Otolith microstructure of Arabesque greenling *Pleurogrammus azonus* a species with a long embryonic period

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Abstract:

We investigated the relationship between morphological development in the embryonic stage and the formation of otolith microstructure in Arabesque greenling *Pleurogrammus azonus*, and then validated that the otolith increments were formed on a daily basis. Increments were more consistent to count and measure in sagittal otoliths from wild caught larvae compared to lapillar otoliths, and we suggest that sagittal otoliths as suitable for otolith microstructure analysis. This study clarified the formation time of three prominent increments on the sagittal otolith. First prominent increment (approx. 17 μm in otolith radius (OR)) was coincident with the time of eye-pigmentation in the embryonic stage. Second (approx. 36 μm OR) was considered to be the hatch increment and the third (approx. 38 μm OR) was at the time of transition from endogenous to exogenous nutrition. The third prominent increment which was the clearest radius (termed below as the check) was used as the starting point for validating the daily increment formation in sagittal otolith, and also there was no significant difference in the check radius among reared (6, 8, and 10 ^{o}C) and wild caught larvae. The relationship between number of days after hatching and the number of increments formed after check was significant and the slope of the regression line was not different from 1, validating the assumption that growth increments are formed on a daily basis in sagittae of *P. azonus*. The check was formed on the otolith at the transition from endogenous to exogenous nutrition, and the number of days required prior to its formation varied with water temperature. For the species in which the check is formed at the nutritional transition from endogenous to exogenous feeding, the relationship between number of days to check formation and water temperature is essential to estimate the hatch date of wild caught individuals.

Keywords: Otolith microstructure, Arabesque greenling, *Pleurogrammus azonus*, Long embryonic period, Validation

1. INTRODUCTION

Year-class strength is affected by survival during the early life history of fishes (Houde, 1987). Otolith microstructure is useful for determining the early life histories of fishes, since many life history events, such as completion of eye pigmentation, mouth opening, hatching, transition from endogenous to exogenous nutrition, and metamorphosis, are reflected by changes in the width of increments and in the elemental composition of otoliths (Jones, 1992). The formation of otolith growth increments theoretically enables age determination with a high accuracy, often to the daily level and such studies are perhaps the most common application of microstructure examinations (Campana and Neilson, 1985). In order to estimate age of specimens from field samples, validation needs to be undertaken to determine the age when the first increment is formed and the accuracy of the increment counts. The age at the first increment deposition is best determined through laboratory rearing (Jones, 1992).

In general, fish with long embryonic periods start forming increments in the otoliths before hatching (Geffen, 1983; Tsukamoto and Shima, 1990; Narimatsu and Munehara, 1997; Morales-Nin, 2000; Joh *et al.*, 2008; Nakaya *et al.*, 2008). However there is no detailed information on the relationship between morphological development during the embryonic period and the formation of the otolith microstructure.

Arabesque greenling *Pleurogrammus azonus* is one of the most important fishery resources in northern Japan, and since 2010 its population size has decreased markedly around Hokkaido (Takashima *et al.*, 2016). In the Sea of Japan and Okhotsk Sea around Hokkaido, Arabesque greenling recruit to the fishery stock before becoming 1-year-old and a high proportion of the fishery catch consists of 0- and 1-year-old fishes (Hoshino *et al.*, 2009; Takashima *et al.*, 2013). Therefore, the early determination of

year class strength is indispensable for the fisheries management of this species. A number of early life studies enabling the determination of year class strength have already been carried out: main spawning grounds are located in coastal reefs in the Sea of Japan around Hokkaido (Irie, 1983; Fig. 1) and they spawn in water temperatures ranging from 10–13 ^{o}C (Kambara, 1957: from early October to early November in the Sea of Japan off northern Hokkaido, and from middle November to middle December in the Sea of Japan off southern Hokkaido). The eggs (2.3–2.7 mm in diameter) are demersal and adhere strongly to one another to form egg masses, and the incubation period is about two months at a water temperature of approximately 10 ^{o}C (Yusa, 1957). The larvae have been collected by plankton net in the surface layer in the Sea of Japan around Hokkaido and Tsugaru Strait from January to February (Suzuki *et al.*, unpublished data; Toyonaga *et al.*, unpublished data, Fig. 1). However, little information is available on age and growth in the early life stage of this species.

This study investigated the relationships between morphological development and the formation of otolith microstructure especially in the embryonic stage and validated that otolith increments were formed daily.

