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ORIGINAL RESEARCH

Experimental study on the influence of water temperature on the otolith formation of the marbled flounder (*Pseudopleuronectes yokohamae*)

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Abstract The marbled flounder (*Pseudopleuronectes yokohamae*) is a widely distributed, economically important demersal species in Japan. A study into the growth of marbled flounder in six sampling areas around Japan also found significant differences in otolith nucleus size (diameter of otolith opaque core area) among areas, warm areas showed the bigger nucleus size of otolith, and cold areas showed the smaller size. Based on the physical environmental factors, it was suggested that water temperature affected the nucleus size among these sampling areas. To investigate the factors that influenced the formation of opaque and translucent zones in otolith nucleus, we conducted a rearing experiment using juvenile fish at 122 days post hatch (dph). Fish were reared at salinity of 33‰ and different water temperatures (15, 20, 25 **°C**) from 138 to 213 dph. The otolith growth was correlated positively to water temperature. We also measured the relative greyscale values of the opaque and translucent zones during the period of rearing experiment in each otolith, which showed a negative correlation with water temperature. The results showed that there was a significant relationship between water temperature and otolith formation. The otolith of the marbled flounder will grow larger and have a higher matrix density at higher water temperatures.

Keywords Otolith . Marbled flounder . Water temperature . Body size . Greyscale value

Introduction

Fish otoliths are widely used in age determination and fish growth analyses, however, the mechanisms in the formation of opaque and translucent zones are still unknown (Katayama 2018). In particular, the otolith nucleus may show some differences in growth compared with other parts of the otolith because of the endogenous factors in the early life stages of juvenile fish, this means that genetic factors and physical environmental conditions may both influence the formation of otolith structures (Blacker et al. 1974; Campana et al. 1985). Water temperature is an important factor that influences fish feeding and growth, and the formation of otoliths is also affected by fish growth (Kusakabe et al. 2017). However, the effect of water temperature on the formation of otolith nucleus is still unclear. In some species, opaque zones were found to form during summer, while translucent zones formed during winter (Mann-Lang et al. 1996), such as Bluegills (*Lepomis macrochirus*), which formed as opaque zones in warm water, and translucent zones in cool water (Harold et al. 1989). In other species, such as the marbled flounder or Silver croaker (*Argyrosomus argentatus*), opaque zones formed during winter, while translucent zones formed during summer (Takaaki 1957; Katayama 2018).

The marbled flounder is a very important commercial species in Japan. In a previous study, we researched the age and growth of marbled flounder among different marine areas in Japan using the otolith surface

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reading method (Hong et al. 2019). The growth of otolith nucleus was also determined by measuring the distance between the alizarin mark and edge of the otolith along a specified axis. The results showed that fish collected from warm areas had a larger otolith nucleus size than those from cold areas. Based on the results of previous studies, the spawning season in cold areas was later than that in warm areas, but the formation period of different nucleus zones in cold and warm areas was almost the same because of the relatively lower summer water temperature in cold areas.

This study aimed to investigate whether water temperature affected the formation of otolith nucleus using rearing experiments in laboratory conditions, and to compare the differences between otoliths by measuring greyscale values.

Materials and methods

Samples

Three hundred cultured juveniles with an average total length (TL) of 44.4 mm and an average weight of 1.2 g at 122 dph were sent to the laboratory (Faculty of Agriculture at Tohoku University) from May 2019. They were accommodated in two 100 - L tanks. The 300 juveniles were acclimatized for 14 days, and then, they were used for the following chemical marking and rearing experiment.

