Doctoral Thesis

Modification and application of stable isotope analysis for ecological study of ayu fish

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Abstract

Stable isotope analysis has been used to elucidate the feeding habits, migration and food web structure of organisms. However, basic knowledge of stable isotope analysis is still needed to apply the analysis to new situations in the field studies. The overall objectives of this thesis were to collect basic knowledge necessary for the field application of stable isotope analysis to a land-locked potadromous fish, ayu fish (*Plecoglossus altivelis altivelis*) in Lake Biwa, and to apply stable isotope analysis to actual ecological studies of ayu fish.

In Chapter 1, the background and overall purpose of this study were described. The relevance of each chapter in this study within the body of knowledge based on previous studies was also described.

In Chapter 2, trophic discrimination factors (TDFs) and lipid correction equations for multiple tissues of ayu fish were determined in an experimental setting. The results showed that TDF was 0.8‰ to 5.6‰ higher in muscle tissue after lipid-elimination than in untreated muscle tissue, and depended on the C/N ratio of the untreated muscle tissue. Following the models reported in previous studies, regression analysis with the C/N ratio of the untreated samples yielded a lipid correction equation specific to ayu fish muscles. On the other hand, the variance in lipid content was smaller in ovaries and the effect of lipids was constant. The TDF for δ^{13}C value, with the effect of lipid removed, differed among muscle (2.4‰), mucus (1.0‰) and ovary tissues (1.9‰). The TDFs for δ15N value differed among muscle (2.5‰), mucus (1.5‰) and ovary tissues (1.6‰). I concluded that The TDFs and lipid correction equations presented in this chapter allow accurate estimates of the feeding habits and migratory ecology of ayu fish. TDFs and lipid correction equations determined here enabled appropriate stable isotope analysis of ayu fish in the field.

In Chapter 3, the isotopic changes of multiple tissues of ayu fish during the growing and reproductive stages were compared in an experimental setting. The results showed that isotopic changes in the muscle and mucus tissues occurred faster in the growing stage than in the reproductive stage. The isotopic change observed in the muscle tissue during the reproductive stage was caused mainly by whole-body growth, whereas allometric growth and/or catabolic turnover accounted more for the isotopic changes in mucus tissue during both stages. The Isotopic change rate was slower in

muscle, mucus, and ovary tissues, in that order, probably in accordance with the allometric growth and/or catabolic turnover of each tissue. As the first report of the isotopic change in the fish ovary tissue, my investigation showed that the timing of drastic changes differed between the $\delta^{13}C$ and $\delta^{15}N$ values, which suggests that the $\delta^{13}C$ and $\delta^{15}N$ values observed in the ovary tissue may reflect changes in diets and/or habitats on different time scales. The results I presented here allowed for better prediction of physiological and environmental changes in ayu fish across all growth stages based on accurate applications of multiple-tissue isotope analysis to ayu fish over all stages. Such approaches can be applied to any fish species with better accurate when the variability of isotopic changes between and within stages are considered.

In Chapter 4, I addressed the proportional change and site-selection variation between river- and lake-produced eggs of ayu fish in the Lake Biwa water system were examined by distinguishing spawned eggs with carbon and nitrogen stable isotope ratios. The $\delta^{15}N$ values of spawned eggs decreased with time during the 3-month reproduction season. This result implies that there was a shift from lake-produced eggs to river-produced eggs during the reproductive season, based on the observation that adult fish in the lake have eggs with distinctly higher $\delta^{15}N$ values in their ovaries than those in the tributaries. This interpretation was also supported by the change in $\delta^{13}C$ values of the spawned eggs. Furthermore, eggs with lower $\delta^{15}N$ and higher $\delta^{13}C$ values tended to be spawned at less variable depths, suggesting that females spawning river-produced eggs selected the spawning sites from a narrower range than lake-produced eggs. I concluded that stable isotope ratios of spawned eggs can be indicators of the relative contributions of female originated from habitats with different food chains, and it enables us to compare comparisons of spawning characteristics between types of egg.

In Chapter 5, I examined the effect of river droughts on the number of river- and lake-produced eggs of ayu fish in Lake Biwa, by collecting spawned eggs quantitatively from 11 tributaries and using stable isotope analysis to distinguish collected eggs. River droughts were observed in 7 out of 11 tributaries of Lake Biwa. The frequency of droughts was greater in the rivers with wider basin and higher riverbed. The number of eggs, river-produced eggs and lake-produced eggs were explained by the total length of the mainstream as expected, with only a few exceptions. More importantly, an interaction between total length and the number of river droughts had a significant impact on the

number of river-produced eggs, suggesting that river droughts reduce the number of spawned eggs that are expected to increase with total length of the mainstream. On the other hand, the relationship between the number of eggs and lake-produced eggs and the factors of river droughts was not detected. I concluded that if the frequency of river droughts increases in the future, this could have an impact on the population size of ayu fish, and in turn on fisheries catches, recreation and food web structure.

Finally, in Chapter 6, I summarised the findings of studies presented in Chapters 2 to 5. I believe that these studies have contributed to the increased precision of stable isotope analysis for ecological study of ayu fish also applicable to ecological studies of other fish. In these studies, I have provided several novel insights associated with reproductive ecology of ayu fish, these include the spawning shift of eggs during a reproductive season from the lake-produced to river-produced ones and the negative impact of river droughts on reproduction. Further field studies are desired for the sustainable use of ayu fish resources from the field.

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Chapter 1

General Introduction

The importance of biodiversity conservation is increasingly recognised around the world. There is a need to develop and improve methods for rapidly understanding the ecology of individual species of organisms. For example, stable isotope analysis has been used to elucidate the feeding habits, migration and food web structure of organisms (Hobson, 1999). However, the basic knowledge of stable isotope analysis is still needed to apply the analysis to new situations in the field. The overall objectives of this thesis were to collect basic knowledge necessary for the field application of stable isotope analysis to a land-locked potadromous fish, ayu fish (*Plecoglossus altivelis altivelis*) in Lake Biwa, and to apply stable isotope analysis to actual ecological studies of ayu fish.

Stable isotope analysis is a powerful tool for understanding the feeding habits and migratory ecology of animals and provides important information for the management of biological resources (Peterson and Fry, 1987; Post, 2002). There is a known rule of thumb that the stable isotope ratios of carbon and nitrogen (hereafter, $\delta^{13}C$ and $\delta^{15}N$) of animals obtained by this analysis will vary by a fixed value (trophic discrimination factors; hereafter, TDFs) from the stable isotope ratios of their prey (DeNiro & Epstein, 1978; DeNiro & Epstein, 1981; Peterson & Fry, 1987). Recently, it has been reported that TDFs differ between species and tissues (Pinnegar & Polunin, 1999; Caut et al, 2009). One reason for the variations is the lipid content in tissues. Since the lipid content in tissues affects the $\delta^{13}C$ value, several lipid correction equations have been proposed to correct the effect of lipid content on $\delta^{13}C$ value by the C/N ratio (McConnaughey & McRoy, 1979; Fry, 2002; Post et al., 2007; Logan et al., 2008; Hoffman & Sutton, 2010). However, it has been found that lipid correction equation can also be estimated more accurately if a species- or tissue-specific lipid correction equation is obtained (McConnaughey & McRoy, 1979; Fry, 2002; Logan et al., 2008). In Chapter 2, TDFs and lipid correction equations for multiple tissues of ayu fish were determined in order to use stable isotope analysis appropriately in ayu fish studies in the field. In this chapter, the TDFs for muscle, mucus, and ovary tissues were calculated from a laboratory rearing experiment in which ayu fish were fed a uniform feed until it reflected the stable isotope ratio of the

feed, and the correspondence between the stable isotope ratio and the C/N ratio was examined to determine the lipid correction equations for muscle and ovary to correct for the C/N ratio. The determination of ayu-specific TDFs and lipid correction equations may enable more accurate estimates of the feeding habits and migration ecology of ayu fish.

The usefulness of isotope ratios of tissues such as mucus and plasma, which reflect short-term dietary habits when compared to traditionally used tissues such as bone and muscle, has recently been demonstrated in isotope ecology (Heady & Moore, 2013). Also, multiple-tissue approaches have been employed for two decades in isotopic studies of diet shifts of various animals, such as seals (Kurle & Worthy, 2002), birds (Ruts et al. 2010), and fishes (MacNeil et al., 2005, 2006; Heady & Moore, 2013). Multiple-tissue approaches can also enable estimations of the timing of immigration from other habitats (isotopic clock). However, the rate at which stable isotopic ratios reach steady-state values reflecting diet (isotopic change rate) may vary even within a single type of tissues of a single species depending on body size and growth stage. Research in this area has not been well established. In Chapter 3, the isotopic change of multiple tissues of ayu fish at different growth stages was monitored for the first time to the best of my knowledge. To focus on inter-tissue differences, diet-switch experiments were conducted to compare the isotopic change rate of muscles, mucus and ovaries between growing and reproductive stages of ayu fish.

In Chapter 4, incorporating the results from Chapter 2, stable isotope analysis was applied to clarify the spawning characteristics of different migration patterns of land-locked ayu fish in Lake Biwa. Ayu fish seasonally migrates between the lake and its tributaries, but its migration pattern is variable within the population — although almost all individuals of the population reproduce in the tributaries and die in the year they were born, the timing of upstream-migration from the lake can vary (Azuma, 1973a, b, c; Tsukamoto et al., 1987). In short, they hatch out from demersal, adhesive eggs broadcasted in the rapids in the lower reach of tributaries from September to November in autumn. Immediately after hatching, they drift down to the lake, where they prey on zooplankton. After several months (ranging from five to 12 months), some migrate in spring and start preying on attached algae in the tributaries until autumn, some migrate upstream in autumn immediately before reproduction, and the remainder migrate at intermediate times (Azuma, 1973b, c). It is crucial to

quantitatively understand the times and places where the eggs are produced for appropriate resource management because ayu fish play an important role in the local fishery, constituting 50% of the total catches in this lake (MAFF, 2016). However, these contributions have not been quantitatively measured due to the lack of suitable methodology. The objective of this chapter was to quantitatively examine the proportional change and site-selection variation between river- and lake-produced eggs of ayu fish in the Lake Biwa water system by distinguishing spawned eggs using stable isotope analysis. Stable isotope ratios of spawned eggs were expected to provide essential information on the origin of the parent fish, which could not be traced even by molecular techniques, to reveal the roles of intraspecific variations in the lifecycle of the fish.

In Chapter 5, the effect of river droughts on the number of river- and lake-produced eggs of ayu fish in Lake Biwa was examined in the field, by collecting spawned eggs quantitatively from the 11 tributaries and using stable isotope analysis to distinguish collected eggs. Recently, river droughts have been frequently observed in tributary rivers of Lake Biwa, where the river channel dries up due to insufficient surface water flow. The river droughts temporarily reduce the suitable habitats for aquatic organisms, mainly due to the lack of sufficient surface water and additionally due to the water temperature rise (Closs & Lake, 1996). The river drought can also severely impact the life cycle of migratory fish species that have evolved on the basis of the continuity of the water system (Bearmish & Northcote, 1989). In Japan, the Ministry of the Environment requests administrators to keep rivers constantly discharging a certain amount of surface water, but many local governments are not even able to assess the current situation. To the best of my knowledge, the current status of river droughts in tributaries of Lake Biwa has not studied or reported, and hence, there are no studies on the impact of river droughts on fish. The three objectives of this chapter were as follows: (1) to understand the current status of river droughts (frequency and scale), (2) to find out the characteristics of rivers prone to river droughts, and (3) to clarify the effects of river droughts on the number of river- and lake-produced eggs of ayu fish by distinguishing spawned eggs using stable isotope analysis. Understanding the current status of river droughts and its impact on resident fish species is essential information for future river management. Also, understanding the impact of river droughts on the number of river- and lake-produced eggs has implications for stock management and

conservation of ayu fish.

Finally, in Chapter 6, the findings of Chapters 2 to 5 were summed up in this concluding chapter of the thesis. Taken together, I believe that these studies have contributed to the optimization of stable isotope analysis for ecological study of ayu fish and augurs well for its application in other fish populations.

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Chapter 2

Examination of trophic discrimination factors and lipid corrections in muscle, ovary and mucus tissues of ayu fish (*Plecoglossus altivelis altivelis*) using carbon and nitrogen stable isotope analyses

1. Introduction

Stable isotope analysis is a powerful tool for understanding animal diets and migratory ecology and provides important information for managing biological resources (Peterson & Fry 1987; Post 2002). It is a rule of thumb that the carbon and nitrogen stable isotope ratios (δ^{13}C and δ^{15}N, respectively) of animals obtained by this analysis show that the value that increases stepwise from the isotope ratio of a prey organism (trophic discrimination factors; hereafter, TDFs) (DeNiro & Epstein, 1978; DeNiro & Epstein, 1981; Peterson & Fry, 1987). This empirical evidence is used to estimate animal feeding habits and migratory ecology. Recently, isotope ratios of tissues with different rates of turnover (e.g., bone, muscle, liver, plasma, mucus, etc.) have been used to provide dietary information at different time scales (e.g., days, weeks, months), and thus there is an increasing interest in the combination of multiple tissue isotope analyses (MacNeil et al., 2005; Heady & Moore, 2013).

Although TDFs have been thought to be constant values (DeNiro & Epstein, 1978; Minagawa & Wada, 1984; Fry & Sherr, 1989), they have recently been found to vary among species and tissues (Pinnegar & Polunin, 1999; Caut et al., 2009). Thus, the interpretation of isotope ratio data in wildlife requires understanding the differences in TDFs among species and tissues. In addition, the lipid content in tissues affects the δ^{13}C value. Because lipids and proteins have different TDFs for δ^{13}C values, when analysing species and tissues with variable lipid content, the δ^{13}C value varies independently of the feeding habits and movement of the target species (Focken & Becker, 1998). Therefore, it is desirable to remove the lipids prior to performing isotope analysis. On the other hand, because lipids do not contain nitrogen and the δ^{15}N value changes irregularly with lipid elimination, it is more appropriate to avoid lipid elimination for the analysis of δ^{15}N value (Sotiropoulos et al., 2004). It is optimal to analyse lipid-free samples for δ^{13}C values and untreated samples for δ^{15}N values separately; however, this may not be practical when dealing with a large

number of samples because of the increases in cost and labour. As a solution to this problem, lipid correction equations have been proposed to correct the effect of lipid content in tissues on $\delta^{13}C$ values by the C:N ratio (McConnaughey & McRoy, 1979; Fry, 2002; Post et al., 2007; Logan et al., 2008; Hoffman & Sutton, 2010). Some previous studies have shown that species- and tissue-specific lipid correction equations are more accurate in estimating $\delta^{13}C$ values than generalized lipid correction equations for all fish species and tissues (Logan et al., 2008, Hoffman & Sutton, 2010).

Ayu fish (*Plecoglossus altivelis altivelis*) are one of the most useful locally caught freshwater fish in Japan, with a catch of approximately 7,500 t in 2015, constituting approximately 11% of the total catches in inland water fisheries (MAFF, 2016). Although many studies have been conducted on the management of ayu fish (Kusuda, 1963; Azuma, 1970; Aizawa et al., 1999), the only studies using stable isotope analysis have been focused on the trophic level of ayu fish as part of the analysis of its food web structure (Yamada et al., 1998). The determination of tissue-specific TDFs and lipid correction equations will lead to a better understanding of the feeding habits and migration of released individuals, which will in turn contribute to the study of ayu fish by stable isotope analysis. In this study, the TDFs of muscle, mucus, and ovary tissues were calculated for ayu fish raised on one feed. Compared to muscle, which has been commonly used in stable isotope analysis, mucus reflects a relatively short period of feeding habits because of its high turnover rate (Church et al., 2009). Ovary tissue can be used to estimate habitat and feeding habits during the reproductive stage and may be a tool to elucidate the spawning ecology of ayu fish, who have a life history of polymorphism, grow ectopically, and spawn sympathetically (Azuma, 1973). The relationship between the C/N ratio and the $\delta^{13}C$ values of muscle and ovary tissues, except for lipid-free mucus, was examined to determine the lipid correction equations to correct for the effect of the lipid content of the tissues using the C/N ratio.

2. Material and Methods

2. 1. Aquarium experiment

Ayu fish (*P. a. altivelis*) individuals used in this investigation belong to a lineage that has been bred successively at the Gifu Prefectural Research Institute for Fisheries and Aquatic Environments in

central Japan (32°22′17 N″, 136°48′ 22″ E) after collecting them from Lake Biwa (34°58′55.6″ N, 135°54′22.0″ E–35°30′52.1″ N, 136°09′49.1″ E) in 2001. The trial fish were raised in the indoor rearing room of the institute in a 2.4 m diameter circular tank in which water with a salinity of 0.5% was circulated, from the fall of 2015 until May 2016 when we started experimental rearing. The diets during the feeding period were a mixture of rotifers and blended diets in an arbitrary ratio. The water was gradually desalinated in the trial fish tank for 1 week before the experiment. On May 23, 2016, 71 individuals were transferred to a 3,000-L rectangular FRP tank and reared in groundwater at a water temperature of 16–17 ℃. During the experimental rearing, the trial fish were fed once daily during the day with commercial fish feed (Ayu soft EPC-3, Nosan Corp., Yokohama, Japan, containing 46% crude protein, 3% crude lipid, and 2.5% crude fibre), which was approximately 7% of the total weight of the trial fish at start of the experiment. The feed was stirred well before the start of experimental rearing to ensure equal isotope ratios, and then three samples were collected for isotope analysis. On September 12, 2016, 112 days after the start of experimental rearing, 39 individuals were collected and frozen at −22 ℃. By storing each individual specimen in a separate package, we prevented mucus from getting mixed in with the other specimens. During the experimental rearing period, the body weight increased from 5.8 g (3.9–7.7 g, minimum–maximum) to 61.1 g (34.1–81.8 g), and the tissue formed prior to experimental rearing was diluted to less than one-ninth of the total. The isotope ratios in the trial fish were judged to have reached a steady state, reflecting the value of the feed, and the differences between the isotope ratios of ayu fish and the feed were used as the TDFs.

2. 2. Sample collection and lipid extraction for isotope analysis

Each frozen individual was thawed and as quickly as possible, mucus, muscle, and ovary (female only) tissues were collected in that order. Mucus was collected by stroking from the trunk to the tail with a 25-mm diameter glass microfiber filter (Whatman GF/F, GE Healthcare) according to the method of previous studies (Maruyama et al., 2016). Immediately after collection and drying, any impurities (e.g., scale and skin) were removed from the filter with tweezers. Muscle tissue was collected from the lateral muscles using dissecting scissors. Ovary tissue was rinsed after removal.

These excised tissues and feed were dried in a drying machine at 60 ℃ for at least 48 hours. Muscle, ovary, and feed were dried, crushed, and stirred using a mortar and pestle. Half of muscle, ovary, and feed, which may contain lipids, were treated to eliminate lipids by immersing these samples in a chloroform-methanol mixture for 24 hours (Folch et al., 1957; Bligh & Dyer, 1959). After lipid elimination, the samples were again dried, crushed, and stirred for at least 48 hours. Mucus contained no lipids and was therefore not treated for lipid elimination (Shephard, 1994). The powder samples before and after dewaxing and the filter from which mucus was collected were wrapped in a tin cup for isotope analysis.

2. 3. Isotope analysis

δ^{13}C, δ^{15}N, and C:N ratios were measured using a Delta V Advantage mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) connected to a Flash EA 1112 elemental analyser (Thermo Fisher Scientific) through a Conflo IV interface (Thermo Fisher Scientific) at Ryukoku University, Japan. Repeated samples of alanine and histidine standards were used for calibration and quality control for the three tissues and the feed. δ^{13}C and δ^{15}N were presented as relative deviations from the standards (carbon: Vienna Pee Dee Belemnite [VPDB], nitrogen: atmospheric nitrogen), as shown in the following equation

$$\delta X = (R_{\text{sample}} / R_{\text{standard}}) - 1,$$

where X is ^{13}C or ^{15}N and R is the ratio of the number of heavy isotopes to the number of light isotopes (^{13}C/^{12}C or ^{15}N/^{14}N).

2. 4. Effects of the lipid elimination on δ^{13}C values of muscle and ovary tissues

The effects of lipid elimination on δ^{13}C and δ^{15}N values were examined in lipid-containing muscle and ovary tissues. Differences in the mean values of δ^{13}C and δ^{15}N between lipid-free and untreated samples were verified by Welch's t-test, and differences in variance were verified by the F test.

