Research article

Changes in Resistance against Bacterial Cold-Water Disease and in Leukocyte Composition along with Sexual Maturation in Ayu *Plecoglossus altivelis*

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ABSTRACT—In the present study, we investigated changes in resistance against bacterial cold-water disease and in leukocyte composition along with sexual maturation in ayu. Fish were reared under long photoperiod (LP) and the natural day length (ND) during September to November. Ayu sampled before starting the experiment was used as an initial control (IN). Gonad-somatic index significantly increased in ND group, but not in LP group, compared with IN group. Cumulative survival rate after challenge with 2.7×10^4 CFU of *Flavobacterium psychrophilum* was significantly higher in LP group (90%) than that in ND group (0%). Gene expression levels for lymphocyte markers (IgM, IgD, CD3 ϵ and TCR β) in the trunk kidney were significantly lower in ND group compared with the values in IN group. Consistently, the decrease of IgM and CD8 α mRNA positive cells in the trunk kidney of ND group was also shown by *in situ* hybridization. Further, flow cytometry revealed that percentage of B lymphocytes in the trunk kidney leukocytes were significantly decreased in ND group than in the other groups. These data suggest that sexual maturation is the major cause of the decrease in disease resistance and of the lymphocytopenia, in ayu during the spawning season.

Key words: Lymphocytopenia, T lymphocyte, B lymphocyte, Thrombocyte, *Plecoglossus altivelis*, *Flavobacterium psychrophilum*, BCWD

Ayu *Plecoglossus altivelis* is an amphidromous fish species that has 1-year life span. Ayu larva hatches in middle or lower reaches of a river, immediately migrates to the coastal area of a sea, and the juvenile fish run-up to the upstream. Adult fish starts sexual maturation after the summer solstice, spawn in the middle or lower reaches of the river during autumn season and die after the spawning. Ayu is the most important fish species for freshwater fisheries in Japan, production output amounts more than 44% of the total value of the freshwater fishery and aquaculture production in 2017 (<https://www.e-stat.go.jp/stat-search/file-download?statInfId=000031831322&fileKind=0>). However, infectious diseases such as bacterial cold-water disease (BCWD) (Wakabayashi *et al.*, 1994), edwardsiellosis (Sakai *et al.*, 2008) and atypical cellular gill disease (Wada *et al.*,

2011) cause a severe loss of fish production in ayu fisheries in Japan.

There is a close relationship between sexual maturation and disease resistance/immune activities in teleost fish. Sexually mature brown trout were more frequently or more severely infected by species of *Ichthyophthirius*, *Scyphidia*, *Gyrodactylus* and *Saprolegnia* compared with immature fish (Pickering and Christie, 1980). The prevalence of *Flavobacterium psychrophilum* in ayu was increased more than 90% during the spawning season (Kumagai *et al.*, 2010). Further, *Edwardsiella ictaluri* was most frequently isolated from ayu caught in a river during the spawning season (Hassan *et al.*, 2012). In salmonid fish, lymphocytopenia induced by elevated level of serum cortisol has been suggested as a cause of the high susceptibility to infectious disease of sexually matured brown trout (Pickering, 1984; Pickering and Pottinger, 1987). Hou *et al.* (1999) showed that the numbers of B cells and serum IgM levels in rainbow trout

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were decreased during the spawning season, along with elevations in plasma cortisol (Hou *et al.*, 1999). Bactericidal activity of the serum also decreases during spawning season in rainbow trout (Iida *et al.*, 1989). In addition, nonspecific immune activities such as phagocytic activity, serum bactericidal activity and lysozyme activity are suppressed along with sexual maturation in ayu (Minami *et al.*, 2018). In this way, innate and adoptive immune activities are decreased along with the sexual maturation, so that sexually matured fish are highly susceptible to infectious diseases in spawning season.

