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Title	Importance of experienced thermal history: effect of acclimation temperatures on the high-temperature tolerance and growth performance of juvenile marbled flounder
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Citation	Journal of Thermal Biology , 97 : 102831
Issue Date	2021-04-14
DOI	10.1016/j.jtherbio.2020.102831
Self DOI	
URL	https://ir.lib.hiroshima-u.ac.jp/00052421
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Relation	



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

3

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

5

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18

19 ABSTRACT

20

21 Experienced thermal history often affects the temperature tolerance of fish; however, the
22 effect of thermal history on growth performance is unclear. To contribute to effective stocking
23 (release of hatchery-reared juveniles in the field), we conducted four laboratory experiments
24 using juvenile marbled flounder (*Pseudopleuronectes yokohamae*, around 30 mm standard
25 length and 0.3 g body wet weight) acclimated at 12 °C and 24 °C for approximately 2 weeks
26 to investigate the effects of acclimation temperature on high-temperature tolerance, food
27 consumption, and growth performance. The acclimation to 24 °C increased tolerance to high
28 temperatures, as shown in a 24-h exposure experiment and in a temperature elevation
29 experiment. The 50% lethal temperature (upper incipient lethal temperature) was estimated to
30 be 25.9 °C and 29.0 °C for the 12 °C and 24 °C acclimation groups, respectively. In
31 subsequent experiments, we tested the effects of high and low temperature acclimation on the
32 food consumption and growth performance of two size groups of juveniles (28.7 ± 2.0 and
33 34.5 ± 2.9 mm, mean \pm SD), that were reared at temperatures ranging from 14 °C to 23 °C.
34 The optimal temperature for growth was 20 °C and did not differ between the acclimation
35 temperatures or between the size groups. However, food consumption and growth
36 performance were suppressed by acute temperature changes. Specifically, feeding and growth
37 were lower in the 24 °C-acclimated group than in the 12 °C-acclimated group when exposed
38 to 14 °C, which is close to the natural water temperature at release in the field. These results
39 suggest that experienced thermal history does not affect the optimal temperature but can affect
40 the growth performance of juveniles. To maximize the post-release growth of hatchery-reared
41 juveniles, the influence of thermal history should be taken into consideration and acute
42 thermal changes before release should be avoided.

43

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

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46

47 1. Introduction

48

49 Water temperature is one of the most important factors that regulate distribution, feeding,
50 and growth of aquatic ectotherms. The rise in sea temperature associated with global warming
51 has affected the geographic distribution and population structure of fish (Roessing et al.,
52 2004; Kuwahara et al., 2006; Shultz et al., 2016). Furthermore, temperature rise can cause an
53 increase in mortality or a change in growth performance of fish in nature or in captivity. Such
54 species-specific thermal responses are essential information to understand recruitment
55 dynamics or to improve efficiency in aquaculture.

56 It is well known that thermal responses such as temperature tolerance can be affected by
57 the previous history of experienced temperature (Reynolds and Casterlin, 1979; Jobling,
58 1981). Individuals that have experienced high temperatures show higher lethal temperatures
59 than those that have experienced low temperatures (Fry et al., 1946; Becker and Genoway,
60 1979; Tsuchida and Setoguma, 1997). In contrast, the temperature that is finally preferred,
61 defined as the final preferendum, is constant regardless of acclimation temperatures (Fry,
62 1947; Jobling, 1981; Díaz et al., 2007). Fish growth can be maximized under species-specific
63 optimal temperatures (Fonds et al., 1992; Iwata et al., 1994; Burel et al., 1996; Björnsson et
64 al., 2001; Handeland et al., 2008), and the final preferendum has been recognized to
65 correspond with the optimal temperature for growth (Kellogg and Gift, 1983; Khan et al.,
66 2014). However, this finding is not necessarily applicable to all species (Schram et al., 2013;
67 Mortensen et al., 2020). Furthermore, to our knowledge, effects of previously experienced
68 temperatures on growth performance have scarcely been studied. Understanding the
69 influences of thermal history on growth performance is important for stock enhancement or
70 restocking programs. Juveniles are often cultured under warmed conditions for efficient
71 growth, but they are released in the field with natural low temperature conditions after
72 reaching sufficient size for release. If growth of released juveniles is suppressed by the low
73 temperature at the release site, post-release mortality may become greater and result in the
74 decreased effectiveness of stocking.

75 The purpose of the present study was to clarify the effects of acclimation temperature on
76 the high-temperature tolerance, feeding, and growth of juvenile fish. The temperature
77 tolerance has been evaluated using static and dynamic methods in laboratory experiments
78 (Cox, 1974; Becker and Genoway, 1979; Reynolds and Casterlin, 1979; Bennett and Judd,
79 1997; Tsuchida and Setoguma, 1997), and the former method was used for estimating the
80 upper incipient lethal temperature (UILT), whereas the latter method was used to determine

81 the critical thermal maximum (CTMax). The UILT is the high temperature at which 50% of
82 population can survive indefinitely (Fry et al., 1946; Brett, 1956; Jobling, 1981; Kir et al.,
83 2017). The CTMax is the temperature with loss of equilibrium and is determined by raising
84 the temperature at a constant rate (Cowles and Bogert, 1944; Jobling, 1981).

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

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

115

116 2. Materials and methods

117

118 2.1. Animal husbandry and experimental setup

119

120 Laboratory experiments for high-temperature tolerance and growth of juveniles were
121 conducted in 2017 and 2018, respectively. We used approximately 200 individuals of
122 hatchery-reared juveniles of marbled flounder each year ($n = 400$ in total). These juveniles
123 were produced by the Hiroshima City Fisheries Promotion Center. For juvenile production,
124 wild fish caught in Hiroshima Bay (34.3°N , 132.4°E) were brought to the laboratory, and
125 ovulation was induced in females through the use of human chorionic gonadotropin. After
126 24–48 h, eggs were obtained by strip-spawning and fertilized artificially in the laboratory.
127 Marbled flounder spawn adhesive demersal eggs, therefore, the eggs can be attached to the
128 wall or bottom of the tanks. Fertilized eggs were accommodated in five 1000-L tanks (≤ 1.2
129 million eggs per tank) in a flow-through system (5000 L of UV sterilized seawater per day).
130 Larvae hatched at approximately 3 mm on day 6–8 after fertilization at $11\text{--}13^{\circ}\text{C}$. They were
131 transferred to two 50-kL tanks and fed rotifers (*Branchionus plicatilis* sp. complex) twice per
132 day at a density of 5 rotifers mL^{-1} until 20 d old. The fish were also fed *Artemia* spp. nauplii
133 twice per day at a density of 100–2000 individuals per fish from 15 to 80 d old and
134 commercial pellets ad libitum from 70 d old. The fish were reared under the flow-through
135 system under temperatures of $11\text{--}15^{\circ}\text{C}$. Juveniles were transferred to the laboratory of the
136 Hiroshima University and then accommodated in a 500-L tank filled with running seawater on
137 April 11, 2017 (90 d old) and April 10, 2018 (81 d old). The density of juveniles (200
138 individuals in a 500-L tank, i.e., 250 individuals m^{-2}) was lower than the stressful density of
139 884 individuals m^{-2} (Sugimoto et al., 2007).