2. 5. Lipid correction of δ^{13}C values in muscle and ovary tissues using C:N ratio

The lipid correction equation for explaining the change in $\delta^{13}C$ values with and without lipid elimination from the C:N ratio of the samples was determined by calculating the nonlinear regression analysis between the C:N ratio of the sample before lipid elimination and the difference of the $\delta^{13}C$ values ($\delta^{13}C_{lipid\text{-}free}$–$\delta^{13}C_{bulk}$) between the sample before and after lipid elimination. Three lipid correction models presented in previous studies were applied (McConnaughey & McRoy, 1979; Fry, 2002; Logan et al., 2008), and coefficients specific to ayu fish were determined using nonlinear regression analysis by the least-squares method. The significance of each of the three models was verified by a likelihood ratio test with a model described only by constants. The Akaike Information Criterion (AIC) was used as an index to compare the predictive power of the three lipid correction models. For tissues that had significant lipid correction equations, the prediction of the lipid correction equations with the smallest AIC and the general lipid correction equations for all fish species in a previous study were compared (Logan et al., 2008). In the other words, $\delta^{13}C$ values were calculated by substituting $\delta^{13}C$ values before correction ($\delta^{13}C_{bulk}$) and the actually measured C:N ratio into both lipid correction equations and compared using Welch's t-test.

2. 6. Comparison of TDFs

TDFs were calculated as the difference between the mean of the isotope ratios of the feed and the isotope ratios of each tissue. To compare TDFs, the values of the samples without lipid elimination were used to calculate the TDFs of the $\delta^{15}N$ because lipids do not contain nitrogen. The TDFs of $\delta^{13}C$ were calculated using the values of the lipid-free samples, except for mucus, which does not contain lipid. The isotope ratios of the feed were also used, with $\delta^{15}N$ as the value of the untreated samples and $\delta^{13}C$ as the value of the lipid-free samples. To test for the differences in TDFs between the tissues, a one-way ANOVA analysis was performed with the independent variable as the tissues and the dependent variable as $\delta^{15}N$ or $\delta^{13}C$. When the difference was significant, post-hoc comparisons were made using the Tukey-Kramer test.

2. 7. Statistical test

R ver. 3.2.1 software was used for the statistical tests (R Development Core Team, 2015). The t.test

function was used for Welch's t-test, var.test function for the *F* test, nls function for nonlinear regression, ANOVA function for likelihood ratio test, aov function for ANOVA, Tukey HSD function for the Tukey-Kramer test, and a significance level of 0.05.

3. Results

3. 1. Change in isotope ratio due to lipid eliminate

The $\delta^{13}C$ and $\delta^{15}N$ values of the muscle tissue were significantly different depending on whether they were lipid free or not (Welch t-test; $\delta^{13}C$: t = 10.233, df = 40.629, $P < 0.001$; $\delta^{15}N$: t = 14.897, df = 74.922, $P < 0.001$; Table 1). There were also significant differences in $\delta^{13}C$ and $\delta^{15}N$ in ovary tissue with and without lipid elimination ($\delta^{13}C$: t = 25.726, df = 26.784, $P < 0.001$; $\delta^{15}N$: t = 3.478, df = 29.479, $P < 0.01$; Table 1).

The variance of $\delta^{13}C$ value in the muscle tissue was decreased by lipid elimination (*F* test; $F_{38, 38} = 28.873$, $P < 0.001$). In ovary tissue, there no significant change in variance ($F_{15, 15} = 0.4853$, $P > 0.05$).

3. 2. Lipid correction equations for muscle and ovary tissues

The maximum and minimum of C:N ratios for muscle tissue were 14.3 and 3.6, respectively, and the maximum and minimum differences between $\delta^{13}C_{lipid\text{-}free}$ and $\delta^{13}C_{bulk}$ were 5.6‰ and 0.8‰, respectively (Fig. 1A). The difference between $\delta^{13}C_{lipid\text{-}free}$ and $\delta^{13}C_{bulk}$ was larger for samples with higher C:N ratios, and statistically significant regression equations were obtained for all three correction models (likelihood ratio test; Model 1: $F_{36, 38} = 653.27$, $P < 0.001$; Model 2: $F_{37, 38} = 1163.6$, $P < 0.001$; Model 3: $F_{37, 38} = 973.37$, $P < 0.001$; Fig 1A). The AIC was smallest for Model 1 (Table 2). The standard deviation of $\delta^{13}C$ corrected by Model 1 was 0.2‰ (*F* test; $F_{38, 38} = 29.299$, $P < 0.001$), and the variance was significantly smaller than the uncorrected value and comparable to the variance of the lipid-free samples (Table 1).

The maximum and minimum of C:N ratios for the muscle tissue were 5.4 and 5.1, respectively, and the maximum and minimum differences between $\delta^{13}C_{lipid\text{-}free}$ and $\delta^{13}C_{bulk}$ were 2.8‰ and 1.8‰, respectively (Fig. 1B). The ovary tissue had a smaller variance in C:N ratio ($F_{15, 38}$

= 0.0040, $P < 0.001$) and a smaller variance in the difference between $\delta^{13}C_{lipid\text{-}free}$ and $\delta^{13}C_{bulk}$ than muscle tissue. As a result, none of the correction models for the ovary tissue was significant (likelihood ratio test; Model 1: $F_{13, 15} = 0.3662$, $P > 0.05$; Model 2: $F_{14, 15} = 0.6386$, $P > 0.05$; Model 3: $F_{14, 15} = 0.6358$, $P > 0.05$; Fig 1B). Thus, the lipid correction equation of the ovary tissue leaves only the constant, $\delta^{13}C_{lipid\text{-}free} - \delta^{13}C_{bulk} = 2.3$.

In muscle tissue for which significant lipid correction equations were obtained, there was no significant difference between the predicted values (–19.5 ± 0.2) when using the coefficients specific to ayu fish obtained in this study and the values (–19.5 ± 0.2) after correction using previously presented lipid correction equations for fish in general (Logan et al., 2008) (Welch t-test; $t = -0.814$, df = 68.003, $P > 0.05$).

3. 3. Comparison of the enrichment factors of $\delta^{13}C$ and $\delta^{15}N$ between the tissues

The TDFs of $\delta^{13}C$ and $\delta^{15}N$ of well-mixed commercial fish feed (Ayu soft EPC-4) used as the feed were −21.9‰ ± 0.1‰ and 8.0‰ ± 0.2‰, respectively. The TDF for $\delta^{13}C$ was significantly different among the three tissues (ANOVA, $F_{2, 51} = 400.6$, $P < 0.001$; Table 1), with significant differences between muscle and ovary tissues (Tukey-Kramer, $P < 0.001$), muscle and mucus tissues ($P < 0.001$), and ovary and mucus tissues ($P < 0.001$). The TDFs for $\delta^{15}N$ were also significantly different among the three tissues ($F_{2, 51} = 137.4$, $P < 0.001$). There was a significant difference between muscle and ovary tissues (Tukey-Kramer, $P < 0.001$) and muscle and mucus tissues ($P < 0.001$) but not between ovary and mucus tissues ($P > 0.05$).

4. Discussion

4. 1. Effect of lipids and lipid eliminating on $\delta^{13}C$ and $\delta^{15}N$ and correction

The effect of C:N ratio on the $\delta^{13}C$ of ayu muscle was found to be higher in samples with higher lipid content. In isotope ecology, differences in $\delta^{13}C$ between lake and river producers (1.9‰–6.8‰; Yamada et al., 1996) and between planktonic and attached producers (maximum 7‰; France, 1995) are used to discriminate between food chains, however the effect of lipids found in this study was up to 5.6‰, indicating the potential for significant misinterpretation of diet and migration based on

δ^{13}C. However, lipid elimination reduced the variance of δ^{13}C. As in previous studies (Kiljunen et al., 2006; Logan et al., 2008; Hoffman & Sutton, 2010), this may be due to the fact that lipids in the samples have a different TDF than proteins do, and the need for lipid elimination and correction equations was again demonstrated in ayu fish. When the lipid correction equation specific to ayu muscle presented in this study was applied, the variance of the corrected δ^{13}C was as small as that of the lipid-free samples. The effect of the lipid correction equation obtained in this study is not much different from that of previously lipid correction equations for fish in general; however, the effect of lipids on δ^{13}C values differs depending on species and tissue (Logan et al., 2008; Hoffman & Sutton, 2010), thus it is important to use lipid correction equations specific to species and tissue.

In the ovary tissue, the variance of the C:N ratio was smaller than in muscle tissue, and none of the lipid correction equations were statistically significant. The variance of the δ^{13}C of the untreated samples was also smaller than that of the muscle. Thus, it is possible to correct for the effect of lipid in ovary tissue using only a constant (2.3‰). This is the first time that a lipid correction equation for ovary tissue has been investigated in fish, and it should be tested in other fish species in the future.

The mean values of δ^{15}N in lipid-free samples were 0.8‰ higher in muscle tissue and 0.3‰ higher in ovary tissue than in untreated samples. The absence of nitrogen in lipids, the absence of significant dispersion of δ^{15}N in both tissues prior to lipid elimination, and the irregularity of δ^{15}N upon lipid elimination (Sotiropoulos et al. 2004) suggest that the values of δ^{15}N from untreated samples should be used. On the other hand, the mechanism of the irregular change in δ^{15}N by lipid elimination is still unknown and requires further study.

For the interpretation of δ^{13}C and δ^{15}N of ayu muscle and ovary obtained in the field, it is desirable to use degreased samples for δ^{15}N and untreated samples for δ^{15}N. In other words, it would be optimal to analyse δ^{13}C and δ^{15}N separately, but this would double the cost and effort and may not be practical for studies involving large numbers of samples. In such cases, samples can be analysed without lipid elimination, and the lipid correction equation obtained in this study can be applied to calculate and examine the δ^{13}C equivalent to that of lipid-free samples and the δ^{15}N without the irregular changes caused by lipid elimination.

4. 2. Differences in TDFs of $\delta^{13}C$ and $\delta^{15}N$ between tissues

Analysis of δ^{13}C and δ^{15}N in lipid-free and untreated samples yielded TDFs specific to ayu muscle, ovary, and mucus. In early isotope ecology, prey-predator TDFs of 0‰–1‰ for δ^{13}C (DeNiro & Epstein, 1978; Fry & Sherr, 1989), 3‰–4‰ for δ^{15}N (DeNiro & Epstein, 1981; Minagawa & Wada, 1984; Post, 2002) were applied to a variety of species and tissues. Recently, however, it has been reported that the TDFs vary among species and tissues (Caut et al., 2009), and it is desirable to determine the TDFs for each species and tissue type experimentally in advance.

The differences in TDFs among the three tissues analysed in this study were in agreement with those reported in previous studies (Pinnegar & Polunin, 1999; Caut et al., 2009). In previous studies on fish, the TDFs of muscle ranged from 0.2‰–2.5‰ for δ^{13}C (lipid free) and −1.0‰–5.6‰ for δ^{15}N (without lipid elimination) (Caut et al., 2009), while the TDFs of mucus ranged from −0.6‰–1.1‰ for δ^{13}C and 0.9‰–1.9‰ for δ^{15}N (Shigeta et al., 2017). The TDFs of ayu fish found in this study were higher at δ^{13}C than in previous studies for both muscle and mucus tissues and were almost identical at δ^{15}N. The TDFs of ovary tissue provided new knowledge because there were no reported data for both elements. In the isotope analysis of ayu fish, the use of tissue-specific TDFs would increase the reliability of interpretation of isotope ratios. However, it should be noted that the TDFs obtained in this study are dependent on the composition of the artificial feed, because the TDFs vary with the protein content and the amino acid composition of the feed and the target organisms.

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6. Figures and Tables

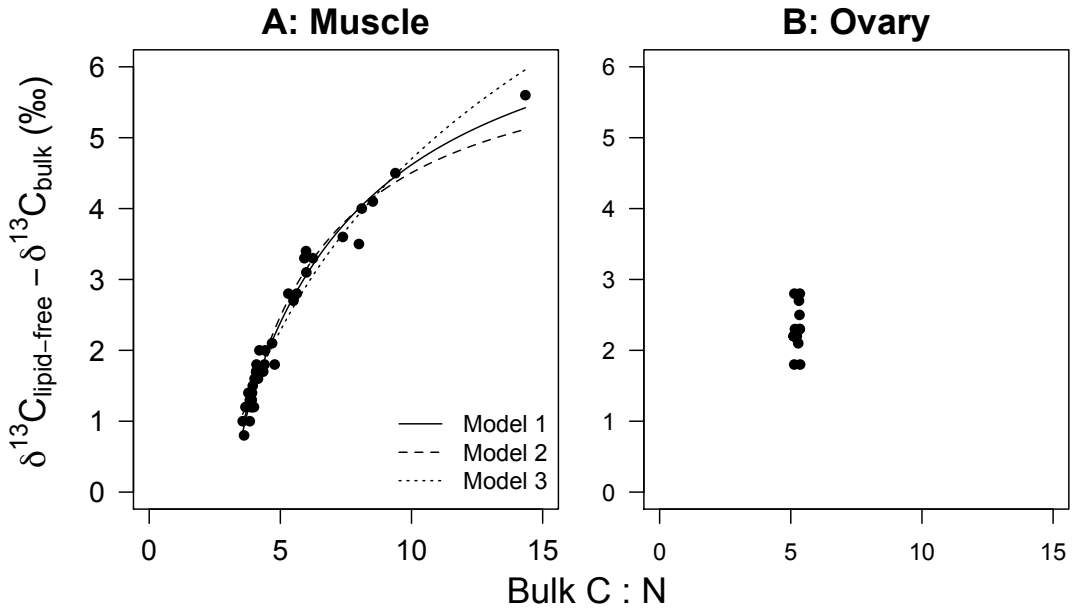

Figure 1. Differences in $\delta^{13}C$ values between lipid-free and bulk tissues ($\delta^{13}C_{lipid\text{-}free}$–$\delta^{13}C_{bulk}$) in muscle tissue (A, n = 39) and in ovary tissue (B, n = 16) of ayu fish (*Plecoglossus altivelis altivelis*) as a function of bulk C:N ratio. Regression curves followed three previously reported models for lipid correction (Model 1, McConnaughey & McRoy, 1979 modified by Logan et al., 2008; Model 2, Fry, 2002; Model 3, Logan et al., 2008)

Table 1. C:N ratios and discrimination factors of muscle, ovary, and mucus tissues of ayu fish (*Plecoglossus altivelis altivelis*) for the $\delta^{13}C$ and $\delta^{15}N$ values before and after lipid elimination and model correction. Means ± standard deviations are shown.

		C:N ratio		$\delta^{13}C$ discrimination factor (‰)			$\delta^{15}N$ discrimination factor (‰)	
Tissue	n	Untreated	Lipid elimination	Untreated	Lipid elimination	Model 1 correction	Untreated	Lipid elimination
Muscle	39	4.6 ± 1.7	3.0 ± 0.3	0.2 ± 1.3	2.4 ± 0.3	2.4 ± 0.2	2.5 ± 0.2	3.3 ± 0.2
Ovary	16	4.8 ± 0.5	3.2 ± 0.3	–0.4 ± 0.2	1.9 ± 0.3	NS	1.6 ± 0.3	1.9 ± 0.2
Mucus	37	3.2 ± 0.3	NA	1.0 ± 0.2	NA	NA	1.5 ± 0.4	NA

n, sample size. NS, model not significant. NA, not applicable (mucus not containing lipid).

Table 2. Three models previously reported to correct the effect of lipid content on the $\delta^{13}C$ value and the parameter estimates (± standard errors) by curve fitting to data of muscle tissue of ayu fish (*Plecoglossus altivelis altivelis*)

Model	Equation	Estimates	d.f.	*F*	AIC
1	$\delta^{13}C_{lipid\text{-}free} - \delta^{13}C_{bulk} = \frac{a \times C{:}N + b}{C{:}N + c}$	a = 7.584 ± 0.541***	36, 38	653.3***	–12.7
		b = –22.012 ± 1.077***			
		c = 1.657 ± 0.848***			
2	$\delta^{13}C_{lipid\text{-}free} - \delta^{13}C_{bulk} = p - \frac{p \times f}{C{:}N}$	p = 6.531 ± 0.133***	37, 38	1163.6***	–9.3
		f = 3.102 ± 0.034***			
3	$\delta^{13}C_{lipid\text{-}free} - \delta^{13}C_{bulk} = \beta_0 + \beta_1 \ln(C{:}N)$	β_0 = –3.340 ± 0.180***	37, 38	973.4***	–3.2
		β_1 = 3.492 ± 0.112***			

Models 1, 2, and 3 are based on McConnaughey and McRoy (1979) modified by Logan et al. (2008), Fry (2002), and Logan et al. (2008), respectively. $\delta^{13}C_{lipid\text{-}free}$, $\delta^{13}C_{bulk}$, and C:N are measured values. Asterisks (***) indicate significance levels ($P < 0.001$). *F* values and d.f. (degrees of freedom) refer to likelihood ratio tests against the null mode.

Chapter 3

Turnover rates for muscle, mucus, and ovary tissues of ayu fish (*Plecoglossus altivelis altivelis*) in multiple stages determined through carbon and nitrogen stable isotope analyses

1. Introduction

Animals migrate for various reasons, including better access to feeds, safer reproduction, and predation avoidance (Dingle & Drake, 2007). Because loss and fragmentation of suitable habitats in each life stage are the main causes of declines in many species, understanding feeding habits and migration routes is a key issue for biological conservation and resource management (Caughley, 1994). However, tracing feeding habits and migration routes of freshwater and marine animals requires great effort. Stable isotope analysis is an effective method for estimating the feeding habits and migratory routes between habitats (Hobson, 1999). Carbon and nitrogen isotope ratio values (hereafter, $\delta^{13}C$ and $\delta^{15}N$, respectively) are used as powerful indicators for understanding the feeding habits and migration ecology of animals and have provided important information for resource management (Peterson & Fry, 1987; Post, 2002; Fry, 2006), based on the stepwise increases in $\delta^{13}C$ and $\delta^{15}N$ values between trophic levels (trophic discrimination factors; hereafter, TDFs) (DeNiro & Epstein, 1978, 1981; Minagawa & Wada, 1984).

The usefulness of quickly responding tissues has been recently revealed in isotope ecology (Heady & Moore, 2013). Quickly responding tissues, such as mucus and blood plasma, reflect more recent feeding habits than conventionally analysed tissues, such as bone and muscle (MacAvoy et al., 2001; Church et al., 2009; Heady & Moore, 2013). Furthermore, an isotopic clock was proposed to estimate the residence time of immigrants (Heady & Moore, 2013). Based on the difference in turnover rate between slowly responding (bone and muscle) and quickly responding tissues (mucus and blood plasma), an isotopic clock utilizes the differences in isotope ratios between multiple tissues to calculate the time elapsed since diet-switch or migration. Thus, the turnover rates in multiple tissues have been investigated intensively with diet-switch experiments using several fish species (Maruyama et al., 2016; Shigeta et al., 2017). However, the turnover rate might be variable within a species by body size or life stage, depending on the growth rate. Isotopic data can lead to

misunderstandings without knowing the ontogenetic change in turnover rate.

We used a landlocked population of ayu fish (*Plecoglossus altivelis altivelis*) in Lake Biwa, the greatest lake in Japan, for this investigation. This population plays an important role in the local fishery, constituting 50% of total catches in the lake (MAFF, 2016). However, partially due to the variable migration pattern of this fish within the population, resource management of ayu fish is difficult and, in fact, its capture has decreased over 3 decades (Azuma, 1973; Tsukamoto et al., 1987). While almost all individuals of the population are known to reproduce in the tributaries in autumn and die within a year, immediately after reproduction, they have various timing of upstream-migration from spring to autumn after the planktivorous stage in the lake in winter (Azuma, 1973; Tsukamoto et al., 1987; Sawada et al., 2020). Based on the isotopic differences between riverine and lacustrine food webs (Yamada et al., 1998), a previous investigation analysed stable isotope ratios of spawned eggs and revealed proportional change and site-selection variation between river- and lake-produced eggs (Sawada et al., 2020). Accurate isotopic clock analysis would reveal when and where conservation efforts should be concentrated for better resource management. For example, if the settlement of released ayu fish from farms can be tracked using isotopic clocks, fisheries would be able to find the best place and time to release the fish. Turnover rates in multiple tissues in multiple life stages are therefore required for this fish.

Thus, we conducted controlled diet-switch aquarium experiments to compare $\delta^{13}C$ and $\delta^{15}N$ change rates between the growing and reproductive stages of ayu fish from Lake Biwa. Muscle and mucus tissues were analysed in both stages as slowly and quickly responding tissues, respectively. Ovary tissue was also analysed only in the reproductive stage. Together with isotopic changes, we determined the TDFs, which are known to differ between tissues (Pinnegar & Polunin, 1999; Caut et al., 2009).

