Research article

Subsequent Changes in the Thymus of Ayu *Plecoglossus altivelis* Reared from Juveniles with Temperature-induced Differential Thymus Growth

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ABSTRACT—The temperature of water used to rear juvenile stages of ayu affects the development of the thymus. However, subsequent differences in the thymus in adults after rearing juveniles at different temperatures are unclear. We analyzed thymus development of ayu grown at a constant water temperature of 15°C from juveniles reared at different water temperatures. Juvenile ayu weighed at 0.6 g and 1.4 g were reared at different temperatures (10°C, 12°C, 15°C, 18°C and 22°C) for 64 days and 54 days, respectively, and then held at 15°C for analysis of the thymus development. Thymus volume was measured by computed tomography and thymus development of experimental fish was evaluated as the ratio of thymus volume to body length. The thymus volume ratios of juveniles immediately after rearing at lower water temperatures (10°C, 12°C) were higher than in those reared at the higher temperatures (18°C, 22°C). These levels of thymus volume ratio were preserved in grown ayu continuously reared at 15°C. After the summer solstice, the thymus began to atrophy and no longer exhibited differences among the experimental groups. Thus the thymus formed at the juvenile stage retains its morphological properties until the summer solstice.

Key words: computed tomography, lymphoid organ, thymus, fish, teleost, juvenile, seed production, temperature

Ayu *Plecoglossus altivelis* is an important species of freshwater fish in the Japanese fishery. Infectious diseases, including bacterial cold-water disease (Kumagai, 2016), edwardsiellosis (Sakai *et al.*, 2008) and atypical cellular gill disease (Wada *et al.*, 2011), cause serious damage to not only aquaculture production of ayu, but population of ayu in rivers. The development of vaccines and disease-resistant seed for prevention of these infectious diseases therefore requires an understanding of the immune system of ayu.

The thymus is a primary lymphoid organ for maturation and production of T cells (Bowden *et al.*, 2005; Rombout *et al.*, 2005). Although studies of T-cell function in teleosts are ongoing, T cells are believed to be responsible for adaptive immunity of teleosts (Dickerson and Findly, 2017; Yamaguchi *et al.*, 2019). The importance of the thymus in the fish immune response has been verified by studies using thymectomized fish.

Marbled rockfish *Sebastes marmoratus* thymectomized at 44 days post-hatch showed a decrease in the numbers of lymphocytes in the pronephros and spleen, and suppression of the antibody response after the stimulation with sheep red blood cells (Nakanishi, 1991). Rainbow trout *Oncorhynchus mykiss* infected with *Cryptobia salmostica* after thymectomy retained low numbers of T-like cells in the blood even in fish vaccinated against *C. salmostica* before the parasite challenge (Feng and Woo, 1996). Therefore, the thymus probably plays an important role in the development of immune responses associated with T-cell function.

Miwa *et al.* (2003) reported that development of the thymus of ayu is affected by rearing water temperature during the juvenile stage for 3 months from about 0.5 g body weight. The thymus was significantly smaller in juvenile ayu reared at higher water temperature (17°C–18°C) than in those reared at lower water temperatures (13°C–14°C). However, subsequent differences of the thymus in adult ayu grown from juveniles with poorly developed thymuses after rearing at higher water

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temperatures are unclear. Because the thymus probably is an important organ for immunity in ayu, it is necessary to understand morphological development from the juvenile stage through the young to adult stages.

Measurement of thymus volume is necessary for studying thymus development. To date, thymus volume have been estimated by using serial histological sections (Miwa *et al.*, 2003). However, this method is laborious and time-consuming and is unsuitable for analysis of large numbers of samples. In this study, we established an efficient method for measuring thymus volume by computed tomography (CT) scan imaging. Furthermore, morphological changes of the thymus were examined in young and adult stages of ayu reared from juveniles with well and poorly developed thymuses using the CT imaging.

Materials and Methods

Preparation of fish for analysis of thymus volume

The schedule of rearing periods and sampling days in this study are shown in Fig. 1.

Experiment 1

Seventh generation hatchery-reared ayu, which was derived from wild fish collected from upstream migrating populations in the Edogawa River and held at the Gunma Prefectural Fisheries Experimental Station, were used for this experiment. Juveniles (average body length: 4.2 cm, average body weight (BW): 0.6 g) were divided into five groups, which were held in separate tanks at a density of about 250 fish per 10⁶ L water. The five groups of fish were assigned to tanks maintained for 64 days at constant temperatures of either 10°C, 12°C, 15°C, 18°C or 22°C. The temperature of each tank was then adjusted to 15°C over 8 hours and held at 15°C until analysis of thymus volume in the fish. During all rearing periods, water flow rate was maintained at 5 L min⁻¹ and fish were fed a commercial diet at a rate of 3% BW day⁻¹. Fish were collected at 0, 19,

54, 82 and 113 days after the experimental temperature treatments. The numbers of fish sampled at each collection time were 10, 3, 5, 5 and 5, respectively.