Alizarin complexone marking

In order to mark the beginning of rearing experiment on the otolith of the marbled flounder, we used alizarin complexone (ALC) for immersion marking the samples. Alizarin complexone was dissolved in artificial seawater (Instant Ocean, Aquarium Systems, France) at a concentration of 35mg/L. The mean salinity of sea water was 33‰. The fish were immersed in 40L artificial seawater for 24 h. Before the immersion treatment, the fish were starved for 24 h, and during the treatment, the fish were fed with an exceeding amount of forage. After the alizarin marking treatment, all the water was exchanged to clean seawater. Fish were reared for another 2 days to avoid the acute death caused by the ALC, and the juveniles were used for rearing experiments. (Liu et al. 2009)

Rearing experiment

In order to investigate the influence of water temperature on the formation of opaque and translucent otoliths, we conducted a 75 - day rearing experiment from June 16 to August 30, 2019. The juveniles were transferred to five aquarium tanks (20 individuals per tank), with artificial seawater. The water temperature in tanks 1-5 was set to 15, 20, 25, 15 and 25 °C, respectively, using waterproof coolers (model TK-60, Takara Industrial Corporation, Japan). The temperature threshold of adult marbled flounder is 28 °C (Takahashi et al. 1987), and in the rearing experiment we carried in 2018, the mortality of juveniles was very high at water temperatures over 26 °C. Therefore, we chose 25 °C as the highest water temperature in the rearing experiment. All tanks were continuously aerated with air stones, and water was exchanged twice a week at 70% tank volume.

Out of these five tanks, the water in three tanks was continuously maintained at 15, 20 or 25 °C (groups 1, 2 and 5, respectively). In order to clarify otolith formation at different water temperatures, for the other two tanks, the water temperature was changed once a month as follows: 25 – 15 – 25 °C and 15 – 25 – 15 °C (groups 3 and 4, respectively) (Fig. 1). All the juveniles were fed twice a day during the daytime (9:00 am and 15:00 pm) with excess amounts of commercial dry pellets (Otohime, Marubeni Nisshin Feed, Japan), which contained 53% crude protein, 8% crude fat, 3% crude fiber, 16% crude ash, 2.3% calcium and 1.5% phosphorus.

After the 75 - day rearing experiment, all the juveniles were frozen for 24 h. The total length (TL) of all the samples was measured to nearest millimetre (mm) without shrinkage correction. The sex was not determined because the juvenile gonads were invisible (Goto et al. 1999). The otoliths were removed, cleaned with ethanol, and stored dry in well plates for observation and microscope slide preparation.

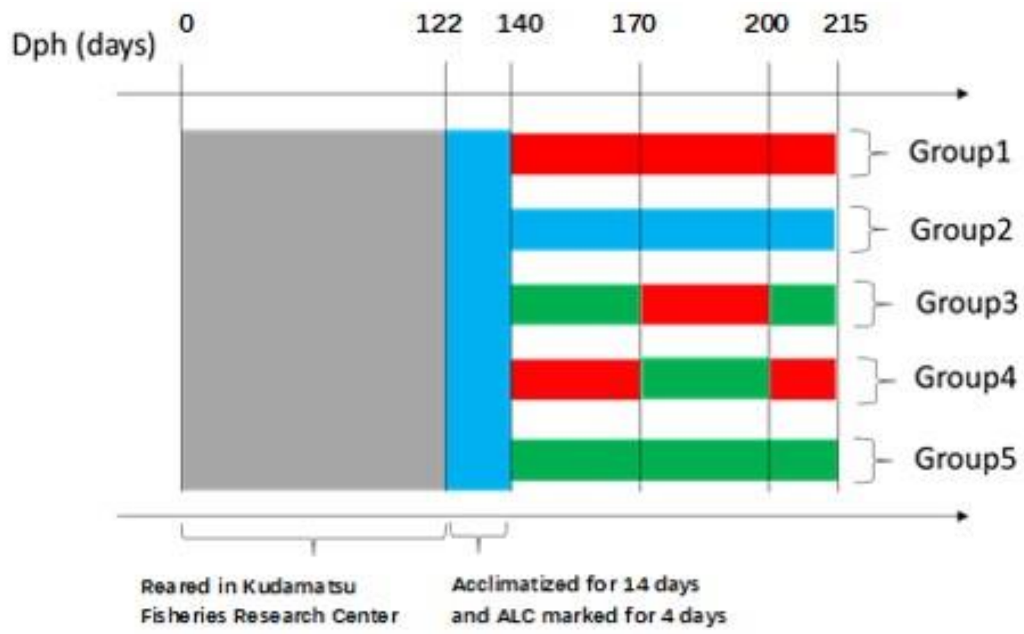

Fig. 1 Experimental settings of water temperatures (°C) and period (days) in the rearing experiment of juvenile marbled flounder

Each step of experiment for juvenile marbled flounder was at 122 dph. All the samples were acclimatized for 14 days and ALC marked for 4 days before experiment started. 5 groups were reared from 140 dph to 215 dph. Temperature settings: 15 (red); 20 (blue); 25 °C (green).