2. Material and Methods

2. 1. The study fish

Ayu fish (*P. a. altivelis*) individuals used in this investigation belong to a lineage that has been bred successively at the Gifu Prefectural Research Institute for Fisheries and Aquatic Environments in

central Japan (32°22’17” N, 136°48’ 22” E) after collecting them from Lake Biwa (34°58’55.6” N, 135°54’22.0” E–35°30’52.1” N, 136°09’49.1” E) in 2001. According to the conventional methods used at this institute, our fish (16th generation) were raised in a circular tank measuring 2.4 m in diameter with circulating water adjusted to a salt concentration of 0.5% from hatching in autumn of 2016 to the start of the experiment in June 2017. The fish were fed feed prepared by mixing several compounded feeds at an arbitrary ratio during the raising period. We have no data on the isotope ratios of the mixed feeds; thus, we cannot calculate TDFs for the fish during this period.

2. 2. Experimental design for comparison of the two stages

We conducted 2 diet-switch experiments mainly to compare isotopic change rates in multiple tissues between the growing and reproductive stages. As ayu fish is revealed to spawn from late August to early November by yearly investigations of spawned eggs in 11 rivers around Lake Biwa (Terai et al., 2020), we defined in this study the reproductive stage from early August, including one-month preparatory period. The growing stage was then defined as the period before the reproductive stage. Experiment 1 was conducted during the growing stage, in which we monitored the $\delta^{13}C$ and $\delta^{15}N$ values in muscle and mucus tissues, as slowly and quickly responding tissues, respectively. Experiment 2 was performed during the reproductive stage, during which we also monitored the $\delta^{13}C$ and $\delta^{15}N$ values in ovary tissues as quickly responding tissue, in addition to muscle and mucus tissues. TDFs for muscle, mucus, and ovary tissues were determined when the $\delta^{13}C$ and $\delta^{15}N$ values reached steady states in Experiment 1.

2. 3. Experiment 1 to obtain isotopic change rates and trophic discrimination factors in multiple tissues during the growing stage

This experiment was conducted to determine the isotopic change rates in muscle and mucus tissues for the growing stage. The water in the tank used to raise the fish was gradually desalinated from 0.5% salt concentration to freshwater for one week before the experiment. Afterwards, 26 individuals were sampled from the same tank and stored frozen at –20℃ as initial samples for the experiment. Another 245 individuals of the fish were transferred to a 3,000-L rectangular FRP tank.

Pittag (BIO9, 134.2kHz) was embedded in the cavum abdominis under anaesthesia (FA-100, DS Pharma Animal Health, Osaka, Japan) for individual identification, and the standard length (mm) and wet weight (g) of individuals were measured to the nearest 0.1 mm and 0.01 g, respectively. The experiment was performed with aerated subsurface water maintained at approximately 16–18℃ between June 8 and August 29.

From day 0 (June 9, 2017), we fed the fish Hikari-Iroage-Kotsubu commercial fish feed (Kyorin Corp., Hyogo, Japan; hereafter, feed A), containing 35% crude protein, 3% crude lipid, 5% crude fibre, and 13% crude ash by dry weight, five times daily until satiation. Approximately 9 kg of feed A were mixed thoroughly in a bucket prior to the experiment in order to ensure that the isotopic composition of feed A was consistent throughout the experiment. Five portions of feed A were randomly sampled at the end of the mixing process to examine the variations in stable isotope ratios. Three individuals of the fish species were sacrificed on days 3, 10, 15, 18, 21, 25, 28, 32, 35, 46, 53, 60, 67, and 81. Individual fish samples were kept in separate plastic zip-lock bags to prevent inter-sample contamination. The samples were frozen at –20℃ and transported to Ryukoku University, Japan, where isotope analysis was performed.

We sampled muscle and mucus tissues from 15 individuals on day 57 to calculate TDFs. The tissues formed before the diet-switching to feed A were diluted to less than approximately one-fifth of the whole, because body weight increased from 3.09 g (1.70–5.09 g, min–max) on day 0 to 17.89 g (12.02–23.88 g) on day 57. Thus, we considered that isotopic composition reached a steady state before day 57. We also collected ovary tissue on days 57 (from 7 females), 71 (3), and 85 (8). Repeated sampling of ovary tissue allowed us to examine the effect of ovary maturation on its isotopic composition. These samples corresponded to the initial samples of Experiment 2.

2. 4. Experiment 2 to obtain isotopic change rates in multiple tissues during reproductive stage

In order not to miss the timing of ovary maturation, Experiment 2 was originated 3 times, and each one is hereafter referred to as the early, middle, and late session of the reproductive stage. The isotopic change rates in muscle and mucus tissues were also determined for comparison with those in the growing stage only in the early session.

We transferred 54, 45, and 52 individuals to three 550-L rectangular FRP tanks on August 5 (early session), August 19 (middle session), and September 2 (late session), respectively, after the isotope ratios in the individuals' muscle and mucus tissues reached steady states with feed A in Experiment 1. Three days after fish transfer (day 0), the fish diets were switched from feed A to another well-mixed commercial fish feed (Ayu soft EPC-3, Nosan Corp., Yokohama, Japan; hereafter, feed B), which contains 46% crude protein, 3% crude lipid, and 2.5% crude fibre by dry weight. We sacrificed 15, 10, and 15 individuals, respectively, on day 0 of the early, middle, and late sessions, to measure the initial isotope ratios. Among them, ovary tissue was collected only from females (7, 3, and 8 individuals, respectively). Three individuals were sacrificed on days 3, 7, 10, 14, 17, 21, 24, 28, 31, 42, and 47 in the early session. Three individuals were sacrificed on days 2, 3, 6, 7, 10, 14, 17, 21, 24, 28, and 33 in the middle session. Furthermore, three individuals were sacrificed on days 1, 2, 3, 6, 7, 9, 10, 13, 14, and 19 from the start of the experiment, during the late session. Fish samples were kept and transported in the same manner as for those in Experiment 1.

2. 5. Tissue collection and isotope analysis

Fish samples of Experiments 1 and 2 were deforested to measure the standard length (SL) with slide callipers to the nearest 0.1 mm, and wet weight (WW) to the nearest 0.01 g. Muscle and mucus tissues were then collected from the samples of Experiment 1 and the early session of experiment 2. Ovary tissue was collected from all three sessions of Experiment 2.

Mucus was wiped directly from the body surface of each thawed fish sample using half of a 25-mm-diameter GF/F glass microfiber filter (GE Healthcare, Buckinghamshire, UK) and dried at 60℃ for 48 h over, as described in a previous investigation (Maruyama et al., 2015; Sawada et al., 2018). Each filter was cleaned with forceps before and after drying to remove any scale or skin fragments. We did not conduct the lipid extraction from the mucus, which contains no lipid components (Shephard, 1994). Muscle tissue was removed from the upper lateral section after mucus sampling, and ovary tissue was extracted from the females. These tissues were dried at 60℃ for 48 h, and ground to a fine powder. The effect of variable lipid content on the $\delta^{13}C$ values of the muscle and ovary tissue was corrected after stable isotope analysis based on the C/N ratio of each

sample, according to the tissue-specific correction models for this species (Sawada et al., 2018). Samples of feeds A and B were treated in the same manner as for the muscle and ovary tissues, except that lipids were removed using 2:1 chloroform/methanol solution (Bligh & Dyer, 1959) prior to carbon stable isotope analysis since correction models specific to these feed samples were not available. We used samples before lipid extraction for nitrogen stable isotope analysis of feeds A and B.

Carbon and nitrogen stable isotope analyses were performed using a Delta V Advantage mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) connected to a Flash EA 1112 elemental analyser (Thermo Fisher Scientific) through a Conflo IV interface (Thermo Fisher Scientific) at Ryukoku University, Japan. Alanine (δ^{15}N, 1.6‰ ± 0.2‰; δ^{13}C, –19.6‰ ± 0.2‰) and histidine (δ^{15}N, -7.6‰ ± 0.2‰; δ^{13}C, –10.7‰ ± 0.2‰) standards were repeatedly analysed between every 12 samples to calibrate and quality control the muscle, mucus, and ovary tissues. δ^{13}C and δ^{15}N values were expressed as $\delta X = (R_{sample}/R_{standard}) - 1$, where X is ^{13}C or ^{15}N; R_{sample} corresponds to the ^{13}C:^{12}C or ^{15}N:^{14}N ratio of the measured samples; and $R_{standard}$ is the ^{13}C:^{12}C ratio of Vienna Pee Dee Belemnite or ^{15}N:^{14}N ratio of atmospheric nitrogen. Analytical errors in the δ^{13}C and δ^{15}N values were less than ± 0.3‰.

2. 6. Data analysis

Isotopic changes occur as a result of catabolic turnover and growth of the focal tissue (i.e. muscle, mucus. and ovary tissues). The δ value at time t (δ_t) after diet-switching can be expressed as shown below (Hesslein et al., 1993):

$$\delta_t = \delta_{final} + (\delta_{initial} - \delta_{final}) \times e^{-(k+c)\times t}, \quad (1)$$

where $\delta_{initial}$ and δ_{final} are δ values at the initial time (t = 0) and at the final time (t = infinity) respectively, k is the growth rate (per day), and c is the catabolic turnover constant (per day). Because the tissues with apparently different growth rates are compared, we divided the growth rate (k) into whole-body growth (k') and allometric growth rate specific to each tissue type (a) in this investigation, and changed Equation 1 as follows:

$$\delta_t = \delta_{final} + (\delta_{initial} - \delta_{final}) \times e^{-(k'+a+c)\times t}. \quad (2)$$

δ_t was obtained as the $\delta^{13}C$ and $\delta^{15}N$ values of each sample. k' of each individual was calculated as ($k' = \ln(W/W_0)/t$), where W and W_0 were body wet weight at sampling and diet switching, respectively. In Experiment 1, $\delta_{initial}$, δ_{final}, and $a + c$ were obtained by curve fitting using the exponential model shown above, because a previous investigation showed different TDF for different diets (McCutchan et al., 2003). $\delta_{initial}$ and $a + c$ were only obtained by curve fitting using the exponential model above in Experiment 2, because generating the estimates of δ_{final} for the post switch feed was expected to take long periods of time for muscle tissue on experiment 2. The half-lives of isotopic change (t_{half}) were calculated as follows:

$$t_{half} = \ln 2 / (k' + a + c). \quad (3)$$

Nonlinear regressions were performed using the nls function in R ver. 3.5.0 software with a significance level of 0.05.

We linearized Equation 1 by taking the natural logarithm as shown below (Cerling et al., 2007) in order to statistically compare isotopic changes between stages, as well as between tissues:

$$\ln [(\delta_t - \delta_{final}) / (\delta_{initial} - \delta_{final})] = -(k + c) \times t. \quad (4)$$

The left side of Equation 4, defined as a reaction progress variable (Cerling et al., 2007; Criss, 1999), was calculated using the extracted data until the day when $(\delta_t - \delta_{final}) / (\delta_{initial} - \delta_{final})$ first became zero or negative. An advantage of the linearization model over the exponential-fit model is that multiple experiments can be conducted together using the normalization process inherent in this approach (Cerling et al., 2007). Isotopic change rate $k + c$ was statistically compared between different types of tissues by the linear model with tissue type (muscle, mucus, or ovary tissues), time (day), and the interaction terms of time with tissue type as fixed factors. Furthermore, the isotopic change rate $k + c$ was statistically compared between different stages by the linear models of each tissue, with stage type (growing or reproductive stage), time, and the interaction terms of time with stage type as fixed factors. Linear model analysis was conducted using the lm function in the R ver. 3.5.0 software package with a significance level of 0.05.

TDFs of the muscle, mucus, and ovary tissues were calculated as the increase from the mean δ values of feed A to those in each tissue when isotopic compositions reached steady states in Experiment 1 (as described above). We used Tukey's multiple comparison tests to compare the

estimates of TDFs between the muscle, mucus, and ovary tissues. Furthermore, we used the one-sample *t*-test to compare the estimates of TDFs between this investigation and the previous one (Sawada et al., 2018). Tukey's test and one-sample *t*-test were performed with R ver. 3.5.0 software with a significance level of 0.05.

3. Results

3. 1. Experiment 1: isotopic change rates and trophic discrimination factors in multiple tissues during growing stage

Ayu fish individuals grew well during the experiment in the growing stage (Figure 1a, e). Whole-body growth rate (k') was calculated as 0.0337 to 0.0355.

Both the δ^{13}C and δ^{15}N values of all tissues examined gradually decreased after diet-switch during the experiment in the growing stage (Figure 2). Nonlinear regressions (Equation 2) showed the sums of allometric growth and catabolic turnover rates ($a + c$) were significantly different from zero for the δ^{15}N changes ($p < 0.001$), but not for the δ^{13}C changes ($p > 0.05$), in muscle and mucus tissues. The half-lives of the δ^{13}C and δ^{15}N values of the muscle and mucus tissues were calculated from the isotopic change rate ($k' + a + c$) (Table 1). The reaction progress variables (Equation 4) for changes in the δ^{13}C and δ^{15}N values in the growing stage decreased significantly over time (Table 2). The interaction terms of tissue type over time in the δ^{13}C and δ^{15}N values showed a positive effect on the changes in the reaction progress variables, indicating that isotope changes were faster in mucus than in muscle tissue.

The wet weight of the ovary tissues, which were sampled 3 times to calculate TDFs, was 0.02 ± 0.01 g ($n = 7$), 0.72 ± 0.60 g ($n = 3$), and 3.93 ± 2.48 g ($n = 8$), at the beginning of the early, middle, and late sessions of Experiment 2, respectively. The C/N ratio of the ovary tissue was 3.83 ± 0.22 ($n = 7$), 5.76 ± 0.56 ($n = 3$), and 5.33 ± 0.19 ($n = 8$), at the same sampling dates, respectively. Mean δ^{13}C values of muscle and mucus tissues, which were collected to calculate TDFs, were –20.0‰ ± 0.1‰ ($n = 15$) and –21.6‰ ± 0.3‰ ($n = 15$), respectively. Mean δ^{13}C values of the ovary tissue sampled at the beginning of the early, middle, and late sessions were –19.4‰ ± 0.3‰ ($n = 7$), –21.1‰ ± 0.5‰ ($n = 3$), and –20.9‰ ± 0.1‰ ($n = 8$), respectively. The mean δ^{13}C value of feed A

was –23.7‰ ± 0.3‰.

Mean δ^{15}N values for muscle and mucus tissues, which were collected to calculate TDFs, were 8.9‰ ± 0.2‰ (n = 15) and 6.9‰ ± 0.5‰ (n = 15), respectively. Mean δ^{15}N values of the ovary tissue sampled at the beginning of the early, middle, and late sessions were 7.8‰ ± 0.4‰ (n = 7), 7.3‰ ± 0.3‰ (n = 3), and 7.2‰ ± 0.3‰ (n = 8), respectively. Mean δ^{15}N value of feed A was 4.9‰ ± 0.2‰.

TDFs of δ^{13}C and δ^{15}N values were calculated to be significantly different between tissues and sessions (Tukey's multiple comparison tests; Table 3). One-sample t-tests showed that the TDFs of both δ^{13}C and δ^{15}N values for all tissues obtained in this investigation were significantly higher than those reported by Sawada et al. (2018) ($p < 0.05$), except for the TDF of δ^{13}C value for the ovary in the middle session ($p > 0.05$).

3. 2. Experiment 2: Isotopic change rates in multiple tissues during the reproductive stage

Ayu fish individuals grew during all sessions in the reproductive stages, despite their short duration (Figure 1b–d, f–h). Whole-body growth rates (k') in the early, middle, and late sessions were 0.0203–0.0223, 0.146, and 0.0123, respectively.

The initial wet weight and C/N ratio of the ovary tissue for the early session were 0.02 ± 0.01 g and 3.83 ± 0.22 (n = 7), and the final wet weight and C/N ratio were 12.09 ± 2.24 g and 5.23 ± 0.10 (n = 3), respectively (Figure 3). For the middle session, the initial wet weight and C/N ratio of the ovary tissue were 0.72 ± 0.60 g and 5.76 ± 0.56 (n = 3), and the final wet weight and C/N ratio were 9.23 g and 5.31 (n = 1) (Figure 3). The initial wet weight and C/N ratio of the ovary tissue were 3.93 ± 2.48 g and 5.33 ± 0.19 (n = 8) for the late session, and the final wet weight and C/N ratio were 9.44 ± 2.03 g and 5.34 ± 0.28 (n = 4) (Figure 3).

The mean δ^{13}C value of feed B was –20.8‰ ± 0.3‰ (mean ± SD, n = 5), hence the difference in δ^{13}C values between feed A and feed B before and after the diet-switch was 2.9‰. The mean δ^{15}N value of feed B was 9.2‰ ± 0.3‰ (mean ± SD, n = 5), hence the difference in δ^{15}N values between feed A and feed B before and after diet-switch was 4.3‰.

Both the δ^{13}C and δ^{15}N values of all tissues examined in the early session gradually

increased during the early session in the reproductive stage (Figure 4a, d). Nonlinear regression (Equation 2) showed the sums of allometric growth and catabolic turnover rates ($a + c$) significantly different from zero for the $\delta^{13}C$ and $\delta^{15}N$ changes in mucus and ovary tissues in the early session ($p < 0.001$), except the $\delta^{13}C$ change in the ovary tissue. The sums of allometric growth and catabolic turnover rates ($a + c$) were not significantly different from zero for the $\delta^{13}C$ and $\delta^{15}N$ changes in the muscle tissue. The half-lives of the $\delta^{13}C$ and $\delta^{15}N$ values of the muscle, mucus, and ovary tissues in the early session were calculated based on the isotopic change rate ($k' + a + c$), except for the $\delta^{13}C$ value of ovary tissue (Table 1). In the middle and late sessions, both the $\delta^{13}C$ and $\delta^{15}N$ values of the ovary tissue gradually increased after diet-switch (Figure 4b, c, e, f). Nonlinear regressions (Equation 2) showed that the sums of allometric growth and catabolic turnover rates ($a + c$) were significantly different from zero for the $\delta^{13}C$ and $\delta^{15}N$ changes in the ovary tissue ($p < 0.001$). The half-lives of the $\delta^{13}C$ and $\delta^{15}N$ values of the ovary tissue in the middle and late sessions were calculated based on isotopic change rate ($k' + a + c$) (Table 1).

The reaction progress variables (Equation 4) for changes in $\delta^{13}C$ and $\delta^{15}N$ values during the early session decreased significantly over time (Table 4). Furthermore, the reaction progress variable for changes in $\delta^{13}C$ was significantly lower in ovary tissue than in mucus tissue. The interaction terms of tissue type with time in the $\delta^{13}C$ value showed a positive effect on the changes in the reaction progress variables, thus indicating that isotopic change was faster in mucus tissue than in muscle and ovary tissues. In $\delta^{15}N$ value, the negative effect of time was greater in muscle tissue, but reduced in ovary tissue, indicating that isotopic change was faster in the order of ovary, mucus, and muscle tissues.

The interaction terms of the session with time had negative effects on the changes in the reaction progress variable of the $\delta^{13}C$ value when the reaction progress variables were compared between sessions (Table 5), thus indicating that isotopic change was significantly rapid in the middle and late sessions, but not in the early session. The reaction progress variables for changes in the $\delta^{15}N$ value of the ovary tissue significantly decreased over time even during the early session. Positive effects of the interaction terms of the session with time on the changes in the reaction progress variables of the $\delta^{15}N$ value indicated that isotopic changes were faster in the early session than in the

middle and late sessions.

3. 3. Difference in isotopic change rates in muscle and mucus tissues between growing and reproductive stages

The growth rate of individuals was higher in the growing stage than in the reproductive stage ($p < 0.001$). The reaction progress variables for changes in the $\delta^{13}C$ and $\delta^{15}N$ values in the muscle tissue decreased significantly over time both in the growing and reproductive stages (Table 6). The interaction terms of the stage showed a positive effect on the changes in the reaction progress variables over time, indicating that isotopic change was faster in the growing stage than in the reproductive stage.

The reaction progress variables for changes in the $\delta^{13}C$ and $\delta^{15}N$ values in mucus decreased significantly with time both in the growing and reproductive stages (Table 6). For the $\delta^{15}N$ value, the interaction terms of the stage with time showed a positive effect on the changes in the reaction progress variables, thus indicating that isotopic change was faster in the growing stage than in the reproductive stage. The effects of the interaction term of the stage over time were not significant for the $\delta^{13}C$ value.