Experiment 2

Fourth generation hatchery-reared ayu, which was derived from wild fish collected from the Suruga Bay and held at the Yamanashi Prefectural Fisheries Technology Center, were used for this experiment. Juveniles (average BW: 1.4 g) were divided into three groups and held in separate tanks at a density of about 220 fish per 10⁶ L. The three fish groups were assigned to tanks maintained for 54 days at constant water temperatures of either 12°C, 15°C or 18°C. The temperature of each tank was then adjusted to 15°C over 8 hours and held at 15°C until analysis of thymus volume in the fish. During all rearing periods, water flow rate was maintained at 5 L min⁻¹ and fish were fed a commercial diet at a rate of 3% BW per day. Fish were collected at 0, 35, 63, 99 and 176 days after the experimental temperature treatments. The numbers of fish sampled at each collection time were 10, 5, 5, 5 and 5, respectively.

CT scanning and calculation of thymus volume

Sampled fish were euthanized by overexposure to eugenol (FA100, DS Pharma Animal Health Co., Ltd.) and then fixed in 10% phosphate-buffered formalin. Pretreatment of the sample for CT scanning was performed by a modification of the method described by Rosemary *et al.* (2009). The head of each fish, including thymus tissue, was separated and immersed in 2.5% phosphomolybdic acid for 6 days under dark conditions, followed by three 5-min washes with distilled water. The head samples were imaged using an X-ray CT system for laboratory animals (Latheta LCT-100A, Hitachi Ltd.) with a slice thickness of 0.1 mm. Image data were saved in a file format according to the digital imaging and communications medicine (DICOM) standard. The volume of thymus was calculated using an open source image analysis software Horos v2.2.0 (<https://>

Fig. 1. Schedule of rearing and sampling of fish in this study. White box: period of rearing fish under different experimental water temperatures. Grey bar: period of rearing fish at 15°C subsequent to the experimental temperature treatments. Arrowheads: sampling days for analysis of thymus volume.

horosproject.org/). The CT system could not occasionally detect poorly developed thymus. The lowest volume of thymus detectable by this system was 0.001 mm³ because each image was synthesized from sections of thickness of 0.1 mm in the axial, coronal and sagittal planes. Therefore, in calculating the thymus volume ratio, we assigned a value of 0.001 mm³ for the thymus volume of fish whose thymus was not detectable by the CT system.

Tissue sectioning and measurement of thymus volume

Juvenile ayu (average body weight: 1.3 g) were divided into two groups held in separate tanks at a density of about 150 fish per 10⁶ L. The two groups were maintained for 56 days at constant temperatures of 12°C and 18°C, respectively. Sampled fish were euthanized by overexposure to FA100 and then fixed in a 10% phosphate-buffered formalin solution. The head of each fish, including thymus tissue, was separated and embedded in paraffin using routine histological techniques. Transverse sections were cut through the tissue every 200 µm and stained with hematoxylin and eosin (H & E). The cross-sectional area of thymus tissue within each section was measured using ImageJ software (<https://imagej.nih.gov/ij/>), and the total volume of each thymus was estimated as follows (Miwa *et al.*, 2003):

$$V = 0.2 \sum a$$

(V: thymus volume, a: cross-sectional area of the thymus)

Estimation of thymus development

Miwa *et al.* (2003) showed a clear positive correlation between thymus volume and body length in wild ayu that were sampled before the day length started to shorten. To eliminate errors associated with fish size, we evaluated thymus development using the ratio of thymus volume to body length. Statistical significance was determined using Student's *t*-test for comparison of paired data, or with analysis of variance followed by Scheffe's test for multiple comparisons (BellCurve for Excel, Social Survey Research Information Co., Ltd.). Values of $p < 0.05$ were considered to be statistically significant.

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.

Results

Thymus volume calculated from CT images

CT images indicated the thymus as a bright region

showing high X-ray absorption in the upper part of opercular cavity, which is the position of the thymus observed in histological sections (Fig. 2A). Three-dimensional images reconstructed from CT data demonstrated the morphological characteristics and the volume of the thymus (Fig. 2B).