Microscope slides preparation and otoliths observation

Otoliths were mounted on a glass slide with epoxy resin for 24 h, then the slide was polished to the core of the otolith using sandpaper to reveal the nucleus and outer margins of the otolith.

The mounted glass slides were observed using a compound fluorescent microscope (model BX60, Olympus Optical Co. Ltd., Japan) attached to a power supply unit for mercury burner (model BH2-RFL-T3, Olympus Optical Co. Ltd., Japan). The fluorescence mark was visible under UV light with a WB filter, and the formation of the otolith during the rearing experiment was considered as the part between the outer margin and the fluorescence mark produced by ALC. The growth of the otolith was measured as the distance from the otolith margin to its ALC mark using an encoded stereomicroscope (model M165C, Leica Microsystems, Germany). A digital camera attached to the microscope was used to capture images of the otoliths (Hong et al. 2019).

To assess whether the translucent and opaque zones differed among these five experimental groups, we used software ImageJ (National Institutes of Health Image) to determine the greyscale value of the opaque-translucent zones of all the otoliths, and compared them with the relative greyscale value. The relative greyscale was calculated using the following equation:

$$Gr = \frac{G - Gt}{Go - Gt}$$

where Gr is the relative greyscale value, Gt is the lowest greyscale value of the whole otolith, Go is the highest greyscale value of the whole otolith, and G is the mean greyscale of the growth part of the otolith nucleus in the rearing experiment.

Data analysis

To investigate and compare the relationships among different groups, one-way ANOVA was performed

to test the difference in the data among different experimental conditions using R software (R Foundation for Statistical Computing, Vienna, Austria). Post-hoc analysis was conducted using Tukey's HSD test. For these analyses, a p value of < 0.05 was considered significant.

Results

Difference in somatic and otolith growth of juveniles among groups

Because of the mortality level in our rearing experiment, after the 75 – day rearing experiment, only those fish that survived were recorded as the valid sample in this research. Group 1 had 11 individuals, Group 2 had 10, Group 3 had 17, Group 4 had 14, and Group 5 had 14 fish.

In order to determine the increment of the fish body, we measured the average TL before the experiment started. The increment in the averaged TL of groups 1, 4 and 5 was significantly larger than that of group 2, which was the smallest increment at 8.13 mm. In the rearing experiment, the increment in average TL was relatively larger at lower water temperatures than at higher water temperatures. The largest average increment was found in group 1 at 18.55 mm. In groups 4 and 5, the increment of group 5 was slightly greater than that of group 4, however, the maximum increase was found in group 5 (Table 1).

The growth of otoliths in the rearing experiment was measured as the distance between the edge of the otolith and the alizarin mark (Fig. 2). In contrast to the somatic growth, the otolith growth showed a different trend among the five groups. The largest average increment (0.21 mm) was found in group 5, and the smallest average increment (0.11 mm) was found in group 2 (Table 1).

Formation of otolith translucent - opaque zones during rearing experiment

In order to investigate the formation of otolith opaque and translucent zones, we observed the growth of otoliths from the chemical mark to the end of this experiment. In this study, the surface of the whole otolith showed different appearances among all five groups (Fig. 3).

In group 1, the growth of all the otoliths was opaque. In groups 2 and 5, the growth of otoliths was totally translucent, and in groups 3 and 4, they showed different formation patterns on the surfaces of otoliths. The otoliths in group 3 changed from translucent to opaque and back to translucent zones, while the otoliths of group 4 changed from opaque to translucent and back to opaque zones. From these results, we can infer that in the otolith nucleus, the formation of opaque and translucent zones was influenced by water temperature, where low temperature led to the formation of an opaque zone, and high temperature led to the formation of a translucent part. Moreover, the periodic change in water temperature resulted in the alternate appearance of opaque and translucent zones in the otolith nucleus.