4. Discussion

In this study, diet-switch experiments with ayu fish (*Plecoglossus altivelis altivelis*) were conducted to illuminate patterns of isotopic change. The differences in isotopic change rates between multiple tissues were compared in the reproductive stage. Also, isotopic change rates in multiple tissues between the growing and reproductive stages were compared. These data allowed accurate estimations of isotopic change rates and TDFs which varied among tissues for a wide range of use in ecology of ayu fish.

4. 1. Difference in isotopic change rates between muscle, mucus, and ovary tissues

Changes in the $\delta^{15}N$ value were faster in the order of ovary, mucus, and muscle tissues during the reproductive stage, while changes in the $\delta^{13}C$ value were faster in mucus than in muscle and ovary

tissues.

Isotopic change was faster in mucus than in muscle tissue in any setting of our experiments. This trend corresponded to previous investigations that studied the mucus of juvenile steelhead (Church et al., 2009), 5-year-old catfish (Maruyama et al., 2016), 3 species of freshwater cyprinid fishes (Shigeta et al., 2017), and a migratory goby (Maruyama et al., 2015). To the best of our knowledge, we confirmed for the first time that this trend is common both in the growing and reproductive stages.

Conversely, our results on the isotopic change rate in the ovary tissue is the first report to the best of our knowledge. The slower $\delta^{13}C$ change in ovary tissue than in mucus tissue in our results does not simply indicate slow turnover of ovary tissue throughout the reproductive stage, because the $\delta^{13}C$ change rate was variable among sessions during the reproductive stage. The slow $\delta^{13}C$ change in the early session corresponds to the low and variable C/N ratio in the ovary tissue, suggesting asymmetric maturation process between carbon and nitrogen, as discussed in the next section. It should be also mentioned that the C/N ratio in the ovary tissue was lower than the range of lipid-correction formula (Sawada et al., 2018), and hence corrected $\delta^{13}C$ values were not completely reliable.

Our investigation also examined the mechanisms of the isotopic changes, which can be compared between tissues. In the muscle tissue, the contributions of allometric growth and catabolic turnover were shown to be almost zero in the 3 conditions among 4 examined. This result corresponds to previous studies (Hesslein et al., 1993, Maruyama et al., 2001) which showed that isotopic change can be explained mainly by the whole-body growth in the most cases. On the other hand, the isotopic changes were explained more by the allometric growth and/or catabolic turnover than by the whole-body growth, except the $\delta^{13}C$ value of the ovary tissue in the early session of the reproductive stage, in mucus and ovary tissues. The contribution of the allometric growth and catabolic turnover rates were 1.7 to 3.3 and 2.3 to 5.9 times that of the whole-body growth rate in the mucus and ovary tissues, respectively. We concluded that the contribution of the allometric growth and catabolic turnover is different in each tissue.

4. 2. Difference in isotopic change rates in ovary tissue during the reproductive stages

As mentioned briefly in the previous section, the isotopic change in the ovary tissue varied among sessions in the reproductive stage. The change in the δ^{15}N value was faster in the early session than in the middle and late sessions, whereas the change in the δ^{13}C value was more slowly in the early session than in the middle and late sessions. Slow δ^{13}C change was surprising, considering that the ovary weight increased approximately 600, 13, and 2 times during the early, middle, and late sessions, respectively. This inconsistency between carbon and nitrogen suggests that the ovarian development begins first with nitrogen (proteins) in the early session, and then shifts to the addition of carbon (lipids). This explanation applies to the low and variable C/N ratio in the ovary tissue in the early session.

The contribution of the allometric growth and catabolic turnover in the ovary tissue was different between the 3 sessions. Their contribution to the δ^{13}C change was greatest in the middle session, when the δ^{13}C change was most rapid. Similarly, their contribution to the δ^{15}N change was the largest in the early session, when the δ^{15}N change was most rapid. These results indicate that a change in the allometric growth and catabolic turnover in ovary tissue caused variable isotopic change rates among sessions during the reproductive stage.

4. 3. Difference in isotopic change rates between growing and reproductive stages

Our experiments showed that the isotopic changes in the muscle and mucus tissues were faster in the growing stage than in the reproductive stage, except for the δ^{13}C value in the mucus tissue. The half-lives of the muscle tissue in the reproductive stage (33 to 36 days) were 2 to 3 times those in the growing stage (10 to 23 days). The half-lives of the mucus tissue in the reproductive stage (8 to 13 days) were also two times that in the growing stage (5 to 9 days). These results imply that the interpretation of isotope ratios from the field can be more accurate by examining the isotopic change rate in each developmental stage of the target fish.

The contribution of allometric growth and catabolic turnover in muscle tissue was hardly confirmed, when compared to whole-body growth, except for the δ^{15}N value in the growing stage. Therefore, the reason for the slower isotopic change in the reproductive stage than in the growing

stage was considered to be the decrease in the whole-body growth rate over time. Such explanation supports a review by Vander-Zanden et al. (2015), who compared turnover rates of several previously studied animals and found that the half-life generally increases with body size. In contrast, in mucus tissue, the contribution of allometric growth and catabolic turnover in the growing and reproductive stages was 1.2 to 3.5 and 1.7 to 3.3 times higher than the whole-body growth, respectively. The slower isotopic change in the reproductive stage than in the growing stage was considered because of the decrease in all 3 factors, namely, the whole-body growth, allometric growth, and catabolic turnover.

4. 4. Inconsistency of trophic discrimination factors between the studies

TDFs of the δ^{13}C and δ^{15}N values were calculated for muscle, mucus, and ovary tissues in this investigation. In traditional isotope ecology, TDF was considered common between animals at 0–1‰ for δ^{13}C value (DeNiro & Epstein, 1978; Fry & Sherr, 1989) and 3–4‰ for δ^{15}N value (DeNiro & Epstein, 1981; Minagawa & Wada, 1984; Post, 2002). TDF variations between species (McCutchan et al., 2003; Caut et al., 2009) and between tissue types (Tieszen, 1983; Heady & Moore, 2013) have recently become widely recognized. Furthermore, TDF in a specific tissue from a species may differ between distinctive feed types (Adams & Sterner, 2000; McCutchan et al., 2003). Thus, careful consideration of variability in TDF is required for accurate estimates of changes in diet.

The differences in TDFs among tissues in this investigation were consistent with previous studies (Pinnegar & Polunin, 1999; Caut et al., 2009). A previous investigation (Sawada et al., 2018) performed a single-diet experiment of the same fish species and calculated the TDFs of the muscle (δ^{13}C: 2.4‰ ± 0.3‰, δ^{15}N: 2.5‰ ± 0.2‰), mucus (1.0‰ ± 0.2‰, 1.5‰ ± 0.4‰), and ovary tissues (1.9‰ ± 0.3‰, 1.6‰ ± 0.3‰). The TDFs of all 3 tissues were higher in both δ^{13}C and δ^{15}N values in this investigation than in the previous investigation. The difference in the TDFs between the two studies ranged 0.4–2.4‰ (min–max). The only difference between the two studies is the feeds (less crude protein and more crude ash were contained in the feed in the present investigation), thereby the TDFs may have changed by the composition difference. Such differentiation in TDF in a specific

tissue has been reported between distinctive feed types (Adams & Sterner, 2000; McCutchan et al., 2003). Further studies are required to clarify the relationship between TDF and feed components.

The TDF of the δ^{15}N value in the ovary tissue in the early session was higher than that of the middle and late sessions. This result may be explained by differences in the development status of the ovary tissue, as was suggested by the C/N ratio of the ovary tissue being lower in the early session than in the middle and late sessions. In this sense, the ovary tissue may not have reached a steady state. Thus, the development status of the ovary tissue should be noted when using the TDF in the ovary tissue.

4. 5. Implications for multiple-tissue approaches using stable isotope analysis

In this investigation, our experiments showed differences in isotopic change rates between multiple tissues, which are required to adopt the multiple-tissue approach to the target fish in the field (Kurle & Worthy, 2002; Heady & Moore, 2013). We also clarified considerable differences in isotopic change rates between growing and reproductive stages, which indicates that stage-specific isotopic change rates would allow accurate interpretation of the field data in the respective seasons. More interestingly, the timing of drastic changes in the δ^{13}C and δ^{15}N values in the ovary tissue were found to be different during the reproductive stage, which implies that the δ^{13}C and δ^{15}N values in the ovary tissue reflect diet and/or habitats of different time scales.

The target fish of our present investigation, namely ayu fish (*Plecoglossus altivelis altivelis*) landlocked in Lake Biwa, is known to migrate seasonally between the lake and its tributaries, but its migration pattern varies within the population; although almost all individuals of the population reproduce in the tributaries and die in the year they were born, they have various timing of upstream-migration from the lake (Azuma, 1973; Tsukamoto et al., 1987; Sawada et al., 2020). With more accurate estimates of the isotopic change rates in the respective seasons obtained in our results, isotopic ratios of each fish tissue could be indicators that reflect the isotopic characteristics of the place and diets that they ingest at the respective timing. Moreover, differences in isotope ratios between different tissues can be indicators of immigration timing (isotopic clock; Heady & Moore, 2013). In case of ayu fish, which play the most important role in the local fishery,

the fate of individuals grown in various places, such as rivers, lakes, and farms, are useful information. Application of isotope analysis may clarify when and where released individuals from farms settle in rivers.

This is one of the first studies to report differences in isotopic change rates between and within stages in a single fish species. Multiple-tissue approaches have been used for two decades in studies of diet shifts of various animals, such as seals (Kurle & Worthy, 2002), birds (Ruts et al. 2010), and fishes (MacNeil et al., 2005, 2006; Heady & Moore, 2013). Multiple-tissue approaches can also enable estimations of the timing of immigration from other habitats (isotopic clock). Our present investigation showed that these approaches can be more accurate when the variability of isotopic change rates between and within stages or seasons is considered.

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6. Figures and Tables

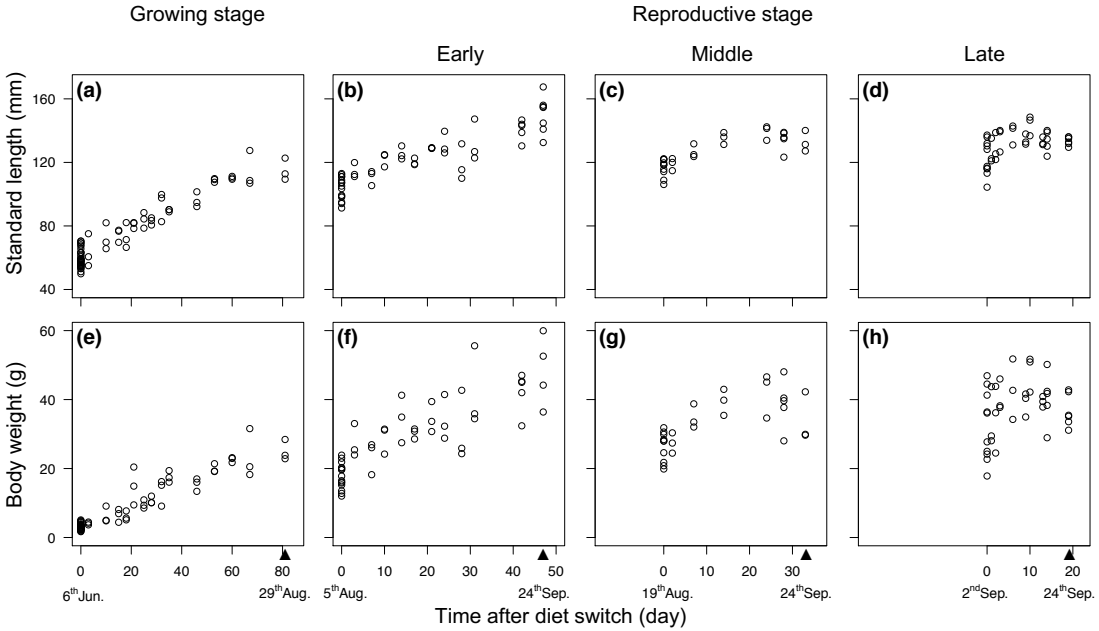

Figure 1. Changes in standard length (a–d) and wet body weight (e–h) of ayu fish (*Plecoglossus altivelis altivelis*) after the diet-switches in its growing stage (a, e) and reproductive stage (early: b, f; middle: c, g; late: d, h). Filled triangles indicate the final day of each experiment.

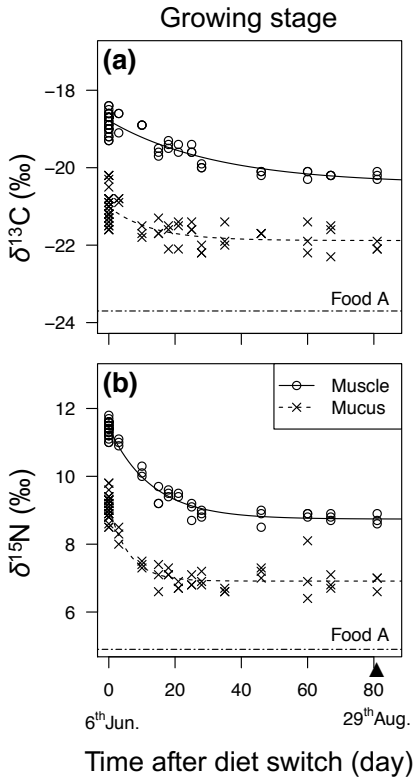

Figure 2. Changes in the δ^{13}C and δ^{15}N values (a and b, respectively) in the muscle and mucus tissues (circles and crosses, respectively) of ayu fish (*Plecoglossus altivelis altivelis*) after the diet-switch from hatchery foods to food A (horizontal line) in its growing stage. Solid and dashed curves indicate significant results of nonlinear regression for muscle and mucus tissues, respectively. Triangle indicates the final day of the experiment.

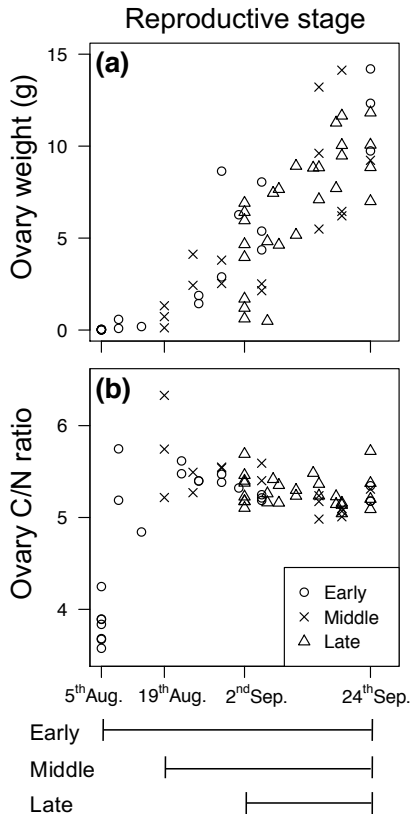

Figure 3. Changes in wet weight (a) and C/N ratio (b) in the ovary tissue of ayu fish (*Plecoglossus altivelis altivelis*) in early, middle, and late sessions (circles, crosses, and open triangles, respectively) after the diet-switches in its reproductive stage. Three bars at bottom show the periods of the early, middle, and late sessions.

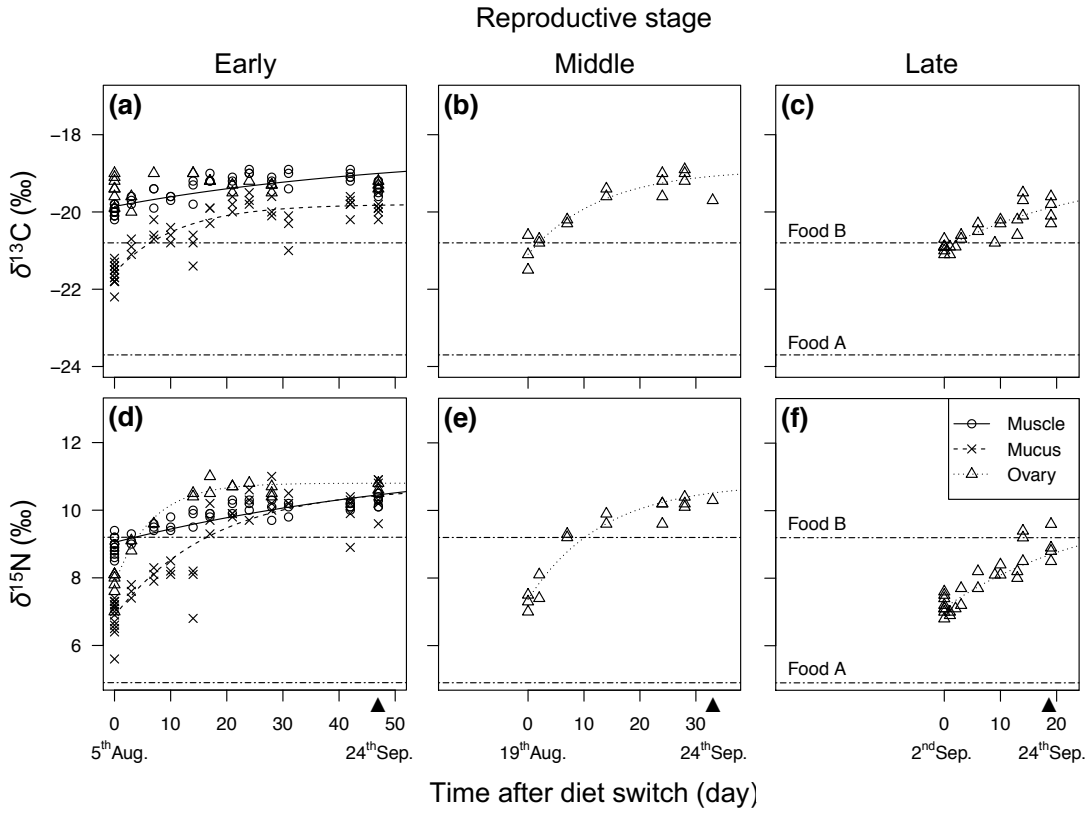

Figure 4. Changes in the δ^{13}C and δ^{15}N values (a–c and d–e, respectively) in the muscle, mucus, and ovary tissues (circles, crosses, and open triangles, respectively) of ayu fish (*Plecoglossus altivelis altivelis*) after diet-switches from foods A to B (horizontal lines) in its reproductive stage (early: a, d; middle: b, e; late: c, f). Solid, dashed, and dotted curves indicate significant results of nonlinear regression for muscle, mucus, and ovary tissues, respectively. Filled triangles indicate the final day of each experiment.

Table 1. Sum of allometric (tissue-specific) growth rate and catabolic turnover rate ($a + c$) and half-lives (t_{half}) of $\delta^{13}C$ and $\delta^{15}N$ values in the muscle, mucus, and ovary tissue of ayu fish (*Plecoglossus altivelis altivelis*) during the diet-switch experiments in growing and reproductive stages. Growth rate (*k'*) was calculated using individual body weight changes.

Stage	Tissue	Session	*N*	k' (day^{-1})	$\delta^{13}C$		$\delta^{15}N$	
					$a + c$ (day^{-1})	t_{half} (day)	$a + c$ (day^{-1})	t_{half} (day)
Growing stage	Muscle		75	0.0337 ± 0.0030	–0.0035 ± 0.0086	23	0.0404 ± 0.0066***	10
	Mucus		71	0.0355 ± 0.0028	0.0432 ± 0.0373	9	0.1230 ± 0.0314***	5
Reproductive stage	Muscle	Early	54	0.0203 ± 0.0010	0.0008 ± 0.0017	33	–0.0009 ± 0.0010	36
	Mucus	Early	54	0.0203 ± 0.0010	0.0665 ± 0.0120***	8	0.0348 ± 0.0049***	13
	Ovary	Early	23	0.0223 ± 0.0017	–0.0108 ± 0.0081	NA*	0.1321 ± 0.0202***	5
	Ovary	Middle	16	0.0146 ± 0.0030	0.0561 ± 0.0106***	10	0.0563 ± 0.0068***	10
	Ovary	Late	27	0.0123 ± 0.0015	0.0286 ± 0.0044***	17	0.0277 ± 0.0032***	18

Mean or estimates ± standard errors are shown with significance levels (***: $p < 0.001$). *N* sample size.

*Not available because the C/N ratios of the samples were out of the range for the tissue-specific correction formula (Sawada et al. 2018)

Table 2. Summary of the linear models that explain the reaction progress variables ($\ln[(\delta_t - \delta_{final}) / (\delta_{initial} - \delta_{final})]$), calculated from changes in the $\delta^{13}C$ and $\delta^{15}N$ values of ayu fish (*Plecoglossus altivelis altivelis*) during the diet-switch experiment in growing stage to compare mucus and muscle tissue.

