT1 into the nucleus. The deletion or modification of the NLS motif in mammals showed blockage of the nuclear translocation of STAT1 induced by

exogeneous IFN-γ (Subramaniam et al., 1999). Indeed, deletion of the C-terminal region of rainbow trout IFN-γ impaired the biological activity in induction of gene expression of IFN gamma inducible protein 10 (Zou et al., 2005).

Table 4.7. Amino acid identity of IFN-γ among teleost and mammalian species.

Species	GeneBank accession number	Amino acid identity (%)
Orange spotted grouper IFN-γ (*Epinephelus coioides*)	AFM31242.1	70
Rock bream IFN-γ (*Oplegnatus fasciatus*)	QDE10278.1	70
Japanese flounder IFN-γ (*Paralichthys olivaceus*)	BAG50577.1	62
Atlantic salmon IFN-γ (*Salmo salar*)	NP_001117030.1	30
Common carp IFN-γ (*Cyprinus carpio*)	CAJ51088.1	29
Rainbow trout IFN-γ (*Oncrohynchus mykiss*)	NP_001118092.1	28
Zebrafish IFN-γ-1 (*Danio rerio*)	BAD72865.1	23
Channel catfish IFN-γ-1 (*Ictalurus punctatus)*	AAZ40506.1	21
Mouse IFN-γ (*Mus musculus*)	NP_620235.1	21
Human IFN-γ (*Homo sapiens*)	NP_000610.2	28

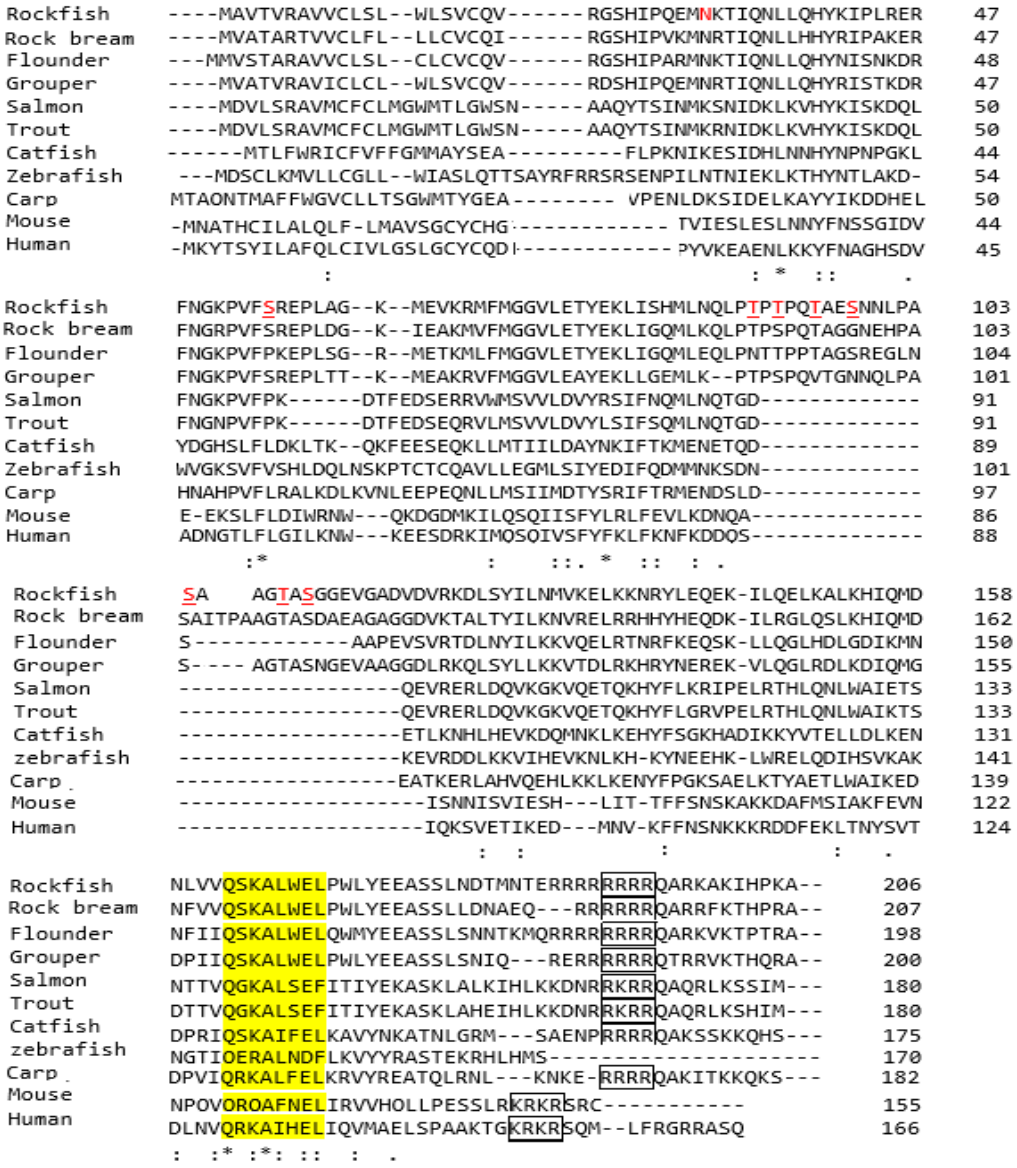

Fig. 4.11. Multiple sequence alignment of teleost and mammal IFN-γ amino sequences. Conserved residues within all the sequence are marked with asterisks (*), while double (:) and single dots (.) indicated the similarities between the amino acids.The signature IFN-γ motif was highlighted in yellow colour and NLS region were boxed. The predicted N-linked glycosylation sites were bold in red and O- linked glycosylation sites were bold in red and underlined. The corresponding GeneBank accession numbers: Rockbream (*Oplegnathus fasciatus*, QDE10278.1), Japanese Flounder (*Paralichthys olivaceus*, BAG50577.1), Orange-spotted Grouper (*Epinephelus coioides*, AFM31242.1), Atlantic Salmon (*Salmo salar*, CAH56503.1), Rainbow Trout (*Oncorhynchus mykiss*, NP_001153976.1), Channel catfish (*Ictalurus punctatus*, AAZ40506.1), Zebrafish (*Danio rerio*, BAD72865.1), Common carp (*Cyprinus carpio*, CAJ51089.1), Mouse (*Mus musculus*, NP_032363.1) and Human (*Homo sapiens*, NP_000610.2).

