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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.

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-17β, 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

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Table 1
Primers used in this study.

Primer name	Sequence (5′–3′)
G-CSF F	CCTGACCCTCGACCCCTCCA
G-CSF R	AAGGCCTTGAAGGTGGGGGC
CD83 F	TCTGCCCCTCCTGGTTCCGA
CD83 R	GCAGGTCTGGAGCGAGAGGTG
TNFα F	GAAGGCTTCTCTGTCGGTAACC
TNFα R	CCCCTGCTTTTGATAACGATCT
IL-8 F	GGAGCTGACCTTCGCTGCCA
IL-8 R	TGGCCTGTCTGCTTCAGCGT
Lysozyme G F	AGCATGTCCGTCAAGCCGCT
Lysozyme G R	GCACCCTGGACGCTCCCATA
SOCS1 F	AAAGAGCCTGAGCCTTCCCTAC
SOCS1 R	CTGGAATTTGCTCGACCTTCTC
SOCS3 F	TGACAACCGACATTTCTTCACC
SOCS3 R	TGCTGGAATCACACTGGACAC
EF1α F	GCTGCCGGCTCCTTCAC
EF1α R	AGATCTGTCCAGGGTGGTTCA

GCSF, granulocyte colony-stimulating factor; TNF, tumor necrosis factor; IL, interleukin; SOCS, suppressor of cytokine signaling; EF, elongation factor.

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 17July). 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: [gonad weight/fish body weight] × 100. Blood was collected from each fish by venipuncture with a syringe. After coagulation, the blood samples were centrifuged at 3000 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 × *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.

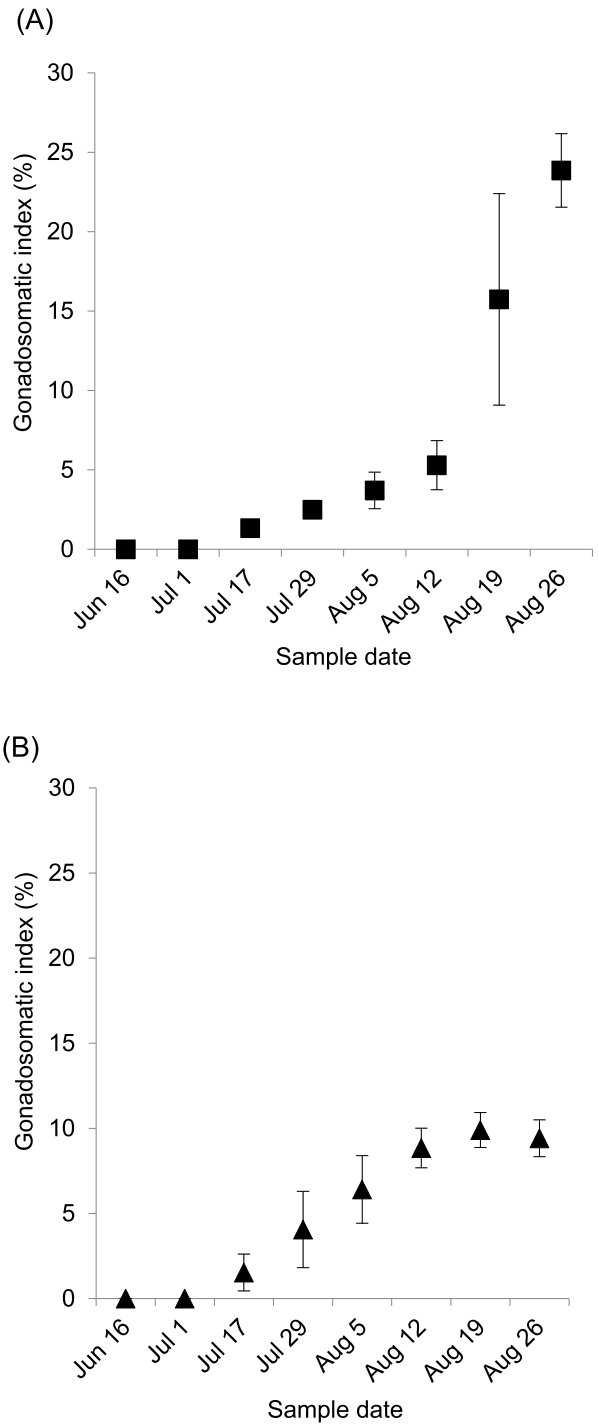

Fig. 1. Changes in gonadosomatic index (GSI) of female ayu (A) and male ayu (B) during June–August 2015. Error bars represent standard deviation.

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