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**Maturation-associated changes in the non-specific immune response against
Flavobacterium psychrophilum in Ayu *Plecoglossus altivelis***

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ABSTRACT

In this study, we investigated maturation-associated changes in non-specific immune responses of ayu against *Flavobacterium psychrophilum*. The gonadosomatic index was minimum on 16 June, began to increase on 17 July, and reached the maximum value during August. The highest phagocytic rate (16.3%) was observed on 16 June, which decreased significantly to 5.6% on 26 August. The number of viable bacteria after the serum treatment was highest during August, suggesting that bactericidal activity of the serum decreased along with the sexual maturation. Gene expression levels of interleukin-8, and tumor necrosis factor- α in the spleen did not change significantly during this period, whereas the level of suppressor of cytokine signaling (SOCS)3 was significantly higher on 26 August than that on 16 July ($p < 0.05$). These results suggest that phagocytic activity of trunk kidney leukocytes and serum bactericidal activity against *F. psychrophilum* decreased with sexual maturation, and that SOCS3 may be related to the decrease in non-specific immune activity in ayu.

Keywords: *Plecoglossus altivelis*, *Flavobacterium psychrophilum*, sexual maturation, non-specific immunity, suppression of cytokine signaling

1. Introduction

Ayu *Plecoglossus altivelis* is the most economically important fish species in Japanese freshwater fisheries as a culinary delicacy and a popular game fish. Ayu have been cultured since 1904, and these fish are used for food and released into rivers as game fish targets. Production output amounts to one-third of the total value of the freshwater fishery and aquaculture production in Japan (<http://www.e-stat.go.jp/SG1/estat/Xlsdl.do?sinfid=000023620693>, accessed 23 June 2017). However, bacterial diseases, such as bacterial cold water disease caused by *Flavobacterium psychrophilum* [1], edwardsiellosis caused by *Edwardsiella ictaluri* [2], and bacterial hemorrhagic ascites caused by *Pseudomonas plecoglossicida* [3] cause significant losses of fish.

Ayu has an approximate 1-year life span. Ayu larvae hatch in freshwater, migrate to the sea, and the juveniles return to freshwater habitats. Mature fish spawn during autumn in the lower reaches of a freshwater system and immediately die after spawning. The prevalence of *F. psychrophilum* has been reported to increase to > 90% between October and November [4]. *E. ictaluri* is most frequently isolated from river ayu in September and October [5]. Physiological changes associated with sexual maturation and/or ageing are considered the reason for the higher susceptibility of ayu to pathogens

during summer and autumn.

Leukocyte immune responses are suppressed in salmonids during the spawning season, along with elevated cortisol and testosterone levels. Sexually mature fish have high plasma cortisol titers and generate relatively fewer antibody-producing cells of peripheral blood leukocytes in chinook salmon *Oncorhynchus tshawytscha* [6] and rainbow trout *O. mykiss* [7]. Administering testosterone and cortisol reduces the plaque forming responses in primary cultured chinook salmon leukocytes [8]. In addition, steroid hormones, such as cortisol, testosterone, estradiol-17 β , and 11-ketotestosterone reduce the number of IgM-secreting cells and specific antibody production *in vitro* [9]. The *in vitro* immunosuppressive effect of cortisol is also observed in common carp *Cyprinus carpio* [10]. However, the association between these immune responses and maturation remains unknown in the annual fish species ayu.

The non-specific innate immune system is thought to be more important than acquired immunity in ayu because it is a short living fish. Neutrophils account for 60–80% of **trunk** kidney leukocytes in ayu and display unusually high respiratory burst activities compared with those of other fish species [11,12]. In contrast, the percentage of B cells is only 4% of peripheral blood leukocytes [13], and only a few IgM- and IgT-mRNA-positive cells are detectable in the **trunk** kidney of ayu [14]. We hypothesized that

the suppressed non-specific immune responses are the cause for the high infection rate in sexually mature ayu. Therefore, we investigated the role of maturation-associated changes in the non-specific immune response of ayu against *F. psychrophilum* in this study.

2. Materials and methods

2.1 Bacteria propagation

F. psychrophilum strain GMA0330 isolated from wild diseased ayu in Gunma Prefecture [15] was used for this study. The bacteria were cultured on modified cytophaga (MCY) agar or broth at 15°C for 48 h [16]. The bacterial cultures were serially diluted and incubated on MCY agar at 15°C to count colony forming units (CFU).