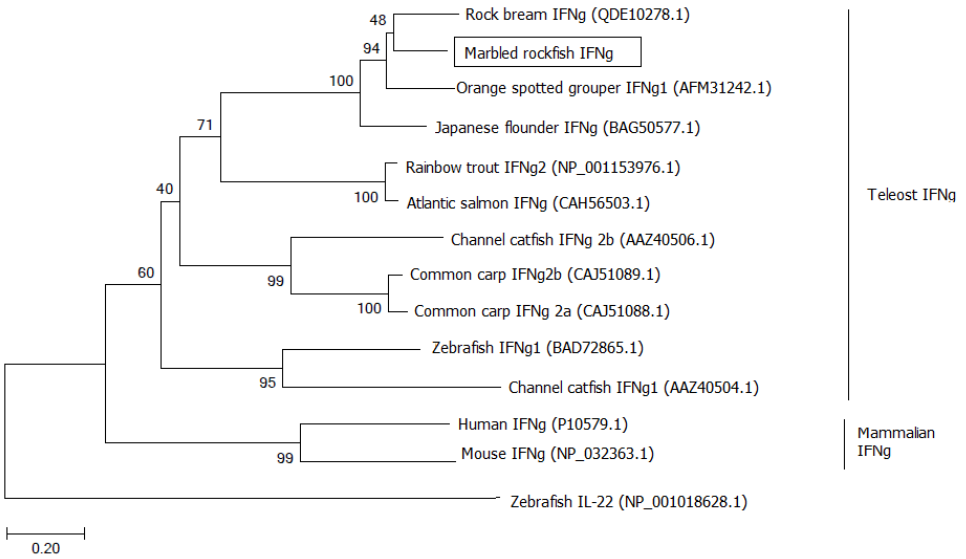

Fig. 4.12. Phylogenetic tree analysis of the SebIFNγ with other known teleost IFNγ molecules. The SebIFNγ was cluster into teleost IFNγ molecules. Zebrafish IL-22 was used as an outgroup. The sequences were aligned using CLUSTAL W and the tree was generated using neighbour-joining method in MEGA 7.0. The bootstrap values are shown at the branches, and the branch length scale indicated below the tree represented genetic distance.

4.4 SUMMARY

T cell markers (CD4, CD8β, CD3ε, CD28) and cytokines (IL-1β and IFNγ) were cloned and characterized through multiple alignments of amino acid sequences and phylogenetic analysis for each target genes. The primary structures for each characterized genes were highly conserved among the teleost. T cell markers and IFN γ in marbled rockfish showed high homology with that in orange-spotted grouper. By contrast, SebIL-1β showed high homology with that in large yellow croaker. These characterized genes are useful for studying the immune responses to *Ochroconis* infection in marbled rockfish.

CHAPTER 5

Analysis of immune relevant genes expression in various organs of intact fish, activated leukocytes with immune stimulants, and in spleen of the infected fish

5.1 INTRODUCTION

In chapter 2 and 3, I proposed that occurrence of *Ochroconis* infection in marbled rockfish was age-dependent, the younger fish was more susceptible to the infection. The histopathological changes in the younger fish were characterized by intense inflammatory response in several organs, while the older fish showed low or no inflammatory responses. In chapter 4, some immune relevant genes of marbled rockfish were cloned and characterized. These genes could be used for studying the immune response by analysis of the gene expression in fish stimulated with antigens and infected with pathogens.

Previous studies showed that early control to bacterial infection depends on the secretion of cytokines through the generation of a protective Th1 response (Kalinski, 2012; Babadjanova et al., 2015). Th1 cells govern the protective response against pathogens such as bacteria, fungi and protozoa (Ashfaq et al., 2019). Production of i cytokines at the early stages of bacterial infection is a critical factor for host protection and bacterial clearance (Medzhitov & Janeway, 1997; Pashine et al., 2005). Several studies reported that secretion of cytokines such as IFN-γ, TNF-α and IL-12, provide immediate control over *Francisella tularensis* replication in the case of intracellular infection (Elkins et al., 2003; Duckett et al., 2005).

Th1 cell-mediated immunity is classified as "protective" responses to fungal infection, which involves the production of cytokines such as IFNγ which stimulates macrophage activation (Traynor & Huffnagle, 2001; Blanco & Garcia, 2008). In the adaptive cell-mediated immune response, IFNγ is mainly produced by NK cells, CD4+ T cells (Th1) and CD8+ T cells (CTL) in response to MHC-presented antigens (Robertsen, 2006). IFNγ produced by T and NK cells is a key cytokine both in the innate and adaptive immunity to invasive fungal infection. It stimulates migration, adherence, and antifungal activity of neutrophils and macrophages against *Candida albicans, Aspergillus fumigatus* and *Fusarium solani* (Lyman et al., 1994; Gaviria et al., 1999; Antachopoulos & Roilides, 2005).

In this chapter, three objectives were set to evaluate differences in the immune response of younger and older marbled rockfish to *Ochroconis* infection. First, expression of the genes (CD4, CD8β, CD3ε and CD28) in several organs of healthy marbled rockfish was analyzed to confirm whether these genes are expressed in T cells. Second, gene expression of T cell markers and cytokines (IFN-γ and IL-1β) in the leukocytes activated with immunostimulants was examined to demonstrate that these genes have similar functions as immune relevant genes in mammals or other fish species. Third, the gene expression in a lymphoid organ of younger and older marbled rockfish after the injection with *O. humicola* was studied to find the difference of the immune response between these fish.

5.2 MATERIALS AND METHODS

5.2.1 Tissue collection from healthy fish

Five healthy marbled rockfish (77 ± 2.2 mm, 10.7 ± 1.4 g) were used. After fish were euthanized by overexposure to FA100, kidney, spleen, thymus, gills, intestine, brain and liver were removed. The tissue samples were then placed in 1mL of Trizol reagent (Invitrogen, Carlsbad CA, USA) for RNA extraction.

5.2.2 Preparation and stimulation of kidney leukocytes

Three marbled rockfish (77 ± 2 mm, 9.5 ± 0.7 g) were used in this study. Head kidney tissue was aseptically removed from fish and placed in the minimal essential medium (MEM; Nissui Pharmaceutical Co., Tokyo, Japan) adjusted to pH7.0 and supplemented with 5% fetal bovine serum (FBS; JRH Bioscience, KS, USA), 14 mM Hepes, 1.4 mM $NaHCO_3$, and 4 mM L-glutamine. The kidney leukocytes were collected by gently pressing the tissue through a 150-gauge mesh stainless steel sieve in MEM medium.