2. MATERIALS AND METHODS

2.1. Sampling of the wild larvae

Pleurogrammus azonus larvae were collected with a plankton net (mouth diameter: 80 cm; mesh aperture: 330 μm) at ca. 1 m depth from the sea surface in the sampling area off Matsumae (Fig. 1) by the T/S *Ushio-maru*, Hokkaido University during 14–15 January and 15–16 February, 2013. Theilacker (1980) and Fox (1996) reported that lengths of fish larvae shrunk more when subjected to abrasion in capture nets compared

with fixing or preservation of undamaged larvae. In 80–90% ethanol solution, there is no typical shrinkage (Theilacker, 1980; Oozeki *et al.*, 1991), and Joh *et al.* (2003) suggested that 90% ethanol is a better overall preservative, particularly for > 5mm NL fish larvae. In this study all larvae collected were preserved in 90% ethanol solution (ethanol : distilled water = 9 : 1) and we used individuals that during collection were only minimally damaged. Water temperature was measured with a CTD (SBE-19 plus, Sea-Bird Electronics Inc., Bellevue, USA) at some stations in the sampling area.

2.2. Laboratory experiments

Exp. 1: Observations of embryonic development and otolith growth

We firstly investigated in otoliths of pre-hatch embryos, the relationship between the occurrence and timing of prominent increments with the morphological development and otolith size. Broodstock of *P. azonus* were caught with trap net and angling in the sampling area off Matsumae (Fig. 1). Eggs were obtained by stripping the females. Sperm were obtained from the testis by dissecting the males. The eggs (diameter: 2.3 ± 0.11 mm (mean ± standard deviation (SD)) and sperm were mixed in a bowl, and then transferred to a hatching jar. Temperature has been considered as one of the major factors controlling development rate of many species (Heath, 1992). Rearing temperatures that were representative of the conditions during the spawning period in the Sea of Japan, coast of Hokkaido (Kambara, 1957; Irie, 1983) were set as constant at 8, 10, and 12 ºC. The eggs were incubated in 30 L tanks at each water condition. The rearing water temperature and salinity were monitored (Table 1) by making daily measurements using a conductivity meter (YSI Pro30, YSI/Nanotech Ltd.). The light condition was 12 hours light (08:00–17:59) and 12 hours dark (18:00–07:59).

115 Embryonic development stages and growth patterns (otolith formation, eye
116 pigmentation, and mouth opening) were identified based on Yusa (1957).
117 Over 20 eggs per daily sampling for each rearing condition were observed for the
118 embryonic development stages and growth patterns (otolith formation, eye pigmentation,
119 and mouth opening) until hatching.
120 Exp. 2: Observations of larval and otolith development, yolk-sac absorption, and
121 feeding activity
122 Secondly, we investigated in otoliths of hatched larvae the relationship between the
123 occurrence and timing of prominent increments with the morphological development
124 and otolith size. Eggs were incubated at 8 ºC until hatching. After hatching, larvae
125 were reared in 10 L cylindrical tanks with set temperature conditions (6, 8, and 10 ºC:
126 representative of the conditions during larval period in the Sea of Japan, coast of
127 Hokkaido (Suzuki *et al.,* unpublished)) in 150 L water baths. The light conditions
128 were the same as in Exp. 1. The larvae were fed rotifers which had been enriched with
129 Super Capsule Powder (Chlorella Industry Co. Ltd., Fukuoka, Japan), two times a day
130 at 08:00 and 15:00 through the rearing period. Three to five larvae were sacrificed
131 every day up to the stage when they had consumed their yolk-sac in order to measure
132 the yolk-sac volume (see section 2.3). Furthermore, we observed the frequency of
133 occurrence of feeding at each rearing temperature up until almost all of the larvae had
134 started feeding.
135 Exp. 3: Somatic growth and otolith increment validation
136 Thirdly, we investigated whether otolith micro-increments are deposited daily after
137 hatching by rearing larvae in a 200 L circular tank at 10.2 ± 1.12 ºC (mean ± SD). The
138 fish were fed rotifers cultured with Marine Glos EX (Marine Tech, Co. Ltd., Aichi,

Japan; 1–25 days after hatching; DAH), *Artemia* sp. nauplii enriched with Super Capsule Powder (20–70 DAH), and frozen mysid (66–70 DAH). The light conditions were the same as Exp. 1. Five to twenty fish were randomly collected at 10 day intervals until 70 DAH.