Comparison of thymus volume ratio obtained from CT scanning and tissue sectioning

In ayu reared at 12°C, which formed the largest thymus, the volume ratio obtained from CT data tended to be higher than that obtained from the tissue sections (Fig. 3). The thymus volume ratio of ayu reared at 12°C was significantly higher than in those reared at 18°C in both CT and histological analyses (Fig. 3). Estimations of thymus development between groups reared at different rearing temperatures were similar between CT data and tissue specimens.

Changes of thymus volume ratio with body growth

Experiment 1

The body sizes of fish at each sampling day were not significantly different among groups reared at different water temperatures, except for those sampled at day 0 (Table 1). At day 0, fish reared at 22°C were smaller than those reared at 10°C or at 12°C. At day 0, the thymus volume ratios of ayu reared at 10°C and 12°C were significantly higher than those of ayu reared at 15°C, 18°C and 22°C (Fig. 4). There was no significant difference of the thymus volume ratio between the groups reared at 10°C and 12°C. The thymus volume ratio of ayu reared at 18°C and 22°C remained low (≤ 0.05) during the whole experimental period. Differences in thymus volume ratio between ayu reared at low (10°C) and high water temperatures (18°C and 22°C) continued up to day 82 (Fig. 4), and ayu reared at 10°C maintained a high thymus volume ratio for a long period after the end of the experimental temperature treatment. The thymus volume ratios of ayu reared at 10°C were not significantly different during the period from day 0 to day 82, but were lower at day 113. The ratios in the groups reared at 12°C and 15°C were not significantly different during the period from day 0 to day 54, but were lower at day 82 (Fig. 5). In ayu reared at 18°C and 22°C, the thymus volume ratio remained low throughout the whole period, except that the ratio transitorily increased at day 54 in those reared at 22°C.

Experiment 2

The body size of fish was not significantly different among groups reared at different water temperatures at corresponding sampling days (Table 2). At day 0, the thymus volume ratios of ayu reared at 12°C and 15°C were significantly higher than that of ayu reared at 18°C (Fig. 6). This difference in volume ratio between ayu

Fig. 2. CT and histological images of the thymus. A: The thymus located at the upper part of opercular cavity (arrows). B: Three-dimensional images reconstructed from the CT data. The thymus is colored blue.

Fig. 3. Thymus volume ratio of ayu reared at different water temperatures (12°C, 18°C). A: Results from CT data ($n=10$). B: Results from histological analysis ($n=5$). Thymus volume ratio = Thymus volume (mm^3)/Body length (cm). *indicates a significant difference from the value at 18°C ($p<0.05$). Values are means $\pm$ SD.

Table 1. Body sizes of fish used in Experiment 1.

sampling days after the finish of temperature control		rearing water temperature									
		10°C		12°C		15°C		18°C		22°C	
		BL (cm)	BW (g)	BL (cm)	BW (g)	BL (cm)	BW (g)	BL (cm)	BW (g)	BL (cm)	BW (g)
Day 0	Average	6.8	3.5	6.7	3.3	6.4	3.0	6.4	3.2	6.1*	2.7**
	Standard deviation	0.4	0.5	0.3	0.4	0.3	0.4	0.3	0.5	0.1	0.3
Day 19	Average	6.6	3.7	6.3	3.8	6.4	3.5	6.6	3.6	6.2	3.1
	Standard deviation	0.2	0.5	0.2	0.2	0.1	0.1	0.1	0.1	0.2	0.5
Day 54	Average	9.1	9.4	9.1	9.0	8.9	8.5	8.8	8.0	9.1	9.1
	Standard deviation	0.4	1.5	0.2	0.9	0.3	1.0	0.2	0.6	0.2	0.8
Day 82	Average	11.0	16.9	10.7	14.8	10.8	16.3	10.8	16.4	10.9	15.3
	Standard deviation	0.2	1.2	0.4	1.8	0.4	1.3	0.4	2.1	0.4	1.6
Day 113	Average	11.5	20.0	11.2	17.6	12.1	22.0	11.7	20.3	11.9	21.3
	Standard deviation	0.5	3.5	0.3	2.6	0.1	0.7	0.7	3.7	0.4	2.5

* indicates a significant difference from body length at Day 0 of fish reared at 10°C and 12°C.

** indicates a significant difference from body weight at Day 0 of fish reared at 10°C.

Fig. 4. Comparison of thymus volume ratios among ayu reared at different water temperatures (Experiment 1). Fish were sampled at days 0, 19, 54, 82 and 113 during rearing at 15°C following the period of experimental temperature control. Thymus volume ratio = Thymus volume (mm³)/Body length (cm). Alphabets indicate a significant difference ($p < 0.05$). Values are means $\pm$ SD.

