

Importance of experienced thermal history: effect of acclimation temperatures on the high-temperature tolerance and growth performance of juvenile marbled flounder

Short title: High-temperature tolerance and growth of a flatfish

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ABSTRACT

Experienced thermal history often affects the temperature tolerance of fish; however, the effect of thermal history on growth performance is unclear. To contribute to effective stocking (release of hatchery-reared juveniles in the field), we conducted four laboratory experiments using juvenile marbled flounder (*Pseudopleuronectes yokohamae*, around 30 mm standard length and 0.3 g body wet weight) acclimated at 12 °C and 24 °C for approximately 2 weeks to investigate the effects of acclimation temperature on high-temperature tolerance, food consumption, and growth performance. The acclimation to 24 °C increased tolerance to high temperatures, as shown in a 24-h exposure experiment and in a temperature elevation experiment. The 50% lethal temperature (upper incipient lethal temperature) was estimated to be 25.9 °C and 29.0 °C for the 12 °C and 24 °C acclimation groups, respectively. In subsequent experiments, we tested the effects of high and low temperature acclimation on the food consumption and growth performance of two size groups of juveniles (28.7 ± 2.0 and 34.5 ± 2.9 mm, mean \pm SD), that were reared at temperatures ranging from 14 °C to 23 °C. The optimal temperature for growth was 20 °C and did not differ between the acclimation temperatures or between the size groups. However, food consumption and growth performance were suppressed by acute temperature changes. Specifically, feeding and growth were lower in the 24 °C-acclimated group than in the 12 °C-acclimated group when exposed to 14 °C, which is close to the natural water temperature at release in the field. These results suggest that experienced thermal history does not affect the optimal temperature but can affect the growth performance of juveniles. To maximize the post-release growth of hatchery-reared juveniles, the influence of thermal history should be taken into consideration and acute thermal changes before release should be avoided.

Keywords: flatfish; acclimation effects; thermal tolerance; food consumption; growth

1. Introduction

Water temperature is one of the most important factors that regulate distribution, feeding, and growth of aquatic ectotherms. The rise in sea temperature associated with global warming has affected the geographic distribution and population structure of fish (Roessing et al., 2004; Kuwahara et al., 2006; Shultz et al., 2016). Furthermore, temperature rise can cause an increase in mortality or a change in growth performance of fish in nature or in captivity. Such species-specific thermal responses are essential information to understand recruitment dynamics or to improve efficiency in aquaculture.

It is well known that thermal responses such as temperature tolerance can be affected by the previous history of experienced temperature (Reynolds and Casterlin, 1979; Jobling, 1981). Individuals that have experienced high temperatures show higher lethal temperatures than those that have experienced low temperatures (Fry et al., 1946; Becker and Genoway, 1979; Tsuchida and Setoguma, 1997). In contrast, the temperature that is finally preferred, defined as the final preferendum, is constant regardless of acclimation temperatures (Fry, 1947; Jobling, 1981; Díaz et al., 2007). Fish growth can be maximized under species-specific optimal temperatures (Fonds et al., 1992; Iwata et al., 1994; Burel et al., 1996; Björnsson et al., 2001; Handeland et al., 2008), and the final preferendum has been recognized to correspond with the optimal temperature for growth (Kellogg and Gift, 1983; Khan et al., 2014). However, this finding is not necessarily applicable to all species (Schram et al., 2013; Mortensen et al., 2020). Furthermore, to our knowledge, effects of previously experienced temperatures on growth performance have scarcely been studied. Understanding the influences of thermal history on growth performance is important for stock enhancement or restocking programs. Juveniles are often cultured under warmed conditions for efficient growth, but they are released in the field with natural low temperature conditions after reaching sufficient size for release. If growth of released juveniles is suppressed by the low temperature at the release site, post-release mortality may become greater and result in the decreased effectiveness of stocking.

The purpose of the present study was to clarify the effects of acclimation temperature on the high-temperature tolerance, feeding, and growth of juvenile fish. The temperature tolerance has been evaluated using static and dynamic methods in laboratory experiments (Cox, 1974; Becker and Genoway, 1979; Reynolds and Casterlin, 1979; Bennett and Judd, 1997; Tsuchida and Setoguma, 1997), and the former method was used for estimating the upper incipient lethal temperature (UILT), whereas the latter method was used to determine

the critical thermal maximum (CTMax). The UILT is the high temperature at which 50% of population can survive indefinitely (Fry et al., 1946; Brett, 1956; Jobling, 1981; Kir et al., 2017). The CTMax is the temperature with loss of equilibrium and is determined by raising the temperature at a constant rate (Cowles and Bogert, 1944; Jobling, 1981).

We used hatchery-reared juveniles of marbled flounder (*Pseudopleuronectes yokohamae*) as a model organism. This species is a commercially important pleuronectid flatfish that inhabits the coastal area of eastern Asia. The size of adult individuals usually ranges from 180 to 460 mm standard length (SL). Hatchery-reared juveniles have been released in Japanese waters at sizes ≥ 20 mm SL (Howell and Yamashita, 2005). This species spawns mainly in December–January (Tomiya et al., 2021), and juveniles appear in the shallow area in March–May (Joh et al., 2009; Hata et al., 2016). It has been reported that hatchery-reared juveniles of 21 mm SL achieve maximum growth at ambient temperatures of 12–14 °C, whereas those of 31–47 mm SL show maximum growth at 20 °C (Kusakabe et al., 2017; Tomiyama et al., 2018). There is a gap between the optimal temperature and the ambient temperature in the wild: in Hiroshima Bay, western Japan, hatchery-reared juveniles of 30 mm SL have been released in March or April when water temperature is approximately 13 °C. If juveniles were reared at approximately 20 °C before release for efficient growth of juveniles, the temperature difference between before and after the release may inhibit post-release growth of juveniles. Thus, the effects of previously experienced temperatures on the growth at given temperatures should be elucidated. Another motivation for testing the effects of acclimation temperature was the phenomenon observed in Tomiyama et al. (2018); in their experiment conducted in April, hatchery-reared juveniles of 21 mm SL that had been reared under 13 °C showed maximum growth at 12–14 °C, low food consumption at 20 °C, and no survival at 24 °C. In contrast, in the experiment conducted in June, hatchery-reared juveniles of 20 mm SL that had experienced 15–20 °C temperatures showed maximum food intake at 24 °C and survived at 24 °C and 26 °C. These results suggested that the high-temperature tolerance, feeding, and growth of juvenile marbled flounder change greatly not by body sizes but according to previously experienced temperatures.

In the present study, we tested three hypotheses: (1) high-temperature tolerance is greater in juveniles acclimated to higher temperatures; (2) optimal temperature for feeding and growth is affected by acclimation temperatures; (3) growth performance is suppressed by acute temperature changes. Specifically, we discuss the temperature condition during the culture of juveniles for maximizing post-release growth performance of juveniles, leading to successful stock enhancement programs.

2. Materials and methods

2.1. Animal husbandry and experimental setup

Laboratory experiments for high-temperature tolerance and growth of juveniles were conducted in 2017 and 2018, respectively. We used approximately 200 individuals of hatchery-reared juveniles of marbled flounder each year ($n = 400$ in total). These juveniles were produced by the Hiroshima City Fisheries Promotion Center. For juvenile production, wild fish caught in Hiroshima Bay (34.3°N , 132.4°E) were brought to the laboratory, and ovulation was induced in females through the use of human chorionic gonadotropin. After 24–48 h, eggs were obtained by strip-spawning and fertilized artificially in the laboratory. Marbled flounder spawn adhesive demersal eggs, therefore, the eggs can be attached to the wall or bottom of the tanks. Fertilized eggs were accommodated in five 1000-L tanks (≤ 1.2 million eggs per tank) in a flow-through system (5000 L of UV sterilized seawater per day). Larvae hatched at approximately 3 mm on day 6–8 after fertilization at $11\text{--}13^{\circ}\text{C}$. They were transferred to two 50-kL tanks and fed rotifers (*Branchionus plicatilis* sp. complex) twice per day at a density of 5 rotifers mL^{-1} until 20 d old. The fish were also fed *Artemia* spp. nauplii twice per day at a density of 100–2000 individuals per fish from 15 to 80 d old and commercial pellets ad libitum from 70 d old. The fish were reared under the flow-through system under temperatures of $11\text{--}15^{\circ}\text{C}$. Juveniles were transferred to the laboratory of the Hiroshima University and then accommodated in a 500-L tank filled with running seawater on April 11, 2017 (90 d old) and April 10, 2018 (81 d old). The density of juveniles (200 individuals in a 500-L tank, i.e., 250 individuals m^{-2}) was lower than the stressful density of 884 individuals m^{-2} (Sugimoto et al., 2007).

Prior to each experiment, juveniles were divided randomly into two groups (100 individuals per group in each year) and acclimated to temperatures of 12°C or 24°C . These temperatures were considered as the two furthestmost extremes for rearing juveniles (Kusakabe et al., 2017; Tomiyama et al., 2018). The juveniles at 12°C and 24°C were designated as the low-temperature acclimation (LA) group and high-temperature acclimation (HA) group, respectively.