Photoperiod and water temperature are the most important factors for controlling sexual maturation in teleost fish (de Vlaming, 1972). In ayu, photoperiod is the dominant factor for the reproduction cycle: Ayu sexually mature in short light period and the maturation is suppressed in long photoperiod (Shiraishi, 1965). The suppression of sexual maturation makes life span of the fish longer, since ayu is an annual fish species. Therefore, ayu are usually reared under the long photoperiod in culture pond in order to prolong shipment period (Fushiki, 1968; Hirano *et al.*, 1980). In addition, ayu reared under the long photoperiod are often used for vaccination and challenge test (Masunari *et al.*, 2001). However, immunological properties of ayu reared under the long photoperiod are largely unknown. In the present study, we aimed to investigate the disease resistance against BCWD and the proportion of leukocyte subpopulation, using ayu reared under long photoperiod and natural day length. Present results may be useful for immunological studies including development of vaccine for ayu aquaculture.

Materials and Methods

Fish rearing

Ayu were reared in 5- or 50-ton tanks with ground-water at 15°C–16°C in Gunma Prefectural Fisheries Experimental Station. The fish were maintained with flow-through water conditions under the natural day length until July 11, 2016 (20 days after the summer solstice) and reared at long day photoperiod at 24 h light/0 h dark condition for 69 days. They were divided into two different groups as follows. One group was reared at the long photoperiod (LP group) and the other group was reared under the natural day-length (ND group). Fish were weighed, anesthetized in eugenol or 2-phenoxyethanol, and dissected at 1 day before starting experiment (an initial control, IN group), 39 days and 61 days after the starting point (Fig. 1A). The gonadal tissue was collected and gonad somatic index (GSI) was calculated as follows: $[\text{gonad weight}/\text{fish body weight}] \times 100$. Significant differences among the groups were assessed using ANOVA and Ryan's post-hoc test.

The fish rearing experiment were performed again in 2017, in order to investigate difference of leukocyte

properties between IN and LP/ND group by flow cytometry. The fish were maintained with flow-through water conditions under the natural day length until the summer solstice (June 21) in 2017 and reared at the long day photoperiod for 100 days. They were also divided into two groups (LP and ND group) and sampled at 3 days before starting experiment (IN group) and 39 days after the starting point (Fig. 1B). GSI was also calculated as described above and significant differences among the groups were assessed using ANOVA and Ryan's post-hoc test. ($n = 2-6$).

Challenge test

Flavobacterium psychrophilum strain GMA0330 isolated from wild diseased ayu in Gunma Prefecture (Arai *et al.*, 2007) was cultured on modified cytophaga (MCY) agar or broth at 15°C for 48 h (Wakabayashi and Egusa, 1974). The bacterial cultures were serially diluted with MCY broth and incubated on MCY agar at 15°C for 48 h to count colony forming units (CFU). Ayu reared in each group ($n = 19-20$) were intraperitoneally challenged with 2.7×10^4 or 2.7×10^5 CFU of *F. psychrophilum* at 61 days after the starting point. The mortalities were recorded for 18 days. The cumulative survival curves were generated by Kaplan-Meier method (Kaplan and Meier, 1958) and significant differences among the groups were assessed using ANOVA and Ryan's post-hoc test.

Gene expression analyses

The spleen was removed after the anesthesia and stored in RNA later at -80°C until use. Total RNA was extracted using ISOGEN (Nippon Gene) and first-strand cDNA was synthesized with 2 μg of the total RNA using MMLV reverse transcriptase (Thermo Fisher Scientific), following the manufacturer's instructions. IgM (GenBank Accession Number, AB703441), IgT (AB921957), IgD (AB921959), CD3 ϵ (LC535224), CD8 α (LC535225), and T cell receptor (TCR) β (LC535226) gene expression level in the spleen was determined by quantitative real-time PCR (qPCR). Gene specific primers (Table 1) were designed using Primer3Plus software (<http://primer3plus.com/cgi-bin/dev/primer3plus.cgi>, accessed on March 23, 2020). The reaction mixture containing cDNA sample was prepared using THUNDERBIRD SYBR qPCR Mix (Toyobo), following the manufacturer's instructions. qPCR was performed using a LightCycler 480 II (Roche Diagnostics), following the manufacturer's instructions. The gene expression level was calculated from a standard curve for each gene and the gene expression level of elongation factor 1 α (EY510389) was used as an internal control. The gene expression values were taken as the mean values of six individuals and presented as the fold-change relative to the value of each gene in IN group, which was set to 1. Significant differences among the groups were assessed using