2.2 Fish rearing conditions

A domesticated stock of ayu *P. altivelis*, that had been maintained by intrastock breeding for 45 and 46 generations at the Gunma Prefectural Fisheries Experimental Station (mean body weight = 16.6 g on 16 July 2015), was used in this study. Fish were reared in 5 or 50-ton tanks with flow-through water conditions under natural day length and water temperature of 15°C –16°C. Fish were fed every day with standard fish pellets

at the rate of 3% of fish body weight. Apparently healthy ayu were used in the experiments shown in below.

2.3 Sampling procedure

Ayu (45 generations) were collected on 16 June, 1, 17 and 29 July, and 5, 12, 19, and 26 August 2015. Five fish were collected randomly while their sex could not be distinguished (16 June and 17 July). Three males and three females were collected randomly after their sex could be distinguished (after 17 July). The fish were weighed and anesthetized in FA 100 (final concentration = 20 ppm, DS Pharma Animal Health, Osaka, Japan). The gonads were removed and weighed to calculate the gonadosomatic index (GSI). GSI (%) was expressed as follows: $[\text{gonad weight/fish body weight}] \times 100$. Blood was collected from each fish by venipuncture with a syringe. After coagulation, the blood samples were centrifuged at 3,000 rpm for 10 min, and the serum was collected and stored at -80°C until use. The trunk kidney was dissected from each fish and smashed on a 79 μm nylon mesh in RPMI 1640 (Nissui, Tokyo, Japan). The cell suspensions were centrifuged at $400 \times g$ for 5 min and resuspended in the medium. The cell suspensions were immediately subjected to the phagocytic assay described below. The spleen was collected from each fish and stored in RNA later (Thermo Fisher Scientific, Waltham,

MA, USA) at -80°C until use.

In addition, ayu (46 generations) were collected on 16 June ($n = 3$, sex was unidentified) and 16 September 2016 ($n = 8$, 4 females and 4 males), and GSI was calculated, as described above. The liver was collected in RNA later and stored at -80°C until use.

2.4 Phagocytosis assay

Phagocytosis assay was performed as previously described in Wiklund and Dalsgaard (2003) [17]. Briefly, *F. psychrophilum* (1 mg wet weight) collected from MCY agar was added to 3.0×10^5 trunk kidney cells and incubated at 18°C for 30 min. The mixture was spread on a glass slide, and the slide was stained using May-Grunwald's stain solution (Nacalai tesque, Kyoto, Japan) and Giemsa's stain solution (Nacalai tesque, Kyoto, Japan), according to the manufacturer's instructions. More than 1,000 leukocytes were observed at random under the microscope and the phagocytic rate was expressed as follows: phagocytic rate (%) = [number of leukocytes with phagocytized bacteria/number of observed leukocytes] $\times 100$.

2.5 Colorimetric assay for serum bacteriolytic activity

A colorimetric assay was used to determine serum bacteriolytic activity as described previously by Ito et al. [18]. Lyophilized *Micrococcus luteus* cells ATCC No. 4698 (Sigma-Aldrich, St. Louis, MO, USA) were stained with Remazol Brilliant Blue R solution and suspended in phosphate buffer (50 mM KH₂PO₄-NaOH, pH 7.0). Ayu serum was diluted 1:10 and inactivated by heating at 44°C in a water bath for 20 min. The inactivated serum was added to the stained *M. luteus* and incubated at 40°C for 4 h. After adding the stop solution (1 M NaOH), the supernatant was separated and collected by centrifugation. Absorbance (OD, 600 nm) was measured with the MPR-A4i microplate reader (TOSOH, Tokyo, Japan).

2.6 Serum bactericidal activity

F. psychrophilum prepared as described above was added to serum (without inactivation), mixed thoroughly, and incubated at 15°C for 6 h. The serum-treated bacteria were collected by centrifugation, and the number of CFUs of bacteria was determined on an MCY agar plate as described above.