2.3 Measurements of otolith microstructure and yolk-sac volume estimation

Eggs and larvae preserved in 90% ethanol solution obtained from Exps. 1–3 were used. We measured the notochord length (NL) of preflexion and flexion larvae of *P. azonus* and the standard length (SL) of postflexion larvae to the nearest 0.1 mm using an electric slide caliper under a stereo microscope, with both measures being referred to as body length (BL) in this report. Otoliths were extracted and prepared for the daily increment validation process. Notochord length (NL), yolk-sac sizes ($Volume = 3/4\ \pi\ (L/2 \times H/2 \times W/2)$, where L, H and W are yolk-sac length, height and width, respectively) were measured to the nearest 0.1 mm under a stereo-microscope. Lapillar and sagittal otoliths were removed from each larva and mounted on a glass slide with epoxy resin. All counts, measurements, and observations of otoliths were conducted with a binocular microscope connected to a video monitor. Otoliths were observed with translucent light at 1000 x magnification (100 x objective lens with 10 x eye-piece lens). Otolith radius (the longest distance from the center of the core to the edge of the sagitta) and daily increments were counted and measured using otolith microstructure analysis software (ARP Ver. 5.27, Ratoc System Engineering Co. Ltd., Tokyo, Japan). These measurements were repeated three times by different researchers and adopted when two or more counts of the number of increments agreed. The mean increment width was used as the data point for the individual.

163

164 **3. RESULTS**

165 *3.1. Otolith features of wild larvae of P. azonus*

166 Mean larval size of wild *P. azonus* was 8.5 ± 0.96 mm NL (± standard deviation
167 (SD)) collected from the sea surface with 9.4–9.7 ^{o}C and salinity 33.9–34.0 during
168 14–15 January, 2013. During 15–16 February, 2013, mean larval size was
169 11.4 ± 1.88 mm NL with 8.0–9.0 ^{o}C and 34.0–34.2. A pair of lapillar and sagittal
170 otoliths were visible in each larva but the asteriscus could not be located. Multiple
171 primordia (Fig. 2a,b) were observed in lapilli (23%) and sagittae (9%) from 90 pairs of
172 otoliths. These multiple primordia tended to lead to some abnormalities in the
173 increment structure, however their occurrence was low in sagittae. Therefore, sagittal
174 otoliths were used for further observations of daily increment analysis.

175 There were three prominent increments on the sagittal otoliths. The first prominent
176 increment was 17 ± 2.6 μm (mean ± SD in otolith radius (OR)), second was
177 36 ± 1.9 μm OR, and third was 38 ± 1.5 μm OR (Fig. 2c). The second prominent
178 increment was harder to differentiate in comparison with the first and third, therefore
179 the increments which are outside of the third prominent were counted and measured.
180 Mean increment width that formed the 2–7 rings outside of the third prominent
181 increment was 0.8 ± 0.16 μm (± SD).

182

183 *3.2. Laboratory experiment*

184 Exp. 1: Embryonic development, and otolith growth

185 The number of days required to complete morphological developments was different
186 among the rearing water temperatures (Table 2). Otolith formation in 8, 10, and 12 ^{o}C

was first completed from 23, 13, and 11 DAF, respectively. The first eye pigmentation day occurred on 32, 21, and 20 DAF, mouth opening was observed on 49, 36, and 35 DAF, and newly hatched larvae were first observed on 58, 46, and then 39 DAF in 8, 10, and 12 °C, respectively. Newly hatched larvae had eyes with guanine pigmentation and open mouths. Notochord lengths (mean ± SD) of newly hatched larvae were 9.6 ± 0.33 mm for larvae reared at 8 °C, 9.6 ± 0.16 mm for larvae reared at 10 °C, and 9.5 ± 0.48 mm for larvae reared at 12 °C. There was no significant difference in the NL among the three rearing temperatures (8, 10, and 12 °C: one way-ANOVA, $p = 0.42$).

The equations between DAF and sagittal otolith radius (OR) were as follows (8 °C: $OR = 0.65DAF - 1.50$ ($r^2 = 0.93$, $p < 0.001$, $N = 21$); 10 °C: $OR = 0.72DAF + 1.86$ ($r^2 = 0.95$, $p < 0.001$, $N = 16$), and 12 °C: $OR = 1.01DAF - 3.78$ ($r^2 = 0.93$, $p < 0.001$, $N = 30$); Fig. 3). Otoliths grew more rapidly at higher water temperature (ANCOVA, $p < 0.001$).

The first prominent increment at approx. 17 μm OR corresponded to the start of the eye-pigmentation process (8–12 °C, 20–32 DAH, 17–19 μm OR). The second prominent increment (approx. 36 μm OR) did not correspond to mouth opening (8–12 °C, 35–49 DAH, 28–32 μm OR) but it corresponded to hatching (8–12 °C, 39–58 DAH, 35–36 μm OR).