The erythrocytes were removed with the hypotonic lysis method modified from Hu et al. (2018). The erythrocytes were disrupted by mixing the kidney cell suspension with 500 µL sterile distilled water and 5 µL DNase (Takara, Shiga, Japan) for 30s. Five mL MEM was then added to prevent cell destruction. The kidney leukocytes were then washed by centrifugation at 350 *g* for 5 min at 4ºC. Freshly prepared kidney leukocytes were seeded into 24

well culture plates at 10^6 cells/mL. These cells were stimulated with 25 μg/mL LPS or 10 μg/mL poly I:C for 6 h, 12 h and 24 h. After the incubation, the cells were re-suspended in 1 ml of Trizol reagent for RNA extraction.

5.2.3 Collection of spleen from fish injected with *O. humicola*

Marbled rockfish were obtained in different months from a commercial fish hatchery, and their body sizes were 52 ± 1.5 mm, 3.6 ± 0.4 g on October, and 77 ± 2.2 mm, 10.9 ± 1.3 g on December. The fish obtained in October and in December were categorized as the younger and older fish, respectively.

O. humicola NJM 1503 was cultured on PYGS agar (0.125 % peptone, 0.125 % yeast extract, 3 % glucose, and 1.2 % agar in artificial seawater) at 25ºC for three weeks. Conidia were suspended from the fungal colony with phosphate buffer saline (PBS) by gently scratching using at 1.0 μL inoculation loop (Sarstedt AG & CO., Nümbrecht, Germany). The collected conidia were counted using a haemocytometer (Erma, Tokyo, Japan) and adjusted to a concentration of 1×10^5 conidia per 50 μL of PBS.

Fish were intraperitoneally injected with the 50 μL conidia suspension and 50 μL PBS for challenge and control group, respectively. Five fish from each category were sampled at 5, 7 and 10 days post-injection (d.p.i.) and sacrificed with overexposure to FA100 (10 % eugenol solution, Tanabe Co. Ltd, Tokyo, Japan). The spleen was removed and placed in 1 ml of Trizol reagent for RNA extraction.

5.2.4 RNA extraction and cDNA preparation

Total RNA was extracted from tissues samples using Trizol reagent (Invitrogen, Carlsbad CA, USA). In brief, tissues samples were manually disrupted in 1 mL of Trizol using a micro homogeniser. The procedure was then continued following the manufacturer's instruction. Finally, RNA pellets were eluted in 30 µL DEPC water and stored at -80ºC until used. One µg of RNA was used to synthesize first strand of cDNA in each sample using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) following the manufacturer's instruction.

5.2.5 Evaluation of immune gene expression by real-time PCR

Real-time PCR was performed using 7500 real-time PCR system (Applied Biosystems, CA, USA). Marbled rockfish β-actin gene was used as the reference gene. Briefly, the mixture for quantitative-real time PCR (qRT-PCR) was prepared using a cDNA corresponding to 1µg of total RNA, KOD SYBR qPCR mix reagent (TOYOBO) and specific primers for each target genes in a total volume of 20 µL. The PCR conditions were 98ºC for 2 min, followed by 40 cycles of 98ºC for 10 s, 55ºC (CD4, CD3ε, CD28, IFN-γ and β-actin) or 60ºC (IL-1β and CD8β) for 10 s, 68ºC for 30 s. The specific primers used for qRT-PCR were listed in Table 5.1.

Triplicate reactions were performed for each template cDNA. Relative standard curves for genes of interest were constructed using a serial dilution

series with 10^8 - 10^1 copies/μL of each cDNA clone inserted into pGEM vector, to estimate the transcript copy number of expressed genes. The expression levels for each gene were normalized to the expression of a house-keeping gene (β-actin).

5.2.6 Statistical analysis

For expression analysis of T cell marker genes in several organs of healthy marbled rockfish, the fold data were indicated as the mean ± SD, relative to the gene expression in the brain as a reference indicator. Statistical significance was estimated using Kruskal Wallis followed by Steel-Dwass multiple comparison test ($p<0.05$).

For gene expression analysis in the leukocytes after immune stimulation, the fold data were indicated as mean ± SD, relative to the 0 hours. Statistical significance was estimated using ANOVA, followed by Dunnet multi comparison test ($p<0.05$).

For gene expression analysis in the spleen of injected fish, the fold data were indicated as mean ± SD, relative to gene expression in the spleen of PBS-injected fish at the same sampling time. Statistical significance was estimated using student paired *T*-test ($p<0.05$).

5.2.7 Ethics statement

All animal experiments were performed under the guidelines of the Animal Experiments Committee at Nippon Veterinary and Life Science University, and all experimental protocols were endorsed by the committee according to authorization 2019K-39.

Table 5.1. Primer used for real-time PCR analysis.

Primer	Sequence (5' → 3')
Seb β actin F	AGGGAAATCGTGCGTG
Seb β actin R	ATGATGCTGTTGTAGGTGGT
Seb CD4 RT F	CTCACTGATCATCACAAACATC
Seb CD4 R2	GAGAGGCCCGGATTTAGACGTATAC
Seb CD8β F	GTGGACGAAAAACGCATC
Seb CD8β R	GAAATAAGTCCAACCAGCGGC
Seb CD28 F2	CACACTCTCAGTGCAAAAGATC
Seb CD28 R2	CTCCAGTGAGCATGAAAGTGTC
Seb CD3e RT F2	CAGGTGACTTGTCTCTATCCCATC
Seb CD3e RT R2	CATTATTTTGGACATGTTGTTGAC
Seb IL-1β F	GGTGACCGAAACCATCGTC
Seb IL-1β R2	GTAGAAGAGGAACCGGTCCATG
Seb IFNg2 F	GGAGGTGAAGAGGATGTTTATG
Seb IFNg2 R	CCATCTGGATGTGTTTGAG

5.3 RESULTS

5.3.1 Gene expression of T cell markers in several organs

The expression levels of SebCD4, SebCD8 and SebCD3ε genes in the thymus was the highest among the organs (Fig. 5.1, 5.2 & 5.3). The spleen also showed comparatively high levels in the expression of these genes. In the levels of SebCD4 gene expression, the gills, kidney, intestine and liver were not significantly different from the spleen. On the other hand, comparatively high levels of SebCD8 and SebCD3ε gene expression were observed in the intestine, and also the gills for SebCD3ε genes. By contrast, the level of SebCD28 gene expression was the highest in the liver (Fig. 5.4). The expression level of SebCD28 in the thymus was not significantly different from other organs such as the spleen, kidney, intestine and gills. The brain showed the lowest levels in the gene expression of all T cell markers.