Greyscale value of otoliths among different groups

From the results of otolith formation in this research, water temperature can affect the formation of opaque and translucent zones in the otolith nucleus. Groups 2 and 5 both showed similar results with the formation of translucent zones during the rearing experiment. However, the growth of otoliths in groups 2 and 5 showed a significant difference. The average growth in group 5 was much greater than that in group 2. The growth of the other groups also varied. We utilized the greyscale value measured using ImageJ to investigate the difference between these groups.

The average relative greyscale value of otoliths in group 1 had the greatest value among these five groups at 0.470, while group 5 showed the lowest value at 0.023. The results of the greyscale analyses (Table 2) showed that, for the same zones of the otoliths, higher water temperatures caused a lower greyscale value, while at low water temperatures, the otoliths had a higher greyscale value.

Based on the results of statistical analysis, we can see that different groups reared at the same water temperature had the same form in the growth of otolith, and the relative greyscale value did not show any difference. Although there was no significant difference found between group 2 and the high temperature period of group 3, we can also see that the relative greyscale value was obviously higher (Fig. 4).

Table 1 Fish body length and otoliths size (mean and SD) of marbled flounder in 5 groups (mm)

Group	Body size	Somatic increment	Otolith size	Otolith increment
1	62.95 ±4.53	18.55	0.99 ±0.08	0.14
2	52.53 ±4.27	8.13	0.96 ±0.04	0.11
3	55.27 ±3.92	10.87	0.97 ±0.05	0.15
4	59.76 ±6.33	15.36	0.99 ±0.06	0.18
5	61.73 ±6.13	17.33	1.02 ±0.06	0.21

The variations in body size among each group were tested by ANOVA. The growth of fish showed a significant difference in these five groups ($F = 80.05$ ***, $P < 0.001$). Pairwise comparisons were conducted using Tukey's HSD test, The p- values were: 1 vs. 4, 1 vs. 5, 2 vs. 3 and 4 vs. 5, $P > 0.05$; the other pairs, $P < 0.001$.