Explanatory variables	Coefficients for ayu fish	
	$\delta^{13}C$	$\delta^{15}N$
Intercept	–0.1369 ± 0.0693	0.0041 ± 0.0261
Time (*t*, day)	–0.0733 ± 0.0140***	–0.1467 ± 0.0087***
Tissue: muscle	0.1177 ± 0.0932	0.0034 ± 0.0368
Time × tissue: muscle	0.0413 ± 0.0142**	0.0747 ± 0.0093***
R^2 values	0.722***	0.902***

Estimates ± standard errors are shown with significance levels (**: $p < 0.01$, ***: $p < 0.001$)

Table 3. Trophic discrimination factors (TDFs) of $\delta^{13}C$ and $\delta^{15}N$ values in muscle, mucus, and ovary tissue of ayu fish (*Plecoglossus altivelis altivelis*) at the end of the diet-switch experiment in the growing stage.

Tissue	Time after diet-switch (day)	*N*	TDFs		
			$\delta^{13}C$		$\delta^{15}N$
			Untreated	Lipid-corrected*	
Muscle	57	15	2.9 ± 0.2	3.7 ± 0.1[a]	4.0 ± 0.2[a]
Mucus	57	15	2.1 ± 0.3[c]	−**	2.0 ± 0.5[c]
Ovary (premature)	57	7	2.0 ± 0.3	NA***	2.9 ± 0.4[b]
Ovary	71	3	0.3 ± 0.4	2.6 ± 0.4[b]	2.4 ± 0.2[c]
Ovary	85	8	0.5 ± 0.1	2.8 ± 0.1[b]	2.3 ± 0.3[c]

*According to the species- and tissue-specific correction formulas by Sawada et al. 2018

**Mucus does not contain lipids (Shephard, 1994)

***Not available because the C/N ratios of the samples were out of the range for the tissue-specific correction formula (Sawada et al. 2018)

Different alphabets indicate significant differences based on Tukey's multiple comparison tests.

Table 4. Summary of the linear models that explain the reaction progress variables ($\ln[(\delta_t - \delta_{final}) / (\delta_{initial} - \delta_{final})]$), calculated from changes in the $\delta^{13}C$ and $\delta^{15}N$ values of ayu fish (*Plecoglossus altivelis altivelis*) during the diet-switch experiment in the early session to compare mucus, muscle, and ovary tissue.

Explanatory variables	Coefficients for ayu fish	
	$\delta^{13}C$	$\delta^{15}N$
Intercept	–0.0299 ± 0.1117	0.0945 ± 0.0703
Time (*t*, day)	–0.0931 ± 0.0139***	–0.0828 ± 0.0060***
Tissue: muscle	–0.0833 ± 0.1465	–0.1415 ± 0.0938
Tissue: ovary	–0.3674 ± 0.1793*	–0.0980 ± 0.1281
Time × tissue: muscle	0.0783 ± 0.0144***	0.0684 ± 0.0065***
Time × tissue: ovary	0.0936 ± 0.0153***	–0.0739 ± 0.01831***
R^2 values	0.348***	0.760***

Estimates ± standard errors are shown with significance levels (*: $p < 0.05$, ***: $p < 0.001$)

Table 5. Summary of the linear models that explain the reaction progress variables ($\ln[(\delta_t - \delta_{final}) / (\delta_{initial} - \delta_{final})]$), calculated from changes in the $\delta^{13}C$ and $\delta^{15}N$ values of ovary tissue of ayu fish (*Plecoglossus altivelis altivelis*) during the diet-switch experiments to compare the early, middle, and late sessions.

Explanatory variables	Coefficients for ayu fish	
	$\delta^{13}C$	$\delta^{15}N$
Intercept	–0.3973 ± 0.1577*	–0.0035 ± 0.0653
Time (*t*, day)	0.0006 ± 0.0071	–0.1567 ± 0.0106***
Session: Middle	0.3835 ± 0.2718	–0.0919 ± 0.0998
Session: Late	0.4145 ± 0.2151	0.0010 ± 0.0835
Time × session: Middle	–0.0843 ± 0.0177***	0.0956 ± 0.0113***
Time × session: Late	–0.0413 ± 0.01613*	0.1173 ± 0.0118***
R^2 values	0.389***	0.923***

Table 6. Summary of the linear models that explain the reaction progress variables (ln [($\delta_t - \delta_{final}$) / ($\delta_{initial} - \delta_{final}$)]), calculated from changes in the $\delta^{13}C$ and $\delta^{15}N$ values of muscle and mucus tissue of ayu fish (*Plecoglossus altivelis altivelis*) during the diet-switch experiments to compare the growing and reproductive stages.

Explanatory variables	Coefficients for ayu fish			
	Muscle		Mucus	
	$\delta^{13}C$	$\delta^{15}N$	$\delta^{13}C$	$\delta^{15}N$
Intercept	–0.0192 ± 0.0418	0.0075 ± 0.0239	–0.1369 ± 0.0881	0.0041 ± 0.0610
Time (*t*, day)	–0.0320 ± 0.0014***	–0.0720 ± 0.0030***	–0.0733 ± 0.0179***	–0.1467 ± 0.0204***
Stage: reproductive stage	–0.0939 ± 0.0690	–0.0544 ± 0.0370	0.1070 ± 0.1510	0.0904 ± 0.1012
Time × stage: reproductive stage	0.0172 ± 0.0026***	0.0576 ± 0.0032***	–0.0198 ± 0.0235	0.0639 ± 0.0215**
R^2 values	0.832***	0.881***	0.432***	0.748***

Estimates ± standard errors are shown with significance levels (**: $p < 0.01$, ***: $p < 0.001$)

Chapter 4

Isotope analysis reveals proportional change and site-selection variation of river- and lake-produced eggs of a landlocked migratory fish

1. Introduction

Fish occupying a relatively high position in the food web have a major impact on aquatic communities through top-down effects (Carpenter *et al.*, 1985; DeMelo *et al.*, 1992), yet are themselves strongly affected by other organisms through bottom-up effects (McQueen *et al.*, 1989; Ware & Thomson, 2005). At the same time, among aquatic organisms in general, fish often exhibit distinctive migration behaviours (McDowall, 1988; Winemiller & Rose, 1992). A large proportion of freshwater fish has life histories involving migration between rivers, lakes and/or the sea (Gross *et al.*, 1988; Healey, 1991), with some exhibiting polymorphism (Healey, 1991; Hindar *et al.*, 1991). Thus, most fish influence and are influenced by each of multiple food chains (e.g., rivers, lakes and/or the sea; Hobson 1999) at the ratios that may be different among populations/morphs within the species. Understanding the relative contribution of each food chain to the focal fish is essential for appropriate resource management. However, obtaining an understanding of the relative importance of each food chain on fish reproduction has proven difficult, partly due to the lack of suitable methodology; molecular or morphological approaches cannot always distinguish the origins of populations (Iguchi & Kuwahara 1999; McCarthy & Waldron, 2000). Hence the contributions of the different food chains remain obscure, particularly in cases of polymorphisms in anadromous and potamodromous fish species.

Nitrogen and carbon stable isotope analyses have been used to trace material flow in food webs (Fry, 2006), based on the stepwise increases in nitrogen and carbon isotope ratios (hereafter, δ^{15}N and δ^{13}C, respectively) between trophic interactions (DeNiro & Epstein, 1978; Minagawa &

Wada, 1984). Because the δ^{15}N and δ^{13}C values of prey organisms sometimes differ among habitats (e.g. rivers, lakes and/or the sea; Hobson 1999), the corresponding values of the focal animals can be used as indicators of their migration history while moving between habitats (Hobson, 1999; Maruyama *et al.*, 2001a), since isotopic incorporation takes place over a long period (several months in well-studied muscle tissue; Peterson & Fry, 1987; Hesslein *et al.*, 1993). Importantly, the δ^{15}N and δ^{13}C values of fish eggs quantitatively reflect what the maternal fish ate when the eggs were maturing in the ovaries (Grey, 2001; Ito *et al.*, 2015). Thus, nitrogen and carbon stable isotope analyses of the spawned eggs can be expected to reveal which food chain the maternal fish had been depending on during egg production.

The population of ayu fish (*Plecoglossus altivelis altivelis*) landlocked in Lake Biwa, central Japan, seasonally migrates between the lake and its tributaries, but its migration pattern is variable within the population — although almost all individuals of the population reproduce in the tributaries and die in the year they were born, they have various timing of upstream-migration from the lake (Azuma, 1973a, b, c; Tsukamoto *et al.*, 1987) [Figure 1]. In short, they hatch out from demersal, adhesive eggs broadcasted in the rapids in the lower reach of tributaries from September to November in autumn. Immediately after hatching, they drift down to the lake, where they keep on preying on zooplankton. After several months (ranging from five to 12 months), some migrate in spring and start preying on attached algae in the tributaries until autumn, some migrate in autumn immediately before reproduction, and the remainder migrate at intermediate times (Azuma, 1973b, c). Thus, the relative contributions of the riverine and lacustrine food chains are expected to be different between individuals within the population. It is crucial to quantitatively understand the times and places, where the eggs are produced for appropriate resource management because of *P. altivelis altivelis* playing an important role in the local fishery, constituting 50% of the total catches in this lake (Ministry of Agriculture, Forestry and Fisheries, 2016). However, these contributions

have not been quantitatively measured due to the aforementioned lack of suitable methodology.

The objective of this study is to quantitatively examine the proportional change and site-selection variation between river- and lake-produced eggs of *P. altivelis altivelis* in the Lake Biwa water system, by distinguishing spawned eggs using stable isotope analysis. A recent study performed in the same lake-river system showed that the δ^{15}N value of lake-produced eggs in the ovary of the fish was 7‰ higher than that of river-produced eggs (Ito *et al.*, 2015), reflecting the difference between isotope ratios of their main foods in the two food chains, which originally derives from eutrophication and/or denitrification in the lake (Yamada *et al.*, 1996). The δ^{13}C values are also expected to differ between the lake- and river- produced eggs, because zooplankton (fish prey) in the lake have lower δ^{13}C values than the benthic invertebrates (ecological equivalents) in the tributaries (Yamada *et al.*, 1998; Maruyama *et al.*, 2001b). Thus, the contributions of the two food chains could be expected to be quantitatively measured via isotope ratios. An understanding of the relative contribution of riverine and lacustrine food chains to the fish could help improve resource management in this lake.

2. Materials and Methods

2. 1. Sample collection

Spawned eggs of *P. altivelis altivelis* were collected from riverbeds in the lower reaches of 16 tributaries of Lake Biwa, namely the Ado, Amano, Ane, Chinai, Echi, Ishida, Kisen, Mano, Momose, Oura, Seri, Shiotsuoo, Tenjin, U, Uso, and Wani rivers (Figure 2). Sampling was conducted from September to November in 2015, covering the full reproductive season of the fish. In each river, three sites were identified to cover the spawning reach of the fish throughout the survey period, based on preliminary surveys. At each site, the spawned eggs were sampled from three areas of 20 × 10 cm, by collecting all pebbles onto which spawned eggs were attached. At the same time, current

velocity and water depth were measured by a propeller current meter CR-11 (Cosmo Riken Ltd., Osaka, Japan) and rulers, respectively. The pebbles bearing the spawned eggs were brought back to the laboratory, and stored at –40 °C until used for stable isotope analysis.

2.2. Stable isotope analysis

Preliminary analysis revealed that, for accurate carbon and nitrogen isotope analysis, each sample needs about 0.5 mg of dry tissue weight, which corresponds to ten eggs of the fish; thus, each sample could have contained eggs spawned by multiple females. Ten eggs were picked from the pebble samples, cleaned with distilled water, put in a tin capsule as a single sample, and dried at 60 °C for 48 h. Afterward, the eggs were crushed with tweezers in the tin capsule, which was folded. Instead of extracting lipids from the egg samples, the effect of variable lipid content on the $\delta^{13}C$ values of the spawned eggs was corrected using the C:N ratio of each sample, according to the correction model [$\delta^{13}C_{lipid\text{-}free} - \delta^{13}C_{bulk} = 2.3$] specific to the eggs of this species (Sawada *et al.* 2019).

$\delta^{15}N$ and $\delta^{13}C$ were measured using a Delta V Advantage mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) connected to a Flash EA 1112 elemental analyser (Thermo Fisher Scientific) through a Conflo IV interface (Thermo Fisher Scientific) at Ryukoku University, Japan. Repeated samples of alanine and histidine standards were used for calibration and quality control for samples of the spawned eggs. Samples that contained an insufficient amount of nitrogen were excluded from further analyses. $\delta^{15}N$ and $\delta^{13}C$ were expressed as $\delta X = (R_{sample}/R_{standard}) - 1$, where X is ^{13}C or ^{15}N; R_{sample} corresponds to the ^{13}C /^{12}C or the ^{15}N /^{14}N ratio of the measured samples; and $R_{standard}$ is the ^{13}C /^{12}C ratio of Vienna Pee Dee Belemnite or the ^{15}N /^{14}N ratio of atmospheric nitrogen. Analytical errors in δ were less than ± 0.3‰.

2. 3. Statistical analysis

We examined temporal changes in the proportions of river- and lake-produced eggs, and the effect of water current velocity and water depth on these values, using linear models (LM) with the date, current velocity and water depth as explanatory variables and δ^{15}N or δ^{13}C values of the spawned eggs as response variables. The effects of interaction terms of the explanatory variables were also examined, and excluded when their standard errors were too large.

To examine the difference in selectivity of environmental conditions for spawning between the river- and lake-produced eggs, we divided the egg samples into two groups at the medians of the δ^{15}N and δ^{13}C values and compared variations in the current velocity and water depth between the two groups using the *F* tests.

R ver. 3.5.0 software (https://www.r-project.org) was used for the statistical tests. We used the lm and var.test functions for the LM and *F* tests, respectively. The significance level was set at $P < 0.05$.

3. Results

3. 1. Change in stable isotope ratios with date

A total of 84 egg samples were collected for stable isotope analysis [Supporting Information Figure S1]. More than one sample were collected from all of 16 tributaries of Lake Biwa visited. The δ^{15}N values of the spawned eggs decreased significantly with date (LM, $t = -13.45$, $P < 0.001$) [Figure 3(a) and Supporting Information Figure S2]. The effect of current velocity or water depth on δ^{15}N value was not significant ($P > 0.05$). The effect of the interaction terms was excluded from the LM. Thus, δ^{15}N values simply decreased with time [Figure 3(a)–(c)].

The δ^{13}C values of the spawned eggs increased significantly with date ($t = 4.89$, $P < 0.001$) [Figure 3(d) and Supporting Information Figure S2]. The effect of current velocity or water depth

was not significant ($P > 0.05$). The effect of the interaction term between date and current velocity was significantly negative for the δ^{13}C values ($t = -2.77$, $P < 0.01$). Thus, δ^{13}C values increased with time, while the increase was moderated by current velocity [Figure 3(d)–(f)].

3. 2. Positional variation between river- and lake-produced eggs

When the spawned eggs were divided into the two groups according to the δ^{15}N values, the variation in the water depth was significantly larger in the spawned eggs with higher δ^{15}N values than those with lower δ^{15}N values (F test, $F_{45, 44} = 3.95$, $P < 0.001$). Also, the variation in the water depth was larger in the eggs with lower δ^{13}C values than those with higher δ^{13}C values ($F_{45, 44} = 0.21$, $P < 0.001$). The variation in current velocity was not significantly different when the eggs were divided according to either the δ^{15}N or the δ^{13}C values ($P > 0.05$).

4. Discussion

4. 1. Contribution of riverine production to the reproduction

In a previous study at Lake Biwa, δ^{15}N values of ovaries extracted from adult fish of *P. altivelis altivelis* were distinctly lower in individuals that had matured in a tributary (6.1 ± 0.5‰) than in those matured in the lake (12.6 ± 0.9‰) (Ito *et al.*, 2015), reflecting differences in the δ^{15}N values of prey organisms between the lake and tributaries (Yamada *et al.*, 1996; Maruyama *et al.*, 2001b). Therefore, the decrease in the δ^{15}N values of the spawned eggs found in our present study indicates a shift from lake-produced to river-produced eggs within a reproductive season. The moderate increase in the δ^{13}C values of the spawned eggs supports this explanation, because the main feed of the fish in Lake Biwa, namely zooplankton, have relatively low δ^{13}C values (winter: –28‰, other: –23‰; Yamada *et al.*, 1998), whereas the benthic invertebrates in rivers, which occupy the similar trophic position as the fish in rivers, have relatively high δ^{13}C values (–21.8‰; Maruyama *et al.*, 2001b). These isotopic changes in the spawned eggs could not be explain by temporal isotopic

changes of the producers in a location, because the previous studies showed that changes in the δ^{15}N and δ^{13}C values of riverine and lacustrine producers with time were limited in the system (Yamada *et al.*, 1996, 1998; Maruyama *et al.*, 2001b). Thus, we concluded that the proportion of river-produced eggs gradually increased with time.

According to previous studies on the life history of the fish population in the Lake Biwa water system, production of *P. altivelis altivelis* eggs in rivers can be explained by the following two processes. The first is life history variation. The population of the fish in the Lake Biwa water system has a variable life history. Some individuals migrate upstream from the lake in spring (6 months after hatching; spring migrants), while others migrate in autumn (immediately before spawning; autumn migrants). The upstream migration endures continuously from spring to autumn, while almost all individuals reproduce sympatrically in the lower reaches of the tributaries and concurrently in the autumn of the year (Azuma, 1973b, c; Tsukamoto *et al.*, 1987). Thus, the locations where maternal individuals produce eggs are also continuously variable among eggs from fully river-produced eggs to fully lake-produced ones. The second is multiple spawning. A previous study showed that a considerable proportion (55%) of autumn migrants in the Lake Biwa water system re-mature after the first spawning and can repeat spawn up to two to three times within a reproductive season (Matsuyama & Matsuura, 1984). In aquariums, such multiple spawning has been observed primarily in the smaller individuals (Iguchi, 1996), which are mostly composed of autumn migrants. Thus, eggs in the second and later clutches may be derived from river mature females, even in autumn migrants.

The increase in the proportion of river-produced eggs can be satisfactorily explained by life history variation. Azuma (1973b) reported an increase in large individuals (around 100 mm SL) in the latter half of the reproductive season. Considering that spring migrants tend to grow larger than autumn migrants, the changes in isotope ratios in our results correspond to the change in the

size composition of the spawning population. In this case, our results imply that a considerable proportion of spring migrants may be contributing to reproduction. Our results also indicate that variable life history can be maintained only when the reproductive season is sufficiently long. Even when the possible cycle switching between spring and autumn migrants in successive generations is considered (Tsukamoto *et al.*, 1987), the loss of life history variation would impact the whole population in the Lake Biwa water system. Thus, connection along the river from upper to lower reaches should be protected throughout the reproductive season, because the spring migrants grow in the upper reaches and then migrate downstream for reproduction in the lower reaches. Droughts, and the building of dams, may impede reproductive migrations (Yuma *et al.*, 1998).

On the other hand, multiple spawning can also satisfactorily explain the increase in the proportion of river-produced eggs. The river-produced eggs would automatically increase if the multiple spawning is the case, because it is known to take approximately 14–20 days for eggs to mature after the previous spawning (Iguchi, 1996). Under these circumstances, the lower reaches of rivers, where both spring and autumn migrants finally gather for reproduction, are especially important for maintenance of the population. However, in the lower reaches, rivers generally tend to be sandy, muddy, and/or turbid, and hence primary production by attached algae is limited (Vannote *et al.*, 1980). Also, the lower reaches frequently dry up, particularly around Lake Biwa (Yuma *et al.*, 1998). Thus, close attention should be paid to the condition of the lower reaches in order to maintain the population.

It is notable that a number of our egg samples showed intermediate values for the isotope ratios. Intermediate values can be explained by individuals that migrate continuously from spring to autumn. However, because each sample for the isotope analysis contained ten eggs, each sample could have been a combination of river- and lake-produced eggs. Between-river variation in isotope ratios of spring migrants could also explain such intermediate isotope ratios, because the isotope

ratios of attached algae, the main feed of *P. altivelis altivelis* in rivers, differ between rivers (Kohzu *et al.*, 2009). Thus, the relative contributions of the two processes, namely life history variation and multiple spawning, to the increase in the proportion of river-produced eggs are unknown at this point, and further studies are necessary. Nevertheless, this stable isotope-based study shows clearly for the first time that riverine primary production is utilised for the maturity of the fish eggs, particularly in the latter half of the reproductive season.