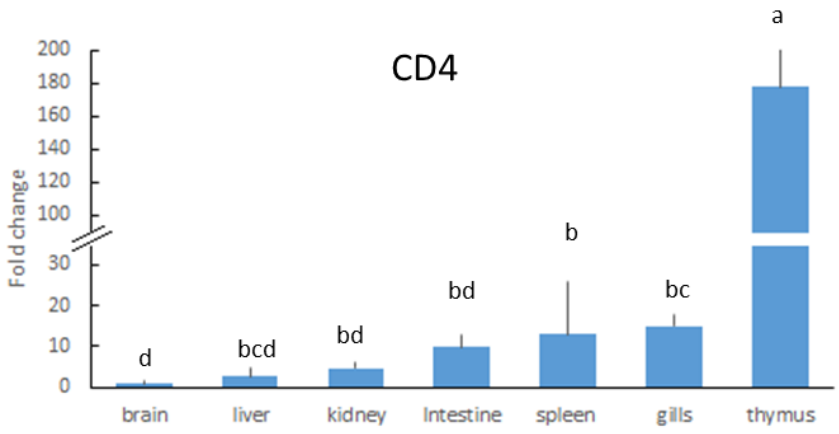

Fig. 5.1. SebCD4 gene expression in various organs of marbled rockfish. The different letters indicate significant differences ($p<0.05$).

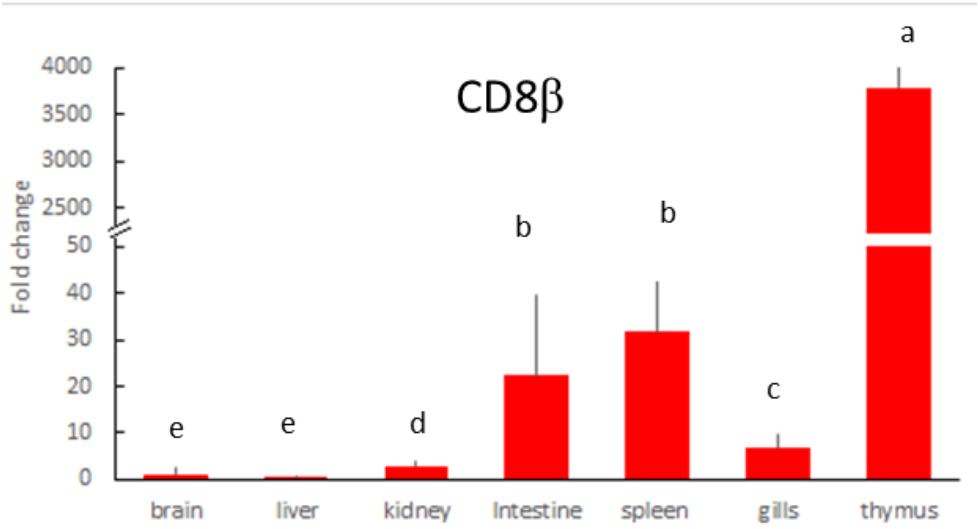

Fig. 5.2. SebCD8β gene expression in various organs of marbled rockfish. The different letters indicate significant differences ($p<0.05$).

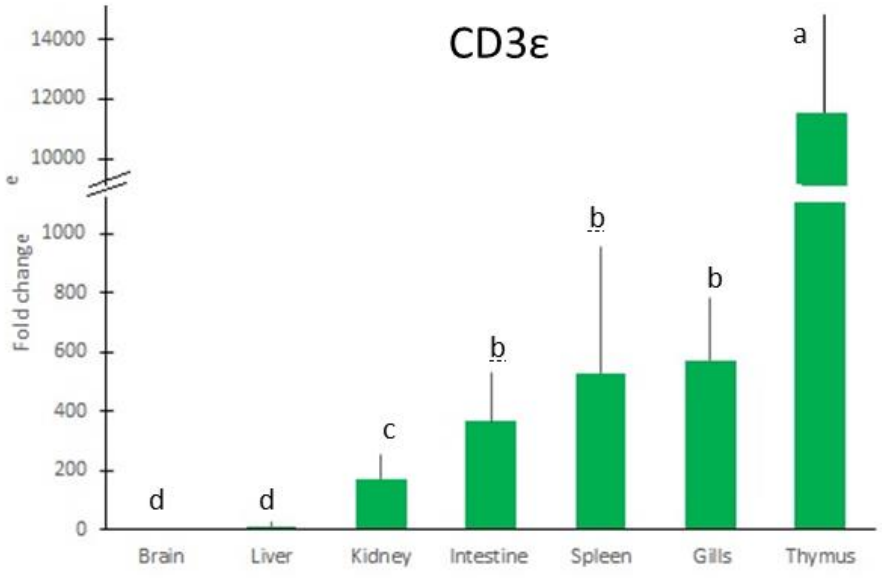

Fig 5.3. SebCD3ε gene expression in various organs of marbled rockfish. The different letters indicate significant differences ($p<0.05$).

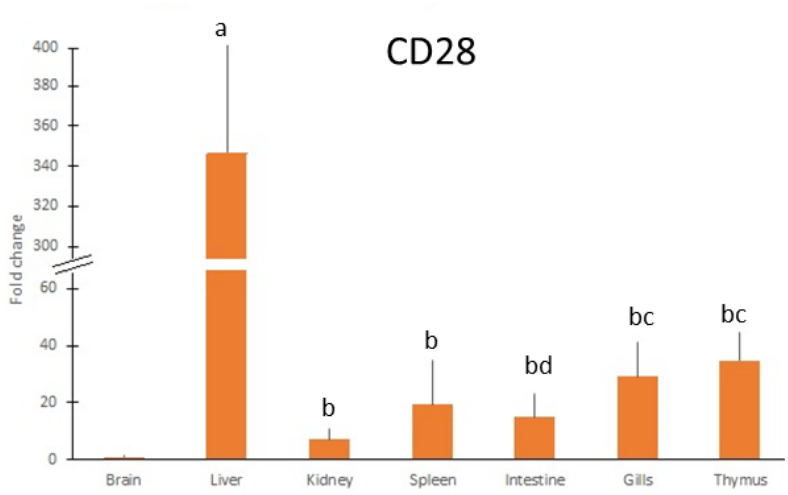

Fig. 5.4. SebCD28 gene expression in various organs of marbled rockfish. The different letters indicate significant differences ($p<0.05$).

5.3.2 Immune relevant gene expression in the activated leukocytes

The gene expression of SebCD4, SebCD8β, SebINFγ and SebIL-1β were examined in kidney leukocytes after stimulated with LPS and poly I:C. The levels of SebCD4 genes in the leukocytes were upregulated 12 h after the stimulation with LPS but no significant upregulation after poly I:C stimulation (Fig. 5.5). The levels of SebCD8β genes in the leukocytes increased at 6 h after both stimulation (Fig. 5.5).

