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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 3

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

55 during summer and autumn.

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

67 The non-specific innate immune system is thought to be more important than
68 acquired immunity in ayu because it is a short living fish. Neutrophils account for 60–
69 80% of trunk kidney leukocytes in ayu and display unusually high respiratory burst
70 activities compared with those of other fish species [11,12]. In contrast, the percentage of
71 B cells is only 4% of peripheral blood leukocytes [13], and only a few IgM- and IgT-
72 mRNA-positive cells are detectable in the trunk kidney of ayu [14]. We hypothesized that

73 the suppressed non-specific immune responses are the cause for the high infection rate in
74 sexually mature ayu. Therefore, we investigated the role of maturation-associated
75 changes in the non-specific immune response of ayu against *F. psychrophilum* in this
76 study.
77
78 **2. Materials and methods**
79 *2.1 Bacteria propagation*
80 *F. psychrophilum* strain GMA0330 isolated from wild diseased ayu in Gunma
81 Prefecture [15] was used for this study. The bacteria were cultured on modified cytophaga
82 (MCY) agar or broth at 15°C for 48 h [16]. The bacterial cultures were serially diluted
83 and incubated on MCY agar at 15°C to count colony forming units (CFU).
84
85 *2.2 Fish rearing conditions*
86 A domesticated stock of ayu *P. altivelis*, that had been maintained by intrastock
87 breeding for 45 and 46 generations at the Gunma Prefectural Fisheries Experimental
88 Station (mean body weight = 16.6 g on 16 July 2015), was used in this study. Fish were
89 reared in 5 or 50-ton tanks with flow-through water conditions under natural day length
90 and water temperature of 15°C –16°C. Fish were fed every day with standard fish pellets

91 at the rate of 3% of fish body weight. Apparently healthy ayu were used in the experiments
92 shown in below.
93
94 *2.3 Sampling procedure*
95 Ayu (45 generations) were collected on 16 June, 1, 17 and 29 July, and 5, 12, 19,
96 and 26 August 2015. Five fish were collected randomly while their sex could not be
97 distinguished (16 June and 17July). Three males and three females were collected
98 randomly after their sex could be distinguished (after 17 July). The fish were weighed
99 and anesthetized in FA 100 (final concentration = 20 ppm, DS Pharma Animal Health,
100 Osaka, Japan). The gonads were removed and weighed to calculate the gonadosomatic
101 index (GSI). GSI (%) was expressed as follows: [gonad weight/fish body weight] × 100.
102 Blood was collected from each fish by venipuncture with a syringe. After coagulation,
103 the blood samples were centrifuged at 3,000 rpm for 10 min, and the serum was collected
104 and stored at −80°C until use. The trunk kidney was dissected from each fish and smashed
105 on a 79 μm nylon mesh in RPMI 1640 (Nissui, Tokyo, Japan). The cell suspensions were
106 centrifuged at 400 × *g* for 5 min and resuspended in the medium. The cell suspensions
107 were immediately subjected to the phagocytic assay described below. The spleen was
108 collected from each fish and stored in RNA later (Thermo Fisher Scientific, Waltham,

109 MA, USA) at −80°C until use.

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

114

115 *2.4 Phagocytosis assay*

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

125

126 *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 KH_2PO_4-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

144 Total RNA was extracted from the spleen and liver using ISOGEN (Nippon
145 Gene, Tokyo, Japan), following the manufacturer's instructions. First-strand cDNA was
146 synthesized with 2 μg of total RNA from each fish using MMLV reverse transcriptase
147 (Thermo Fisher Scientific), following the manufacturer's instructions. CD83 (GenBank
148 Accession Number, LC310721), granulocyte colony-stimulating factor (GCSF)
149 (LC310723), interleukin (IL)-8 (KJ652902), suppressor of cytokine signaling (SOCS) 1
150 (LC310869), SOCS3 (LC218425), and tumor necrosis factor-α (TNF-α) (DD019003)
151 gene expression levels in the spleen were determined by quantitative real-time-PCR
152 (qPCR) analysis. In addition, the mRNA levels for G-type lysozyme (LC310722) and
153 SOCS3 in the liver were also determined by qPCR. The gene expression level of
154 elongation factor 1α (EY510389) in each sample was used as an internal control. Gene
155 specific primers (Table 1) were designed using Primer3Plus software
156 (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi, accessed 23 June 2017). The
157 reaction mixtures containing each cDNA sample were prepared using THUNDERBIRD
158 SYBR qPCR Mix (Toyobo, Tokyo, Japan), following the manufacturer's instructions.
159 qPCR was performed using a LightCycler 480 II (Roche Diagnostics, Manheim,
160 Germany), following the manufacturer's instructions.
161

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).