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

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

149 Japan) and chillers (ZC-200, Zensui Co. Ltd., Japan) with thermostats (NX005, Gex Co. Ltd.,
150 Japan). Then, the temperature of the tank at 20 °C was gradually raised to 24 °C over 24 h to
151 reduce the shock due to the rapid change in temperature (Fig. 1). The tanks were filled with
152 filtered seawater (salinity of 32) and were aerated. The juvenile marbled flounder were fed
153 commercial pellets (Otohime S1, Nisshin Marubeni Feed Co. Ltd., Japan) twice a day via
154 automatic feeders during 13 d of acclimation. These juveniles (107 d old) were used for
155 experiments of high-temperature tolerance from April 28, 2017. Fish were not fed during the
156 experiments. Actual water temperatures at 12 °C and 24 °C were 12.1 ± 0.12 °C and $23.8 \pm$
157 0.51 °C (mean \pm SD), respectively.

158 In 2018, we conducted experiments in April and June. For the experiment in April, 100
159 juveniles were divided into two groups (50 individuals each) and distributed to two 100-L
160 tanks of 12 °C and 24 °C on April 10 in 2018. Juveniles (81 d old) were acclimated to each
161 temperature with feeding commercial pellets twice a day for 12 d before the experiment. The
162 remaining 100 individuals for the experiment in June were kept in a 500-L tank filled with
163 running seawater under natural water temperature, and commercial pellets (Otohime S1) were
164 fed to fish ad libitum. After 36 d on May 16, these juveniles (117 d old) were transferred to
165 two 100-L tanks of 18 °C (50 individuals each), and they were kept with being fed under
166 18 °C for 8 d. Thereafter, the temperatures of the tanks were adjusted to 12 °C and 24 °C
167 within 3 h and feeding continued for 8 d to acclimate the juveniles before the experiment in
168 June (Fig. 1).

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

171

172 2.2. Estimation of UILT

173

174 To estimate UILT, we conducted a 24-h survival experiment (hereinafter referred to as
175 the UILT-test) under four temperatures of 22 °C, 24 °C, 26 °C, and 28 °C. Ten juveniles from
176 each of the LA (mean \pm SD: 27.8 ± 3.2 mm SL, $n = 40$) and the HA (29.4 ± 3.8 mm SL, $n =$
177 40) groups were directly transferred to each 100-L tank that was filled with seawater of each
178 adjusted temperature. The juveniles were placed in a transparent plastic cage (27-cm long,
179 14-cm wide, and 6-cm high) with many 6-mm-diameter holes on the side of the cage for
180 allowing sufficient seawater exchange between the cage's interior and exterior (ESM Fig. S1).
181 A 6-mm mesh nylon modular net was attached to the top to prevent the juveniles from
182 escaping. Two floating cages for two groups (one cage for each group with 10 juveniles) were

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

187 We first carried out the 24-h experiment using both HA and LA groups under 22 °C and
188 24 °C on April 28 (acclimated for 13 d) and then under 26 °C and 28 °C on April 29
189 (acclimated for 14 d). Because all juveniles survived at 22 °C and 24 °C, we additionally
190 placed the surviving LA juveniles into the 26 °C and 28 °C tanks, respectively, to test whether
191 an exposure of only 24 h to relatively high temperatures affects the thermal tolerance of
192 juveniles. These individuals were designated as the "conditioned LA" group (Fig. S2).
193 Juveniles directly transferred from 12 °C were assigned as the "naive LA" group. Juveniles
194 that survived at 28 °C (observed only in the HA group) were subsequently maintained in the
195 same tank with water temperature raised to 30 °C for the 24-h observation of survival on
196 April 30 (Fig. S2). The water temperature in the 22 °C, 24 °C, 26 °C, 28 °C, and 30 °C tanks
197 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,
198 during the experiment. The total number of fish was 80 (10 individuals \times 2 acclimation
199 groups \times 4 temperatures), but a proportion of these fish was used again as the conditioned
200 individuals (10 individuals \times 3 groups, Fig. S2).

201

202 2.3. Estimation of CTMax

203

204 To estimate CTMax, we conducted a temperature-elevating experiment (hereinafter
205 referred to as CTMax-test) where the water temperature was raised from 21 °C at 1 °C every
206 2 h (0.5 °C h⁻¹) in a 100-L tank filled with aerated seawater. This rate could be experienced by
207 wild fish in the field (pers. obs.). In the experiment, 10 juveniles each from the LA group
208 (27.3 ± 3.0 mm SL) and HA group (29.7 ± 3.6 mm SL) were placed in cages (27-cm long,
209 16-cm wide, and 11-cm high) and were directly transferred from each acclimation
210 temperatures to 21 °C on April 28. Survival and opercular movements were observed at every
211 2 h. The CTMax was defined as the temperature with cessation of opercular movement,
212 because it is difficult to identify the loss of equilibrium for flatfish: juveniles increase their
213 swimming frequency with increasing opercular movement under rising temperatures; however,
214 they mostly stay lying down at the bottom without flipping until they cease opercular
215 movement. After cessation of opercular movement, they died immediately. Dead individuals
216 were removed at each observation and their SLs were measured. The water temperature was

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

219

220 2.4. Experiments on feeding and growth

221

222 As mentioned above, we conducted feeding and growth experiments in April and June
223 2018 (hereinafter referred to as F&G expts). In these experiments, six individuals were
224 directly transferred to three floating cages (same sizes as those of the CTMax-test) within
225 each 100-L tank, following Kusakabe et al. (2017). Each cage was divided into two sections
226 (an area of 13.5 cm \times 16 cm per section), and one individual from the LA group was put into
227 one section, whereas one individual from the HA group was put into the other section (Fig.
228 S3). Commercial pellets (Otohime S1 or S2) were fed manually to each individual fish until
229 satiation thrice per day (08:00, 13:00, and 18:00 h). Remaining pellets at 1 h after feeding
230 were collected each time, and the number of ingested pellets was recorded for calculation of
231 food consumption (S1: 0.62 mg per particle; S2: 1.01 mg per particle; Tomiyama et al., 2018).

232 We used eight 100-L tanks at four temperatures (14 °C, 17 °C, 20 °C, and 23 °C) with
233 two replicates (tanks), and a total of 24 individuals per acclimation group was used for each
234 experiment. Experimental temperatures were selected from 14 °C to 23 °C because juveniles
235 around 31 mm exhibited high growth and food consumption at temperatures from 14 to 22 °C
236 (Tomiyama et al., 2018). Temperatures were adjusted by heaters (SH320) and chillers
237 (ZC-200) with thermostats (NX005). In the F&G-expt in April, initial sizes of the LA and HA
238 groups were 29.3 ± 1.8 mm SL and 28.1 ± 1.9 mm SL, respectively. The water temperature
239 for the four temperatures during the experiment was 14.2 ± 0.2 °C, 17.1 ± 0.4 °C, $20.1 \pm$
240 0.5 °C, and 22.8 ± 0.3 °C. In the F&G-expt in June, initial sizes of the LA and HA groups
241 were 34.0 ± 2.3 mm SL and 35.0 ± 3.4 mm SL, respectively. The water temperature for the
242 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
243 individuals were maintained at each temperature without feeding for 24 h, and fed for 7 d
244 from April 23 (94 d old) or June 2 (134 d old), 2018.