Exp. 2: Larval and otolith development, yolk-sac absorption, and feeding activity

Under 6, 8, and 10 °C conditions, the larvae had already absorbed the yolk (with yolk volume < 0.01 mm^3) at 9, 8, and 7 DAH, respectively. The first feeding was initiated 8, 7, and 6 DAH in 6, 8, and 10 °C, respectively. The third prominent increment was observed on 9, 8, and 6–7 DAH in 6, 8, and 10 °C, respectively (Fig. 4).

211 Their sagittal otolith sizes were 38 ± 1.0 μm OR for 6°C (N = 16), 39 ± 1.0 μm OR for
212 8 °C (N = 20), and 38 ± 1.0 μm OR for 10 °C (N = 21), and hereafter called the "check".
213 There was no significant difference in the check radius among the three rearing
214 temperatures (6, 8, and 10 °C) and wild caught larvae (38 ± 1.5 μm OR; one
215 way-ANOVA, p = 0.31).

216 Exp. 3: Somatic growth and otolith increment validation

217 The relationship between BL and DAH is shown in Fig. 5a. Mean BL at 0 DAH
218 larvae was 10.3 ± 0.74 mm (± SD), and 16.5 ± 0.35 mm at 40 DAH. Mean increment
219 width in sagittal otoliths was 0.8 ± 0.24 μm from 6–7 DAH to 40 DAH (Fig. 5b). The
220 minimum width was 0.3 μm, and < 0.35 μm was observed in only 0.3% of increments.
221 After 50 DAH (18.1 ± 0.41 mm BL), the growth increased drastically and all
222 individuals collected at 70 DAH (28.3 ± 1.60 mm BL) had already reached the juvenile
223 stage. Their increment widths increased drastically from 50 DAH (2.0 ± 0.53 μm;
224 mean ± SD) in flexion stage larvae to 70 DAH (3.3 ± 0.84 μm) in juveniles. The third
225 prominent increments (check; 37 ± 1.9 μm OR), which was used as the starting point of
226 otolith increments count, were observed in 6–7 DAH. The relationship between DAH
227 and the number of increments formed after check (NIAC) is $NIAC = 0.99DAH - 6.18$
228 ($r^2 = 0.99$, $p < 0.001$, N = 49; Fig. 6). The slope of the regression line was not
229 different from 1 (*t*-test, p = 0.09).

230

231 **4. DISCUSSION**

232 *4.1. Relationship between the morphological development in embryonic-larval stage*
233 *and the otolith microstructure*

234 Species with long embryonic periods have been reported to start forming increments

235 in the otoliths before hatching (Geffen, 1983; Tsukamoto and Shima, 1990; Narimatsu
236 and Munehara, 1997; Morales-Nin, 2000; Joh *et al.*, 2008; Nakaya *et al.*, 2008). Most
237 of these workers reported that the prominent increment (check) was associated with
238 eye-pigmentation, but has been shown to form at the time of hatching in fat greenling
239 *Hexagrammos otakii* (Joh *et al.*, 2008), which is a closely-related species of Arabesque
240 greenling *P. azonus*. Otolith microstructure observations in *P. azonus* showed that
241 some increments were already formed before hatching. This study clarified the timing
242 of the formation of three prominent increments on sagittal otoliths. The timing of
243 formation of the first prominent increment (approx. 17 μm OR) was coincident with the
244 time when eye-pigmentation occurred in the embryonic stage. The second prominent
245 increment (approx. 36 μm OR) agreed with the day of hatching, and was considered to
246 be the hatch increment, though the second prominent increment was harder to
247 differentiate in comparison with the first and third, while the third (approx. 38 μm OR)
248 occurred at the time of transition from endogenous to exogenous nutrition.

249 Microstructural features formed during the early life stages may be the result of
250 changes in physiological metabolism, stress, and/or growth cycles that are generally
251 associated with these transitions (Campana and Neilson, 1985). Especially the check
252 formation at the first feeding stage might be related to a shift from endogenous nutrition
253 to exogenous feeding and related to circadian activity patterns. It was noted by
254 Campana (1983) that stress can cause an inhibition of calcium uptake from the water
255 which significantly reduces the calcium deposition in the otolith, resulting in check
256 formation. The third prominent increment (approx. 38 μm OR) in sagittal otolith was
257 the most prominent increment and was used as the starting point (check) for validating
258 the daily increment formation for *P. azonus*. This check radius coincided with the