In 2017, we conducted the experiments on high-temperature tolerance (UILT and CTMax). Juveniles were distributed to two 100-L tanks in which the water temperature had already been adjusted to 12°C and 20°C , respectively, using heaters (SH320, Gex Co. Ltd.,

Japan) and chillers (ZC-200, Zensui Co. Ltd., Japan) with thermostats (NX005, Gex Co. Ltd., Japan). Then, the temperature of the tank at 20 °C was gradually raised to 24 °C over 24 h to reduce the shock due to the rapid change in temperature (Fig. 1). The tanks were filled with filtered seawater (salinity of 32) and were aerated. The juvenile marbled flounder were fed commercial pellets (Otohime S1, Nisshin Marubeni Feed Co. Ltd., Japan) twice a day via automatic feeders during 13 d of acclimation. These juveniles (107 d old) were used for experiments of high-temperature tolerance from April 28, 2017. Fish were not fed during the experiments. Actual water temperatures at 12 °C and 24 °C were 12.1 ± 0.12 °C and 23.8 ± 0.51 °C (mean \pm SD), respectively.

In 2018, we conducted experiments in April and June. For the experiment in April, 100 juveniles were divided into two groups (50 individuals each) and distributed to two 100-L tanks of 12 °C and 24 °C on April 10 in 2018. Juveniles (81 d old) were acclimated to each temperature with feeding commercial pellets twice a day for 12 d before the experiment. The remaining 100 individuals for the experiment in June were kept in a 500-L tank filled with running seawater under natural water temperature, and commercial pellets (Otohime S1) were fed to fish ad libitum. After 36 d on May 16, these juveniles (117 d old) were transferred to two 100-L tanks of 18 °C (50 individuals each), and they were kept with being fed under 18 °C for 8 d. Thereafter, the temperatures of the tanks were adjusted to 12 °C and 24 °C within 3 h and feeding continued for 8 d to acclimate the juveniles before the experiment in June (Fig. 1).

All experimental protocols and animal care followed the guidelines of the Hiroshima University Animal Research Committee (number CD001825).

2.2. Estimation of UILT

To estimate UILT, we conducted a 24-h survival experiment (hereinafter referred to as the UILT-test) under four temperatures of 22 °C, 24 °C, 26 °C, and 28 °C. Ten juveniles from each of the LA (mean \pm SD: 27.8 ± 3.2 mm SL, $n = 40$) and the HA (29.4 ± 3.8 mm SL, $n = 40$) groups were directly transferred to each 100-L tank that was filled with seawater of each adjusted temperature. The juveniles were placed in a transparent plastic cage (27-cm long, 14-cm wide, and 6-cm high) with many 6-mm-diameter holes on the side of the cage for allowing sufficient seawater exchange between the cage's interior and exterior (ESM Fig. S1). A 6-mm mesh nylon modular net was attached to the top to prevent the juveniles from escaping. Two floating cages for two groups (one cage for each group with 10 juveniles) were

placed in each 100-L tank to make the later treatment (such as removing dead individuals) easier. Confirmation of survival was conducted visually at 1, 2, 4, 12, 19, and 24 h after the start of the experiment, and the dead juveniles were removed from the cage at each observation to maintain water quality.

We first carried out the 24-h experiment using both HA and LA groups under 22 °C and 24 °C on April 28 (acclimated for 13 d) and then under 26 °C and 28 °C on April 29 (acclimated for 14 d). Because all juveniles survived at 22 °C and 24 °C, we additionally placed the surviving LA juveniles into the 26 °C and 28 °C tanks, respectively, to test whether an exposure of only 24 h to relatively high temperatures affects the thermal tolerance of juveniles. These individuals were designated as the "conditioned LA" group (Fig. S2). Juveniles directly transferred from 12 °C were assigned as the "naive LA" group. Juveniles that survived at 28 °C (observed only in the HA group) were subsequently maintained in the same tank with water temperature raised to 30 °C for the 24-h observation of survival on April 30 (Fig. S2). The water temperature in the 22 °C, 24 °C, 26 °C, 28 °C, and 30 °C tanks was 21.8 ± 0.4 °C, 24.2 ± 0.3 °C, 25.8 ± 0.2 °C, 27.8 ± 0.4 °C, and 30.3 ± 0.3 °C, respectively, during the experiment. The total number of fish was 80 (10 individuals \times 2 acclimation groups \times 4 temperatures), but a proportion of these fish was used again as the conditioned individuals (10 individuals \times 3 groups, Fig. S2).

2.3. Estimation of CTMax

To estimate CTMax, we conducted a temperature-elevating experiment (hereinafter referred to as CTMax-test) where the water temperature was raised from 21 °C at 1 °C every 2 h (0.5 °C h^{-1}) in a 100-L tank filled with aerated seawater. This rate could be experienced by wild fish in the field (pers. obs.). In the experiment, 10 juveniles each from the LA group (27.3 ± 3.0 mm SL) and HA group (29.7 ± 3.6 mm SL) were placed in cages (27-cm long, 16-cm wide, and 11-cm high) and were directly transferred from each acclimation temperatures to 21 °C on April 28. Survival and opercular movements were observed at every 2 h. The CTMax was defined as the temperature with cessation of opercular movement, because it is difficult to identify the loss of equilibrium for flatfish: juveniles increase their swimming frequency with increasing opercular movement under rising temperatures; however, they mostly stay lying down at the bottom without flipping until they cease opercular movement. After cessation of opercular movement, they died immediately. Dead individuals were removed at each observation and their SLs were measured. The water temperature was

recorded at every 2 h and was raised until all the juveniles had died. The total number of fish was 20 (10 individuals \times 2 acclimation groups).

2.4. Experiments on feeding and growth

As mentioned above, we conducted feeding and growth experiments in April and June 2018 (hereinafter referred to as F&G expts). In these experiments, six individuals were directly transferred to three floating cages (same sizes as those of the CTMax-test) within each 100-L tank, following Kusakabe et al. (2017). Each cage was divided into two sections (an area of 13.5 cm \times 16 cm per section), and one individual from the LA group was put into one section, whereas one individual from the HA group was put into the other section (Fig. S3). Commercial pellets (Otohime S1 or S2) were fed manually to each individual fish until satiation thrice per day (08:00, 13:00, and 18:00 h). Remaining pellets at 1 h after feeding were collected each time, and the number of ingested pellets was recorded for calculation of food consumption (S1: 0.62 mg per particle; S2: 1.01 mg per particle; Tomiyama et al., 2018).

We used eight 100-L tanks at four temperatures (14 °C, 17 °C, 20 °C, and 23 °C) with two replicates (tanks), and a total of 24 individuals per acclimation group was used for each experiment. Experimental temperatures were selected from 14 °C to 23 °C because juveniles around 31 mm exhibited high growth and food consumption at temperatures from 14 to 22 °C (Tomiyama et al., 2018). Temperatures were adjusted by heaters (SH320) and chillers (ZC-200) with thermostats (NX005). In the F&G-expt in April, initial sizes of the LA and HA groups were 29.3 ± 1.8 mm SL and 28.1 ± 1.9 mm SL, respectively. The water temperature for the four temperatures during the experiment was 14.2 ± 0.2 °C, 17.1 ± 0.4 °C, 20.1 ± 0.5 °C, and 22.8 ± 0.3 °C. In the F&G-expt in June, initial sizes of the LA and HA groups were 34.0 ± 2.3 mm SL and 35.0 ± 3.4 mm SL, respectively. The water temperature for the four temperatures was 14.2 ± 0.3 °C, 17.2 ± 0.1 °C, 20.5 ± 0.6 °C, and 22.9 ± 0.3 °C. All individuals were maintained at each temperature without feeding for 24 h, and fed for 7 d from April 23 (94 d old) or June 2 (134 d old), 2018.

2.5. Measurements and analyses

To compare the survival between acclimation groups, Fisher's exact test was used for UILT- and CTMax-tests. To obtain UILT, we constructed a generalized linear model (GLM) with a binomial family and logit-link function for the mortality probability of each individual

in the experiment. The initial explanatory variables were the water temperature, acclimation temperature groups, and interactions. Water temperature with the associated mortality probability of 0.5 was assigned as the UILT. The sample sizes were 40 for the LA group (10 individuals \times four temperatures) and 50 for the HA group (10 individuals \times five temperatures including 30 °C). To determine CTMax, we constructed a linear model for the temperature at which each individual ceased opercular movement in the CTMax-test. Initial explanatory variables were acclimation temperature and individual SL. Because death or survival was confirmed every 2 h, the average temperature during the 2 h in which the individual fish died was used. Model selection was performed based on the Akaike information criterion for small sample size (AICc).

Before and after the F&G-expts, all juveniles were anesthetized using 0.1% FA-100 (a pharmaceutical preparation of 10% eugenol; Tanabe Pharmaceutical Co., Japan), and the SL and body wet weight (BW) were measured using a digital caliper (CD67-S20PS, Mitsutoyo Corp., Japan) and an electronic balance (TX4202N, Shimadzu Corp., Japan), respectively. We excluded individuals that died during the experiments (see results) from the analyses. Most of these individuals did not show feeding and seemed to die because of the handling artifacts at measurements.

To test whether daily food intake changes were associated with time and acclimation groups in the F&G-expts, two-factor (day and acclimation group) repeated measures analysis of variance (ANOVA) was performed for each temperature in each experiment.