245

246 2.5. Measurements and analyses

247

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

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

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

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

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

$$274 \quad \text{SGR} = (\ln(\text{BW}_t) - \ln(\text{BW}_0)) / t \times 100$$

$$275 \quad \text{OGR} = (\text{SL}_t - \text{SL}_0) / t$$

$$276 \quad \text{DFI} = \text{total food consumption} / t,$$

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

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

285 temperature. Because the effects of acclimation temperature seemed different between the
286 experiments, the interaction of acclimation temperature and experiment was also added as an
287 initial explanatory variable. This difference might be attributed to the experienced
288 temperatures before acclimation, i.e., around 12 °C in April and 18 °C in June, or to body
289 sizes of juveniles, although body size effect can be tested by incorporating SL_0 as an
290 explanatory variable. Tank was incorporated as a random variable. The model was selected
291 based on the AICc. All statistical procedures were performed using R version 3.5.1 (R Core
292 Team, 2018) with packages lme4 (Bates et al., 2015) and MuMIn version 1.42.1 (Bartoń,
293 2018) for fitting LMM and AICc model selection, respectively.

294

295 3. Results

296

297 3.1. High-temperature tolerance

298

299 The survival rate of juvenile marbled flounder, exposed to constant, elevated
300 temperatures for 24 h, differed between the acclimation groups and temperatures in the
301 UILT-test. The UILT was estimated to be 25.86 °C and 29.04 °C in the LA and HA groups,
302 respectively (Fig. 2). At 26 °C, all individuals of the HA group and conditioned LA group had
303 survived, whereas three individuals (30%) of the naive LA group died at 19 h (Fig. S4). The
304 survival rate after 24 h was not significantly different among the three groups (Fisher's exact
305 test, $p = 0.21$). At 28 °C, all juveniles of the HA group ($n = 10$) had survived, whereas one
306 individual (10%) of the conditioned LA group had died during the 24 h, and nine individuals
307 (90%) of the naive LA group had died within 1 h after the accommodation and the remaining
308 individual had died at 2 h. The survival rate was significantly different among the three
309 groups ($p < 0.001$). At 30 °C, none of the HA individuals that had previously been exposed to
310 28 °C for 24 h had survived for ≥ 5 h after accommodation. None of the individuals in the two
311 acclimation groups died during 24 h at 22 °C and 24 °C. In the GLM for the mortality
312 probability of the individuals, water temperature and the acclimation temperature groups were
313 adopted as explanatory variables, and the interaction between the water temperature and the
314 acclimation temperature groups was excluded from the model (Table 1).

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

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

325

326 3.2. Feeding and growth

327

328 Some individuals out of the 96 individuals died during the experiment (Table S2). No
329 significant difference in survival between the acclimation groups was observed for each
330 temperature in each experiment (Fisher’s exact test, $p > 0.06$). Individuals that survived
331 consumed approximately 10–70 mg of pellets per day (Fig. 4), whereas daily food intake of
332 the dead individuals when they had been alive was extremely small (ranged from 0 to 6.8 mg,
333 mean = 1.7 mg). In the F&G-expt in April, juveniles of the LA group consumed more food
334 than those of the HA group, especially at 14 °C and 17 °C during the first 5 d; however, such
335 a pattern was not observed in the F&G-expt in June. Daily food intake increased as the days
336 progressed in both experiments (two-way repeated measures ANOVA, April: $p < 0.001$ for all
337 temperatures; June: $p < 0.01$ for 14 °C and $p < 0.001$ for other temperatures). A significant
338 difference in the food intake on each day between the acclimation groups was observed only
339 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
340 in HA, $F_{1,10} = 7.71$, $p < 0.05$).

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

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

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

362

363 4. Discussion

364

365 This study demonstrated that the thermal history of early life stages of marbled flounder
366 affects upper thermal tolerance limits and growth performance. The second hypothesis on
367 optimal temperature was rejected; the optimal temperature was not different between the two
368 acclimation temperature groups. The first and third hypotheses were adopted:
369 high-temperature tolerance was greater in juveniles acclimated to higher temperatures, and
370 growth performance was suppressed by acute temperature changes from the acclimation
371 period to the experimental period. The optimal temperature for growth of juveniles was
372 consistently at 20 °C, however, growth suppression was observed for juveniles acclimated to
373 24 °C and reared at 14 °C compared to that in juveniles acclimated to 12 °C and reared at
374 14 °C. These results suggest that juveniles should not be reared around 20 °C, even if it was
375 the optimal temperature, to avoid growth inhibition after release into the wild.

376

377 4.1. High-temperature tolerance

378

379 This study clearly demonstrated that acclimation temperature affects high-temperature
380 tolerance of juvenile marbled flounder. In both UILT- and CTMax-tests, more individuals in
381 the HA group than in the LA group had survived at temperatures higher than 26 °C. The
382 estimated 50% lethal temperature (UILT) and CTMax were significantly higher in the HA
383 group. These tendencies are common in other marine fishes. For example, the UILT for young
384 Schlegel's black rockfish (*Sebastes schlegelii*) that had been acclimated at 15 °C and 28 °C
385 for over 14 d was 28.1 °C and 30.5 °C during 72 h, respectively, and their CTMax was
386 31.2 °C and 34.6 °C, respectively (Tsuchida, 1995; Tsuchida and Setoguma, 1997). The

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

391 The effects of acclimation may be detectable within short periods. Higher survival in the
392 conditioned LA group than in the naive LA group at 26 °C and 28 °C in the UILT-test
393 indicates that just 24 h of intermediate temperature exposure (22–24 °C) provides a buffering
394 effect on temperature tolerance. A similar example was observed in juvenile seahorse
395 (Mascaró et al., 2019); CTMax of individuals experiencing temperature changes from 25 °C
396 to 30 °C at a rate of 1 °C day⁻¹ showed high CTMax (37.8 °C), which was similar to that of
397 juveniles maintained at 30 °C (36.5 °C) and higher than that of juveniles maintained at 25 °C
398 (31.9 °C). Such short-time exposure would affect the thermal responses, although the
399 complete acclimation to different temperatures takes a few weeks when considering metabolic
400 and aerobic processes (Sandblom et al., 2014).

401 No individual in the HA group was able to survive for longer than 5 h at 30 °C. In the
402 dynamic CTMax-test, some individuals were alive at 30.6 °C, but none of the individuals
403 survived at 31.0 °C. Considering the sublethal effects of the CTMax (unable to escape from
404 predation owing to the loss of equilibrium), a temperature of approximately 30 °C is unlikely
405 to support the survival of marbled flounder. This value was consistent with the
406 high-temperature tolerance of adult and immature marbled flounder (211–271 mm total
407 length) caught in Hokkaido, northern Japan (Takahashi et al., 1987). The CTMax is a useful
408 indicator for assessing the potential thermal limit of ectotherms, but it should be noted that the
409 warming rate affects the CTMax (Vinagre et al., 2015; Illing et al., 2020). For marbled
410 flounder, Tsuchida (2002) reported that the CTMax of juveniles (49 mm SL) acclimated to
411 15–28 °C for ≥14 d was 30.7–33.6 °C under a warming rate of 5 °C h⁻¹. This CTMax was
412 higher than that of 29.3–30.5 at 0.5 °C h⁻¹ in the present study. However, a temperature
413 change of 5 °C h⁻¹ is not realistic in the field. Furthermore, methods to obtain CTMax should
414 be improved, as the CTMax in this study was almost equivalent to the lethal temperature.
415 Alternative endpoint definitions such as the temperature induced metabolic rate (Paschke et
416 al., 2018), cessation of locomotory activity, onset of muscle spasms, or loss of righting
417 response (Lutterschmidt and Hutchinson, 1997) may provide more conservative and realistic
418 CTMax estimates, although onset of spasms is not reliable in fish (Becker and Genoway,
419 1979).