259 transition from endogenous to exogenous nutrition and relates to a life history event as
260 found in other species (Hayashi *et al.,* 1989; Maillet and Checkley, 1990; Joh *et al.*,
261 2005).
262
263 *4.2. Validation of daily increment formation*
264 To estimate age in wild caught larvae and juveniles, two pieces of information must
265 be known: the age at first increment formation (check) and the accuracy of the
266 increment deposition rate (Jones, 1992). In this study for *P. azonus*, the third
267 prominent increment was used as the starting point for validating the daily increment
268 formation in sagittal otoliths. There was no significant difference in the check radius
269 among reared (6, 8, and 10 ^{o}C), and wild caught larvae, though the timing of the check
270 formation was different between rearing temperature conditions. Temperature is a
271 major determinant of somatic growth and metabolic rate where higher temperature
272 induces faster somatic growth and metabolic rate up to a certain limit (Hare and Cowen,
273 1995). In the higher temperatures, the check was deposited earlier which seems to be
274 due to the elevated metabolic rate. Life history events such as hatching and first
275 feeding are also regulated by temperature, since it plays an important role in embryonic
276 and larval development stage. The mean increment width of larval *P. azonus* was
277 0.8 ± 0.24 μm (± SD) and also 99.7% of increment measurements were above the
278 resolution limits of light microscopes (0.3 μm, Fox *et al.,* 2003; 0.35 μm, Campana *et*
279 *al.,* 1987). Therefore, it is possible to count the number of increments.
280 In this study, we investigated the relationship between the morphological
281 development and otolith formation in the range of water temperatures experienced by
282 eggs in the field. Life history events (i.e. completion of eye pigmentation, mouth

opening, hatching, transition from endogenous to exogenous nutrition) corresponded to prominent increments, and the otolith size did not vary even if water temperature changed. The present study clarified the duration under 3 different temperature conditions and established the essential basis of growth analysis and hatch date estimation of wild caught *P. azonus* larvae and juveniles. For species with a check formation at a transition from endogenous to exogenous nutrition, understanding of the duration from hatching to the check formation and the effect of the water temperature on its formation is requisite data for the hatch date estimation of wild caught individuals.

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372

373 Fig. 1. Major spawning grounds (Irie, 1983) and horizontal distribution of larvae and
374 juveniles of *P. azonus* in the Sea of Japan and Tsugaru Strait (Suzuki *et al.*,
375 unpublished data; Toyonaga *et al.*, unpublished data). Location of the sampling area
376 of *P. azonus* larvae by T/S *Ushio-maru* off Matsumae on January 14–15 and February
377 15–16 in 2013.

378 Fig. 2. Otoliths of wild caught *P. azonus* larvae that were collected off Matsumae from
379 January to February 2013 (a, b: 9.7 mm in notochord length (NL), c: 12.9 mm NL).
380 Lapillus with multiple primordia (pointed by arrows; a) and sagitta (b). Three
381 prominent increments in the sagittal otolith of a wild caught *P. azonus* larva (c).
382 Otolith radii of first, second and third prominent increments (1^{st}, 2^{nd}, and 3^{rd}) were
383 17 ± 2.6 μm OR, 36 ± 1.9 μm OR, and 38 ± 1.5 μm OR (mean ± standard deviation),
384 respectively.

385 Fig. 3. Relationship between days after fertilization (DAF) and otolith radius (OR) of
386 sagittae. The regression lines for 8 ºC: $OR = 0.65DAF - 1.50$ ($r^2 = 0.93$, $p < 0.001$,
387 N = 21), 10 ºC: $OR = 0.72DAF + 1.86$ ($r^2 = 0.95$, $p < 0.001$, N = 16), and 12 ºC:
388 $OR = 1.01DAF - 3.78$ ($r^2 = 0.93$, $p < 0.001$, N = 30).

389 Fig. 4. Relationship between water temperature and day of check observed (DCO)
390 after hatching. Numerals show the sample sizes.

391 Fig. 5. Relationship between days after hatching and notochord length or standard
392 length of *P. azonus* larvae (a), and changes in daily increment widths on sagittae (b).

393 Fig. 6. Relationship between the number of days after hatching (DAH) and the
394 number of otolith increments after check (NIAC).