The growth performance and food consumption were evaluated by specific growth rate (SGR), observed growth rate (OGR, mm d⁻¹), and daily food intake (DFI, mg d⁻¹ fish⁻¹), following Tomiyama et al. (2018):

$$\text{SGR} = (\ln(\text{BW}_t) - \ln(\text{BW}_0)) / t \times 100$$

$$\text{OGR} = (\text{SL}_t - \text{SL}_0) / t$$

$$\text{DFI} = \text{total food consumption} / t,$$

where SL_t, SL₀, BW_t, and BW₀ represent SL or BW at the end and start of the experiment, respectively, and t is the number of feeding days during the experiment. SGR has been used to assess growth rates of fish in captivity (Lugert et al., 2016), although OGR has frequently been used especially in field studies.

To test the relevance of acclimation temperature on feeding and growth, we constructed linear mixed models (LMM) for SGR, OGR, and DFI. Models were fit using maximum likelihood. Initial explanatory variables were the acclimation temperature, water temperature, SL₀, experiment (in April or June), and interaction of acclimation temperature and water

temperature. Because the effects of acclimation temperature seemed different between the experiments, the interaction of acclimation temperature and experiment was also added as an initial explanatory variable. This difference might be attributed to the experienced temperatures before acclimation, i.e., around 12 °C in April and 18 °C in June, or to body sizes of juveniles, although body size effect can be tested by incorporating SL_0 as an explanatory variable. Tank was incorporated as a random variable. The model was selected based on the AICc. All statistical procedures were performed using R version 3.5.1 (R Core Team, 2018) with packages lme4 (Bates et al., 2015) and MuMIn version 1.42.1 (Bartoń, 2018) for fitting LMM and AICc model selection, respectively.

3. Results

3.1. High-temperature tolerance

The survival rate of juvenile marbled flounder, exposed to constant, elevated temperatures for 24 h, differed between the acclimation groups and temperatures in the UILT-test. The UILT was estimated to be 25.86 °C and 29.04 °C in the LA and HA groups, respectively (Fig. 2). At 26 °C, all individuals of the HA group and conditioned LA group had survived, whereas three individuals (30%) of the naive LA group died at 19 h (Fig. S4). The survival rate after 24 h was not significantly different among the three groups (Fisher's exact test, $p = 0.21$). At 28 °C, all juveniles of the HA group ($n = 10$) had survived, whereas one individual (10%) of the conditioned LA group had died during the 24 h, and nine individuals (90%) of the naive LA group had died within 1 h after the accommodation and the remaining individual had died at 2 h. The survival rate was significantly different among the three groups ($p < 0.001$). At 30 °C, none of the HA individuals that had previously been exposed to 28 °C for 24 h had survived for ≥ 5 h after accommodation. None of the individuals in the two acclimation groups died during 24 h at 22 °C and 24 °C. In the GLM for the mortality probability of the individuals, water temperature and the acclimation temperature groups were adopted as explanatory variables, and the interaction between the water temperature and the acclimation temperature groups was excluded from the model (Table 1).

In the CTMax-test, the temperature at which juveniles ceased opercular movements differed between the acclimation groups. The CTMax was estimated to be 29.32 ± 0.41 °C (mean \pm SD) and 30.53 ± 0.57 °C for the LA and HA groups, respectively. One individual of the LA group died during 16–18 h at 28.0–28.3 °C, and the remaining nine individuals died

during 18–20 h at 28.3–30.6 °C (Fig. 3). Of the HA group, two individuals died during 18–20 h, and the remaining eight individuals died during 20–22 h at 30.6–31.0 °C. The number of individuals that had survived for 20 h (when the temperature had reached 30.6 °C) was significantly different between the LA and HA groups (Fisher's exact test, $p < 0.001$). In the linear model for the temperature with cessation of opercular movement, individual SL was excluded and acclimation temperature was adopted as the explanatory variable ($p < 0.001$).

3.2. Feeding and growth

Some individuals out of the 96 individuals died during the experiment (Table S2). No significant difference in survival between the acclimation groups was observed for each temperature in each experiment (Fisher's exact test, $p > 0.06$). Individuals that survived consumed approximately 10–70 mg of pellets per day (Fig. 4), whereas daily food intake of the dead individuals when they had been alive was extremely small (ranged from 0 to 6.8 mg, mean = 1.7 mg). In the F&G-expt in April, juveniles of the LA group consumed more food than those of the HA group, especially at 14 °C and 17 °C during the first 5 d; however, such a pattern was not observed in the F&G-expt in June. Daily food intake increased as the days progressed in both experiments (two-way repeated measures ANOVA, April: $p < 0.001$ for all temperatures; June: $p < 0.01$ for 14 °C and $p < 0.001$ for other temperatures). A significant difference in the food intake on each day between the acclimation groups was observed only for tanks at 14 °C in April (greater in LA, $F_{1,9} = 19.97$, $p < 0.01$) and at 20 °C in June (greater in HA, $F_{1,10} = 7.71$, $p < 0.05$).

In the F&G-expt in April, juveniles of the LA group showed higher SGR and OGR when reared at 14 °C and 17 °C, than the fish of the HA group; however, OGR at 23 °C was higher in the HA group (Fig. 5). In the F&G-expt in June, DFI of HA juveniles was greater than that of the LA juveniles at all temperatures except 14 °C. Growth performance was greater in the HA juveniles at all temperatures.

The selected LMM showed that SGR, OGR, and DFI were commonly greater in the F&G-expt in April than in June and in the LA group than in the HA group (Table 2). However, the effect of the interaction of acclimation temperature (HA) and experiment (June) was also observed in the selected models. Incorporating the interaction, the coefficients of HA for SGR, OGR, and DFI in April were –1.48, –0.15, and –2.45, respectively, whereas those in June were 1.87 (i.e., 3.35 minus 1.48), 0.01, and 4.10, respectively. This result indicated that SGR, OGR, and DFI were greater in the LA group than in the HA group in April but the contrary

result was observed in June. Experimental temperature was adopted as an explanatory variable in the models for SGR, OGR, and DFI. SGR and OGR were the greatest at 20 °C in April, whereas DFI was the greatest at 23 °C. No significant effect of initial SL was observed for SGR or OGR. The important variables, as suggested from the analysis of deviance, were experimental water temperature and the interaction of acclimation temperature and experiment (April or June). Additionally, the positive effects of the interaction between acclimation temperatures and experimental water temperatures indicated that OGR was greater in the HA group at higher temperatures especially for the F&G-expt in June. DFI was affected by initial SL of flounder, indicating that food intake was greater in larger individuals.

4. Discussion

This study demonstrated that the thermal history of early life stages of marbled flounder affects upper thermal tolerance limits and growth performance. The second hypothesis on optimal temperature was rejected; the optimal temperature was not different between the two acclimation temperature groups. The first and third hypotheses were adopted: high-temperature tolerance was greater in juveniles acclimated to higher temperatures, and growth performance was suppressed by acute temperature changes from the acclimation period to the experimental period. The optimal temperature for growth of juveniles was consistently at 20 °C, however, growth suppression was observed for juveniles acclimated to 24 °C and reared at 14 °C compared to that in juveniles acclimated to 12 °C and reared at 14 °C. These results suggest that juveniles should not be reared around 20 °C, even if it was the optimal temperature, to avoid growth inhibition after release into the wild.

4.1. High-temperature tolerance

This study clearly demonstrated that acclimation temperature affects high-temperature tolerance of juvenile marbled flounder. In both UILT- and CTMax-tests, more individuals in the HA group than in the LA group had survived at temperatures higher than 26 °C. The estimated 50% lethal temperature (UILT) and CTMax were significantly higher in the HA group. These tendencies are common in other marine fishes. For example, the UILT for young Schlegel's black rockfish (*Sebastes schlegelii*) that had been acclimated at 15 °C and 28 °C for over 14 d was 28.1 °C and 30.5 °C during 72 h, respectively, and their CTMax was 31.2 °C and 34.6 °C, respectively (Tsuchida, 1995; Tsuchida and Setoguma, 1997). The

CTMax of the marbled rockfish (*Sebastiscus marmoratus*) became higher (32.8 °C) when fish were acclimated to higher temperatures (25 °C) for two weeks (Kita et al., 1996). Juvenile meagre (*Argyrosomus regius*) exhibited higher temperature tolerance after acclimation to higher temperatures (Kir et al., 2017).

The effects of acclimation may be detectable within short periods. Higher survival in the conditioned LA group than in the naive LA group at 26 °C and 28 °C in the UILT-test indicates that just 24 h of intermediate temperature exposure (22–24 °C) provides a buffering effect on temperature tolerance. A similar example was observed in juvenile seahorse (Mascaró et al., 2019); CTMax of individuals experiencing temperature changes from 25 °C to 30 °C at a rate of 1 °C day⁻¹ showed high CTMax (37.8 °C), which was similar to that of juveniles maintained at 30 °C (36.5 °C) and higher than that of juveniles maintained at 25 °C (31.9 °C). Such short-time exposure would affect the thermal responses, although the complete acclimation to different temperatures takes a few weeks when considering metabolic and aerobic processes (Sandblom et al., 2014).