420 All LA individuals survived at 22–24 °C for 24 h in the UILT-test, which was in contrast
421 to the observation in a previous study (Tomiyama et al., 2018) in which 60-day-old
422 hatchery-reared juvenile marbled flounder (21 mm SL) reared under 13 °C could not survive
423 at 24 °C. Although the reason for the discrepancy is unclear, the presence or absence of
424 feeding may be a related factor. In the present study, the experiment was conducted under a
425 non-feeding condition, but the aforementioned mortality of 60-day-old juveniles at 24 °C was
426 under a feeding condition. For European plaice (*Pleuronectes platessa*) and European
427 flounder (*Platichthys flesus*), the lethal temperature was reported as lower in fed individuals
428 than in unfed individuals, because of the increased consumption of oxygen (Fonds et al.,
429 1992). Another explanation could be the ontogenetic development of thermal tolerances in
430 juveniles. The juveniles used in this study were approximately 28 mm SL and 90 days old,
431 and their high-temperature tolerance may be greater than those of 21 mm SL and 60 days old.
432 Because the lethal temperature also changes depending on the season and the fish age
433 (Golovanov, 2012), further studies are needed to clarify the ontogenetic changes in
434 high-temperature tolerance.

435

436 4.2. Feeding and growth

437

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

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

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

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

481

482 5. Conclusion

483

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

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

495

496 Acknowledgements

497 We thank staff of Setouchi Field Science Centre Takehara Station and members of the
498 Laboratory of Biology of Aquatic Resources, Hiroshima University, for their support in
499 laboratory experiments. We also thank Hiroshima City Fisheries Promotion Center for
500 providing cultured juveniles. The helpful comments from 4 anonymous reviewers are
501 acknowledged. This study was partly supported by the Ministry of Agriculture, Forestry and
502 Fisheries of Japan and by JSPS KAKENHI Grant Number 19K06207. There is no conflict of
503 interest in relation to this study.

504

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- 661
662

663 **Table 1** Results of the generalized linear model for the mortality probability of juvenile
 664 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

665 Residual deviance = 12.2, df = 87.
 666 Analysis of deviance was carried out using Type II likelihood ratio chi-square (LR Chisq)
 667 tests. The response variable was the death or survival of each individual. The initial
 668 explanatory variables were acclimation temperature (AT), water temperature (WT), and their
 669 interaction. Final model was selected on the basis of the Akaike information criterion for
 670 small sample size (AICc, see Table S1).
 671

672 **Table 2** Results of the linear mixed models for specific growth rate (SGR), observed growth
673 rate (OGR), and daily food intake (DFI) of juvenile marbled flounder in the laboratory
674 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

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

685
686

687 Figure legends

688

689 Fig. 1. Thermal history experienced by juveniles before experimentation. Open and solid
690 circles represent the high-temperature acclimation (HA) group and low-temperature
691 acclimation (LA) group, respectively. Juveniles were raised by the Hiroshima City Fisheries
692 Promotion Center until April 11 in 2017 and April 10 in 2018 and were transferred to the
693 laboratory for experiments on the upper incipient lethal temperature (UILT) and the critical
694 thermal maximum (CTMax) in 2017 and feeding and growth experiments (F&G-expts) in
695 2018.