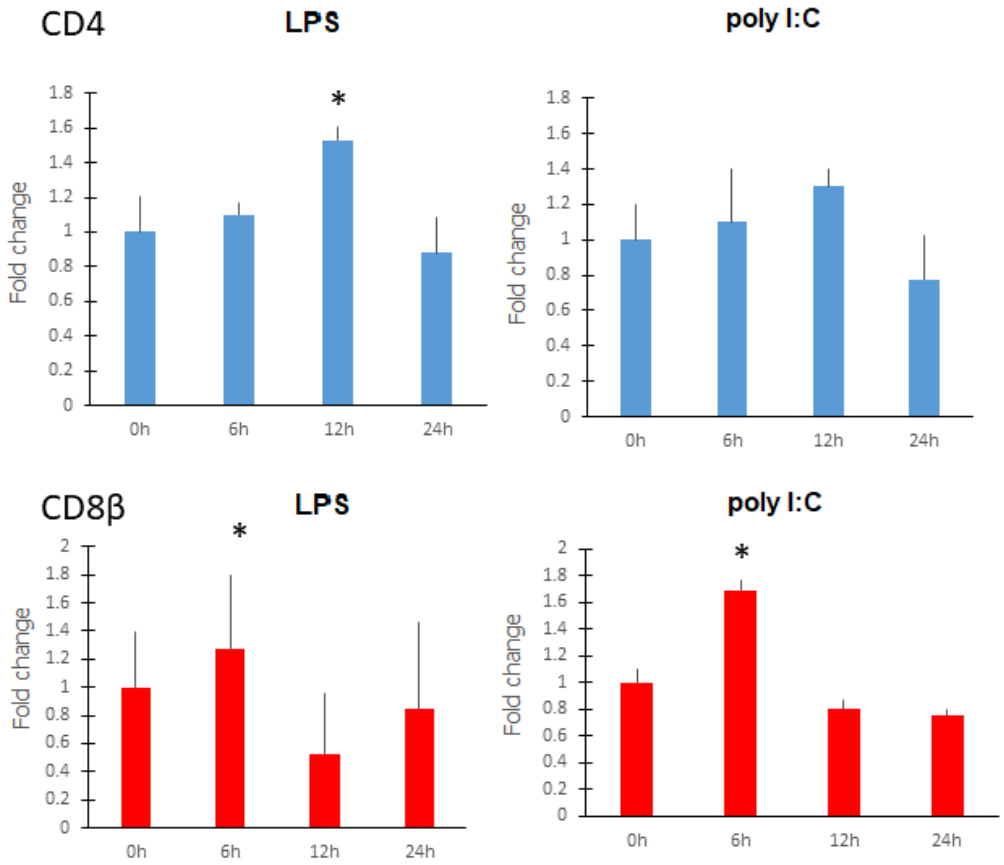

Fig. 5.5. SebCD4 and SebCD8β gene expression in the activated leukocytes by stimulation with LPS and poly I:C. Asterisks (*) indicate a significant difference from the data at 0 h ($p < 0.05$).

The levels of SebIFNγ genes in the leukocytes were upregulated at 12 h and 24 h after the stimulation with LPS and at 6 h and 12 h after that with poly I:C (Fig. 5.6). The levels of SebIL-1β genes in the leukocytes activated by LPS or poly I:C increased after 12 h and 24h or 6 h and 24 h after the stimulation, respectively (Fig. 5.6).

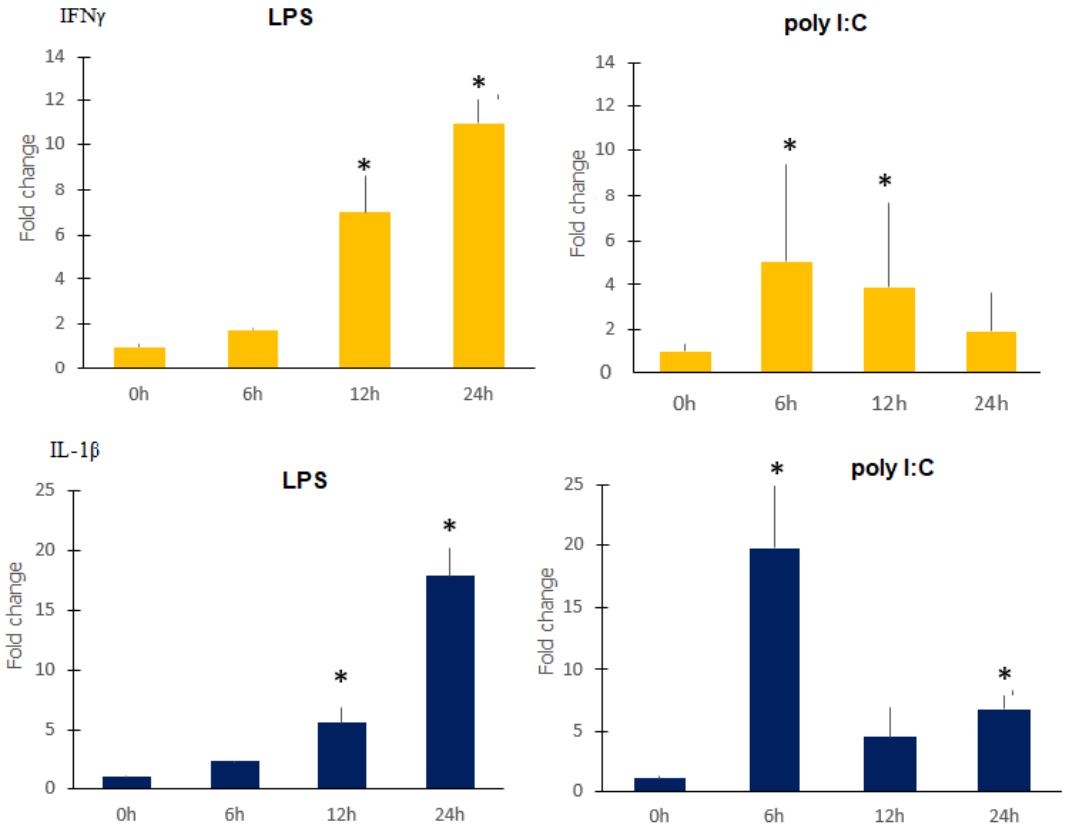

Fig. 5.6. SebIFNγ and SebIL-1β gene expression in the activated leukocytes by stimulation with LPS and poly I:C. Asterisks (*) indicate a significant difference from the data at 0 h ($p < 0.05$).

5.3.3 Immune relevant gene expression in the spleen after injected with *O. humicola*

In the SebCD4 gene, the younger marbled rockfish showed significant increase of the gene expression at 5 and 7 d.p.i and the expression became decrease at 10 d.p.i (Fig. 5.7). In older marbled rockfish, upregulation of SebCD4 gene expression was also observed at 7 and 10 d.p.i (Fig. 5.7), but the expression was induced slower than that in the younger fish.