No individual in the HA group was able to survive for longer than 5 h at 30 °C. In the dynamic CTMax-test, some individuals were alive at 30.6 °C, but none of the individuals survived at 31.0 °C. Considering the sublethal effects of the CTMax (unable to escape from predation owing to the loss of equilibrium), a temperature of approximately 30 °C is unlikely to support the survival of marbled flounder. This value was consistent with the high-temperature tolerance of adult and immature marbled flounder (211–271 mm total length) caught in Hokkaido, northern Japan (Takahashi et al., 1987). The CTMax is a useful indicator for assessing the potential thermal limit of ectotherms, but it should be noted that the warming rate affects the CTMax (Vinagre et al., 2015; Illing et al., 2020). For marbled flounder, Tsuchida (2002) reported that the CTMax of juveniles (49 mm SL) acclimated to 15–28 °C for ≥14 d was 30.7–33.6 °C under a warming rate of 5 °C h⁻¹. This CTMax was higher than that of 29.3–30.5 at 0.5 °C h⁻¹ in the present study. However, a temperature change of 5 °C h⁻¹ is not realistic in the field. Furthermore, methods to obtain CTMax should be improved, as the CTMax in this study was almost equivalent to the lethal temperature. Alternative endpoint definitions such as the temperature induced metabolic rate (Paschke et al., 2018), cessation of locomotory activity, onset of muscle spasms, or loss of righting response (Lutterschmidt and Hutchinson, 1997) may provide more conservative and realistic CTMax estimates, although onset of spasms is not reliable in fish (Becker and Genoway, 1979).

All LA individuals survived at 22–24 °C for 24 h in the UILT-test, which was in contrast to the observation in a previous study (Tomiyama et al., 2018) in which 60-day-old hatchery-reared juvenile marbled flounder (21 mm SL) reared under 13 °C could not survive at 24 °C. Although the reason for the discrepancy is unclear, the presence or absence of feeding may be a related factor. In the present study, the experiment was conducted under a non-feeding condition, but the aforementioned mortality of 60-day-old juveniles at 24 °C was under a feeding condition. For European plaice (*Pleuronectes platessa*) and European flounder (*Platichthys flesus*), the lethal temperature was reported as lower in fed individuals than in unfed individuals, because of the increased consumption of oxygen (Fonds et al., 1992). Another explanation could be the ontogenetic development of thermal tolerances in juveniles. The juveniles used in this study were approximately 28 mm SL and 90 days old, and their high-temperature tolerance may be greater than those of 21 mm SL and 60 days old. Because the lethal temperature also changes depending on the season and the fish age (Golovanov, 2012), further studies are needed to clarify the ontogenetic changes in high-temperature tolerance.

4.2. Feeding and growth

This study demonstrated that optimal temperature for growth would be constant regardless of the acclimation temperatures: the greatest growth in SGR and OGR was observed at 20 °C in both the F&G-expts in April and June, same as that reported in the previous study (Kusakabe et al., 2017). This result is consistent with the fact that the final preferred temperature is not influenced by acclimation temperature (Fry, 1947; Jobling, 1981; Díaz et al., 2007). However, growth performance was obviously different between the acclimation groups. It is notable that SGR was greater in the LA group in April, but in June in the HA group (Table 2). Although the interaction between the acclimation group and water temperature was excluded from the model for SGR, growth rates, especially OGR, were greater in the LA group than in the HA group at 14 °C and 17 °C, whereas these were greater in the HA group than in the LA group at 23 °C in April (Fig. 5). In contrast, growth rates of the LA group were consistently lower than those of the HA group at all temperatures in June. The overall analyses using models did not support the body size effect between the experiments.

Factors causing these variations in the growth performance are unclear; however, one possible explanation is that the rapid thermal change may inhibit growth of juveniles. Actually,

a rapid temperature increase causes the increase in oxygen consumption and cortisol level (Pérez-Casanova et al. 2008). In the F&G-expt in April, water temperature before the acclimation was 12.5 °C, which was close to the acclimation temperature of LA. The rapid shift from 12.5 °C to 24 °C in the HA group might have caused physiological stress. However, thermal change from pre-acclimation (18 °C) to acclimation (± 6 °C) was equivalent between the acclimation groups in the F&G-expt in June (Fig. 1). The reduced feeding and growth in the LA group might be related to the explanation that decreasing temperature causes reduced appetite and growth inhibition in fish (Fu et al., 2018). Moreover, the growth was lower in the LA group than in the HA group at 14 °C despite similar food intake in June, indicating that the feed conversion efficiency was suppressed in the LA group. The influence of temperature changes on metabolism and the digestive system should be further investigated.

The present study showed the importance of thermal history on feeding and growth of juvenile marbled flounder. Feeding and growth were greater in the LA group than in the HA group at 14 and 17 °C in the F&G expt in April, whereas they were greater in the HA group than in the LA group at 20 °C and 23 °C in the F&G-expt in June (Figs. 4, 5). Moreover, the optimal temperature was constant between the acclimation temperatures, but the growth performance was lower in the experiment in June than in April (Fig. 5), indicating the suppression in growth after experiencing rapid thermal changes. However, the thermal history could not explain the different optimal temperatures with similar body sizes, as observed in Tomiyama et al. (2018). As mentioned above, the metabolic and digestion physiology would vary with juvenile size or developmental stages. For example, the temperature optima for the growth of Atlantic cod decrease as the size increases (Imsland et al., 2005). Further studies are expected to reveal the mechanisms underlying the ontogenetic changes in the optimal temperature and thermal acclimation capacity. Additionally, a relatively high mortality of juveniles and short duration of the experiments in the present study should be remedied, although the growth performance of individuals that survived was similar to the growth of juveniles in the previous study (Tomiyama et al., 2018).

5. Conclusion

The results of this study showed that acclimation of juvenile marbled flounder to low water temperature of 12 °C clearly decreased high-water temperature tolerance, namely UILT and CTMax, as compared with that of juveniles acclimated to 24 °C. Food consumption and growth performance were also lower in juveniles that had been acclimated to 12 °C than those

acclimated to 24 °C when exposed to 20 °C or 23 °C, whereas both were greater in juveniles acclimated to 12 °C when exposed to 14 °C. However, optimal temperature for growth of juveniles approximately 30 mm in length was consistently 20 °C regardless of the acclimation temperatures. Because juveniles of this size class have been released in Hiroshima Bay, in March or April when natural water temperature is approximately 13 °C, an efficient juvenile culture system incorporating the optimal temperature for juvenile growth and the effects of thermal history on growth inhibition should be established.

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Table 1 Results of the generalized linear model for the mortality probability of juvenile marbled flounder in the experiment on the upper incipient lethal temperature (UILT-test)

Analysis of deviance				Coefficients			
Error source	df	LR Chisq	<i>p</i>	Parameter	Estimate	SE	<i>p</i>
				Intercept	−423.25	78251.5	0.996
AT	1	34.59	<0.001	AT (HA)	−52.21	9993.5	0.996
WT	1	88.27	<0.001	WT	16.37	3033.0	0.996

Residual deviance = 12.2, df = 87.

Analysis of deviance was carried out using Type II likelihood ratio chi-square (LR Chisq) tests. The response variable was the death or survival of each individual. The initial explanatory variables were acclimation temperature (AT), water temperature (WT), and their interaction. Final model was selected on the basis of the Akaike information criterion for small sample size (AICc, see Table S1).

Table 2 Results of the linear mixed models for specific growth rate (SGR), observed growth rate (OGR), and daily food intake (DFI) of juvenile marbled flounder in the laboratory experiments

Analysis of deviance				Coefficients				
Error source	df	Chisq	<i>p</i>	Parameter	Estimate	SE	<i>t</i>	<i>p</i>
SGR								
				Intercept	6.15	0.48	12.79	<0.001
AT	1	1.25	0.26	AT (HA)*	−1.48	0.53	−2.82	0.006
WT	3	126.38	<0.001	WT (17 °C)*	2.26	0.47	4.77	<0.001
				WT (20 °C)*	4.84	0.49	9.81	<0.001
				WT (23 °C)*	4.66	0.51	9.20	<0.001
Expt	1	3.02	0.082	Expt (June)*	−2.44	0.52	−4.69	<0.001
AT:Expt	1	22.69	<0.001	AT (HA):Expt (June)	3.35	0.70	4.76	<0.001
OGR								
				Intercept	0.37	0.048	7.64	<0.001
AT	1	2.14	0.14	AT (HA)*	−0.15	0.065	−2.30	0.024
WT	3	114.79	<0.001	WT (17 °C)*	0.25	0.057	4.43	<0.001
				WT (20 °C)*	0.32	0.064	5.01	<0.001
				WT (23 °C)*	0.21	0.064	3.25	0.002
Expt	1	7.96	0.005	Expt (June)*	−0.17	0.045	−3.88	<0.001
AT:WT	3	10.30	0.016	AT(HA):WT(17)	0.032	0.081	0.39	0.70
				AT(HA):WT(20)	0.19	0.085	2.20	0.031
				AT(HA):WT(23)	0.23	0.087	2.63	0.010
AT:Expt	1	7.17	0.007	AT (HA):Expt (June)	0.16	0.060	2.68	0.009
DFI								
				Intercept	−35.42	7.43	−4.77	<0.001
AT	1	1.16	0.28	AT (HA)*	−2.45	1.63	−1.50	0.14
WT	3	196.16	<0.001	WT (17 °C)*	9.20	1.51	6.11	<0.001
				WT (20 °C)*	18.26	1.63	11.21	<0.001
				WT (23 °C)*	20.26	1.61	12.60	<0.001
SL ₀	1	47.08	<0.001	SL ₀	1.64	0.24	6.86	<0.001
Expt	1	2.39	0.12	Expt (June)*	−5.49	2.01	−2.73	0.008
AT:Expt	1	8.73	0.003	AT (HA):Expt (June)	6.55	2.22	2.95	0.004

Analysis of deviance was carried out using Type II Wald Chi-square tests. The response variables were SGR, OGR, and DFI of each individual. The initial explanatory variables were acclimation temperature (AT), water temperature (WT), initial standard length (SL₀), experiment (Expt), interaction of AT and WT (AT:WT), and interaction of AT and Expt (AT:Expt). Tank was incorporated as a random variable. Final model was selected on the basis of the Akaike information criterion for small sample size (AICc, see Table S3). *The effect of the high-temperature acclimation (HA) was assessed on the basis of the low-temperature acclimation group, the effects of temperatures (17 °C, 20 °C, 23 °C) were assessed on the basis of 14 °C, and the effect of Expt (June) was assessed on the basis of Expt (April).