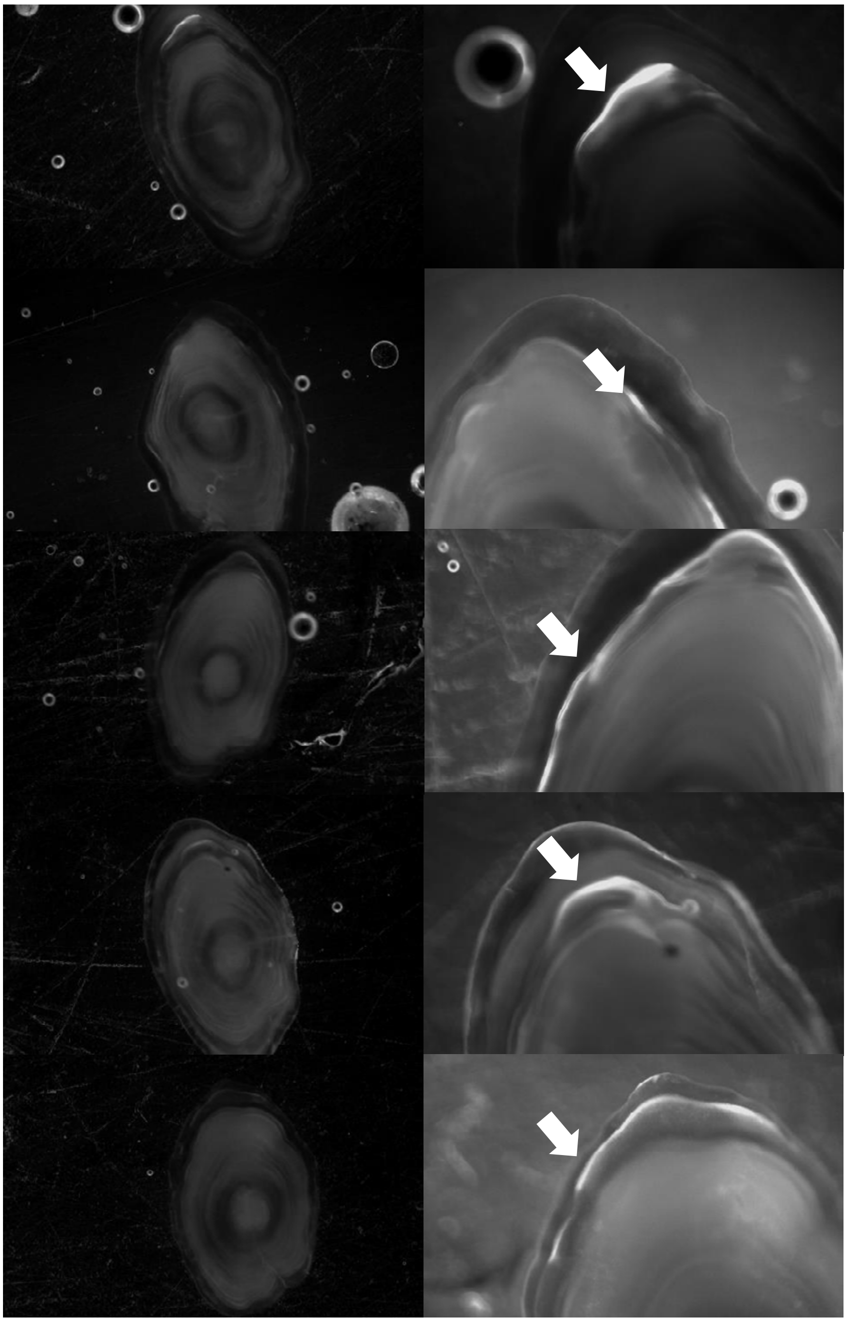

Fig. 2 The otoliths of marbled flounder under UV light (WIG filter). Calibrated images of otoliths were captured in 4x and 10x respectively. The white arrows indicate the ALC marks during experiment.