In the SebCD8β gene, the younger fish increased the level of gene expression at 5 d.p.i and the expression was downregulated at 7 and 10 d.p.i (Fig. 5.7). The levels of gene expression in the older fish showed no significant difference during the experimental period (Fig. 5.7).

In the SebIFNγ, the younger fish significantly upregulated the gene expression throughout the experimental period (Fig. 5.8). In the older fish, upregulation of SebIFNγ gene expression was also observed at 7 and 10 d.p.i (Fig. 5.8), but the expression was induced slower than that in the younger fish.

The expression of SebIL-1β gene showed changes at 10 d.p.i in both the younger and the older fish with different regulation pattern (Fig. 5.8). The expression was significantly downregulated in the younger fish, whereas significantly upregulated in the older fish.

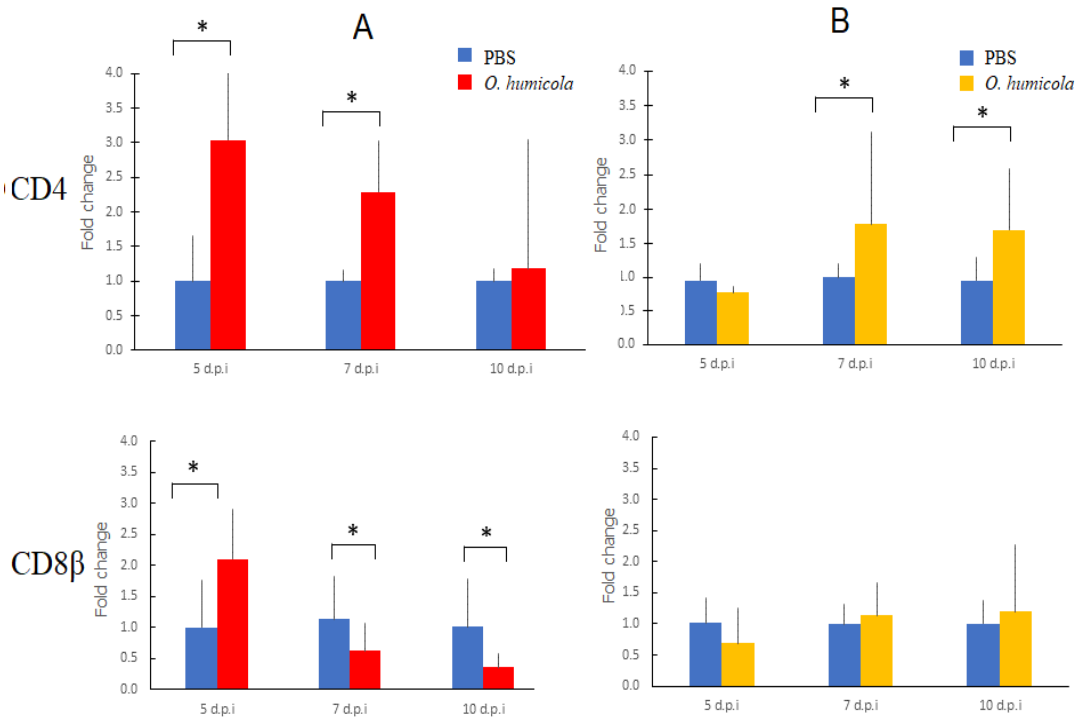

Fig. 5.7. SebCD4 and SebCD8β gene expression in the spleen of injected fish with *O. humicola*. (A): younger fish. (B): older fish. Asterisks (*) indicate a significant difference between *O. humicola*-injected and PBS-injected groups at the same time point ($p<0.05$).

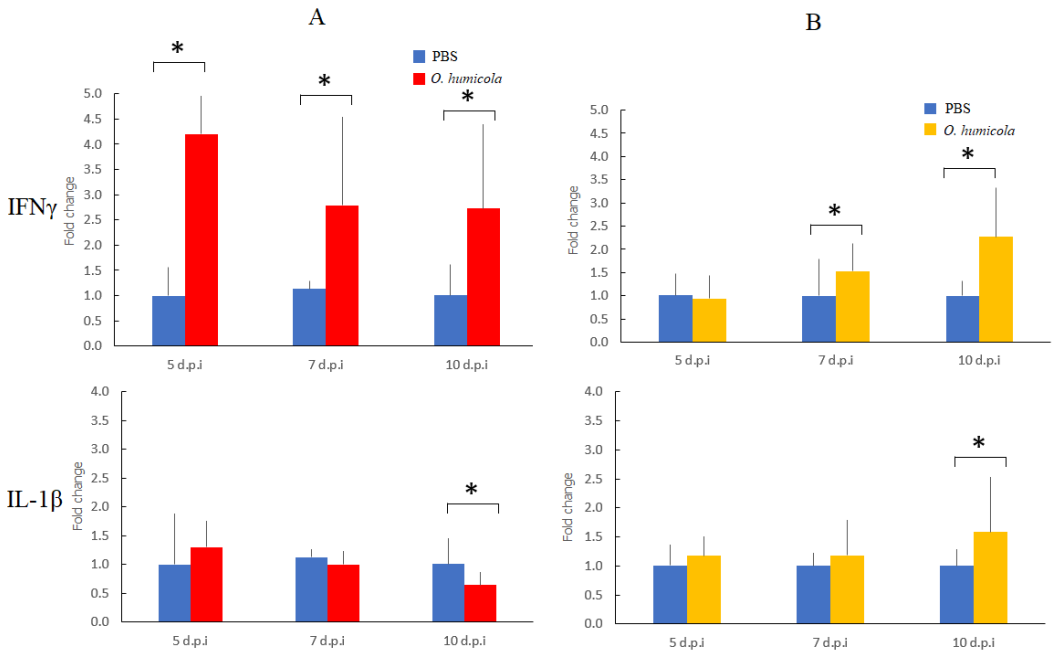

Fig. 5.8. SebIFNγ and SebIL-1β gene expression in the spleen of injected fish with *O. humicola*. (A): younger fish. (B): older fish. Asterisks (*) indicate a significant difference between *O. humicola*-injected and PBS-injected groups at the same time point ($p<0.05$).

5.4 DISCUSSION

5.4.1 The determination of T cell makers

It is well documented that teleost T cells have the functional equivalents and gene homologues to mammalian T cells (Wilson et al., 1998; Boudinot et al., 2001; Edhom et al., 2007; Hansen et al., 2011). The description of several T cell markers including CD4, CD8, CD3 and CD28 suggest that difference T cell subtypes exist in teleost fish (Laing & Hansen, 2011; Castro et al., 2011).