Figure legends

Fig. 1. Thermal history experienced by juveniles before experimentation. Open and solid circles represent the high-temperature acclimation (HA) group and low-temperature acclimation (LA) group, respectively. Juveniles were raised by the Hiroshima City Fisheries Promotion Center until April 11 in 2017 and April 10 in 2018 and were transferred to the laboratory for experiments on the upper incipient lethal temperature (UILT) and the critical thermal maximum (CTMax) in 2017 and feeding and growth experiments (F&G-expts) in 2018.

Fig. 2. Mortality probability of juvenile marbled flounder in relation to the water temperature in the upper incipient lethal temperature (UILT)-test. Open and solid circles represent the high-temperature acclimation (HA) group and low-temperature acclimation (LA) group, respectively. The line shows the prediction by the generalized linear model. Shaded areas show 95% confidence intervals.

Fig. 3. Changes in the survival rate of juvenile marbled flounder (upper panel) with increasing water temperature (lower panel) after the onset of the experiment in the critical thermal maximum (CTMax)-test. Open and solid circles represent the high-temperature acclimation (HA) group and low-temperature acclimation (LA) group, respectively.

Fig. 4. Temporal changes in the daily food intake of juvenile marbled flounder in the feeding and growth experiments (F&G-expts) in April and June. Data are shown as mean \pm standard error (SE). Triangles and circles represent the high-temperature acclimation (HA) group and low-temperature acclimation (LA) group, respectively.

Fig. 5. Boxplots of specific growth rate (SGR), observed growth rate (OGR), and daily food intake (DFI) of juvenile marbled flounder in the feeding and growth experiments (F&G-expts). Boxes show the 25% and 75% quartiles and median, dashed vertical bars show the maximum and minimum values, and open circles show outliers. Open and solid boxes represent the high-temperature acclimation (HA) group and low-temperature acclimation (LA) group, respectively.