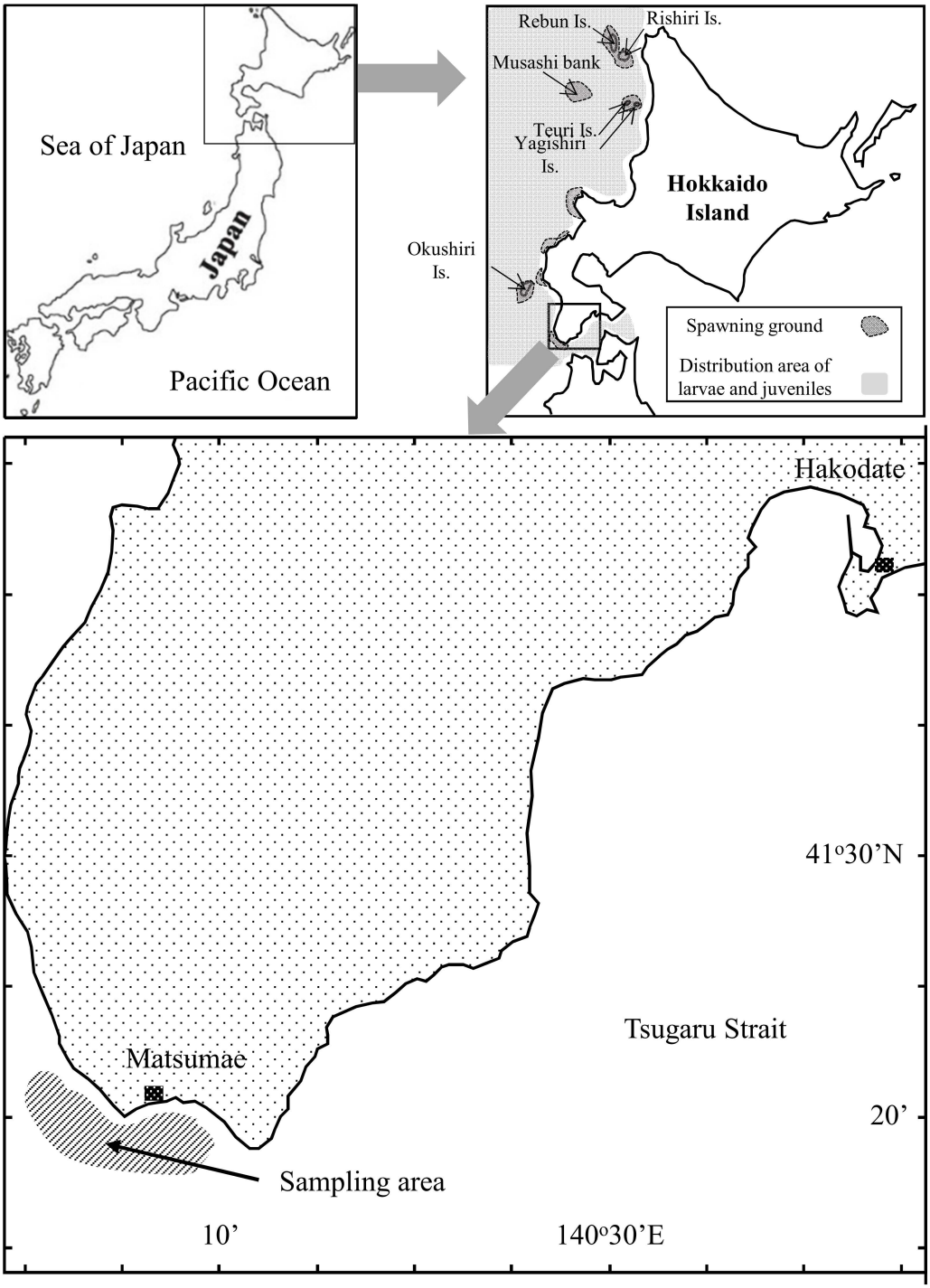

Fig. 1. Marannu *et al.*

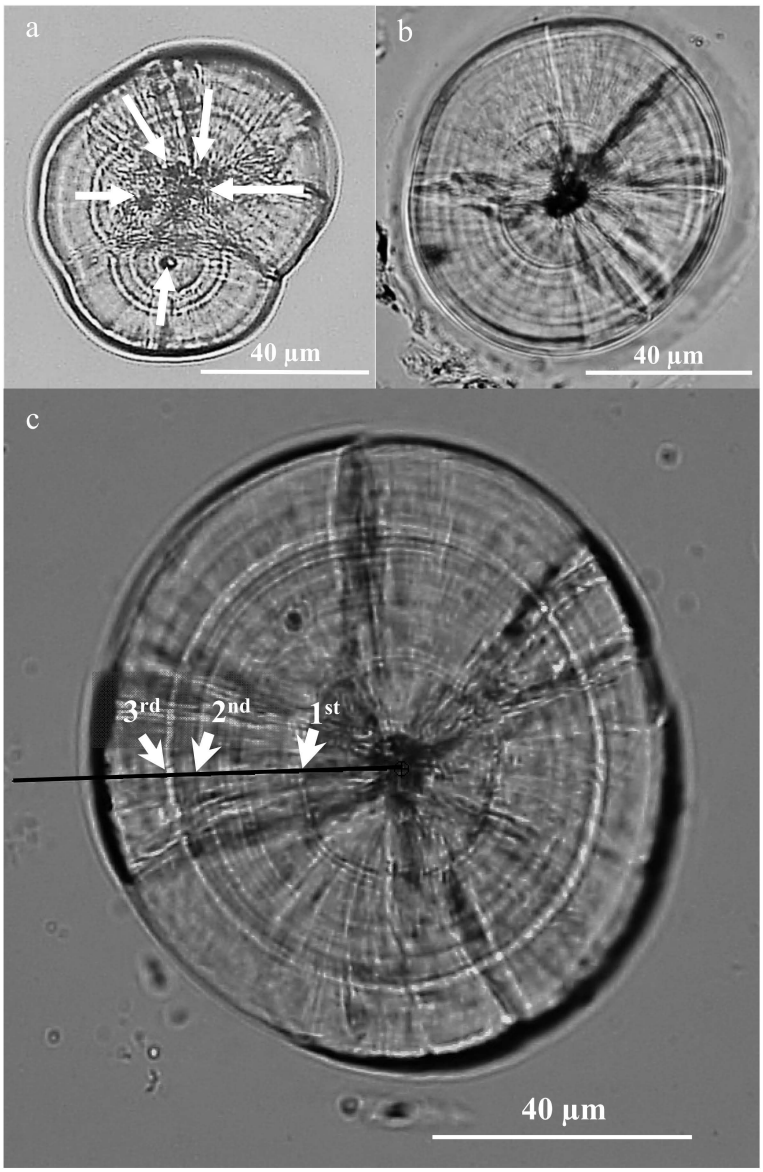