2.7 Expression levels of immune related genes in the spleen and liver

Total RNA was extracted from the spleen and liver using ISOGEN (Nippon Gene, Tokyo, Japan), following the manufacturer's instructions. First-strand cDNA was synthesized with 2 µg of total RNA from each fish using MMLV reverse transcriptase (Thermo Fisher Scientific), following the manufacturer's instructions. CD83 (GenBank Accession Number, LC310721), granulocyte colony-stimulating factor (GCSF) (LC310723), interleukin (IL)-8 (KJ652902), suppressor of cytokine signaling (SOCS) 1 (LC310869), SOCS3 (LC218425), and tumor necrosis factor- α (TNF- α) (DD019003) gene expression levels in the spleen were determined by quantitative real-time-PCR (qPCR) analysis. In addition, the mRNA levels for G-type lysozyme (LC310722) and SOCS3 in the liver were also determined by qPCR. The gene expression level of elongation factor 1 α (EY510389) in each sample was used as an internal control. Gene specific primers (Table 1) were designed using Primer3Plus software (<http://primer3plus.com/cgi-bin/dev/primer3plus.cgi>, accessed 23 June 2017). The reaction mixtures containing each cDNA sample were prepared using THUNDERBIRD SYBR qPCR Mix (Toyobo, Tokyo, Japan), following the manufacturer's instructions. qPCR was performed using a LightCycler 480 II (Roche Diagnostics, Mannheim, Germany), following the manufacturer's instructions.

2.8 Statistical analysis

Significant differences ($p < 0.05$) among the experimental time points in the phagocytosis assay, colorimetric assay, and bactericidal activity test were assessed using one-way analysis of variance and Tukey's *post-hoc* test. Student's *t*-test was used to detect significant differences ($p < 0.05$) in **trunk** kidney and liver gene expression between **the value on 16 June** and the others.

3. Results

3.1 GSI

The GSI increased beginning on 17 July (**the age of ayu was 45 weeks old**), and the maximum value was recorded in late August 2015 (Figure 1A and B). The changes in the GSI values in 2016 were similar to those in 2015 (data not shown).

3.2 Phagocytosis assay

F. psychrophilum was phagocytized by monocytes with a pale blue-stained cytoplasm (Figure 2A) and neutrophils that had segmented nuclei (Figure 2B). The phagocytic rate of these cells was 16.3% on 16 June; it decreased significantly to 10.8% on 17 July and to 6.3% on 5 August (Figure 2C).

3.3 Serum bacteriolytic and bactericidal activities

The colorimetric assay revealed that the bacteriolytic activity of ayu serum was highest on 16 June and then decreased gradually along with the experimental time course (Figure 3). Bacteriolytic activity on 26 August was significantly lower than the value on 16 June ($p < 0.05$). Consistently, serum bactericidal activity in August was lower than that detected during June and July (Figure 4). The number of CFU after the serum treatment was significantly higher on 5 and 19 August, compared with the values during June and July ($p < 0.05$).

3.4 qPCR of the immune related genes

No significant differences in the CD83, G-CSF, IL-8, SOCS1, or TNF α gene expression were detected in the spleen among the eight time points (Figure 5). Whereas, the SOCS3 gene expression level in the spleen was higher in June than that in late August ($p < 0.05$). Since SOCS3 gene is highly expressed also in the liver of teleost fish [19–21], we performed qPCR analysis using the liver samples collected in 2016, to complement the results in the spleen samples in 2015. The SOCS3 gene expression level in the liver on 16 September was 4.7-fold higher ($p < 0.05$) than that on 16 June (Figure 6). G-type

lysozyme gene expression was significantly downregulated 0.6-fold in the liver on 16 June ($p < 0.05$), compared with that on 16 September (Figure 6).

4. Discussion

The biological defenses of ayu have been believed to be weak after sexual maturation, based on experiences of the ayu culture industry. However, little is known about the changes in immune activities in ayu with maturation. In this study, we showed that non-specific immune activities of ayu against *F. psychrophilum* decreased with sexual maturation. The decrease in non-specific immune activities is probably one of the main causes of the high susceptibility to bacterial diseases during summer and autumn in ayu.

Based on the classification of ayu leukocyte subpopulations by Nakada et al., [22] neutrophils and macrophages were the phagocytes containing *F. psychrophilum* in this study. Neutrophils account for 60–80% of total trunk kidney leukocytes in ayu [12] and these cells have an unusually high respiratory burst activity [11]. Macrophages plays important roles protecting against *F. psychrophilum* by producing reactive oxygen species in rainbow trout [23]. Phagocytic rates of leukocytes also decrease during winter in rainbow trout [24]. The increased affinity between leukocytes and maturation hormones

216 is a possible cause for the decreased phagocytic rates [25]. In this study, we showed that
217 the phagocytic rates of **trunk** kidney leukocytes decreased significantly with maturation.
218 Thus, the decrease in the phagocytic rate is probably one of the causes for the decreased
219 resistance against bacterial infection in ayu. Moreover, the increase in the levels of
220 maturation hormones may suppress phagocytic activity in ayu.