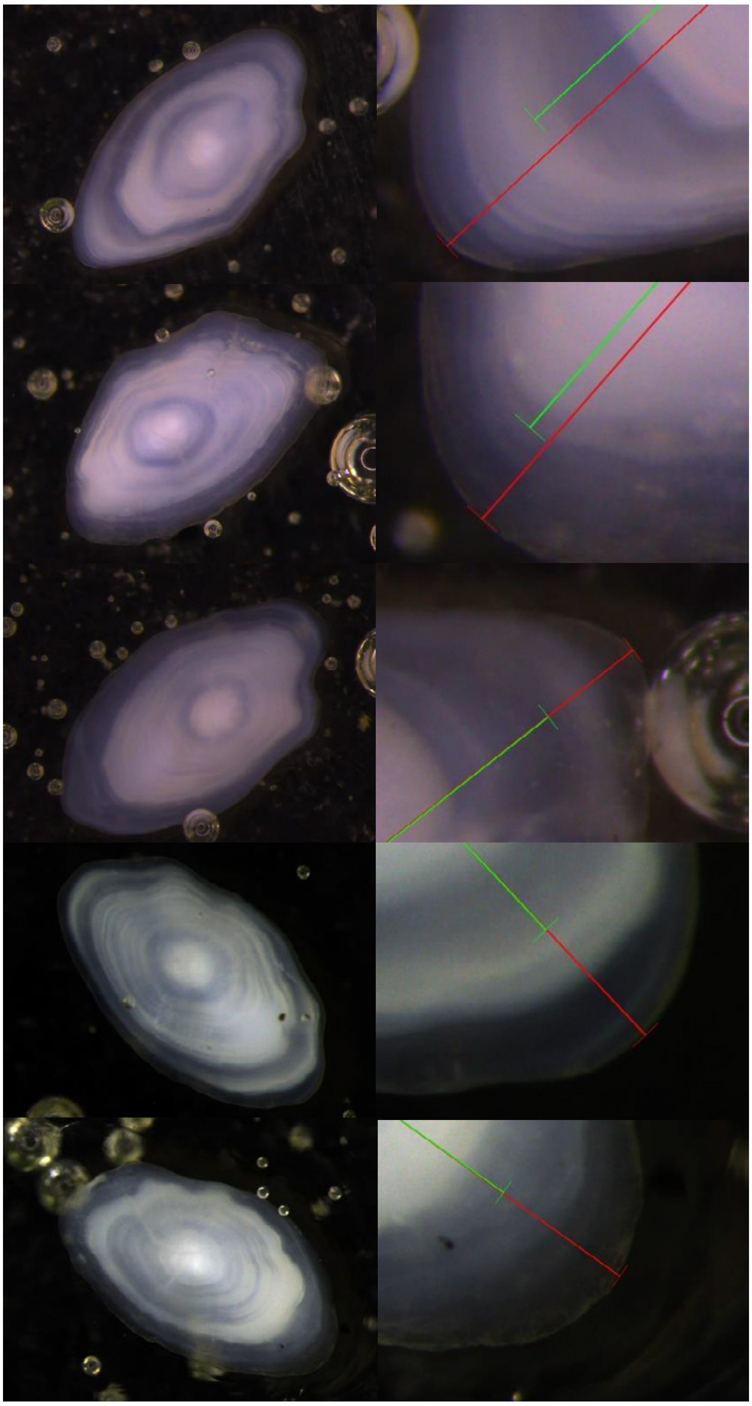

Fig. 3 The otoliths of marbled founder under normal reflecting light. Photographs of each otolith were captured in 4× and 10× magnification. The white arrowhead line indicates the growth of the otolith during the rearing experiment.

Discussion

Fish growth

In the present study, the marbled flounder had the largest growth at 15 °C, and growth had a positive correlation with water temperature when it was higher than 15 °C. Generally, fish somatic growth is affected by the ambient water temperature and feeding, and food consumption is also correlated with water temperature (Takahashi et al. 1987; Wooton et al. 1990; Fonds et al. 1992; Iwata et al. 1994; Imsland et

Table 2 The relative greyscale value of marbled flounder otoliths (mean and SD)

Group	1	2	3H	3L	4L	4H	5
Relative greyscale value	0.470 ± 0.187	0.164 ± 0.051	0.062 ± 0.050	0.406 ± 0.086	0.419 ± 0.125	0.034 ± 0.030	0.023 ± 0.013

The variation of greyscale value among different water temperature was tested by ANOVA. Where Group 3H and 4H were the growth of otolith in high temperature of group 3 and 4, group 3L and 4L were the growth of otolith in low temperature of group 3 and 4. There is significant difference confirmed in these 7 groups ($F = 61.56$ ***, $P < 0.001$). Pairwise comparisons were conducted using Tukey's HSD test, the p values were: 1 vs. 3L, 1 vs. 4L, 2 vs. 3H, 3H vs. 4H, 3H vs. 7, 3L vs. 4L, 4H vs. 7, $P > 0.05$; 2 vs. 4H, 2 vs. 7, $P < 0.05$; the other pairs, $P < 0.001$.