4. 2. Spawning habitat selection

No correlation was observed between current velocity or water depth and the δ^{15}N or δ^{13}C values of the spawned eggs. However, when the spawned eggs were divided into two groups according to the δ^{15}N or δ^{13}C values, the variation in water depth was larger in the spawned eggs with higher δ^{15}N values and lower δ^{13}C values than in those with lower δ^{15}N values and higher δ^{13}C values. This result implies that river-produced eggs tend to be spawned in a narrower range of water depths than lake-produced eggs.

There may be several explanations for the different range of environmental conditions for spawning between river- and lake-produced eggs. First, the river-produced eggs may have been spawned by spring migrants, as discussed above. Spring migrants generally grow larger than autumn migrants (Azuma, 1973c), and this may confer an advantage when competing for the optimum spawning beds, possibly leading to a choice of a narrower range of environmental conditions. Second, when the river-produced eggs are the result of multiple spawning, individuals that spawn second clutches may have advantages over those reaching the spawning bed only recently, due to prior residence effects. This would also lead to the narrower range of environmental conditions for the river-produced eggs. Third, variation in the environmental conditions might have decreased with time. In this case, even if *P. altivelis altivelis* had spawned in the conditions available, the range of

the environmental conditions would decrease with time. Thus, further studies are required to examine whether the change in the range of spawning conditions is active or passive. It is notable, at this point, that discrimination of river- and lake-produced eggs by stable isotope analysis enables comparison of environmental conditions by the two types of egg within a single species.

4. 3. Implications of stable isotope analysis for discriminating spawned eggs

To the best of our knowledge, this study is among the first to successfully discriminate eggs spawned by maternal fish of osmerid fish that had different individual histories between river and lake (Ito *et al.*, 2015), while quite a few studies have reported this on salmonids to separate freshwater-resident from anadromous migrants (Curry, 2005; Charles *et al.*, 2006; Gabrielsson *et al.*, 2012; Kristensen et al., 2011; Briers *et al.*, 2013). This approach is applicable to other fish with variable life histories that depend on a different source of primary production of nitrogen or carbon, such as migration patterns and trophic polymorphisms. For example, different δ^{15}N and δ^{13}C values of eggs in ovaries are reported between life history dimorphisms of brown trout (*Salmo trutta*), each morph that reproduce sympatrically at the same places but grow allopatrically in different places (McCarthy & Waldron, 2000). Discriminating between spawned eggs has the potential to enable comparison of the preference for spawning sites between parent fish with different histories. The technique could also reveal the contributions of each morph to the reproduction of following generations. Despite the importance of spawning sites for the maintenance of the focal population, studies on site preference and the contribution of each morph are often hampered because parent fish often leave the site immediately after spawning. Stable isotope ratios of spawned eggs are expected to provide essential information on parent fish, which could not be traced even by molecular techniques, to reveal the roles of intraspecific variations in the lifecycle of the fish.

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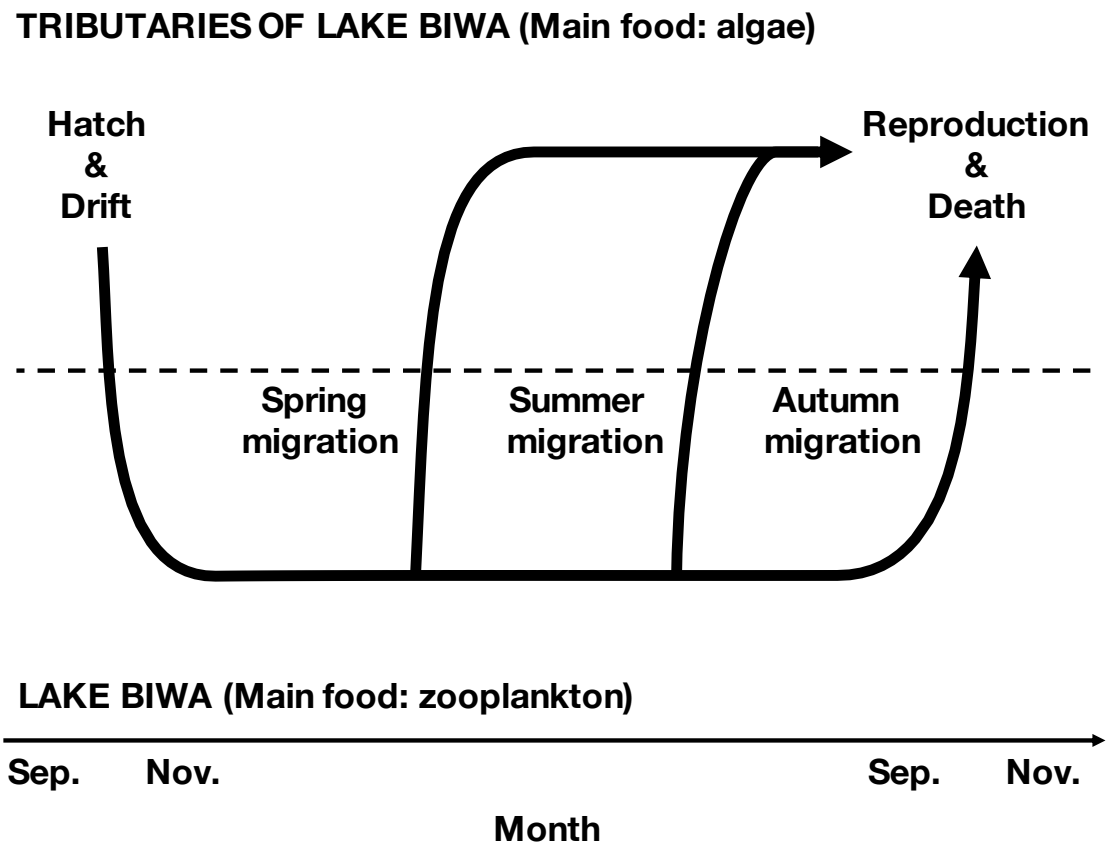

Figure 1. Schematic diagram describing different life histories of spring, summer, and autumn migrants of *Plecoglossus altivelis altivelis* in Lake Biwa (Azuma 1973c)

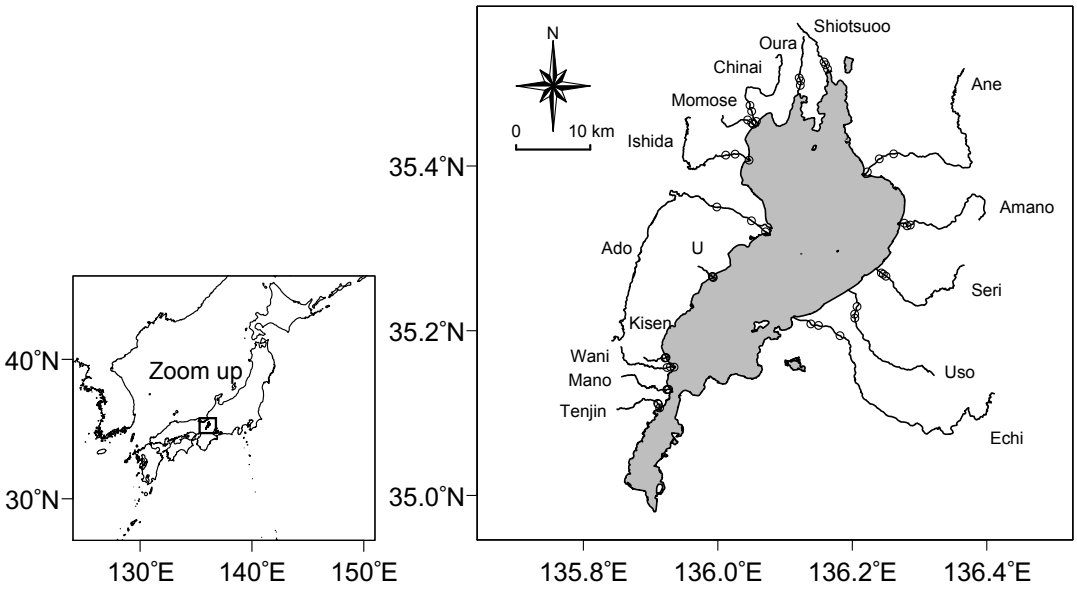

Figure 2. Locations of the survey sites (open circles) along the 16 tributaries of Lake Biwa

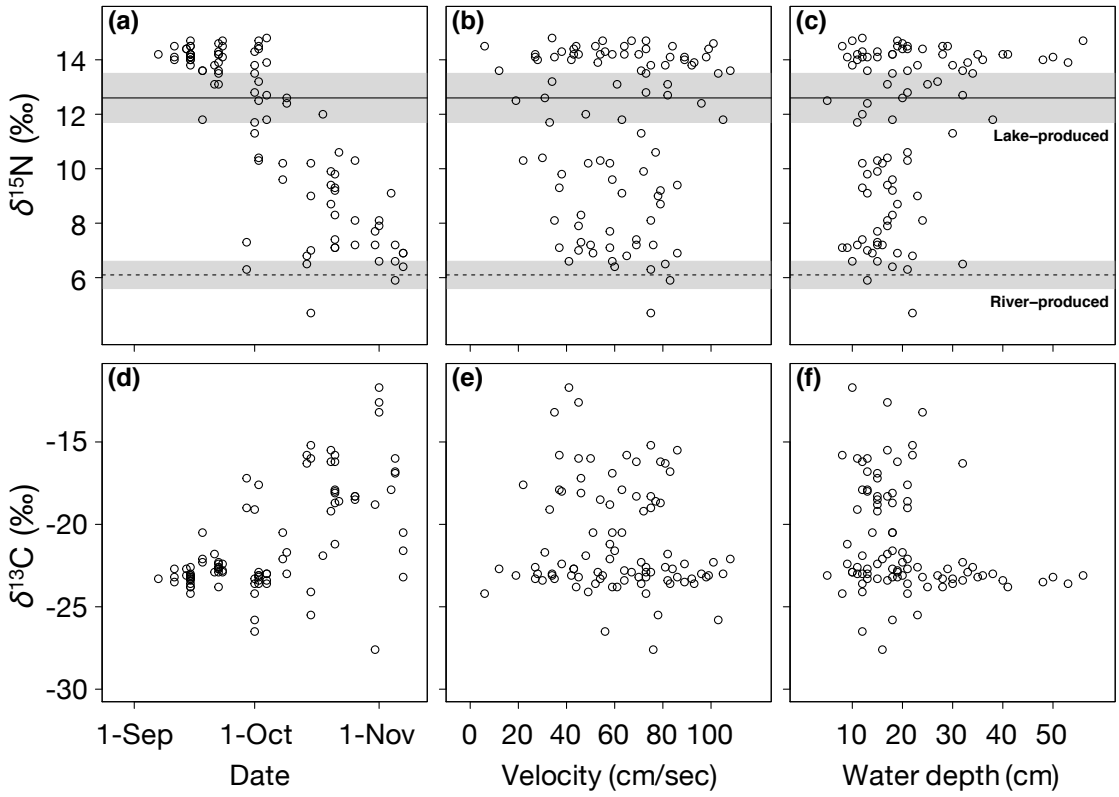

Figure 3. The relationships between δ^{15}N [(a)–(c)] and δ^{13}C [(d)–(f)] values of the spawned eggs of *Plecoglossus altivelis altivelis* in 16 tributaries (pooled) of Lake Biwa and date [(a), (d)], current velocity [(b), (e)] and water depth [(c), (f)]. The solid and dotted lines with grey zones indicate the mean values (with SD) of eggs in ovaries collected from adult fish of lake-maturity and river-maturity, respectively [(a), (b), (c)] (Ito *et al.* 2015).

Chapter 5

Effect of river drought on river- and lake-matured types of ayu fish

Introduction

Rivers connecting the land and the sea provide ecosystem services as seasonal migration routes essential for many aquatic organisms to sustain their life cycles. For example, familiar fisheries resources such as ayu fish (sweet fish), eel and salmon all migrate between rivers and the sea, providing food and recreation for people on land. In recent years, however, river droughts have been frequently observed in rivers in some regions of Japan where the river channel dries up due to insufficient surface water flow. River droughts temporarily reduce the suitable habitats for aquatic organisms due to the lack of surface water and generally higher water temperature (Closs & Lake, 1996). These catastrophic events can even disrupt the life cycle of migratory fish species that have evolved on the basis of the continuity of the water system (Bearmish & Northcote, 1989). The Japanese Ministry of the Environment mandates river administrators to keep rivers continuously discharging a certain amount of surface water, but many local governments are not even able to assess the current situation. Understanding the current status of river droughts and their effect on ayu populations is essential information for future river management.

River droughts are considered to affect many fishes through a variety of processes. In general, the size of the available habitat is known to be an important determinant of fish densities, and the reduction or loss of habitat is thought to affect fish densities (Fausch & Northcote, 1992). For example, a reduction in surface water is thought to cause the loss of rapids, reducing the density of fish that use them as habitat (Closs & Lake, 1996). With the loss of rapids, some fish move to the pools of the river and increase their local density (Matthews & Marsh-Matthews, 2003). Previous studies have reported a number of different impacts of river drought on fish, including stunted

growth and reduced disease resistance through increased density in a reduced habitat size (Bruton, 1985). In addition, the fragmentation of riverine system associated with river droughts is thought to make it difficult to maintain the life cycle of diadromous and potadromous fish (Bearmish & Northcote, 1989). Thus, river droughts not only inhibit the growth of fishes and worsen their disease resistance but might also impact their life cycles by delaying their migration.

In recent years, researchers, fishermen and local residents have unofficially reported frequent river droughts in the tributaries of Lake Biwa. Most tributaries of Lake Biwa have artificially-raised riverbed as a result of repeated embankment for several centuries. In addition, an increase in the quantity of water intake for agricultural use, due to the pursuit of convenience in modern agriculture may have led to increased droughts. Climate change may have also reduced precipitation. These are all possible factors contributing to increase droughts. Yet, no surveys have been conducted to understand and monitor the changes associated with river drought with the exception of a study at Takatoki River, to the best of our knowledge. It has been reported that in the Lake Biwa water system the catches of various fish species migrating between Lake Biwa, inland lakes and rivers have been declining (Kitagawa & Ishigure, 2003; Fujioka, 2013). There are no studies examining whether river drought played a role in reducing fish population size.

The population of ayu fish (*Plecoglossus altivelis altivelis*) landlocked in Lake Biwa, central Japan, play an important role in the local fishery, constituting 50% of total catches in Lake Biwa (MAFF, 2016), its catch has recently decreased. Also, this fish is a migratory fish that attracts attention as an environmental indicator species. Thus, there is no doubt that ayu fish is the first species that should be examined regarding the effects of river droughts. Ayu fish seasonally migrates between the lake and its tributaries, but its migration pattern is variable within the population; although almost all individuals of the population reproduce in the tributaries and die in the year they were born, they have various timing of upstream-migration from the lake (Azuma, 1973a, b, c;

Tsukamoto et al., 1987). In short, they hatch out from demersal, adhesive eggs broadcasted in the rapids in the lower reach of tributaries in autumn, from September to November. Immediately after hatching, they drift down to the lake, where they prey on zooplankton. After several months (ranging from 5 to 12 months), some migrate in spring and start grazing attached algae in the tributaries until autumn, some migrate in autumn immediately before reproduction and the remainder migrate at intermediate times (Azuma, 1973b, c). It is crucial to understand the respective numbers of spawned eggs produced in the rivers and the lake for appropriate resource management. However, these contributions have not been quantitatively measured due to the aforementioned lack of suitable methodology (but see Sawada et al., 2020).

The objectives of this study were to understand the current status of river droughts (frequency and scale), to identify the characteristics of rivers prone to river droughts, and to clarify the effects of river droughts on the number of river- and lake-produced eggs of ayu fish by distinguishing spawned eggs using stable isotope analysis. A recent study performed in the same lake-river system showed that the δ^{15}N value of lake-produced eggs in the ovary of the fish was 7‰ higher than that of river-produced eggs (Ito et al., 2015), reflecting the difference between isotope ratios of their main foods in the two food chains, which originally derives from eutrophication and/or denitrification in the lake (Yamada et al., 1996). The δ^{13}C values are also expected to differ between the lake- and river- produced eggs, because zooplankton (fish prey) in the lake have lower δ^{13}C values than the benthic invertebrates (ecological equivalents) in the tributaries (Yamada et al., 1998; Maruyama et al., 2001b). Recently, changes in the proportions of river- and lake-produced eggs of ayu fish in the Lake Biwa water system were monitored by distinguishing spawned eggs using carbon and nitrogen stable isotope analysis (Sawada et al., 2020). Thus, the contributions of the river- and lake-produced eggs could be quantitatively measured via isotope ratios. Understanding the impact of river droughts on each of the number of river- and lake-produced eggs has implications

for stock management and conservation of this species.

Materials and methods

Survey of the frequency and scale of the river droughts by foot and aerial photography

Surveys of the current status of the river droughts was conducted along 11 tributaries of Lake Biwa once a week from May to November for three years, namely from 2016 to 2018 (Figure 1). The surveys covered the middle and lower reaches of the river where river droughts are likely to occur. Whenever a river drought was found, the latitude and longitude of its upper and lower ends were recorded. In the rivers where access to the river channel was difficult due to the forests (mostly, abandoned bamboos), aerial photographs were taken from the drones (Phantom 4, DJI, Guangdong, China). The characteristics of each river that may affect the occurrence of river droughts, namely total length of mainstream (L), total length of branches (l), catchment area (A), mean width of basin (A/L), average gradient (L/elevation difference), sinuosity (L/linear distance), river density [$(L+l)$/A], and 'relative riverbed height' were calculated on a numerical map using GIS software (QGIS and ArcGIS). 'Relative riverbed height' was computed as the mean elevation difference between the riverbeds and the surrounding lands (30–300 m from the river), measured at intervals of 100 m along the mainstream from the mouth (84 m a.s.l.) to the middle reaches (120 m a.s.l.) of the river, where river droughts are likely to occur. In addition, the permitted quantity of water intake from the rivers for agriculture (mainly, for paddy fields) was collected from the Shiga Prefectural River Basin Policy Bureau. The records of precipitation from April to December measured at stations located within the catchment of the target rivers were collected from the Japan Meteorological Agency.

Collection of adult ayu fish for referential isotope ratios of spawned eggs produced in the lake

The $\delta^{13}C$ and $\delta^{15}N$ values of the spawned eggs was assumed to be equal to the $\delta^{13}C$ and $\delta^{15}N$ values

of the ovary tissue. Adult ayu fish were collected at the lower reach in 11 tributaries of Lake Biwa by cast nets in August, September, and October 2018. The collected ayu fish were frozen at –40 °C until being used for stable isotope analysis. The ovary tissue of each female individual was used for the analysis. The $\delta^{13}C$ and $\delta^{15}N$ values of the ovary tissue may reflect the diet and habitat at maturity, since ovary tissue develop rapidly at maturity. In addition, ayu fish have life history variation of migrating from lake to rivers from spring to autumn, and spawn in the lower reaches in autumn (Azuma, 1973a, b, c; Tsukamoto et al., 1987). The earlier they migrate to the rivers, the further upstream they go (Tsukamoto et al., 1987). Thus, the lower reaches in summer and autumn are dominated by individuals that have just recently migrated from the lake, and hence most of the individuals collected in this survey may have matured in the lake.

An attempt was made to establish a reference value for the identification of spawned eggs from the $\delta^{13}C$ and $\delta^{15}N$ values of the ovary tissue of the individuals collected. As some of the individuals may have matured in rivers, the relationship between the mean and variance of the $\delta^{13}C$ and $\delta^{15}N$ of the ovary tissue in each river were examined. Previous studies have shown that individuals matured in rivers have higher $\delta^{13}C$ value and lower $\delta^{15}N$ value than those maturing in lakes (Ito et al., 2015). This is due to the fact that the ayu fish feeds mainly on zooplankton in lake and on attached algae in rivers. It has also been reported that the $\delta^{13}C$ and $\delta^{15}N$ values of attached algae vary with river conditions, and the $\delta^{13}C$ and $\delta^{15}N$ values of individuals inhabiting rivers vary among rivers and within rivers (Yamada et al., 1996; Kohzu et al., 2009). On the other hand, there are seasonal variations in the isotope ratios of zooplankton in the lake, but the variability between individuals is small (Yamada et al., 1998).