Fig. 2. Marannu *et al.*

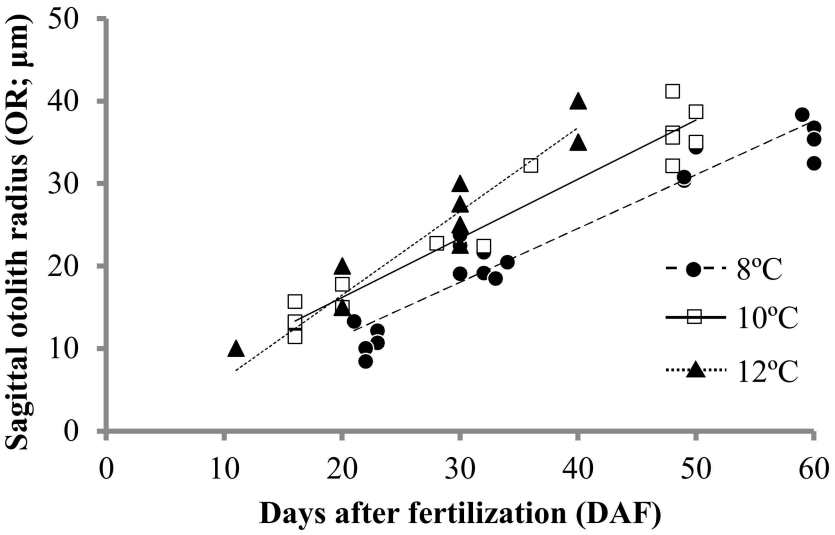

Fig. 3. Marannu *et al.*

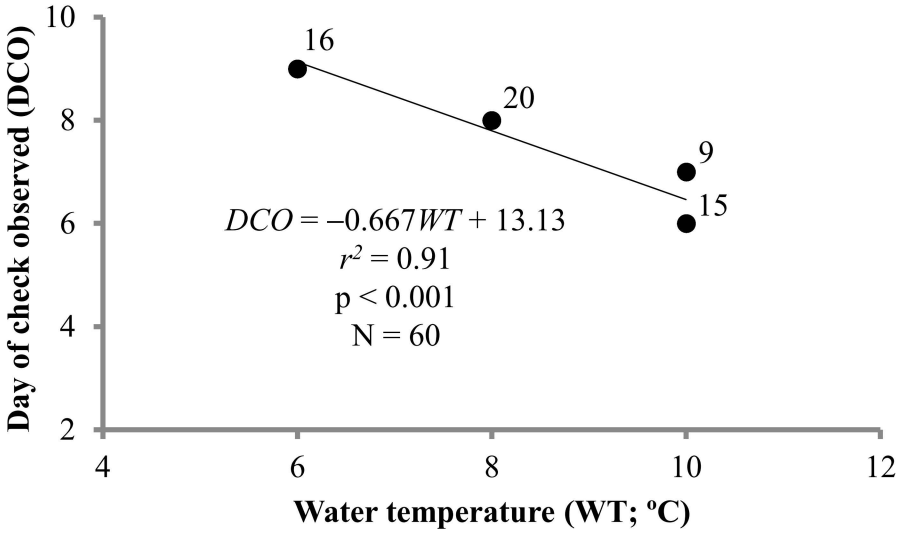