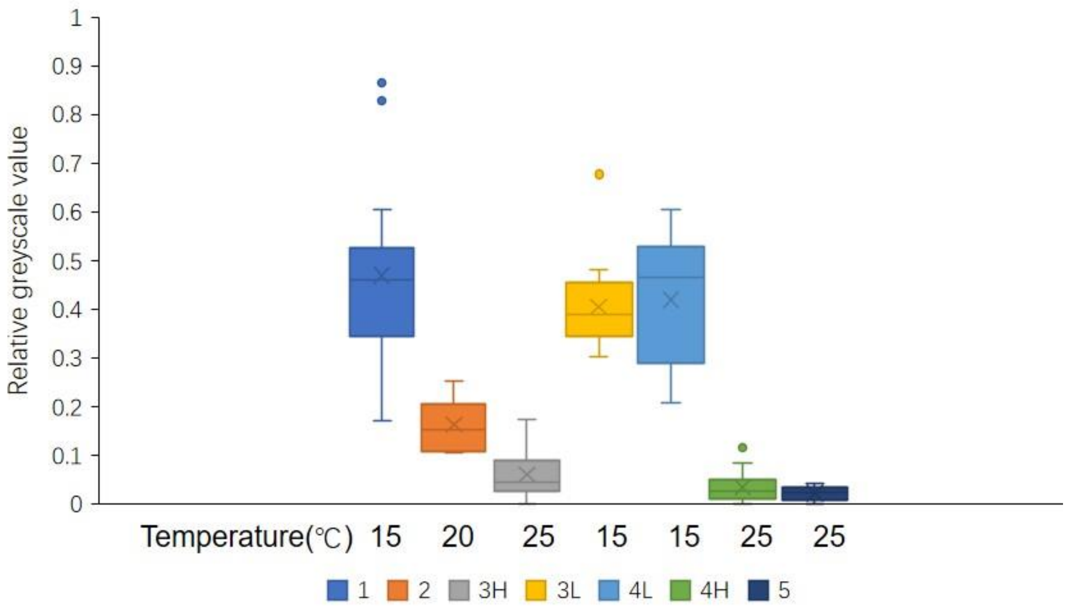

Fig. 4 Relative greyscale value of each part in rearing experiment (mean and SD) in the marbled flounder otolith. Group 3H and 4H were the growth of otolith in high temperature of group 3 and 4, group 3L and 4L were the growth of otolith in low temperature of group 3 and 4. The relative greyscale value in group 2 is obviously higher than group 3H, 4H and 5.

al 1996; Jonassen et al. 1999; Yamashita et al. 2001; Schram et al. 2013; Jph et al. 2013; Laurel et al. 2014; Hata et al. 2016). The growth of marbled flounder increased with temperatures up to the optimal temperature and decreased below 5 and above 25 °C (Takahashi et al. 1987). Feed conversion efficiency was greatest at 20 and 16, and lowest at 26 °C (Kusakabe et al. 2017). Researches into the age and growth of the marbled flounder, suggested that body size at same age was greater in warm areas than in cold areas. However, the fishery pressure was sometimes higher in warm temperatures, and higher fishery pressure would cause a lower density in warm temperatures, so that the fish in warm temperature were able to grow better (Hong et al. 2019). In this study, the growth of juveniles showed a different trend: the lowest growth was at 20 °C, and the highest was at 15 and 25 °C, while the density in each group did not show a significant difference. Based on these results, we suggested that water temperature can affect the growth rate of the marbled flounder, but there are still some more factors can affect it which may explain the difference between the results in this research and others (Kooka et al. 2000).

The formation patterns of otoliths

Otolith growth is different from somatic growth in response to environmental factors (Lombarte et al. 1993). From other studies, otolith growth showed a different extracellular process compared with somatic cellular growth (Simkiss 1974). The shape of otolith is affected by many environmental, genetic and ontogenetic