180

181 *3.3 Serum bacteriolytic and bactericidal activities*

182 The colorimetric assay revealed that the bacteriolytic activity of ayu serum was
183 highest on 16 June and then decreased gradually along with the experimental time course
184 (Figure 3). Bacteriolytic activity on 26 August was significantly lower than the value on
185 16 June ($p < 0.05$). Consistently, serum bactericidal activity in August was lower than that
186 detected during June and July (Figure 4). The number of CFU after the serum treatment
187 was significantly higher on 5 and 19 August, compared with the values during June and
188 July ($p < 0.05$).

189

190 *3.4 qPCR of the immune related genes*

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

198 lysozyme gene expression was significantly downregulated 0.6-fold in the liver on 16
199 June ($p < 0.05$), compared with that on 16 September (Figure 6).
200
201 **4. Discussion**
202 The biological defenses of ayu have been believed to be weak after sexual
203 maturation, based on experiences of the ayu culture industry. However, little is known
204 about the changes in immune activities in ayu with maturation. In this study, we showed
205 that non-specific immune activities of ayu against *F. psychrophilum* decreased with
206 sexual maturation. The decrease in non-specific immune activities is probably one of
207 the main causes of the high susceptibility to bacterial diseases during summer and
208 autumn in ayu.
209 Based on the classification of ayu leukocyte subpopulations by Nakada et al.,
210 [22] neutrophils and macrophages were the phagocytes containing *F. psychrophilum* in
211 this study. Neutrophils account for 60–80% of total trunk kidney leukocytes in ayu [12]
212 and these cells have an unusually high respiratory burst activity [11]. Macrophages plays
213 important roles protecting against *F. psychrophilum* by producing reactive oxygen species
214 in rainbow trout [23]. Phagocytic rates of leukocytes also decrease during winter in
215 rainbow trout [24]. The increased affinity between leukocytes and maturation hormones

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

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

234 [32]. Furthermore, Gordon *et al.* (2016) demonstrated that SOCS3 knockdown
235 significantly enhances the phagocytic capacity of human macrophages, indicating that
236 SOCS3 suppress phagocytic activity of the macrophage [33]. Therefore, suppression of
237 cytokines by SOCS3 might result in decreased neutrophil and macrophage activity in ayu.

238 Lysozyme was initially associated with defense against Gram-positive and
239 Gram-negative bacteria [34]. In fish, lysozyme has broader activity than that in mammals
240 [35]. Lysozyme is also an opsonin that activates the complement system and phagocytes
241 [36]. *F. psychrophilum* is resistant to the action of complement activity present in rainbow
242 trout [37] and ayu sera [38]. In this study, we showed that bacteriolytic and bactericidal
243 activities decreased in serum during August. These data suggest that the decrease in
244 lysozyme activity, rather than complement activity, affected the resistance to *F.*
245 *psychrophilum* by ayu during maturation.

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

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

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390 **Figure legends**

391 Figure 1

392 Changes in gonadosomatic index (GSI) of female ayu (A) and male ayu (B) during June–

393 August 2015. Error bars represent standard deviation.

394

395 Figure 2

396 The change in phagocytic rates of ayu trunk kidney leukocytes against *Flavobacterium*

397 *psychrophilum*. The main populations of trunk kidney leukocytes that phagocytized the

398 bacteria: macrophages (A) and neutrophils (B). Scale bar, 10 μm. Mean phagocytic rate

399 values (n = 6) of trunk kidney leukocytes against the bacteria are shown (C). Error bars

400 represent standard deviation and different letters represent significant differences between

401 the groups ($p < 0.01$).

402

403 Figure 3

404 Changes in serum bacteriolytic activity against *Micrococcus luteus*. Error bars represent

405 standard deviation, and different letters represent significant differences between the

406 groups ($p < 0.05$).

407

Figure 4

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

Figure 5

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

Figure 6

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