Fig. 5. Changes of thymus volume ratio in juveniles reared at different temperatures (Experiment 1). The data were obtained at days 0, 19, 54, 82 and 113 during rearing at 15°C following the period of experimental temperature control. Thymus volume ratio = Thymus volume (mm³)/Body length (cm). * indicates a significant difference from day 0 ($p < 0.05$). Values are means $\pm$ SD.

Table 2. Body lengths (cm) of fish used in Experiment 2.

sampling days after the finish of temperature control		rearing water temperature		
		12°C	15°C	18°C
Day 0	Average	7.9	8.2	7.7
	Standard deviation	0.5	0.3	0.4
Day 35	Average	10.4	10.5	10.5
	Standard deviation	0.2	0.7	0.3
Day 63	Average	12.1	12.1	12.0
	Standard deviation	0.5	0.5	0.7
Day 99	Average	15.6	15.6	14.7
	Standard deviation	1.2	1.2	0.4
Day 176	Average	17.0	17.0	17.8
	Standard deviation	0.9	0.9	0.6

Fig. 6. Comparison of thymus volume ratios among ayu reared at different water temperatures (Experiment 2). Fish were sampled at day 0, 35, 63, 99 and 176 during rearing at 15°C following the period of experimental temperature control. Thymus volume ratio = Thymus volume (mm³)/Body length (cm). Bars with different alphabetic labels are significantly different ($p < 0.05$). Values are means $\pm$ SD. **Fig. 7.** Changes of thymus volume ratio in juveniles reared at different temperatures (Experiment 2). The data were obtained at days 0, 35, 63, 99 and 176 during rearing at 15°C following the period of experimental temperature control. Thymus volume ratio = Thymus volume (mm³)/Body length (cm). * indicates a significant difference from day 0 ($p < 0.05$). Values are means $\pm$ SD.

reared at 12°C and 18°C continued up to day 35. Thymus volume ratios in all groups were not significantly different from day 0 values up to day 63, but were significantly lower after day 99 at 12°C and 15°C (Fig. 7). Thymus volume ratio in ayu reared at 18°C remained low throughout the whole period.

Discussion

In this study, we describe a method for visualization and volume measurement of ayu thymus by CT scanning. The use of CT scanning data facilitates an efficient workflow for measurement of thymus volume on many samples. In fact, we showed that ayu reared at the low water temperatures during the juvenile stage had relatively high thymus volume ratios using the CT scanning. The present results provide independent experimental confirmation of previous observations using different strains of ayu (Miwa *et al.*, 2003; Hara *et al.*, 2006). The thymus volume ratio estimated from CT data tended to be higher than that generated from tissue sections particularly in samples with a large thymus. This artefact may be a result of tissue shrinkage during the paraffin embedding step in the preparation of histological specimens (Stowell, 1941).

At the end of the period of experimental temperature control, differences in thymus volume ratio between

the treatment groups were maintained during the subsequent period in which the fish were bred under the same water temperature of 15°C. In particular, the thymus volume ratio of ayu reared at 10°C remained high for a long period. These results indicate that a large well-developed thymus produced by rearing at a low water temperature during the juvenile stage persists even after the water temperature is increased. In contrast, the thymus volume ratio of ayu reared at higher water temperatures (18°C and 22°C) was low. In Experiment 1, the thymus volume ratio was extremely low (≤ 0.05). This may be a consequence of raising younger fry with immature thymuses in Experiment 1. Poorly developed thymuses produced by rearing at high water temperatures during the juvenile stage did not grow to become larger even after the water temperature decreased.

The differences in the thymus volume ratio among ayu reared at different water temperatures were not observable at day 113 in Experiment 1 or at day 63 in Experiment 2, i.e., from the end of June to the beginning of July. Because the thymus volume ratio in all experimental groups remained constant until the middle of June, the thymus clearly developed in parallel with the growth of the fish body during this period. After the middle of June, the thymus volume ratio began to decrease even though the fish's body size steadily increased, implying atrophy of the thymus. The onset of sexual maturity in ayu accompanies the shortening photoperiod that commences after the summer solstice at the end of June in Japan (Nomura, 1966). In ayu, the thymus atrophies along with the development of the gonads (Honma and Tamura, 1972; Tamura, 1978). Therefore, we speculate that the decrease in volume ratio and atrophy of the thymus was caused by the onset of sexual maturity and not by rearing at 15°C following the period of water temperature control.

In conclusion, a well-developed thymus produced at low temperature in juveniles retained its high thymus volume ratio in young developing stages, even in fish kept at a higher water temperature. By contrast, a poorly developed thymus was not recovered in fish subsequently kept at a lower water temperature. We consider that the development of the thymus at the juvenile stage has practical importance for preserving the immune response associated with T-cell function in ayu.

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