696

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

702

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

707

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

712

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

719

Figure 1

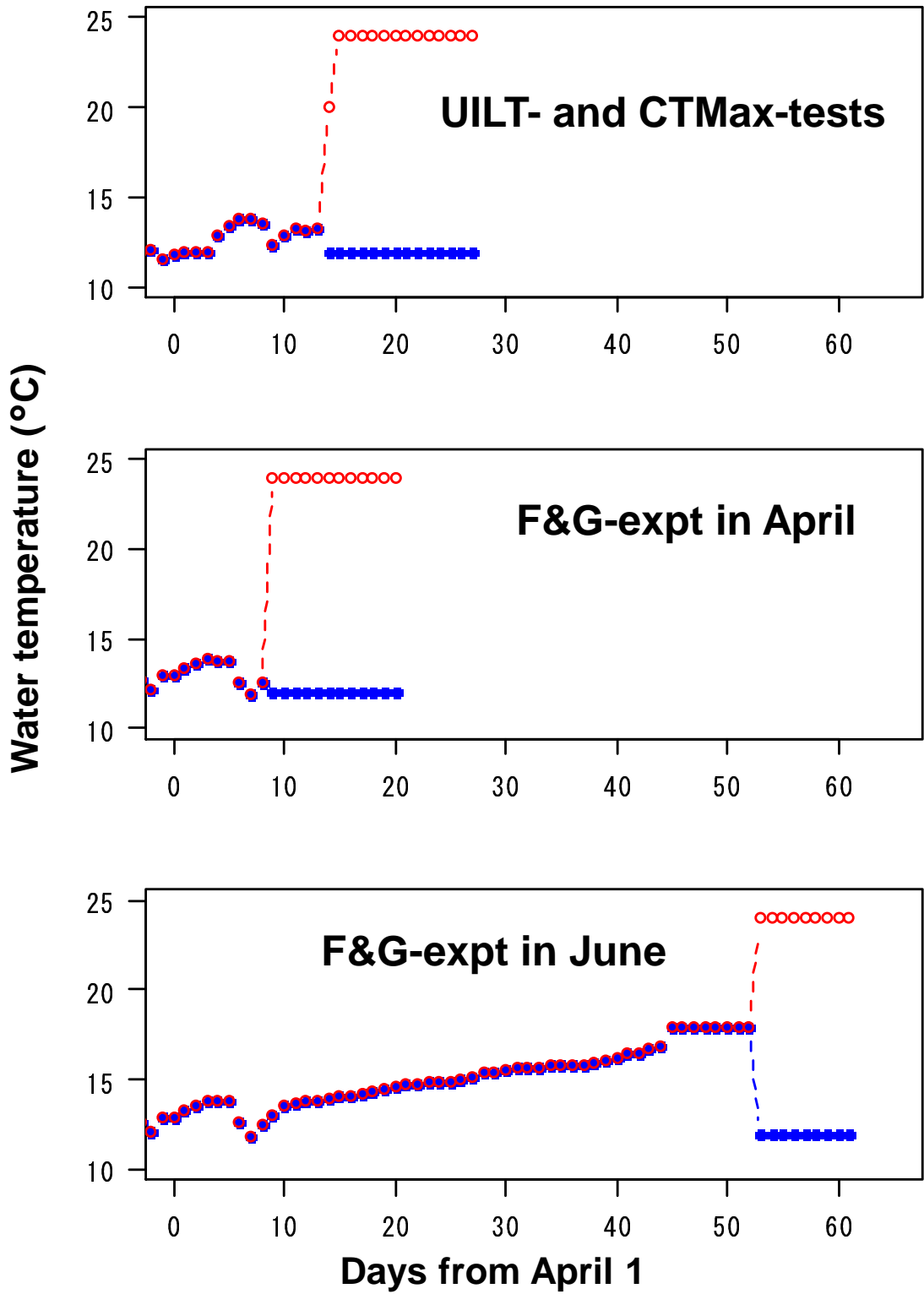


Figure2

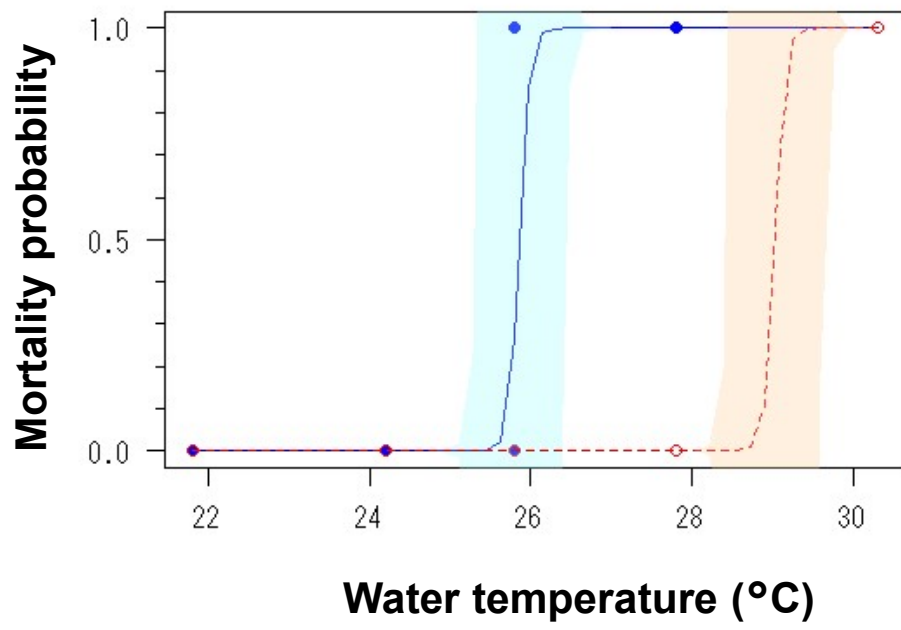


Figure3

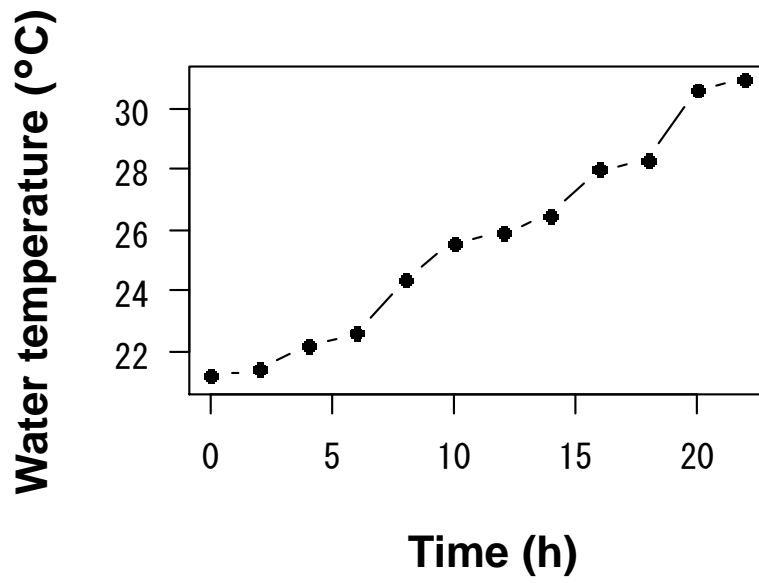
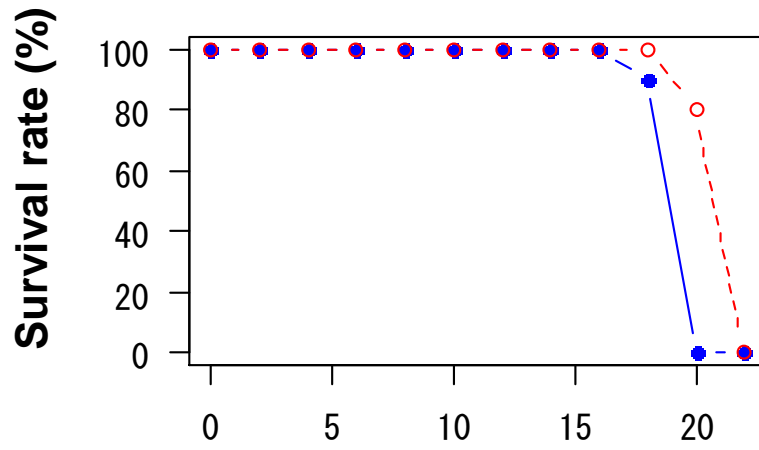