Survey of ayu fish spawned eggs

Surveys of ayu fish spawned eggs were carried out in the lower reaches of the rivers, where ayu fish

spawning has been confirmed, in 11 tributaries of Lake Biwa (Figure 1). The surveys were repeated four to five times in each river from September to December 2018. In each survey, all patches where ayu fish is likely to spawn (pebbles are abundant at the bottom) were searched exhaustively on foot. When such patch was found, all pebbles in a 20 × 10 cm quadrat were collected and checked for the presence of ayu fish eggs. If eggs were found, the eggs in the quadrat were counted, and the area of the patch was roughly measured. The number of total eggs in the patch was estimated as the number of eggs in each quadrat × the area of the patch / the area of the quadrat. This quadrat method has been used for the quantitative monitoring of ayu fish eggs by Shiga Prefectural Institute of Fisheries. Pebbles with eggs on them were brought back to the laboratory and stored at –40 °C for stable isotope analysis.

Stable isotope analysis

Ovary tissue was extracted from female samples of ayu fish collected in the lower reach in 11 tributaries of Lake Biwa. The ovary tissue was dried at 60℃ for 48 h, and ground to a fine powder.

Sawada et al. (2020) revealed that, for accurate carbon and nitrogen isotope analysis, each sample needs ca. 0.5 mg of dry tissue, which corresponds to 10 ayu eggs. Thus, each sample of this study also contained 10 eggs, which might have been spawned by multiple females. Ten eggs were picked from the pebble samples, cleaned with distilled water, put in a tin capsule as a single sample and dried at 60 ℃ for 48 h. Afterward, the eggs were crushed with tweezers in the tin capsule, which was folded.

Instead of extracting lipids from the ovary and egg samples, the effect of variable lipid content on the $\delta^{13}C$ values of the ovary tissue and spawned eggs was corrected using the C/N ratio of each sample, according to the correction model ($\delta^{13}C_{lipid\text{-}free} - \delta^{13}C_{bulk} = 2.3$) specific to the eggs of this species (Sawada et al., 2019).

δ^{15}N and δ^{13}C were measured using a Delta V Advantage mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) connected to a Flash EA 1112 elemental analyser (Thermo Fisher Scientific) through a Conflo IV interface (Thermo Fisher Scientific) at Ryukoku University, Japan. Repeated samples of alanine and histidine standards were used for calibration and quality control for samples of the spawned eggs. Samples that contained an insufficient amount of nitrogen were excluded from further analyses. δ^{15}N and δ^{13}C were expressed as $\delta X = (R_{sample}/R_{standard}) - 1$, where X is ^{13}C or ^{15}N; R_{sample} corresponds to the ^{13}C /^{12}C or the ^{15}N /^{14}N ratio of the measured samples; and $R_{standard}$ is the ^{13}C /^{12}C ratio of Vienna Pee Dee Belemnite or the ^{15}N /^{14}N ratio of atmospheric nitrogen. Analytical errors in δ were less than ± 0.3‰.

Statistical analysis

As a preliminary preparation before the statistical analysis, the number of spawned eggs, river characteristics such as length, total extension, and catchment area, and quantity of water intake from the rivers were $\log_{10}$-transformed to make each distribution type closer to conformity with normality. Because some parameters on river characteristics, permitted quantity of water intake and precipitation may be correlated with each other, their correlations were examined between any combinations. The results showed significant strongly-positive correlations between total length of branches, catchment area, sinuosity and permitted quantity of water intake with respect to length, with a correlation coefficient exceeding 0.8. Thus, the total length was only retained as a representative value for river size and the other parameters were removed. It should be noted that the total length may also indicate the effect of meandering sinuosity and quantity of water intake.

The presence or absence, times and cumulative reach length of droughts were assumed to follow binomial, Poisson, and normal distributions, respectively. A generalized linear model (GLM) or a linear model (LM) with the presence or absence of river droughts as the response variable and

length, mean width of basin, river density, average gradient, relative riverbed height, and precipitation as explanatory variables was used. The link functions of the GLMs with the presence or absence and times of river droughts as objective variables are the logit function and log function, respectively.

Spawned eggs were discriminated into river-produced, and lake-produced eggs based on the isotope ratios of the spawned eggs, using the reference values calculated from ovary tissue of ayu fish collected in this study (see Results and Discussion). However, a sample that failed the analysis were not identified. Number of river-produced and lake-produced eggs was calculated for each river.

The number of all, river-produced, and lake-produced eggs were $\log_{10}$-transformed to make the distribution type to normal. They were assumed to follow normal distribution. LMs with the number of all, river-produced, and lake-produced eggs as objective variables and the length and the factor of river droughts and their interactions as explanatory variables were used. Before conducting the LM, the times and cumulative reach length of droughts were $\log_{10}$-transformed to make the distribution type to normal. In addition, to eliminate multicollinearity among the explanatory variables, the length, and the times and cumulative reach length of droughts were centralized.

R ver. 3.5.0 software (https://www.r-project.org) was used for the statistical tests. We used the glm, lm, and cor.test functions for the GLM, LM, and Pearson's correlation test, respectively. The significance level was set at $P < 0.05$.

Results

Frequency and scale of river droughts

River droughts were observed in 7 out of 11 rivers. Times and cumulative reach length of droughts were not variable for three years in each river, but significantly different among rivers (Figure 2).

The droughts were more likely observed in the rivers with higher relative riverbed height (GLM with a logit link function, $z = 2.21$, $P < 0.05$, Table 1). The droughts were more frequently observed in the rivers with larger mean width of basin (GLM with log link function, $z = 2.40$, $P < 0.05$, Table 2), larger relative riverbed height ($z = 6.62$, $P < 0.001$), and smaller total length of the mainstream ($z = -2.23$, $P < 0.05$). The cumulative reach length of droughts was longer in the rivers with larger mean width of basin (LM, $t = 3.75$, $P < 0.01$), and larger relative riverbed height ($t = 5.94$, $P < 0.001$, Table 2).

Determination of referential isotope ratios of spawned eggs produced in the lake

The ovary tissue of adult ayu fish were collected in 10 rivers, excluding the Otani River. The mean $\delta^{13}C$ and $\delta^{15}N$ values of the ovary tissue in each river was –21.3‰– –16.8‰ and 11.3‰–13.9‰ (minimum–maximum), respectively (Figure 3). The $\delta^{15}N$ value of the ovary tissue decreased significantly with the $\delta^{13}C$ of them ($r = -0.66$, $P < 0.05$). The standard deviation of the $\delta^{15}N$ of the ovary tissue increased significantly with the standard deviation of the $\delta^{13}C$ of them ($r = 0.76$, $P < 0.05$). The standard deviation of the $\delta^{15}N$ of the ovary tissue decreased significantly with the $\delta^{15}N$ of them ($r = -0.81$, $P < 0.01$). The standard deviation of the $\delta^{13}C$ of the ovary tissue increased significantly with the $\delta^{13}C$ of them ($r = 0.73$, $P < 0.05$).

The variation in the $\delta^{13}C$ value of the ovary tissue was smaller with lower the $\delta^{13}C$ value and the variation in the $\delta^{15}N$ value of the ovary tissue was smaller with higher the $\delta^{15}N$ value. Previous studies have shown that lake dwellers have lower $\delta^{13}C$ value and higher $\delta^{15}N$ value than river dwellers (Ito et al. 2015). Furthermore, the $\delta^{13}C$ and $\delta^{15}N$ values of lake dwellers are expected to show less variability than them of river dwellers. Reference values were calculated from the ovary tissue of ayu fish from the Ishida River, U River, and Taki River, where the $\delta^{13}C$ value of the ovary tissue is low, $\delta^{15}N$ value is high and there is little variation in both $\delta^{13}C$ and $\delta^{15}N$ values. Spawned

eggs with the $\delta^{13}C$ and $\delta^{15}N$ values within this criterion of ± 2SD were assumed to be lake-produced eggs, while other spawned eggs were assumed to be river-produced eggs. Using this criterion, the number of spawned eggs was estimated by distinguishing between river- and lake-produced eggs.

The effect of river droughts on the number of spawned eggs of ayu fish

The spawned eggs of ayu fish were found in all 11 rivers, and the estimated total number of spawned eggs of ayu fish was 167,996,800 eggs. When the spawned eggs were identified using the $\delta^{15}N$ value, the number of river-produced eggs, lake-produced eggs, and unidentifiable eggs due to analysis error were 32,896,200, 135,099,000, and 1,600 eggs, respectively (Table 3). Also, when identified using the $\delta^{13}C$ value, river-produced eggs, lake-produced eggs, and unidentifiable eggs were 13,486,300, 154,508,900, and 1,600 eggs, respectively (Table 4).

A comparison of the AICs of the three LMs explaining the number of all eggs showed that the LM, which included the interaction term between the presence or absence of river droughts and river length as an explanatory variable (Model 1), had the lowest AIC (Table 5). The LM (Model 1), showed the lowest AIC, showed that the number of all eggs increased with river length when the presence or absence of river droughts was zero.

A comparison of the AICs of the six LMs explaining the number of the river-produced eggs identified from $\delta^{15}N$ value showed that the LM, which included the interaction term between the times of river droughts and river length as an explanatory variable (Model 5), had the lowest AIC (Table 6). The LM (Model 5), showed the lowest AIC, showed that the number of river-produced eggs increased with river length when the times of river droughts was zero. The positive effect of what river length was shown to be mitigated by an increase in the times of river droughts.

A comparison of the AICs of the six LMs explaining the number of the lake-produced eggs identified from $\delta^{13}C$ value showed that the LM, which included the times of river droughts and river

length as an explanatory variable (Model 3), had the lowest AIC (Table 7). The LM (Model 3), showed the lowest AIC, showed that the number of lake-produced eggs increased with river length when the length of river droughts was zero.

Discussion

This study was carried out to achieve three objectives: to clarify the frequency and scale of river droughts, to identify the characteristics of rivers prone to river droughts, and to clarify the effects of river droughts on the number of spawned eggs of ayu fish. The results of this study provided information on the times, cumulative reach length and location of river droughts in 11 tributaries of Lake Biwa over a three-year period. The river droughts occurred in rivers with a higher relative riverbed height. It was suggested that river droughts reduce the number of spawned eggs of ayu fish maturing in the rivers.

Characteristics of rivers prone to river droughts

A three-year survey of river droughts has revealed the number, cumulative reach length and location of river droughts in 11 tributaries of Lake Biwa. In the rivers where river droughts occurred, river droughts occurred every year. This means that river droughts occur regardless of the slight differences in climate from year to year. The information obtained in this study is novel and is expected to provide necessary information for river management in the future.

River droughts were more likely to occur in rivers raised riverbed. The times of river droughts increased in rivers with radial basin or raised riverbed, and decreased in rivers with length. The cumulative reach length of river droughts increased in rivers with radial basin or raised riverbed. These results suggest that the raised riverbed is a factor that promotes the occurrence of river droughts. As the distance between the original riverbed height and the impermeable layer increases

by raised riverbed, the volume of surface water decreases, which may lead to the occurrence of river droughts. As water tends to flow in low places, it can be expected that the water in the river that has raised riverbed has infiltrated the embankment and flowed out to the land inside the embankment, which is lower than the riverbed.

The times and cumulative reach length of river droughts also increased in rivers with radial basin. Rivers with radial basin are characterised by high peak flows but short flood durations due to the concentration of outflow from tributaries. Conversely, rivers with feather-like basin are characterised by lower peak flows and longer flood durations. It is therefore thought that river droughts are more likely to occur from rivers with radial basin. On the other hand, the number of river droughts decreased with the river length. The shorter the river length, the shorter the time it takes for water to flow through it. Thus, smaller rivers may be more prone to frequent rapids than larger rivers. It should be noted that the length is correlated not only with the total extension and catchment area, which indicate the size of the river, but also with the degree of sinuosity and the quantity of water intake, and the effect of these factors may be reflected through the length. However, it is unlikely that the greater the sinuosity or the greater the quantity of water intake, the fewer the number of river droughts. The river length is therefore considered to be a factor in indicating the size of the river.

The effect of river droughts on the number of spawned eggs of ayu fish

River length had a significant positive effect on the number of all eggs, river-produced eggs and lake-produced eggs (except for some results). Furthermore, significant interaction between total length and the number of river droughts was observed in the number of river-produced eggs. This interaction suggests that the river droughts may reduce the number of spawning eggs that increases with river length. The number of river-produced eggs was less than 20% of the total in the spawning

survey. River droughts may be responsible for the low river-produced eggs. The origin of the ayu fish in Lake Biwa is thought to have been specialized from the ayu fish that migrates between rivers and the sea. Most ayu fish in the sea are thought to complete their migration to rivers in the spring and summer, but as shown by the number of lake-produced eggs in this study, a large number of ayu fish in Lake Biwa grow up in Lake Biwa until just before spawning. The fact that individuals maturing in the lake now constitute the majority of the population in Lake Biwa may be attributed to the fact that their ascent into the river was prevented by river droughts. On the other hand, the relationship between the number of all eggs and lake-produced eggs and the factors of river droughts was not confirmed.

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Figures & Tables

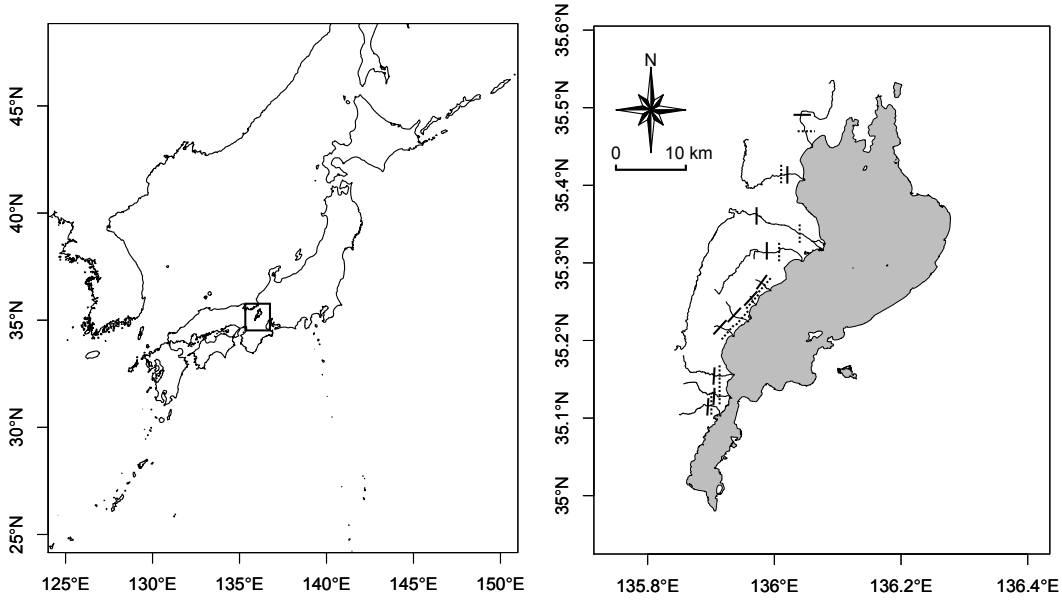

Figure 1. Locations of Lake Biwa in Japan (open square) and the target 11 tributaries of Lake Biwa (From north to south, the Chinai river, the Ishida river, the Ado river, the Kamo river, the U river, the Taki river, the Hira river, the Otani river, the Wani river, the Mano river, and the Tenjin river). The survey of river droughts was conducted in the range from the solid bar to the river-mouth and the spawning survey was conducted in the range from the dotted bar to the river-mouth.

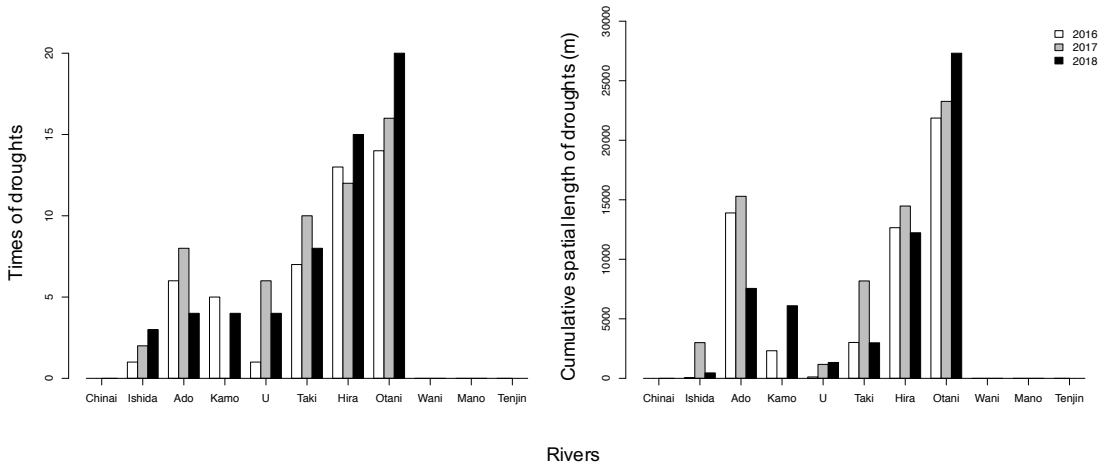

Figure 2. The times and cumulative reach length of river droughts in each year for the 11 tributaries of Lake Biwa. White, grey, and black in the bars represent the results for 2016, 2017, and 2018 years, respectively.

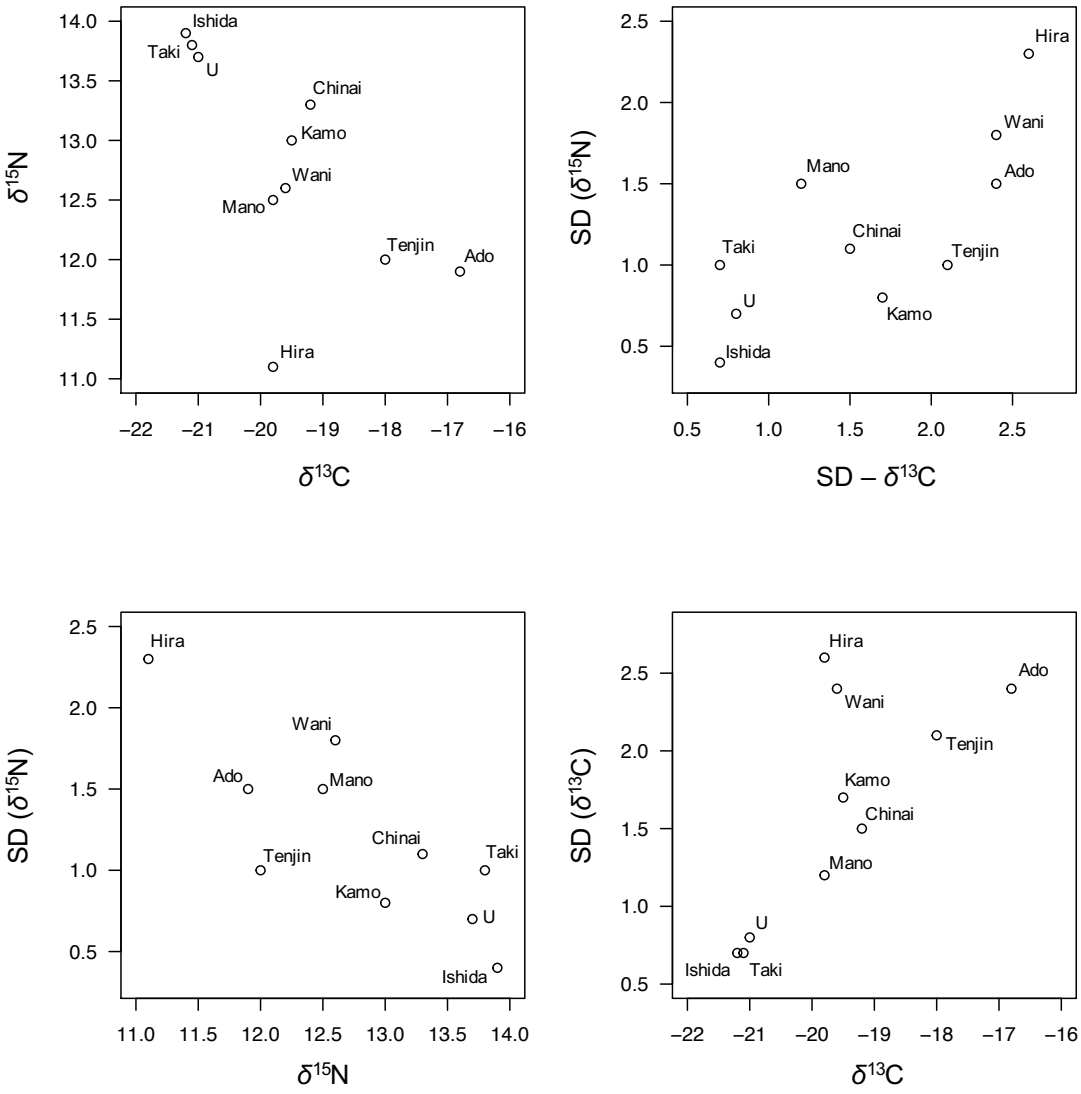

Figure 3. The relationships between δ^{15}N and δ^{13}C, and their standard deviations of the ovary tissue of ayu fish (*Plecoglossus altivelis altivelis*) in the 11 tributaries of Lake Biwa. The subscripts in the figures indicate the river names.