In this study, SebCD4, SebCD8β and SebCD3ε were highly expressed in the thymus. This result was consistent with findings observed in other fish species (Moore et al., 2005; Laing et al., 2006; Dijkstra et al., 2006; Edholm et al., 2007; Sun et al., 2007; Buonocore et al., 2008; Liu, et al., 2008; Øvergård et al., 2009). The fish thymus has been known function for T cell differentiation and development as observed in mammals (Castro et al., 2011; Toda et al., 2011). Since the thymus is a primary lymphoid organ for T cell lymphopoiesis and maturation in teleost (Hansen & Zapata, 1998), the higher thymic expression pattern of SebCD4, SebCD8β, and SebCD3ε has been expected.

SebCD4, SebCD8β, and SebCD3ε were moderately expressed in immune relevant organs, especially in the spleen, gills, intestines, and relatively low expression in the kidney. The expression of these T cells makers in the spleen is reasonable because the spleen has been known as an

important lymphoid organ in fish (Qi et al., 2016). Recent studies have highlighted the importance of the gills (Haugarvoll et al., 2008) and intestine (Boardman et al., 2012) as mucosal immune tissues in fish. Rombout et al. (1998) demonstrated that the T cells were abundantly found in mucosal immune tissues of carp, and these T cells represent a distinct subset from those present in systemic lymphoid tissues.

The most significant variation between SebCD28 and other T cell markers (SebCD4, SebCD8β, and SebCD3ε) was shown in the expression pattern. Expression of SebCD28 gene was highest in the liver but not in the thymus. SebCD28 gene moderately expressed in other lymphoid organs such as thymus, spleen, intestines and gills and this pattern was similar to finding in other fish species. The expression of CD28 gene was detected in lymphoid organs, liver, brain and heart in rock bream (Jeswin et al., 2017), while not detected in the brain, heart and liver of half-smooth tongue sole (Hu et al., 2012).

In humans, CD28 is expressed on CD4 and CD8 T cells, plasma cells, neutrophils and eosinophils (Lee et al., 1990; Boomer & Green, 2010). Recent studies have demonstrated that the existence of tissue-resident lymphocytes (non-circulating lymphocytes) in the liver of mammals and these cells are involved in innate and adaptive immune response (Gasteiger et al., 2015; Wang & Zhang, 2019).

The SebCD4, SebCD8β, and SebCD3ε consistently expressed in lymphoid organs, and showed the highest expression in thymus. Based on the expression pattern in lymphoid organs, it is convincing that SebCD4, SebCD8β,

and SebCD3ε were true gene orthologs and considered as T cell markers for marbled rockfish. With the different expression pattern of SebCD28, it has remained unknown whether CD28 is truly expressed in T cells of marbled rockfish.

5.4.2 Functional behaviour of the immune relevant genes

The expression of immune relevant genes was examined to verify functional behaviour of the genes under the stimulation with LPS and poly I:C, mimicking a pathogen infection. LPS, a component of the cell wall in Gram negative bacteria has been known as a potent immunostimulant in fish (Iwama & Nakanishi, 1996; Nya & Austin, 2010). LPS is beneficial to induce non-specific immune responses, such as activation of complement alternative pathway, phagocytic activity of the macrophage and proliferation of B and T lymphocytes in fish (Nya & Austin, 2010). Poly I:C is a synthetic structural analogue of double-stranded RNA which used to a mimic for viral infection (Matsumoto & Seya, 2008;).

Upregulation of the SebCD8β was observed after LPS and poly I:C stimulation. However, significant responses of SebCD4 genes was observed only after LPS stimulation but not after poly I:C stimulation. A study demonstrated that upregulation CD8β genes in the trunk kidney of Japanese flounder after *Edwardsiella tarda* and viral hemorrhagic septicemia virus infection (Kato et al., 2013). Moreover, CD4 gene expression was induced after LPS stimulation in seabass kidney leukocytes (Buonocore et al., 2008).

Patel et al. (2009) demonstrated that the gene expression of CD4 was not significantly increased at the early stage of infection by Nodavirus and suggesting that CD4 positive cells do not immediately proliferate after viral infection. SebCD4 showed no significant responses after poly I:C stimulation suggesting that 24 hours of experiment period may not be enough to observe the upregulation of CD4 genes. Quickly upregulation of SebCD8β was observed after poly I:C stimulation and this finding was similar as seen in orange-spotted grouper (Xu et al., 2011). Poly I:C can activate cytotoxic T lymphocytes and NK cells, which promoting adaptive immunity (Salem et al., 2009). Taken together these observations, the gene expression analysis of SebCD4 and SebCD8β can be used for estimation of T cell activity in marbled rockfish.

The elevated expression levels of SebIFN-γ were observed at 6 h and 12 h after poly I:C and LPS stimulation, respectively. The gene expression induced by LPS stimulation was slower than that by poly I:C stimulation. Furnes et al. (2009) demonstrated that Atlantic cod IFN-γ gene expression by formalin-killed *Vibrio anguillarum* was lower when compared to poly I:C stimulation, indicating that poly I:C is a potent stimulator for IFN-γ production. Previous works showed poly I:C was able to upregulate IFN-γ gene expression in zebrafish (Igawa et al., 2006) and grass carp (Chen et al., 2010). In the present study, expression of SebIFN-γ gene peaked at 6 h after poly I:C stimulation, thereafter the expression tended to decrease. Verjan et al. (2008) showed transient and quick IFN-γ response in rainbow trout intraperitoneally injected with recombinant hematopoietic necrosis virus glycoprotein.

The expression levels of SebIL-1β showed rapid upregulation from 6 h when treated with LPS and poly I:C but in different manners. The continuous expression was observed in leukocytes stimulated with LPS. On the other hand, the gene expression was downregulated after 12 h in leukocytes stimulated with poly I:C. Gardella et al. (2000) reported positive regulation of IL-1β gene by poly I:C in human dendritic cells and murine macrophages. Zou et al. (1999) demonstrated that LPS is a potent inducer of IL-1β gene in head kidney leukocytes and macrophages of rainbow trout. Previously studies reported upregulation of IL-1β by LPS or poly I:C stimulation in carp head kidney phagocytes (Engelsma et al., 2001), in sea bass blood, head-kidney, spleen, gill and hepatic leukocytes (Scapigliati et al., 2001) and in orange-spotted grouper PBL (Lu et al., 2008). Taken together with current results and similar observation to other fish species, SebIFN-γ and SebIL-1β seem to function in the immune response of marbled rockfish.