Figure4

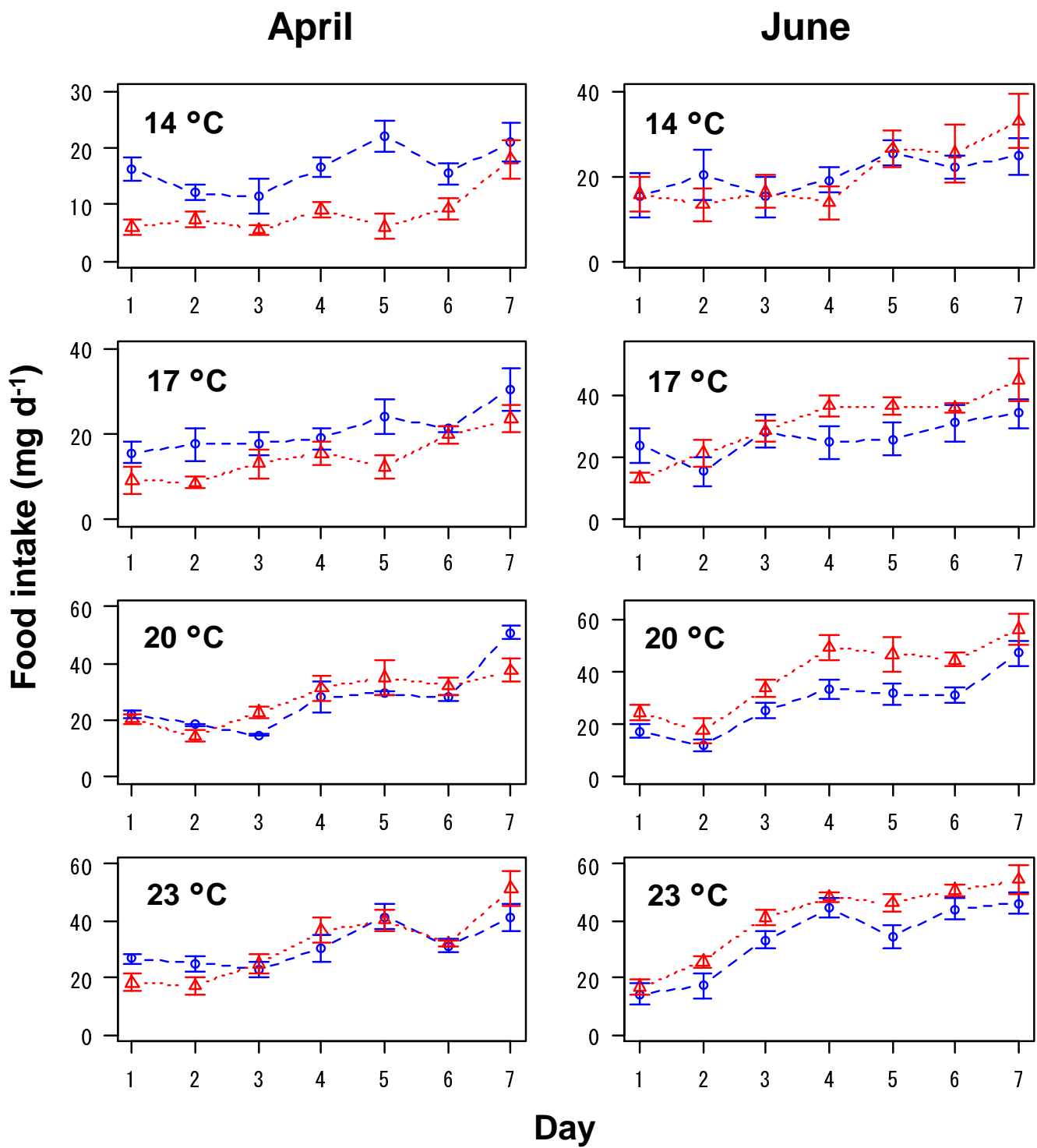


Figure5

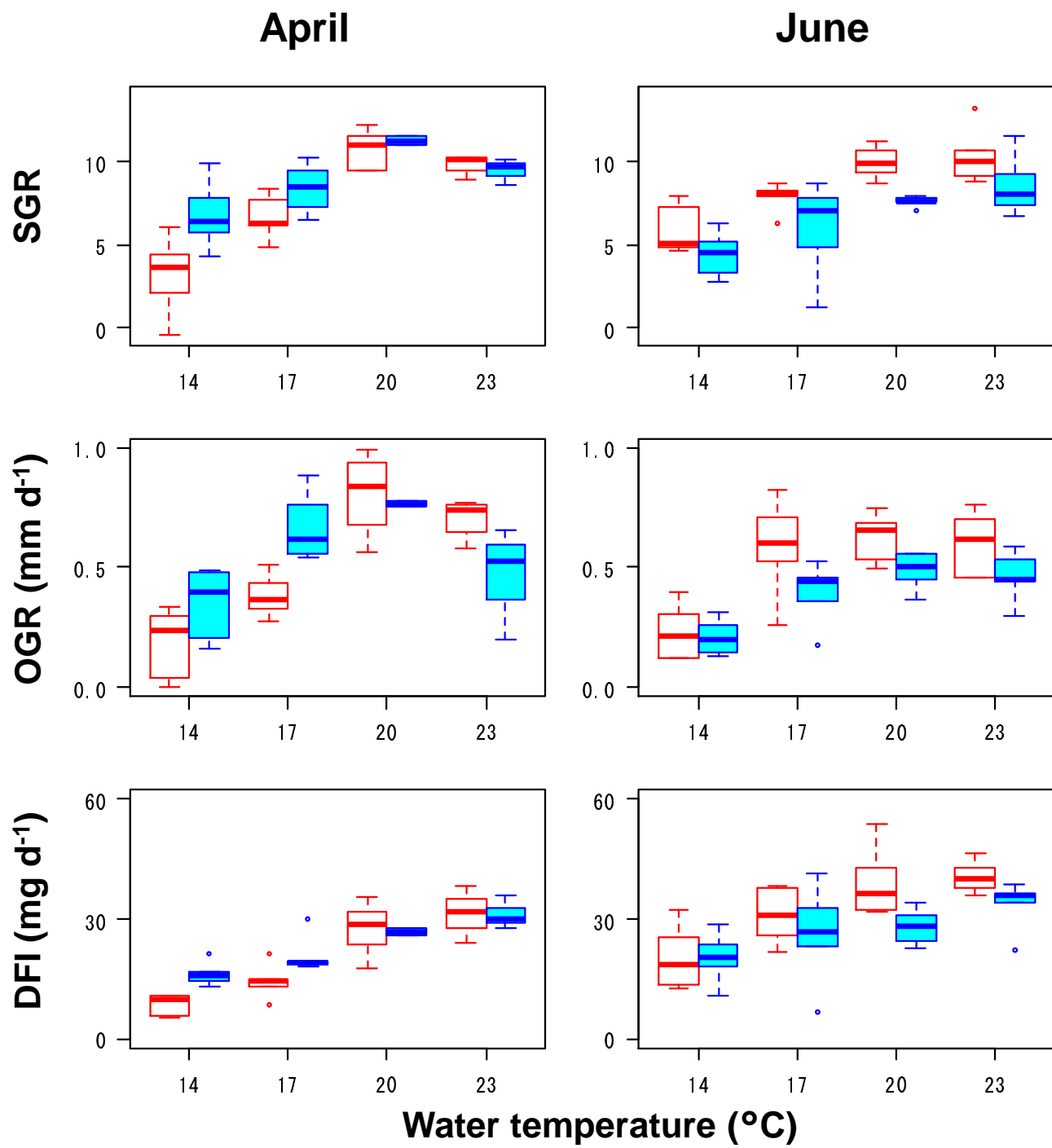


Table S1 Comparison of models for the death/survival of juvenile marbled flounder in the UILT-test

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)