Fig. 1. Schedule for fish rearing and gonad-somatic index (GSI) of initial (IN), long photoperiod (LP) and the natural day length (ND) group. (A) Fish were reared under LP condition for 69 days and then divided into two groups for GSI, gene expression analyses (GEA), *in situ* hybridization (ISH), flow cytometry (FCM) and challenge test in 2016. A black triangle indicates the starting point of the experiment. (B) Fish were reared under LP condition for 100 days and divided into two groups for GSI and FCM in 2017. A black triangle indicates the starting point of the experiment. (C) GSI values for IN, LP and ND (both male and female) at 39 and 61 days after starting the experiment in 2016. Different letters represent significant differences between the groups ($p < 0.01$). NA, not applicable. (D) GSI values for IN, LP and ND (both male and female) at 39 days after starting the experiment in 2017. Different letters represent significant differences between the groups ($p < 0.01$). NA, not applicable.

ANOVA and Ryan's post-hoc test.

In situ hybridization

Digoxigenin-labeled antisense RNA probes for IgM and CD8 α were generated using a DIG RNA labeling kit (Roche Applied Science), following the manufacturer's instructions. The DNA fragments amplified by PCR with the gene-specific primers (Table 1) were cloned into pGEM T-easy vector (Promega) and used as templates for antisense RNA syntheses. A sense RNA probe for IgM was also generated and used as a negative control. The trunk of the body was cut into cross-sectional pieces including the trunk kidney and fixed overnight in

Davidson's fixative (30% ethanol, 10% formalin, 10% acetic acid). The fixed samples were decalcified in 0.1 M phosphate buffered saline containing 0.3 M EDTA for 48 h, embedded in paraffin and sectioned at 3- μ m thickness. The section was deparaffinized and immersed in phosphate buffered saline (PBS, pH = 7.4) for 10 min. After incubation with proteinase K (10 μ g/mL) at 37°C for 15 min, the section was washed with PBS containing 2 mg/ml of glycine for 10 min. The section was acetylated for 20 min and washed twice with 4 $\times$ saline sodium citrate (SSC, pH = 7.0) buffer. Prehybridization was performed with 50% formamide and 2 $\times$ SSC buffer at 42°C for 30 min. Hybridization buffer (ISHR 7

Table 1. Primers used in this study

Primer name	Sequence (5'-3')	Reference
<i>qRT-PCR</i>		
IgM RT F	TCAAAGGAGGAGCTACAAGACAATAA	Kato <i>et al.</i> , 2015
IgM RT R	TGAAAAGTCAATGGCAAAGCA	
IgT RT F	AGAGCGTAACTCTGGCCTGTCT	This study
IgT RT R	CCTCCATTTGATTTGGTAGTCACA	
IgD RT F	CACGCTCACACACGTGTATCC	Kato <i>et al.</i> , 2015
IgD RT R	CTTCGTGGCCCAGTTCAGA	
CD3 ϵ RT F	ACCTGTGCCTGCAACCGACT	This study
CD3 ϵ RT R	TGTGGGGTACAGGGGTGGTGA	
CD8 α RT F	GGCCCCACATCAAAGGATCGC	This study
CD8 α RT R	ACTACCAAGTCGTGCGCCCC	
TCR β RT F	TGGATGATCAGTGGGGTGAAC	This study
TCR β RT R	TGCGTTACGTTGTCCACAC	
EF1 α RT F	GCTGCCGGCTCCTTCAC	Kato <i>et al.</i> , 2015
EF1 α RT R	AGATCTGTCCAGGGTGGTTCA	
<i>RNA probe</i>		
CD8 α F	AGACGGGACAATGAAAGCAC	This study
CD8 α R	GCAGCGTCTGGTTCTAATACG	
IgM F	TCCTGCAGTCTCCGTCTCTT	Kato <i>et al.</i> , 2015
IgM R	TCTTCTGCTGAAGGCGGTAT	