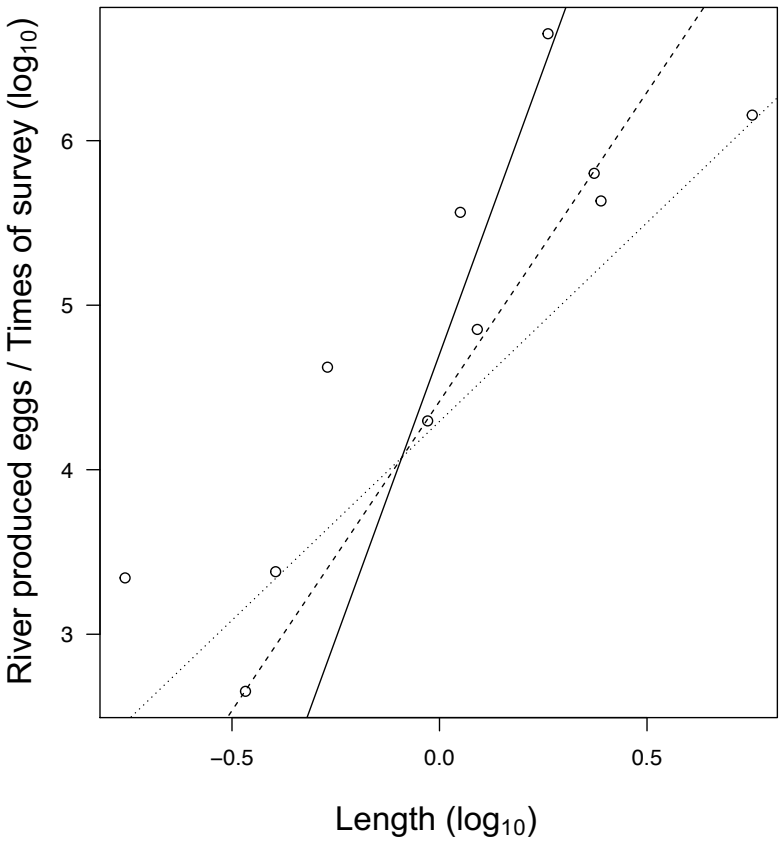

Figure 4. The relationship between the number of the river produced eggs of ayu fish (*Plecoglossus altivelis altivelis*) and the river length in 11 tributaries of Lake Biwa. Solid, dashed, and dotted lines indicate the regression lines during 0, 5, 10 times of river droughts, respectively.

Table 1. Coefficients in the generalized linear model to explain the presence or absence of river droughts

Explanatory variables	Coefficients
Intercept	1.11 ± 1.64
Total length of mainstream	–
Mean width of basin	–
River density	–
Average gradient	–
Relative riverbed height	1.71 ± 0.77*
Precipitation	–

Estimates ± standard errors are shown with significance level (*: $p < 0.05$)

Table 2. Coefficients in the linear model and generalized linear model to explain the times and cumulative reach length of river droughts, respectively

Explanatory variables	Response variables	
	Times of river droughts[1]	Cumulative reach length of river droughts
Intercept	$3.31 \times 10^{-1} \pm 8.10 \times 10^{-1}$	$2.78 \times 10^{-1} \pm 2.49 \times 10^{-1}$
Length	$-5.55 \times 10^{-1} \pm 2.49 \times 10^{-1}$*	–
Mean width of basin	$2.28 \times 10^{-4} \pm 0.95 \times 10^{-4}$*	$2.78 \times 10^{-4} \pm 0.95 \times 10^{-4}$**
River density	–	–
Average gradient	–	–
Relative riverbed height	$2.50 \times 10^{-1} \pm 0.38 \times 10^{-1}$***	$2.68 \times 10^{-1} \pm 0.45 \times 10^{-1}$***
Precipitation	–	–

[1] Poisson distribution was assumed

Estimates ± standard errors are shown with significance levels (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$)

Table 3. Number of spawned eggs of ayu fish (*Plecoglossus altivelis altivelis*) collected from 11 tributaries of Lake Biwa in 2018 year. Identification of spawned eggs was based on the standard of δ^{15}N value in ovary tissue.

（The number of eggs: k）

River	1st			2nd			3rd				4th			5th		
	11st Sep - 19th Sep			26th Sep - 4th Oct			10th Oct - 25th Oct				31st Oct - 14th Nov			21st Nov - 5th Dec		
	River[1]	Lake[2]	Total	River[1]	Lake[2]	Total	River[1]	Lake[2]	Unknown*	Total	River[1]	Lake[2]	Total	River[1]	Lake[2]	Total
Chinai	16000.0	63121.6	79121.6	-	-	-	1809.2	0	-	1809.2	32.0	0	32.0	24.0	0	24.0
Ishida	-	-	-	0	39585.2	39585.2	1620.4	0	-	1620.4	100.0	0	100.0	0	0	0
Ado	4.8	9637.0	9641.8	0	9000.0	9000.0	295.0	0	-	295.0	6610.0	0	6610.0	240.0	0	240.0
Kamo	2400.0	592.0	2992.0	-	-	-	119.5	0	-	119.5	12.8	0	12.8	0	0	0
U	0	119.0	119.0	-	-	-	0	0	1.6	1.6	0	0	0	1.8	0	1.8
Taki	0	1518.2	1518.2	11.0	0	11.0	0	0	-	0	0	0	0	0	0	0
Hira	0	0	0	210.0	0	210.0	0	0	-	0	0	0	0	0	0	0
Otani	0	0	0	12.0	0	12.0	0	0	-	0	0	0	0	0	0	0
Wani	0	2520.0	2520.0	304.0	0	304.0	30.0	0	-	30.0	22.2	0	22.2	0	0	0
Mano	0	0	0	1800.0	0	1800.0	34.0	0	-	34.0	0	0	0	0	0	0
Tenjin	0	0	0	81.0	0	81.0	18.0	0	-	18.0	0	0	0	0	0	0

[1]River-produced eggs, [2]Lake-produced eggs *... Due to an analytical error

Table 4. Number of spawned eggs of ayu fish (*Plecoglossus altivelis altivelis*) collected from 11 tributaries of Lake Biwa in 2018 year. Identification of spawned eggs was based on the standard of $\delta^{13}C$ value in ovary tissue.

（The number of eggs: k）

River	1st			2nd			3rd				4th			5th		
	11st Sep - 19th Sep			26th Sep - 4th Oct			10th Oct - 25th Oct				31st Oct - 14th Nov			21st Nov - 5th Dec		
	River[1]	Lake[2]	Total	River[1]	Lake[2]	Total	River[1]	Lake[2]	Unknown*	Total	River[1]	Lake[2]	Total	River[1]	Lake[2]	Total
Chinai	0	79121.6	79121.6	-	-	-	1654.8	154.4	-	1809.2	32.0	0	32.0	0	24.0	24.0
Ishida	-	-	-	0	39585.2	39585.2	1620.4	0	-	1620.4	100.0	0	100.0	0	0	0
Ado	0	9641.8	9641.8	0	9000.0	9000.0	295.0	0	-	295.0	6610.0	0	6610.0	240.0	0	240.0
Kamo	2400.0	592.0	2992.0	-	-	-	119.5	0	-	119.5	12.8	0	12.8	0	0	0
U	0	119.0	119.0	-	-	-	0	0	1.6	1.6	0	0	0	1.8	0	1.8
Taki	0	1518.2	1518.2	11.0	0	11.0	0	0	-	0	0	0	0	0	0	0
Hira	0	0	0	210.0	0	210.0	0	0	-	0	0	0	0	0	0	0
Otani	0	0	0	12.0	0	12.0	0	0	-	0	0	0	0	0	0	0
Wani	0	2520.0	2520.0	0	304.0	304.0	18.0	12.0	-	30.0	0	22.2	22.2	0	0	0
Mano	0	0	0	120.0	1680.0	1800.0	10.0	24.0	-	34.0	0	0	0	0	0	0
Tenjin	0	0	0	1.0	80.0	81.0	18.0	0	-	18.0	0	0	0	0	0	0

[1]River-produced eggs, [2]Lake-produced eggs *... Due to an analytical error

Table 5. Coefficients in linear models Models 1–3 to explain the number of all eggs of ayu fish (*Plecoglossus altivelis altivelis*).

Explanatory variables	Response variables		
	Model 1	Model 2	Model 3
Intercept	4.80***	5.50***	5.50***
Total length of mainstream	9.93*	1.91*	1.91*
Presence or absence of river droughts	0.66	–	–
Times of river droughts	–	–	–
Cumulative reach length of river droughts	–	–	–
Interaction effect of total length of mainstream and another factor	–8.23	–	–
AIC	32.214	33.500	33.500

Positive and negative coefficient values for interaction effects indicate increased and decreased positive effects of length factors. Estimates ± standard errors are shown with significance levels (*: $p < 0.05$, ***: $p < 0.001$)

Table 6. Coefficients in linear models (Models 1–6) to explain the number of river-produced eggs of ayu fish (*Plecoglossus altivelis altivelis*).

Explanatory variables	Response variables					
	$\delta^{13}C$			$\delta^{15}N$		
	Model 1	Model 2	Model 3	Model 4	Model 5	Model 6
Intercept	3.66***	4.17***	4.32***	4.64***	4.42***	4.68***
Total length of mainstream	6.83*	3.52*	3.33**	7.53*	3.76***	3.54**
Presence or absence of river droughts	0.98			0.00	–	–
Times of droughts	–	0.48		–	–0.39	–
Cumulative reach length of river droughts	–		0.28	–	–	–0.06
Interaction effect of total length of mainstream and another factor	–4.50	–2.87	–1.01	–5.20	–4.32*	–1.24
AIC	24.509	24.260	25.26	23.548	22.333	24.968

Positive and negative coefficient values for interaction effects indicate increased and decreased positive effects of length factors. Estimates ± standard errors are shown with significance levels (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$)

Table 7. Coefficients in linear models (Models 1–6) to explain the number of lake-produced eggs of ayu fish (*Plecoglossus altivelis altivelis*).

Explanatory variables	Response variables					
	$\delta^{13}C$			$\delta^{15}N$		
	Model 1	Model 2	Model 3	Model 4	Model 5	Model 6
Intercept	4.74**	5.10***	5.10***	2.68*	4.13**	4.49***
Total length of mainstream	10.17	1.73	1.99	18.10*	3.92	3.27**
Presence or absence of river droughts	0.11	–	–	2.18	–	–
Times of river droughts	–	–1.02	–	–	–0.02	–
Cumulative reach length of river droughts	–	–	–0.44	–	–	0.11
Interaction effect of total length of mainstream and another factor	–8.23	–	–	–16.16	–5.33	–1.22
AIC	44.11	42.43	42.35	46.48	51.47	52.07

Positive and negative coefficient values for interaction effects indicate increased and decreased positive effects of length factors. Estimates ± standard errors are shown with significance levels (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$)

Chapter 6

Final conclusion

The overall objectives of this thesis were to collect basic knowledge necessary for the field application of stable isotope analysis to land-locked ayu (*Plecoglossus altivelis*) in Lake Biwa, and to further understand reproductive ecology of ayu fish by applying stable isotope analysis. In order to achieve these objectives, trophic discrimination factors, lipid correction equations and turnover rates of multiple tissues of ayu fish were determined by laboratory aquatic experiments in order to apply stable isotope analysis more appropriately in the field. also, isotope analysis was used to clarify the spawning characteristics of ayu fish in different migration patterns. In addition, the effect of river droughts on ayu fish was clarified. These results of this study and future work are summarised below.

In Chapter 2, TDFs and lipid correction equations for multiple tissues of ayu fish were determined in order to use stable isotope analysis appropriately in the field for ayu fish. The results showed that the TDF was 0.8‰ to 5.6‰ higher in muscle tissue after lipid-eliminated than in untreated muscle tissue, and depended on the C/N ratio of the untreated muscle tissue. On the other hand, the variance in lipid content was smaller in ovaries and the effect of lipids was constant. Following the model reported in previous studies, regression analysis with the C/N ratio of the untreated samples yielded a lipid correction equation specific to ayu fish muscles. The TDF for δ^{13}C value, with the effect of lipid removed, differed between muscle (2.4‰), mucus (1.0‰) and ovary tissues (1.9‰). The TDFs for δ^{15}N value differed between muscle (2.5‰), mucus (1.5‰) and ovary tissues (1.6‰). The TDFs and lipid correction equations presented in this chapter allow accurate estimates of the feeding habits and migratory ecology of ayu fish.

In Chapter 3, the isotopic changes of multiple tissues of ayu fish during the growing and

reproductive stages was presented. The results showed that isotopic changes in the muscle and mucus tissues were faster in the growing stage than in the reproductive stage. Isotopic change in the muscle tissue during the reproductive stage was caused mainly by whole-body growth, whereas allometric growth and/or catabolic turnover rather than whole-body growth accounted more for the isotopic changes in mucus tissue during both stages. Isotopic change was slower in muscle, mucus, and ovary tissues, in that order, probably according to the allometric growth and/or catabolic turnover of each tissue. My investigation showed that the timing of drastic changes differed between the δ^{13}C and δ^{13}C values, as the first report of the isotopic change in the fish ovary tissue, which suggests that the δ^{13}C and δ^{15}N values in the ovary tissue may reflect diets and/or habitats of different time scales. These results allowed for accurate applications of multiple-tissue isotope analysis to the target fish over all stages. Such approaches to any fish species are more accurate when considering the variability of isotopic changes between and within stages.

In Chapter 4, the proportional change and site-selection variation between river- and lake-produced eggs of ayu fish in the Lake Biwa water system were examined by distinguishing spawned eggs using stable isotope analysis. The results showed that the δ^{15}N values of spawned eggs decreased with time during the 3-month reproduction season. This result implies that there was a shift from lake-produced eggs to river-produced eggs within a reproductive season, based on the observation that adult fish in the lake had previously been shown to have eggs with distinctly higher δ^{15}N values in their ovaries than those in the tributaries. This explanation was also supported by the change in δ^{13}C values of the spawned eggs. Furthermore, eggs with lower δ^{15}N and higher δ^{13}C values tended to be spawned at less variable depths, suggesting that females spawning river-produced eggs selected the spawning sites from a narrower range. I conclude that stable isotope ratios of spawned eggs can be indicators of the relative contributions of different food chains, and can enable comparisons of reproductive characteristics between types of egg.

In Chapter 5, stable isotope analysis was used to identify spawning eggs and to clarify the effect of river droughts on the number of river- and lake-produced eggs of ayu fish in Lake Biwa. The results showed that river droughts were observed in 7 out of 11 rivers, and the times and cumulative reach length of droughts were differed among rivers. River length had a significant positive effect on the number of all eggs, river-produced eggs and lake-produced eggs (except for some results). Furthermore, an interaction between total length and the number of river droughts was shown on the number of river-produced eggs. This interaction suggests that the river droughts may reduce the number of spawning eggs that increases with river length. On the other hand, the relationship between the number of all eggs and lake-produced eggs and the factors of river droughts was not confirmed. If the times of the river droughts increases in the future, this could have an impact on the population size of ayu fish, with repercussions for catches, recreation and food web structure.

These results of the above studies provide an advancement in the basic knowledge for future application of isotope analysis in ayu and deepen our knowledge of the ecological study of ayu fish. The basic knowledge revealed in this study, such as the trophic discrimination factors, lipid correction equations, and turnover rates for multiple tissues of ayu fish, is important for the application of stable isotope analysis in the field. These findings contribute to the reliability of the results of stable isotope analysis of ayu fish. In particular, the difference in the turnover rate at different growth stages may have provided useful information not only for ayu fish but also for other species. For example, if the growth stage could be taken into account, the residence time of immigrants could be estimated more appropriately when using the isotope clock.

To the best of our knowledge, this study is among the first to successfully discriminate eggs spawned by maternal fish that had different individual life histories (Kristensen et al. 2011; Ito et al., 2015). This approach is applicable to other fish with variable life histories that depend on a

different source of primary production of nitrogen or carbon, such as migration patterns and trophic polymorphisms. For example, different $\delta^{15}N$ and $\delta^{13}C$ values of eggs in ovaries are reported between life history dimorphisms of brown trout (Salmo trutta), each morph that reproduce sympatrically but grow allopatrically in different places (McCarthy & Waldron, 2000). Stable isotope ratios of spawned eggs are expected to provide essential information on parent fish, which could not be traced even by molecular techniques, to reveal the roles of intraspecific variations in the lifecycle of the fish. This study will contribute to the modifying of stable isotope analysis and expand the application of stable isotope analysis for ecological study of ayu fish. Using the identification of spawned eggs by stable isotope analysis, the effects of river droughts on ayu fish were examined. This success suggests that the method of identifying spawners from isotope ratios may be adapted to a variety of fish species in the future, not just ayu. This study contributes to the modifying of stable isotope analysis and expand the application of stable isotope analysis for ecological study of ayu fish.

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1. **Sawada, H.**, Yonekura, R., & Maruyama, A. (2018). Examination of trophic discrimination factors and lipid corrections in muscle, ovary and mucus tissues of ayu (*Plecoglossus altivelis altivelis*) using carbon and nitrogen stable isotope analyses. *Japanese Journal of Ichthyology*, 65, 1–7. doi:10.11369/jji.17-017. [in Japanese]
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3. **Sawada, H.**, Shigeta, K., Kawakami, M., Yuma, M., & Maruyama, A. (2020). Isotope analysis reveals proportional change and site-selection variation of river- and lake-produced eggs of a landlocked migratory fish. *Journal of Fish Biology*, 96, 168–174. doi:10.1111/jfb.14200
4. Tsuji, S., Shibata, N., **Sawada, H.**, & Ushio, M. (2020). Quantitative evaluation of intraspecific genetic diversity in a natural fish population using environmental DNA analysis. *Molecular Ecology Resources*, 20, 1323–1332. doi:10.1111/1755-0998.13200
5. **Sawada, H.**, Fujiwara, S., Tanaka, R., Yonekura, R., & Maruyama, A. (Accepted). Turnover rates for muscle, mucus, and ovary tissues of ayu fish (*Plecoglossus altivelis altivelis*) in multiple stages determined through carbon and nitrogen stable isotope analyses. *Ecology of Freshwater Fish*

List of Conference

International Conference

・Poster presentations

1. Sawada, H., Fujiwara, S., Shigeta, K., Kawakami, M., Yuma, M., & Maruyama, A. (2019). Spawning characteristics of life-history polymorphic landlocked Ayu fish in Lake Biwa as revealed by stable isotope analysis. East Asian Federation of Ecological Societies, Nagoya, Japan
2. Sawada, H., Tsuji, S., Shibata, N., Watanabe, K., Hiraishi, Y., Okayama, S., Yamanaka, H., Imamura, A., & Maruyama, A. (2019). Diurnal change in fish environmental DNA concentration in a river, British Ecological Society Annual Meeting, Belfast, Northern Ireland

Domestic Conference

・Poster presentations

1. **沢田隼**，重田環志，川上将樹，遊磨正秀，丸山敦，「安定同位体比によるアユの産着卵の生活史型と産卵回数の推定」，第 64 回日本生態学会，東京，2017 年 3 月

2. 冨田勢，神松幸弘，山中裕樹，永野昌大，佐藤拓哉，高原輝彦，**沢田隼**，勝原光希，源利文，「ユニバーサルプライマーを用いたサンショウウオ属（*Hynobius*）の環境 DNA 検出」，第 64 回日本生態学会，東京，2017 年 3 月
3. **沢田隼**，藤原壮平，遊磨正秀，丸山敦，「琵琶湖水系に陸封されたアユの安定同位体比からわかること～異なる時間スケールの食性を示す複数組織を組み合わせて～」，第 65 回日本生態学会，北海道，2018 年 3 月
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・Oral presentations

9. **沢田隼**，遊磨正秀，丸山敦，「安定同位体比が示した琵琶湖水系に陸封されたアユの生活史変異とその産卵特性」，第 49 回日本魚類学会，岐阜，2016 年 9 月
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11. **沢田隼**，藤原壮平，遊磨正秀，丸山敦，「安定同位体比で判明した琵琶湖に生息するアユにおける各生活史型の産卵特性」，第 65 回魚類自然史研究会，滋賀，2017 年 11 月