Figure1

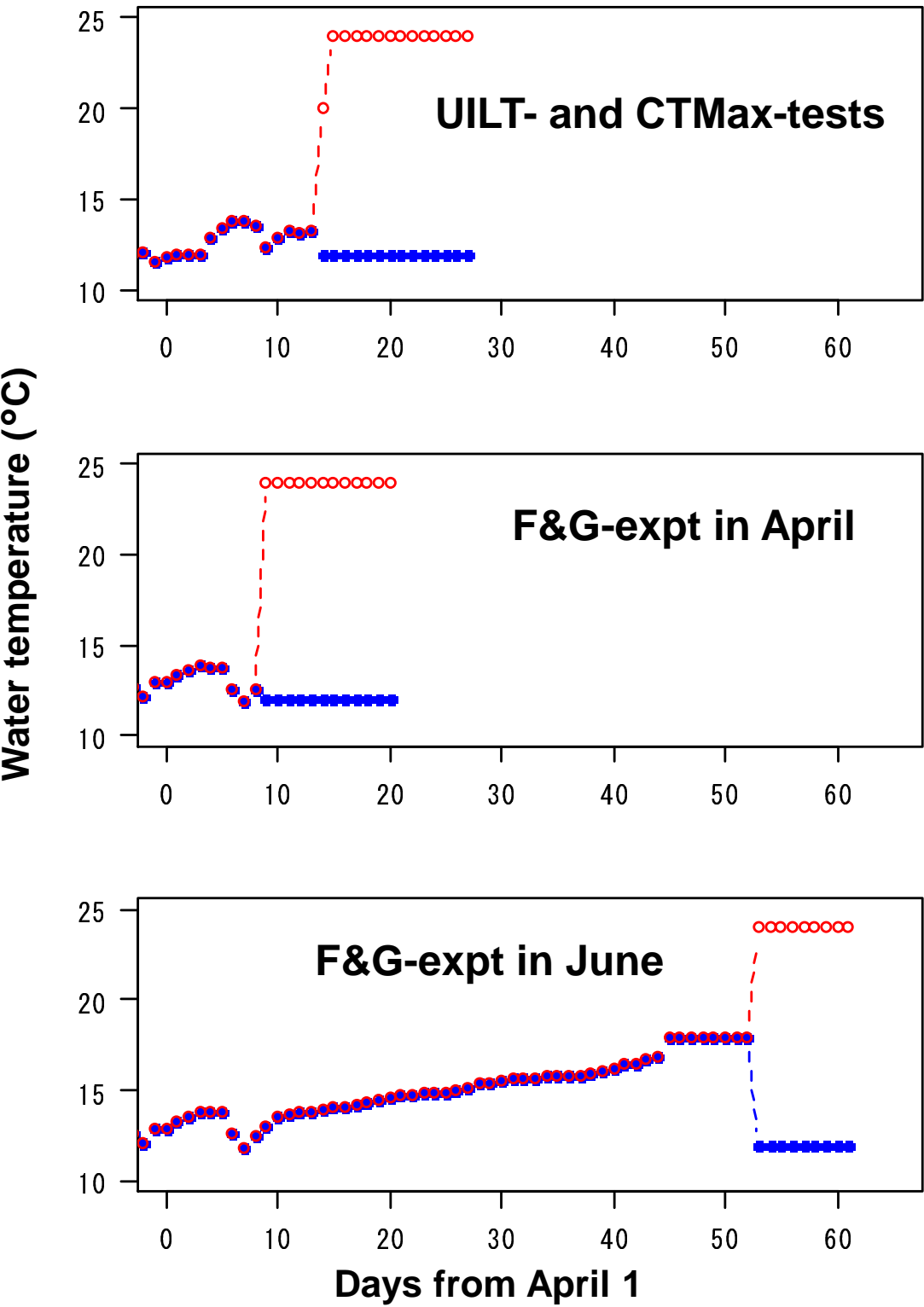


Figure2

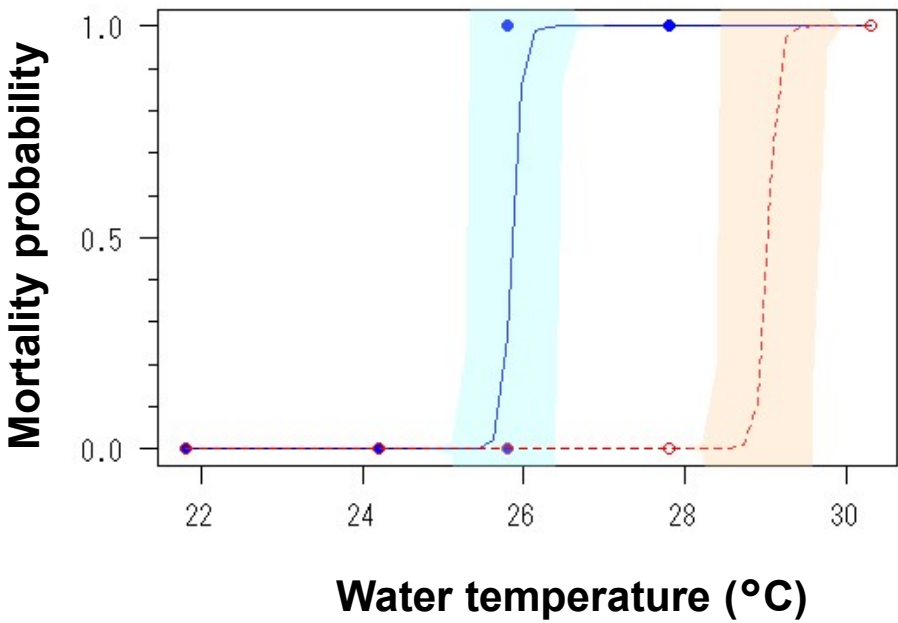


Figure3

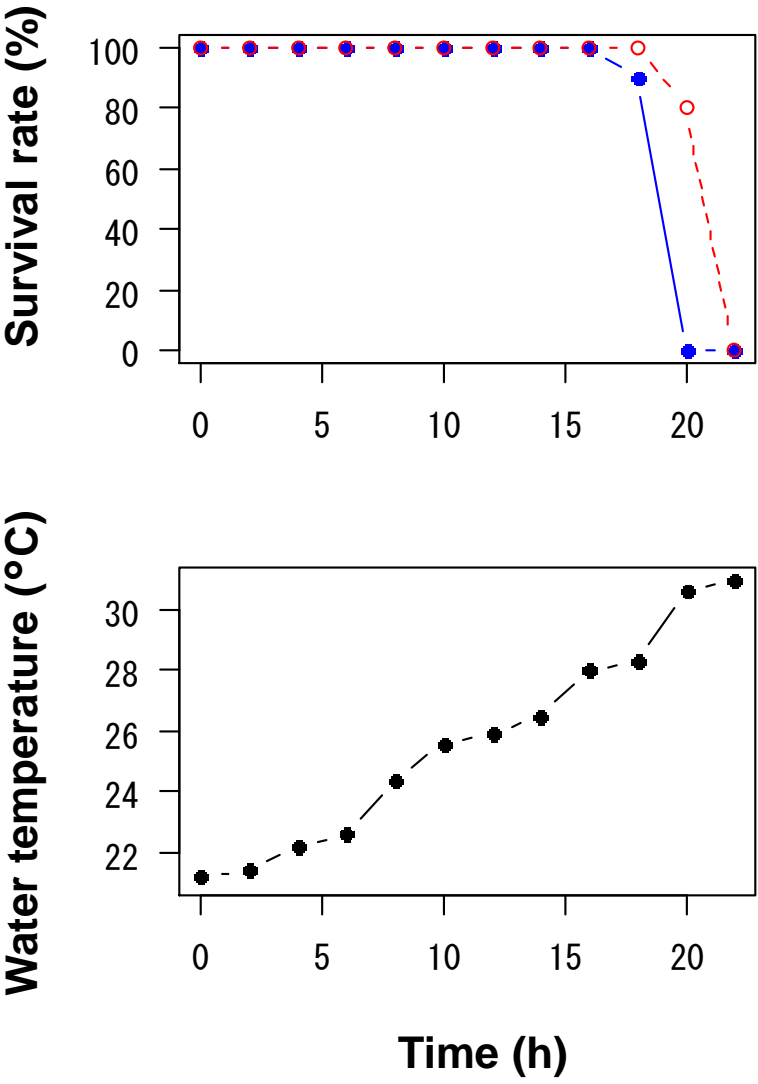


Figure4

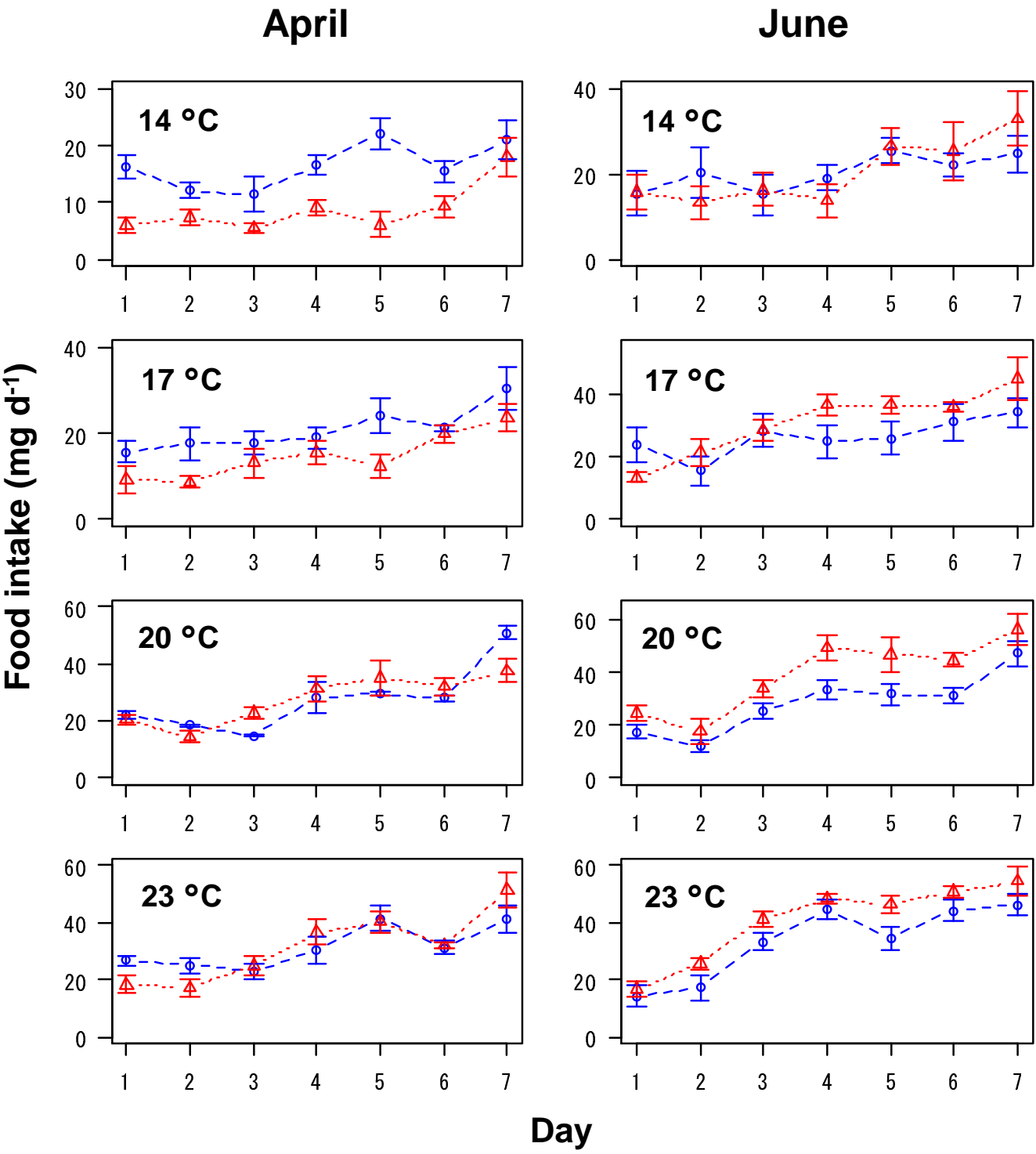


Figure5

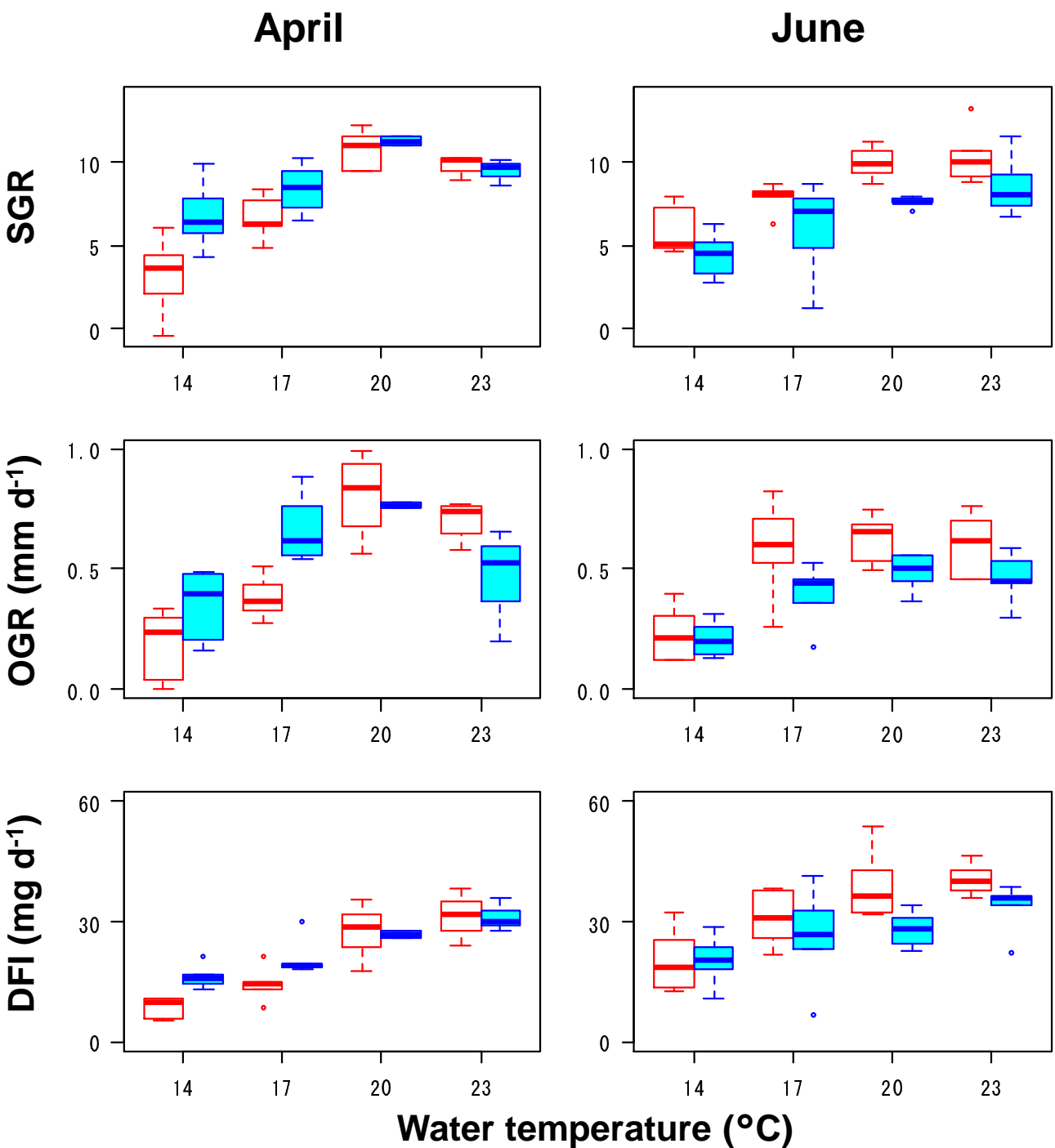


Table S1 Comparison of models for the death/survival of juvenile marbled flounder in the UILT-test