Hybridization Buffer, Nippon gene) containing 1 μ g/mL of the DIG-labeled probe was added to the section and incubated at 42°C for 14–18 h in a moist chamber. After washing with 50% formamide and 2 $\times$ SSC, the section was incubated with 20 μ g/mL of RNase in NTE buffer (0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA) at 37°C for 30 min. After washing with 0.1 $\times$ SSC and Tris buffered saline (TBS), the section was incubated with anti-digoxigenin-AP Fab fragments (Roche Applied Science). Finally, the section was incubated with BM Purple AP Substrate (Roche Applied Science) at 37°C overnight and counterstained with neutral red. Digital images were captured and analyzed with a Nikon OPTIPHOT light microscope (Nikon) and NIS Elements software (Nikon). Ten sections for IgM and 39–71 sections for CD8 α were subjected to the light microscopy, respectively. The experiment was twice repeated using two different individuals.

Flow cytometry

Composition of leukocyte subpopulations in the trunk kidney of fish reared in 2016 were analyzed using a flowcytometry. The trunk kidney was sampled after the anesthesia, and the leukocytes were isolated using percoll (GE Healthcare Life Sciences) with a density of 1.075 g/mL. The trunk kidney leukocytes were suspended in RPMI-1640 medium (Nissui) at a density approximately 1.0×10^7 cells/mL and subjected to flow cytometry as below. In addition, the trunk kidney leukocytes were incubated with either anti-ayu Ig monoclonal antibody (mAb, K1) (Kato *et al.*, 2015), anti-ayu phagocyte mAb (H13) (Kato *et al.*, 2015) or anti-ayu thrombocyte mAb (G30) (Kato *et al.*, 2015) at 4°C for 1 h. After washing twice with RPMI-1640 medium, the leukocytes were incubated with donkey anti-mouse IgG H+L

AlexaFluor 488 (1:1,000 dilution, Thermo Fisher Scientific) at 4°C for 45 min. After staining with DAPI (1 μ g/mL, Dojindo), cell suspension was subjected to flow cytometry using CytoFLEX (Beckman Coulter) and the samples with the cellular viability more than 95% were used for the experiment. For the data analyses, DAPI-positive population was gated out as a dead cell population. Data ($n = 6$) from the cytometers were analyzed with a Kaluza Flow Cytometry Analysis Software (Beckman Coulter). Significant differences between LP group and ND group were assessed using Student's *t*-test.

Flowcytometry with K1 mAb were performed using the trunk kidney leukocytes derived from fish reared in 2017, following the protocol described above. In addition, the number of leukocytes per mg tissue in the trunk kidney was calculated using the flow cytometry. Briefly, the trunk kidney removed from an experimental fish was weighed and suspended in RPMI-1640 medium. The kidney leukocytes were isolated using percoll as above and resuspended in 1 mL of RPMI-1640 medium. After staining with DAPI solution, DAPI-positive dead cells were gated out and the number of live cells per 5 μ L was counted by the flowcytometry. Significant differences among the groups were assessed using ANOVA and Ryan's post-hoc test ($n = 9$ –10).

Results

GSI value

Mean GSI values ($n = 2$ –6) for fish used in this study was shown in Fig. 1C. GSI value of ND group (male and female) was significantly higher than that of IN group at 39 days after the starting point, while there is no significant difference in the value between IN and LP

group at the same time point. Significant difference was also found between IN and ND (female) group, but not between IN and LP group at 61 days after the starting point. Similar results were also obtained in 2017 (Fig. 1D). In addition, there is no significant difference in the body weight of the experimental fish among the three groups.

Challenge test

Ayu reared in LP and ND group was challenged with a high dose or a low dose of *F. psychrophilum* at 61

days after the starting point. Cumulative survival rates of ND group were 0% at both challenge doses, while those of LP group were 65% and 90% at the high and low dose challenge, respectively (Fig. 2). The survival times of LP group were significantly longer than those of ND group at both challenge dose ($p < 0.01$).

Gene expression analyses

Gene expression levels for IgM and IgD significantly decreased in ND group ($p < 0.05$), but not in LP group compared with the levels in IN group (Fig. 3A and B).

Fig. 2. Cumulative survival curves for ayu challenged with *Flavobacterium psychrophilum*.