5.4.3 The relevance between the immune response and age in marbled rockfish

In this study, the difference of immune relevant gene expression between younger and older marbled rockfish under the stimulation with the pathogen *O. humicola* was analyzed. The results about the gene expression showed a unique profile that the younger fish characterized quick and intense responses, but the older fish characterized slow and moderate response. The upregulation of SebCD4 and SebCD8 gene expression occurred in the younger

fish at 5 d.p.i but not in the older fish. At 10 d.p.i, the expression of these genes became dropped to basal levels or lower in the younger fish. By contrast, the older fish started to upregulate the gene expression of SebCD4 from 7 d.p.i and kept the expression until 10 d.p.i. The expression of SebIFNγ gene displayed a similar pattern with SebCD4 gene expression in both younger and older marbled rockfish except for the response at 10 d.p.i.

The present results suggested the activation of helper T cells (Th) cells of marbled rockfish by the injection with *O. humicola*. This is supported by significant upregulation of SebCD4 and SebIFNy genes in the younger and the older fish but in a different manner. The activation of T cells involved gene expression such as CD4 and CD8 together with IFNγ has been reported as Th1 mediated response in Atlantic salmon infected with Salmonid alpha virus subtype 3 (SaV3) (Xu et al., 2012). The primary source of IFN-y is T cells and NK cells in mammals. Channel catfish T and NK cells also synthesize IFN-γ transcripts (Milev-Milovanovic et al., 2006).

The quick response of SebCD4 and SebIFNγ in younger marbled rockfish may correspond to the inflammatory response occurred in the adipose tissues and liver. The upregulation of the SebCD4 and SebIFN-γ transcripts in the spleen of younger fish is probably related to maturation or migration of immune cells to the adipose tissues and liver which showed severe damage by the experimental challenge with *O. humicola* (chapter 3). Also, infiltration of mononuclear cells was observed in the adipose tissues and liver of the younger fish. IFN-γ is an essential cytokine for the activation of mononuclear phagocytes such as macrophages (Wang & Secombes, 2013).

In this study, the differences between younger and older fish were marked at 5 d.p.i and 10 d.p.i. Higher expression of SebCD4, SebCD8 and SebIFN γ were observed at 5 d.p.i in the younger fish, whereas these genes were significantly induced at 10 d.p.i in the older fish. The dull and moderate expression of SebIFN-γ and SebCD4 in older fish seems to contribute the resistant capacity to *O. humicola* infection but the exact mechanism remains unclear. In Atlantic salmon fry challenged with infectious pancreatic necrosis virus (IPVN), the susceptible fish showed high levels of gene expression in many kinds of immune relevant genes, which were leading to eventual apoptosis (Robledo et al., 2016). Furthermore, lower inflammatory response was characterised in resistant individuals of Atlantic salmon challenged with infectious salmon anaemia virus (ISAV), which allowed fish to survive for a more extended period until the clearance of the virus (Jørgensen et al., 2008).

The increased expression of SebIFN-γ and SebCD4 may be responsible for the inflammatory response after *O. humicola* injection in younger marbled rockfish. Reyes-Lopez et al. (2015) reported that the inflammatory response in Atlantic salmon infected with IPNV was observed in susceptible individuals but not in resistant individuals. Several studies reported that increased expression of pro-inflammatory cytokines may be responsible for tissue damage, it has been described for the brain damage associated to the neurodegenerative disease such as viral encephalitis (Brabers & Nottel, 2006; Kim & Joh, 2006; Ghoshal et al., 2007).

Although inflammation is essential in immune response, however, intense or chronic activation of inflammatory processes seriously affects the host (Hussel & Goulding, 2010). Poisa-Beiro et al. (2007) demonstrated that upregulation of cytokine may be responsible for strong neuroinflammatory process in the brain of a susceptible species, like seabass infected with nodavirus. The inflammation has been described as an important factor causing brain damage in the pathogenesis of neurodegenerative diseases (Brabers & Nottel, 2006; Kim & Joh, 2006, Lafon et al., 2006; Sutton et al., 2006). Indeed, 'cytokine storm' and overwhelming inflammatory response have been linked to pathogenesis by highly pathogenic influenza virus (Shinya et al., 2009; Mcbeath et al., 2014). The mortality by *O. humicola* infection in the younger marbled rockfish may be caused by overactivation of CD4 positive T cells and overproduction of IFN-γ, which contribute to the tissues damage.

The difference of SebIL-1β gene expression was marked at 10 d.p.i in both the younger and the older fish, but the expression was not remarkably induced. The expression of SebIL-1β was upregulated in the older fish but downregulated in the younger fish. Up-regulation of SebIL-1β in older fish at 10 d.p.i. might be related to immune responses of intraperitoneal adipose tissues observed in the few older surviving fish which showed encapsulation of fungal hyphae. However, direct evidence on how SebIL-1β contribute to resistance capacity in older were remain unclear. Downregulation of IL-1β at 10 d.p.i in younger fish could indicate a decrease in the innate inflammatory responses at this point. The downregulation of IL-1β gene expression has been observed in rainbow trout the spleen of rainbow trout at 72 h after

Lactococcus garvieae infection (Castro et al., 2019), in the spleen of rainbow trout at 8 days after *Icthyophthirius multifilis* infection (Syahputra et al., 2019), and in rainbow trout macrophages infected with *Renibacterium salmoninarusm* (Grayson et al., 2002).

Expression of IL-1β gene quickly occurred in the liver and kidney of pink salmon at 24 h after the intraperitoneal injection of *Aeromonas salmonicida* (Fast et al., 2007). In the present study, gene expression of IL-1β was not analysed in the early time within 5 d.p.i. Their role of SebIL-1β in the immunological response against *O. humicola* infection still remains unclear.

5.5 SUMMARY

The present study showed that expression of SebCD4, SebCD8β, SebCD3ε occurred primarily in the thymus and other lymphoid organs such as spleen, intestines, gills and kidney. These data suggest that these genes were expressed on T cells and can be identified as T cell markers in marbled rockfish. On the other hand, SebCD28 showed higher 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 upregulated after stimulation with LPS and poly I:C, indicating that these genes have a similar role for immune response. These genes were useful in studying the immune response in marbled rockfish after *O. humicola* injection.

This study showed that the gene expression of SebCD4, SebCD8β, SebIFN-γ and SebIL-1β were upregulated in the spleen of the younger and older marbled rockfish after the *O. humicola* injection. The higher expression pattern of SebCD4 and SebIFN-γ in the younger fish probably initiates the inflammatory reaction in the adipose tissues and liver of the fish infected with *O. humicola* (chapter 3). The expression of these genes could be crucial for the recruitment for immune cells to eliminate infected fungi but this response in the 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.

CHAPTER 6

SUMMARY

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.

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