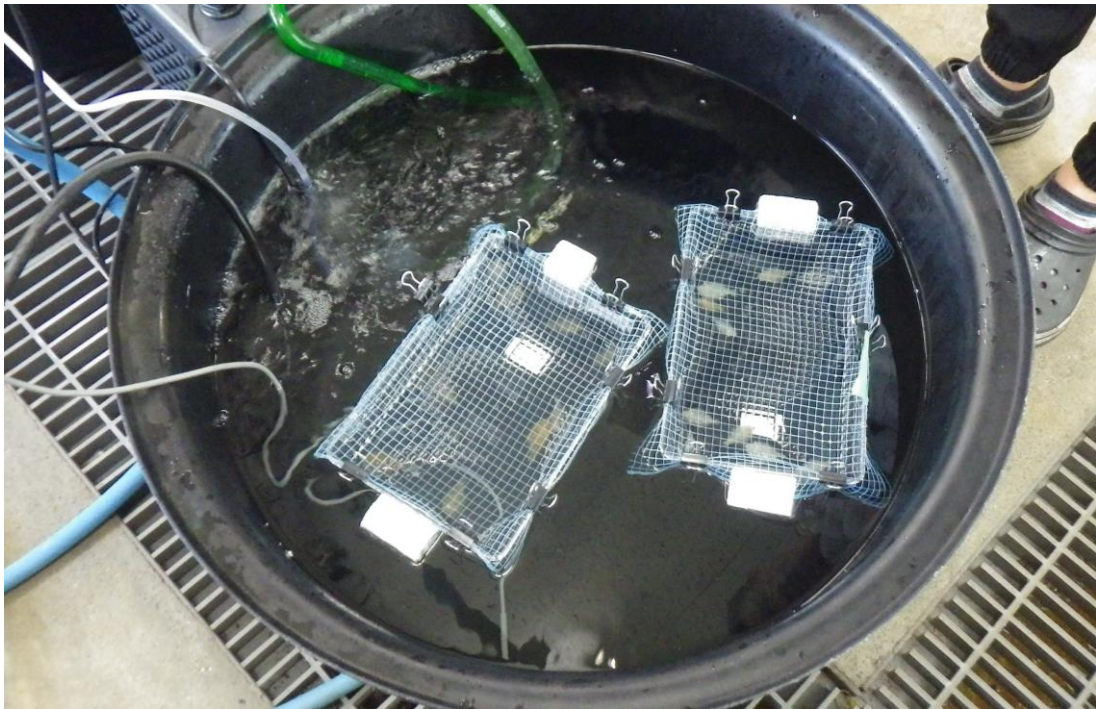


Fig. S1. Photographs of the UILT-test. Floating cages contained 10 individuals per cage. Each cage has many holes on its sides (no hole on its bottom).

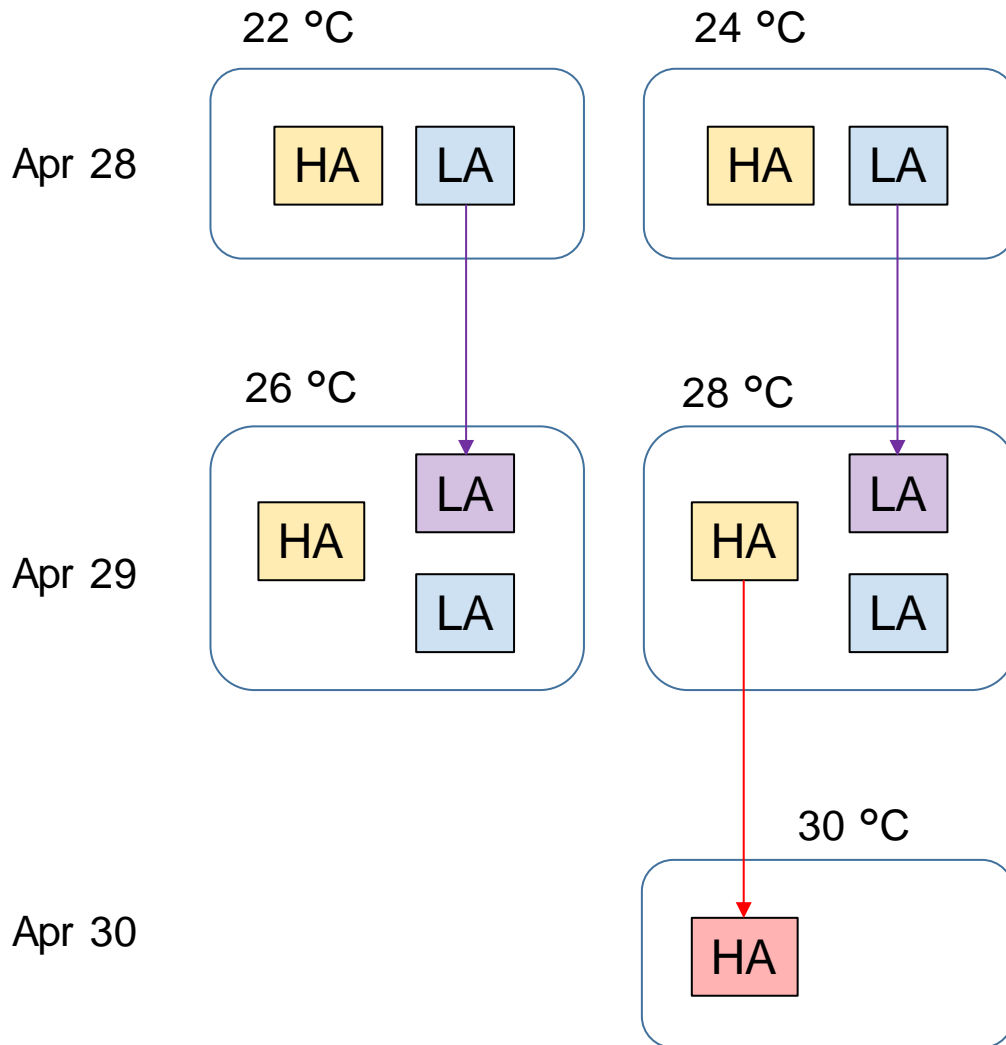
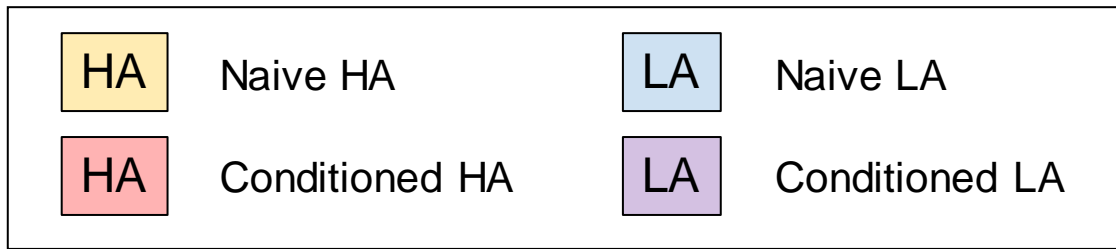


Fig. S2. The flow of UILT-test. HA and LA indicate high-temperature (24 °C) acclimation and low-temperature (12 °C) acclimation groups, respectively. Individuals survived during 24-h exposure at the given temperature were assigned as the conditioned group. Each group has 10 individuals.