Fig. 3. Gene expression analyses for IgM (A), IgD (B), IgT (C), CD3 ϵ (D), TCR β (E) and CD8 α (F). Each dot indicates the gene expression level relative to the mean expression level in IN group, which was set as 1. Horizontal lines indicate the mean expression levels ($n = 5-6$). Different letters represent significant differences between the groups ($p < 0.05$).

Fig. 4. *In situ* hybridization for IgM (A) and CD8 α (B) mRNA in the trunk kidney. Upper, middle and lower panels show the results from IN, LP and ND group, respectively. Arrowheads indicate representative signals stained purple. Bar = 100 μ m.

Although no significant difference was observed, mean value for the IgT gene expression level in ND group was lower than the level in IN and LP group (Fig. 3C). In addition, significant decrease of CD3 ϵ and TCR β gene expression level was also found only in ND group compared with IN group (Fig. 3 D and E, $p < 0.05$). CD8 α gene expression levels were slightly lower in LP and ND group, while there is no significant difference from the value in IN group (Fig. 3F).

In situ hybridization

IgM and CD8 α mRNA signals (stained purple) were found on the interstitial tissue of trunk kidney in IN, LP and ND group by *in situ* hybridization (Fig. 4). The number of signals for IgM in ND group was clearly less than the others. Further, signal intensities of IgM mRNA in ND and LP group were obviously weaker than that in IN group (Fig. 4A). Although the signal intensity

was not different between the groups, the number of the positive cells for CD8 α in the trunk kidney slightly decreased along with the sexual maturation (Fig. 4B). Two to three signals were observed in a trunk kidney section of IN and LP group (Fig. 4B upper and middle case), while no signal in the most sections and only one signal in some sections was found in the trunk kidney section of ND group (Fig. 4B lower case). No purple signal was detected on a trunk kidney section incubated with a sense probe for IgM as a negative control (data not shown).

Flow cytometry

Flow cytometry with forward and side scatter performed in 2016 showed that percentage for lymphocytes in ND group was significantly lower than that in LP group (Fig. 5A). In contrast, percentage of phagocytes in ND group was significantly higher than that in LP group (Fig.

Fig. 5. Flow cytometry for the trunk kidney leukocytes from ayu reared at long photoperiod (LP) and the natural day length (ND) performed in 2016. (A) Dot plot with forward scatter (FSC-A) and side scatter (SSC-A) for the trunk kidney leukocytes in LP (left) and ND (middle) group. Mean percentage (in the population gated as live cells) values for lymphocyte/thrombocyte (L/T) and phagocyte (P) gate are shown in the right panel ($n = 6$). Error bars indicate standard deviation and asterisks show significant differences (** $p < 0.01$). (B-C) Histogram for K1- (B), H13- (C) and G30-positive (D) leukocytes in the trunk kidney in LP (left) and ND (middle) group. Black area surrounded by solid line indicates mAb-treated sample and greyed area surrounded by dotted line shows a negative control (without mAb treatment). Mean percentage (in the population gated as live cells) values for mAb-positive cells are shown in the right panel ($n = 6$). Error bars indicate standard deviation and asterisks show significant differences (** $p < 0.01$).

Fig. 6. Change in percentage of K1-positive cells in the trunk kidney leukocytes (A) and the number of leukocytes per mg tissue of the trunk kidney (B) in 2017. Data are represented as mean values from nine individuals in each group and error bars indicate standard deviation. Different letters represent significant differences between the groups ($p < 0.05$).

5A). In the same way, significantly lower proportion of K1-positive cells (B cells) and significantly higher proportion of H13-positive cells (neutrophils and macrophages) was observed in ND group compared with LP group (Fig. 5B and C). However, no significant difference was observed in percentage of thrombocyte (G30-positive cells) between ND and LP group (Fig. 5D).

Leukocyte samples obtained from experimental fish reared in 2017 were also subjected to flow cytometry (Fig. 6). Proportion of K1-positive cells and the number of leukocytes in the trunk kidney were significantly lower in ND group than in IN group (Fig. 6A and B). Although no significant difference was found, mean percentage of K1-positive cells and the number of leukocytes was slightly lower in ND group, compared with the value in LP group.