Model	df	AICc	Δ AICc	w
AT + WT	3	22.6	0.00	0.739
AT + WT + AT:WT	4	24.7	2.08	0.261
WT	2	38.3	15.72	0.000
(none)	1	133.5	110.87	0.000
AT	2	135.5	112.87	0.000

The top five models are shown.

w: Akaike weight

AICc : Akaike information criterion for small sample size

AT: acclimation temperature (categorical)

WT: water temperature

Table S2 Survival of juvenile marbled flounder in feeding and growth (F&G) experiments

Experiment	Acclimation group	Water temperature (°C)			
		14	17	20	23
April	LA	5 (1)	6	2 (4)	4 (2)
	HA	6	5 (1)	6	4 (2)
June	LA	5 (1)	6	6	5 (1)
	HA	5 (1)	6	6	6

Numerals show the number of survived individuals (the number of died individuals in parentheses). No significant difference was observed between acclimation groups for each temperature in each experiment (Fisher's exact test, $p > 0.06$)

Table S3 Comparison of models for specific growth rate (SGR), observed growth rate (OGR), and daily food intake (DFI) of juvenile marbled flounder in the laboratory experiments

Model	df	AICc	Δ AICc	w
SGR				
AT + WT + Expt + AT:Expt	9	327.0	0.00	0.477
AT + WT + Expt + AT:WT + AT:Expt	12	328.1	1.15	0.269
AT + WT + SL0 + Expt + AT:Expt	10	328.9	1.92	0.182
AT + WT + SL0 + Expt + AT:WT + AT:Expt	13	330.8	3.81	0.071
WT + SL0 + Expt	8	342.9	15.92	0.000
OGR				
AT + WT + Expt + AT:WT + AT:Expt	12	-68.9	0.00	0.475
AT + WT + Expt + AT:Expt	9	-67.2	1.68	0.205
AT + WT + SL0 + Expt + AT:WT + AT:Expt	13	-66.1	2.77	0.119
AT + WT + Expt + AT:WT	11	-64.7	4.12	0.061
AT + WT + SL0 + Expt + AT:Expt	10	-64.6	4.26	0.056
DFI				
AT + WT + SL0 + Expt + AT:Expt	10	514.6	0.00	0.563
AT + WT + SL0 + Expt + AT:WT + AT:Expt	13	517.0	2.40	0.170
WT + SL0	7	518.7	4.12	0.072
WT + SL0 + Expt	8	518.8	4.21	0.069
AT + WT + SL0	8	519.9	5.30	0.040

The top five models are shown.

w: Akaike weight

AICc : Akaike information criterion for small sample size

AT: acclimation temperature (categorical)

WT: water temperature (categorical)

SL0: initial standard length

Expt: Experiment (III or IV)

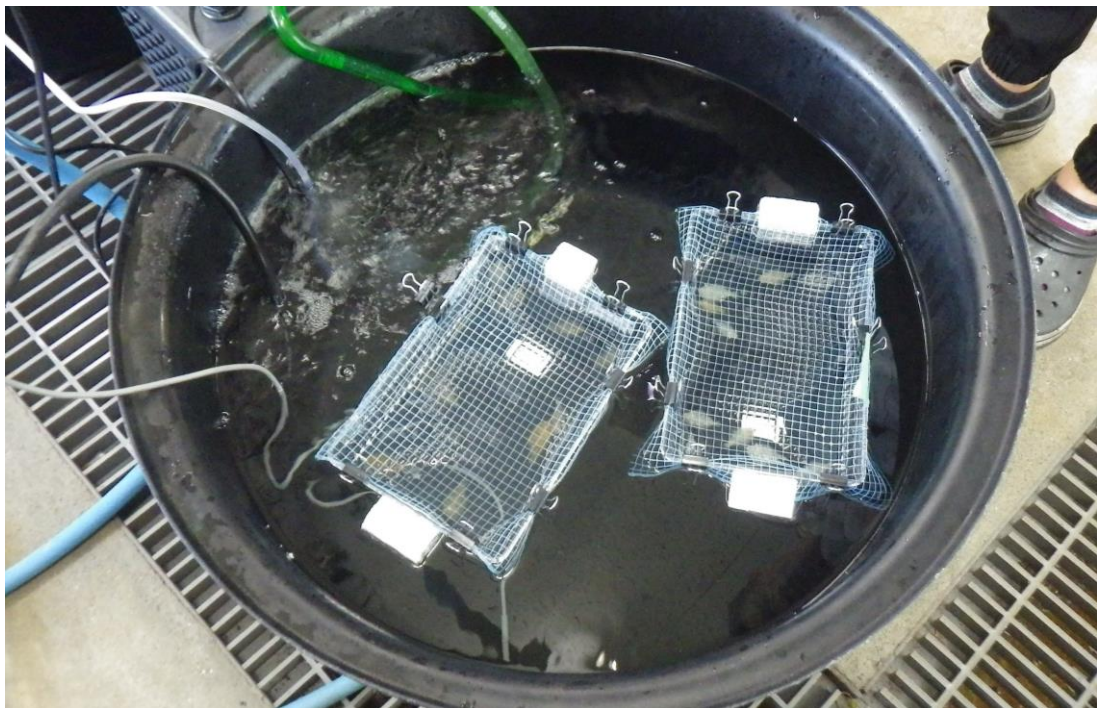


Fig. S1. Photographs of the UILT-test. Floating cages contained 10 individuals per cage. Each cage has many holes on its sides (no hole on its bottom).

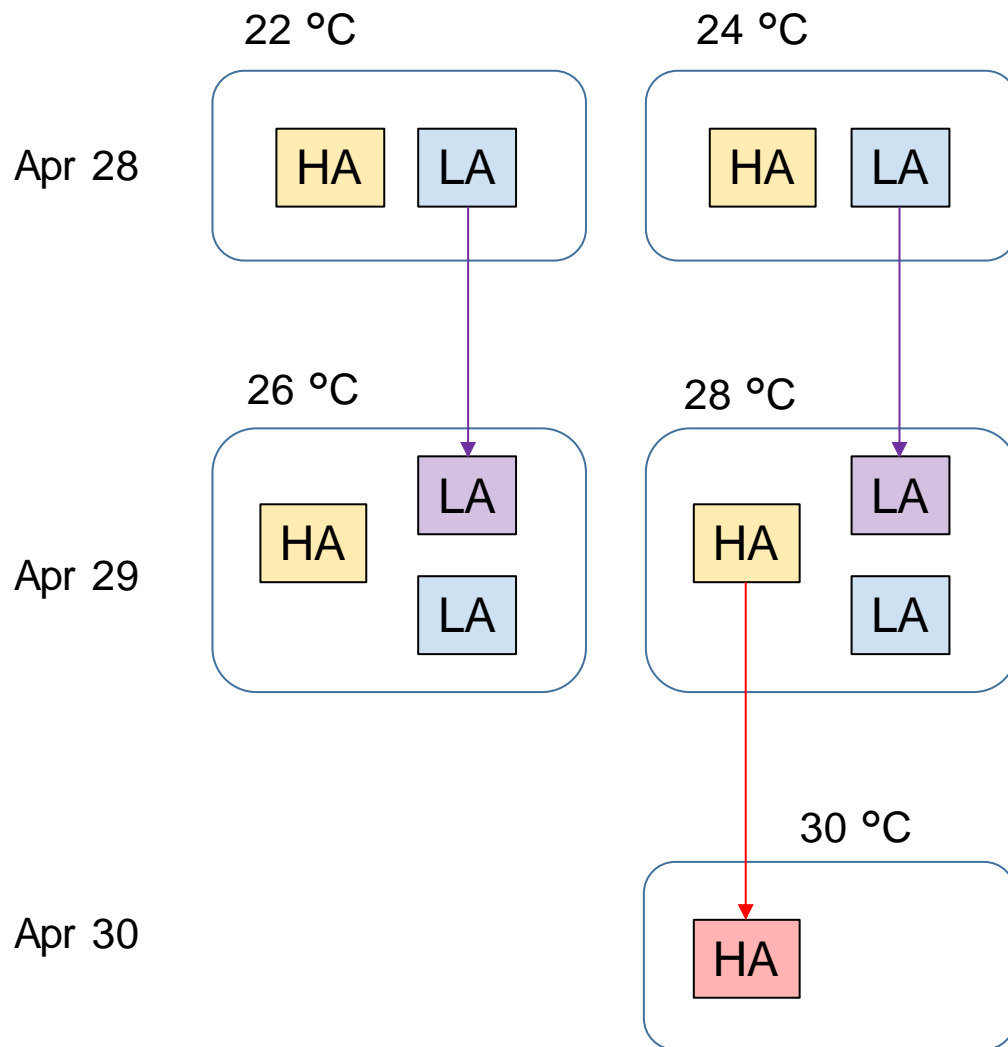
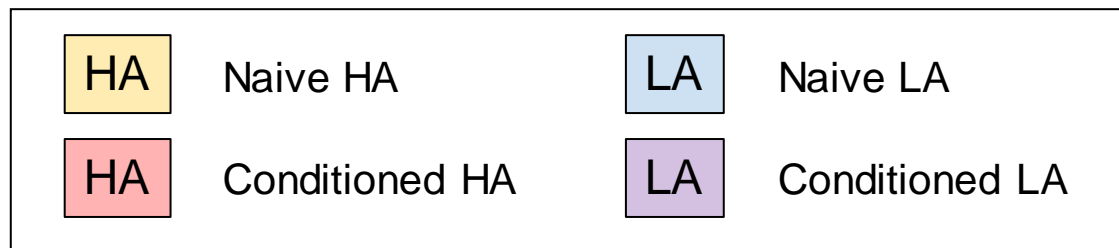


Fig. S2. The flow of UILT-test. HA and LA indicate high-temperature (24 °C) acclimation and low-temperature (12 °C) acclimation groups, respectively. Individuals survived during 24-h exposure at the given temperature were assigned as the conditioned group. Each group has 10 individuals.