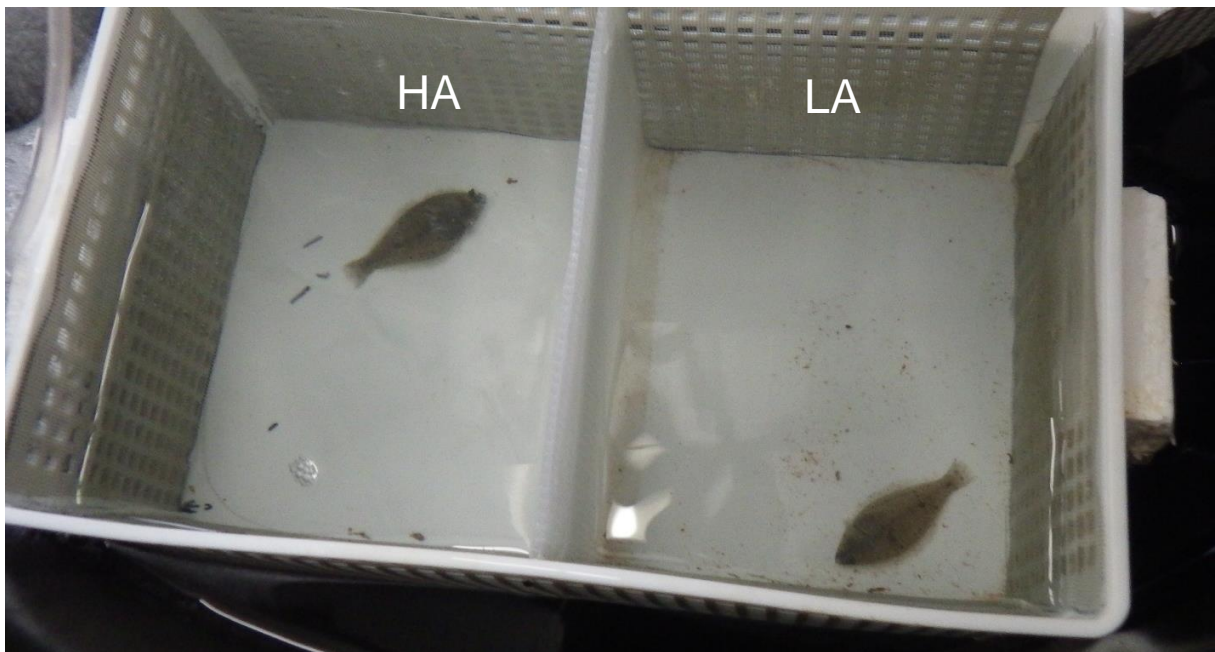
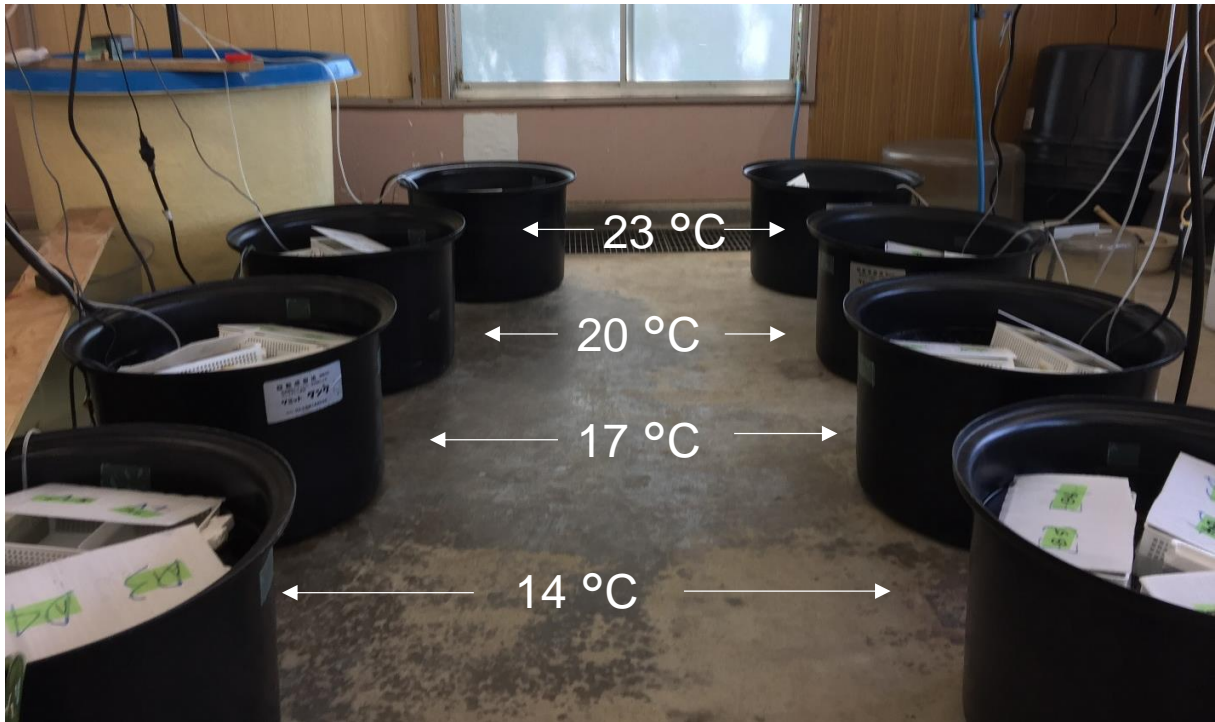


Fig. S3. Photographs of the feeding and growth (F&G) experiment. Upper panel shows aquaria with floating cages that accommodated two fish per cage (separated into two rooms). Lower panel shows a cage containing juveniles from high-temperature (24 °C) acclimation (HA) and low-temperature (12 °C) acclimation (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.

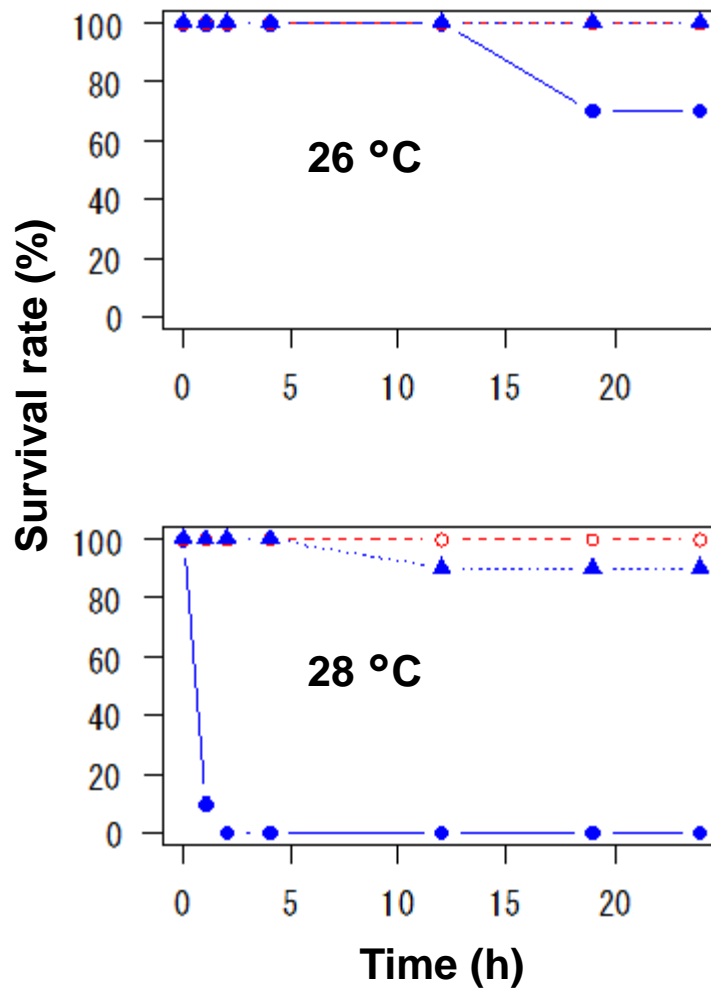


Fig. S4. Changes in the survival rate of juvenile marbled flounder at 26 °C and 28 °C after the onset of experiment in UILT-test. Open circles, solid circles, and solid triangles represent the high-temperature acclimation (HA) group, naive low-temperature acclimation (LA) group, and conditioned LA group,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