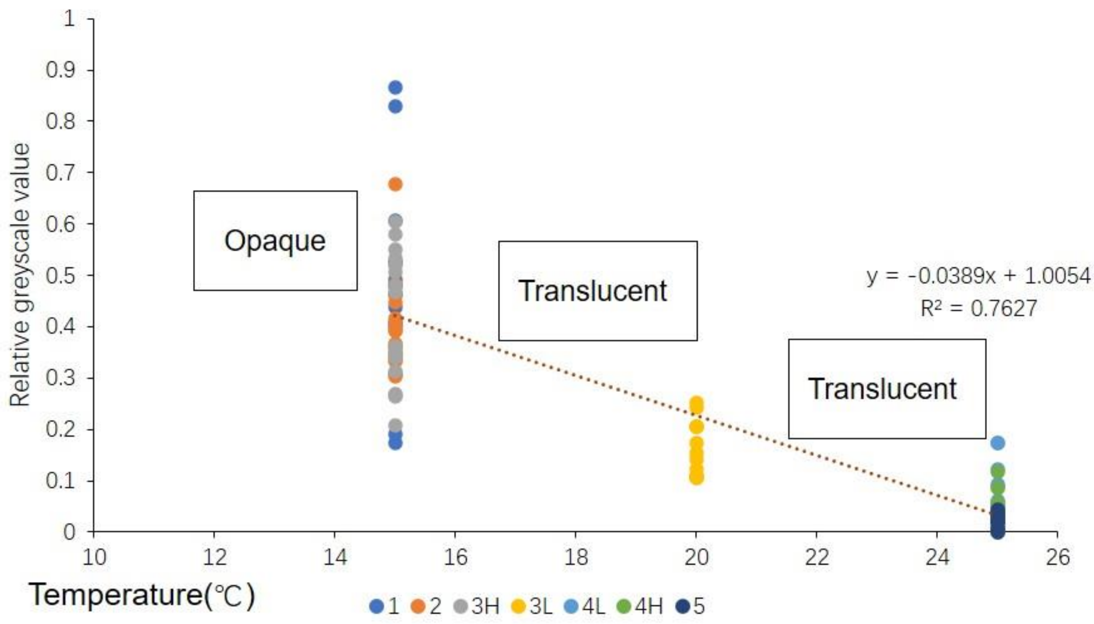

Fig. 5 Relative greyscale value of each part in rearing experiment. Group 3H and 4H were the growth of otolith in high temperature of group 3 and 4, group 3L and 4L were the growth of otolith in low temperature of group 3 and 4. The relative greyscale value was negatively correlated with water temperature, and there was a nearly linear relationship between water temperature and relative greyscale in otolith nucleus.

factors, but the fish body is not such sensitive as otolith, that we can assumed the formation process of otolith is more directly affected by temperature than the metabolic process in somatic growth (Casselman et al. 1990; Campana et al. 1993; Hüssy 2008; Vignon et al. 2010; Capoccioni et al. 2011; Afanasyev et al. 2017; Mahé et al. 2019a; Mahé et al. 2019b). Water temperature regulated the quantity of material deposited during the formation of the otolith, and an increase in the proportion of carbonates was found in otoliths as the temperature increased (Gauldie et al. 1980; Lombarte et al. 1993). In the present research, otoliths in group 2 (20 °C) had the lowest growth, and in the other groups, the growth of otoliths was greater at higher water temperatures. At early life stage, somatic growth sometimes peaks at low temperature, which is different from adult stage, but the growth of otolith in this study showed positive correlation with temperature, which may suggest that the otolith growth is more affected by water temperature than somatic growth.

Difference in macrostructure of opaque – translucent zones

It was reported in other studies that the formation of opaque and translucent zones in otoliths was caused by factors such as the calcium carbonate ratio and elemental ratios (Wright et al. 2002), which led to a different crystal matrix density in fish otoliths. In this study, however, in the otolith nucleus of the marbled flounder, water temperature influenced not only the formation of opaque and translucent zones, but also the greyscale value in the experimental zones. Although the growth in group 2 (20 °C) was slower than for the fish in group 1 (15 °C), a translucent zone was still formed in the otolith nucleus. When the water temperature rose beyond the optimum level which caused a slower growth (25 °C), the otolith nucleus also formed as a translucent zone. The relative greyscale value decreased with the increase in water temperature (Fig. 5). Otoliths at 20 and 25 °C both formed translucent zones. The relative greyscale value in 25 was higher than that at 20 °C, which implied that the higher temperature could make the otoliths denser in nucleus zones.
In order to determine how greyscale varies in different temperature regimes, the analysis of the protein matrix ratio and calcium carbonate ratio should be investigated in future research. Besides water temperature, the effect of salinity on the formation of otolith nucleus in the marbled flounder, as an euryhaline species, also needs to be investigated in the future.

Conflict of interest The authors declare that they have no conflict of interest.

Authors' contributions PH carried out the experimental design, experiments, data analysis and writing of manuscript; SJ contributed to the rearing experiments, data collection, data analysis, and participated in the experimental design; SK carried out the ALC marking experiment, contributed to conception, supervision, experimental design, and helped to draft the manuscript. All authors read and approved the final manuscript.

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