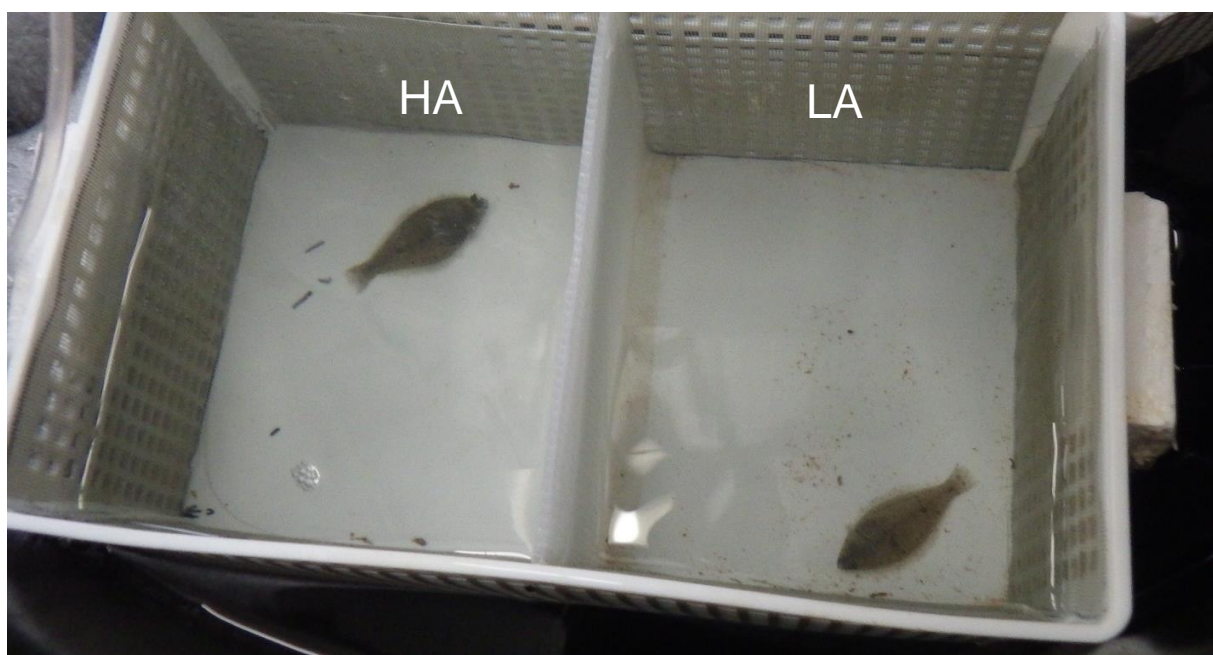
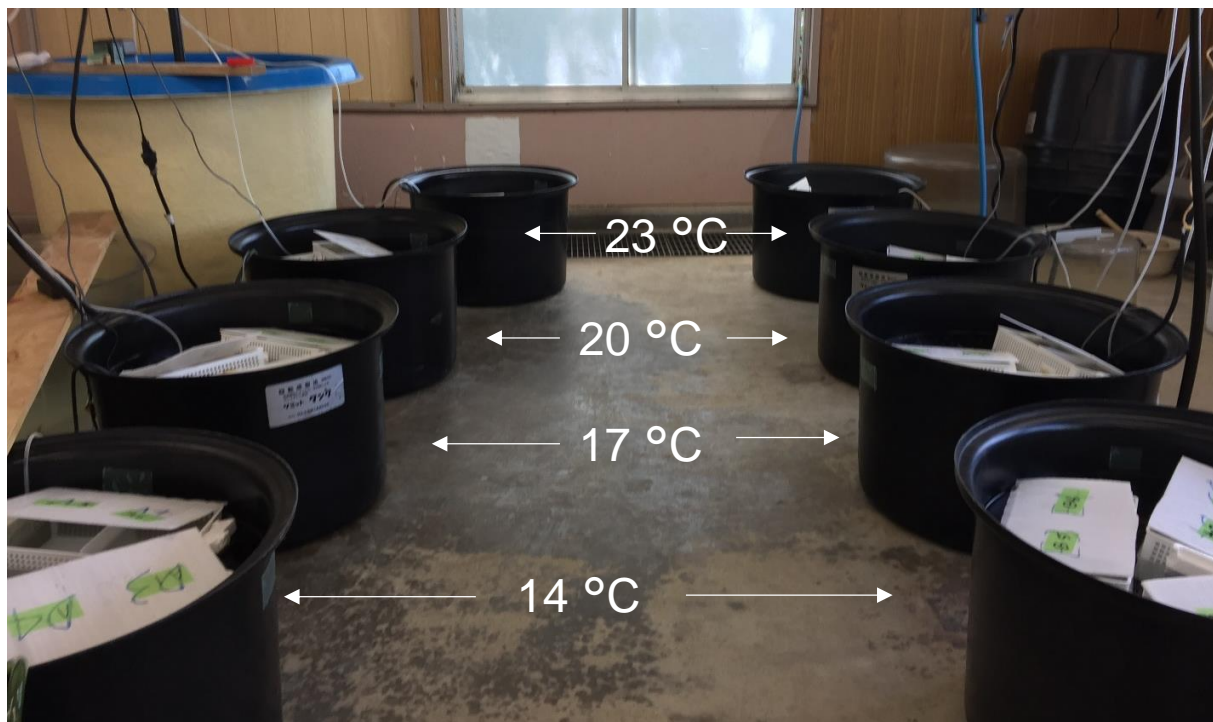


Fig. S3. Photographs of the feeding and growth (F&G) experiment. Upper panel shows aquaria with floating cages that accommodated two fish per cage (separated into two rooms). Lower panel shows a cage containing juveniles from high-temperature (24 °C) acclimation (HA) and low-temperature (12 °C) acclimation (LA) groups. A total of 96 individuals (2 individuals \times 3 cages \times 2 tanks (replicates) \times 4 temperatures \times 2 experiments in April and June) were used. Commercial pellets were fed to each individual and the pellets remaining after 1 h were collected to determine the food intake of each individual.

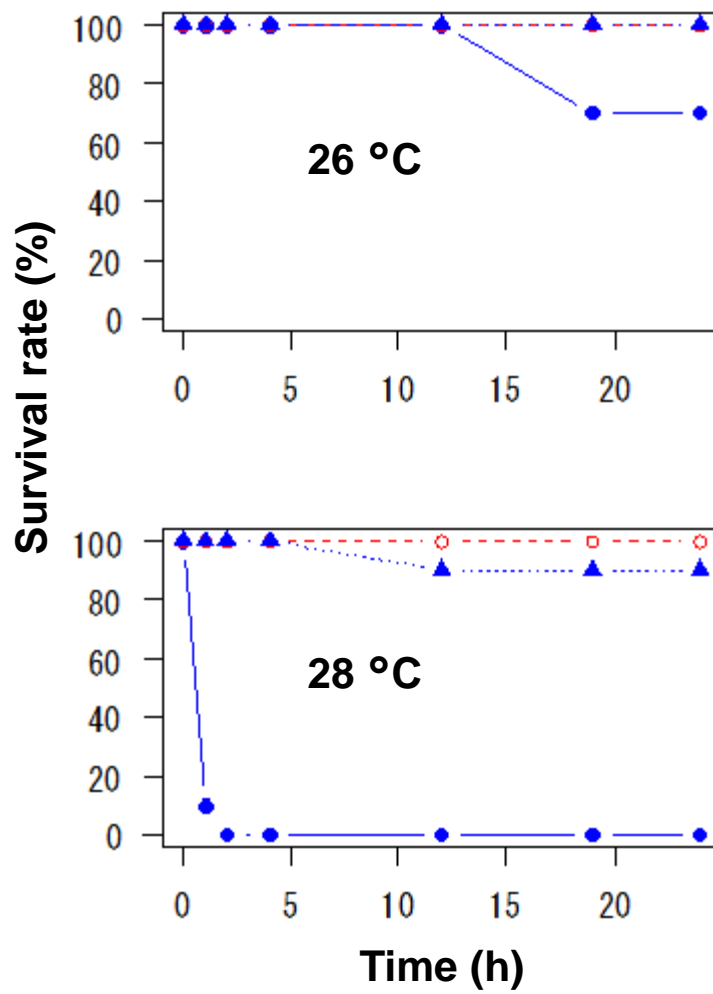


Fig. S4. Changes in the survival rate of juvenile marbled flounder at 26 °C and 28 °C after the onset of experiment in UILT-test. Open circles, solid circles, and solid triangles represent the high-temperature acclimation (HA) group, naive low-temperature acclimation (LA) group, and conditioned LA group, respectively. Results at 22 °C and 24 °C were not shown because all juveniles of HA and LA groups survived during 24 h.