221 CD83 is a marker molecule of mature dendritic cells in mammals [26], and a
222 homolog is highly expressed in phagocytes, such as macrophages and neutrophils, of
223 gilthead seabream *Sparus aurata* [27], turbot *Scophthalmus maximus* [28], and ayu [13].
224 The CD83 gene expression level remained unchanged with maturation in ayu, suggesting
225 that the number of phagocytes remained unchanged during the time course. In addition,
226 no significant change was observed in G-CSF, IL-8, or TNF α gene expression levels,
227 suggesting that maturation did not affect expression of these cytokine genes in ayu.
228 SOCS3 gene expression, but not SOCS1, was significantly upregulated after maturation.
229 SOCS1 and SOCS3 play a key role in the negative regulation of interleukins by
230 suppressing the Janus kinase/signal transducers and activators of transcription
231 (JAK/STAT) pathway in mammals and teleost fish [29–31]. SOCS1 suppresses
232 interferon- γ expression by inhibiting STAT1 phosphorylation, whereas SOCS3
233 suppresses IL-2 and IL-6 expression by inhibiting STAT3 phosphorylation in mammals

[32]. Furthermore, Gordon *et al.* (2016) demonstrated that SOCS3 knockdown significantly enhances the phagocytic capacity of human macrophages, indicating that SOCS3 suppress phagocytic activity of the macrophage [33]. Therefore, suppression of cytokines by SOCS3 might result in decreased neutrophil and macrophage activity in ayu.

Lysozyme was initially associated with defense against Gram-positive and Gram-negative bacteria [34]. In fish, lysozyme has broader activity than that in mammals [35]. Lysozyme is also an opsonin that activates the complement system and phagocytes [36]. *F. psychrophilum* is resistant to the action of complement activity present in rainbow trout [37] and ayu sera [38]. In this study, we showed that bacteriolytic and bactericidal activities decreased in serum during August. These data suggest that the decrease in lysozyme activity, rather than complement activity, affected the resistance to *F. psychrophilum* by ayu during maturation.

In conclusion, phagocytic activity of trunk kidney leukocytes against *F. psychrophilum* decreased during summer and autumn in ayu. Phagocytic activity is probably modulated by upregulation of SOCS3 during maturation. Furthermore, serum bactericidal and bacteriolytic activities also decreased during summer and autumn, with downregulation of lysozyme activity. The decrease in non-specific immune activities

251 may be one of the causes for the high susceptibility of ayu to pathogenic bacteria during
252 summer and autumn.

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388
 389

Figure legends

Figure 1

Changes in gonadosomatic index (GSI) of female ayu (A) and male ayu (B) during June–August 2015. Error bars represent standard deviation.

Figure 2

The change in phagocytic rates of ayu **trunk** kidney leukocytes against *Flavobacterium psychrophilum*. The main populations of **trunk** kidney leukocytes that phagocytized the bacteria: macrophages (A) and neutrophils (B). Scale bar, 10 μ m. Mean phagocytic rate values ($n = 6$) of **trunk** kidney leukocytes against the bacteria are shown (C). Error bars represent standard deviation and different letters represent significant differences between the groups ($p < 0.01$).

Figure 3

Changes in serum bacteriolytic activity against *Micrococcus luteus*. Error bars represent standard deviation, and different letters represent significant differences between the groups ($p < 0.05$).

408

409 Figure 4

410 Changes in serum bactericidal activity against *Flavobacterium psychrophilum*. Colony
411 forming units of *F. psychrophilum* treated with the serum collected at each time point. An
412 increase in the CFU values indicates a decrease in serum bactericidal activity. Error bars
413 represent standard deviation, and different letters represent significant differences
414 between the groups ($p < 0.05$).

415

416 Figure 5

417 Changes in the gene expression levels of CD83, granulocyte colony-stimulating factor
418 (GCSF), interleukin (IL)-8 suppressor of cytokine signaling (SOCS)1, SOCS3, and tumor
419 necrosis factor (TNF) α in the spleen. Error bars represent standard deviation. P-values
420 calculated from the t -test are shown.

421

422 Figure 6

423 Changes in G-type lysozyme and suppressor of cytokine signaling (SOCS)3 gene
424 expression levels in the liver. Error bars represent standard deviation. P-values calculated
425 from the t -test are shown.