Discussion

In the present study, the decrease of disease resistance and the lymphocytopenia were observed in ayu reared under the natural day length during spawning season. Further, rearing under the long photoperiod could suppress lymphocytopenia and decrease of disease resistance of ayu, along with the sexual maturation in the spawning season. Increase of serum cortisol suggested as a cause of lymphocytopenia during spawning season in rainbow trout and brown trout (Pickering, 1984; Pickering and Pottinger, 1987; Hou *et al.*, 1999). Rearing under long photoperiod (light, 16 h; dark, 8 h) suppressed increase of plasma steroid and sexual maturation in male ayu (Chang *et al.*, 1992). The present data suggest that sexual maturation (possibly induced by increase of serum cortisol) is also the major cause for the decrease of disease resistance and for the lymphocytopenia in a monotelic fish species ayu.

Cumulative survival rate in LP group was significantly higher than that in ND group at both challenge dose, showing that sexually matured ayu was more sensitive against BCWD compared with immature fish. *F.*

psychrophilum were frequently detected from ayu caught in Hirose river, Miyagi Prefecture (Kumagai *et al.*, 2010) and twelve rivers in Toyama Prefecture during the spawning season (Muraki and Tago, 2004). In an epidemiological survey in Kagami river, Kochi Prefecture, high copy numbers of *F. psychrophilum* PPIC gene were detected by PCR from the river water during the spawning season (Imajoh *et al.*, 2017). *F. psychrophilum* isolates that has a high virulence to ayu (*gyrA* G/C genotype) were found with a high probability from ayu as well as *Cyprinidae*, *Gobiidae*, *Petromyzontidae* and *Cottidae* in rivers flowing into Biwa Lake during September (Fujiwara-Nagata *et al.*, 2019). Therefore, the decrease of resistance against BCWD is probably a cause for high detection rate of *F. psychrophilum* from sexually matured ayu and from river water during the spawning season. In addition, sexually matured ayu affected with BCWD can be a source of bacterial shedding to other fish species during autumn season.

In this study, lymphocytopenia, decrease both of B and T lymphocytes, was observed in sexually matured ayu. Minami *et al.* (2018) showed that nonspecific immune activities such as phagocytic rate of the trunk kidney leukocytes, serum bactericidal activity and lysozyme activity were suppressed in sexually matured ayu. Suppression of these immune system along with the sexual maturation suggested to be a major cause of decrease of disease resistance against BCWD. Although no significant difference was observed, the number of leukocytes and B cells were tended to decrease in LP group compared with IN group. Gene expression analyses and *in situ* hybridization also detected such a tendency in CD3 ϵ , TCR β , IgM and CD8 α expression level. Generally, immune function declines along with age in mammals. The number of CD4 $^{+}$ T cells, CD8 $^{+}$ T cells and B cells are reduced in older individuals, compared with younger individuals (Sansoni *et al.*, 1993). The thymus, the responsible organ for producing functional CD4 $^{+}$ and CD8 $^{+}$ T cells, are progressively regressed in advanced age in human,

causing the significant decrease of CD4⁺ and CD8⁺ T cells (reviewed by Gui *et al.*, 2012). Age-related thymic involution was also observed in teleost fish medaka *Oryzias latipes* (Ghoneum and Egami, 1982) and flounder *Paralichthys olivaceus* (Liu *et al.*, 2003). Therefore, aging may be a cause for the tendency of lymphocyte decrease in ayu reared at long photoperiod and is also a non-negligible factor for the lymphocytopenia of ayu in the spawning season.

Along with the lymphocytopenia, significant increase of the percentage for phagocytic cell population was observed in sexually matured ayu in 2016. Minami *et al.* (2018) have suggested that the number of phagocytes remained unchanged with maturation in ayu based on the results of gene expression analyses for CD83, G-CSF, IL-8 and TNF α . The increase of percentage for phagocytic cells during sexual maturation in ayu may be caused by significant decrease of lymphocytes, rather than proliferation of the cells.

In conclusion, rearing under the long photoperiod can suppress not only decrease of disease resistance against BCWD but also lymphocytopenia, along with the sexual maturation in ayu. Since aging also possibly affects to lymphocytopenia and decrease of disease resistance, it is suggested that young immature ayu (before the summer solstice) is the most suitable for immunological studies including development of vaccines in ayu.

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