Fig. 4. Marannu *et al.*

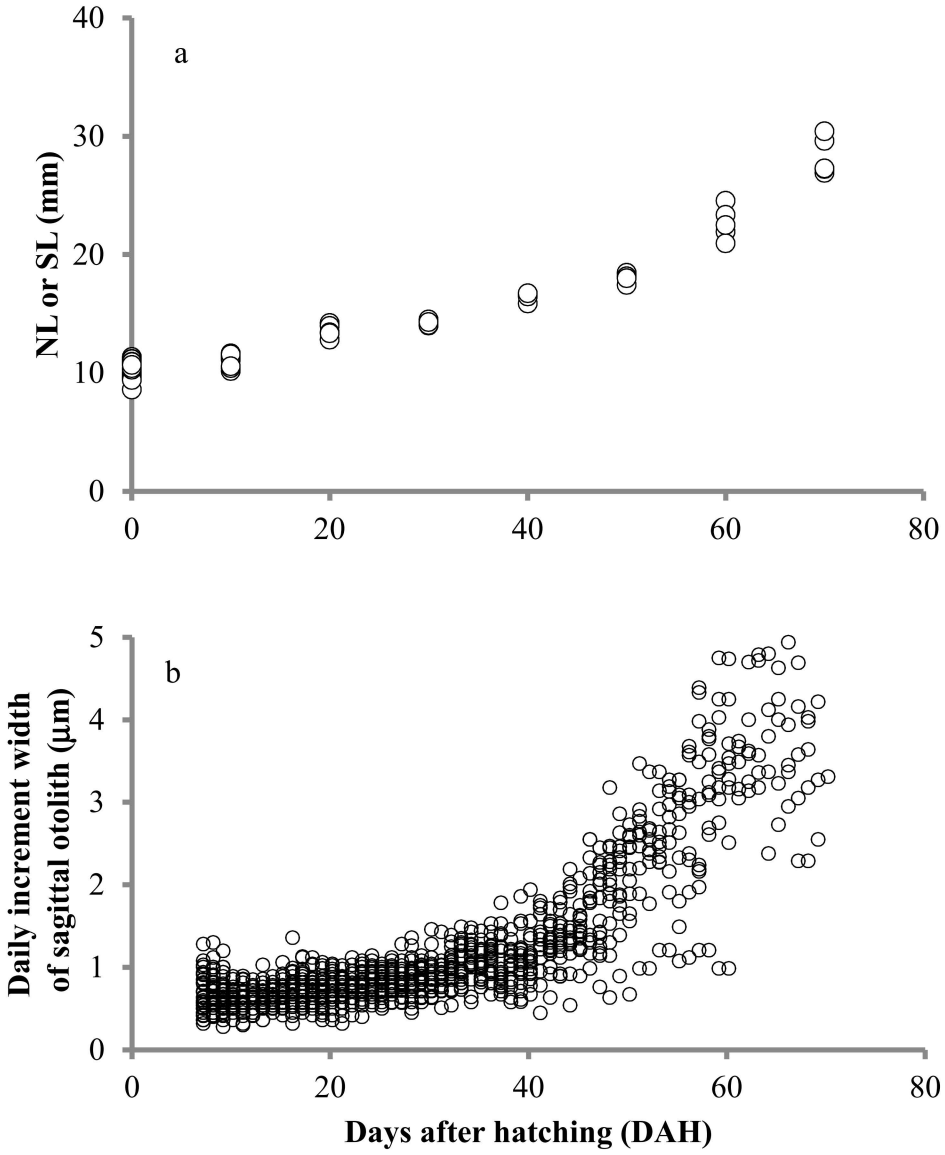

Fig. 5 Marannu *et al.*

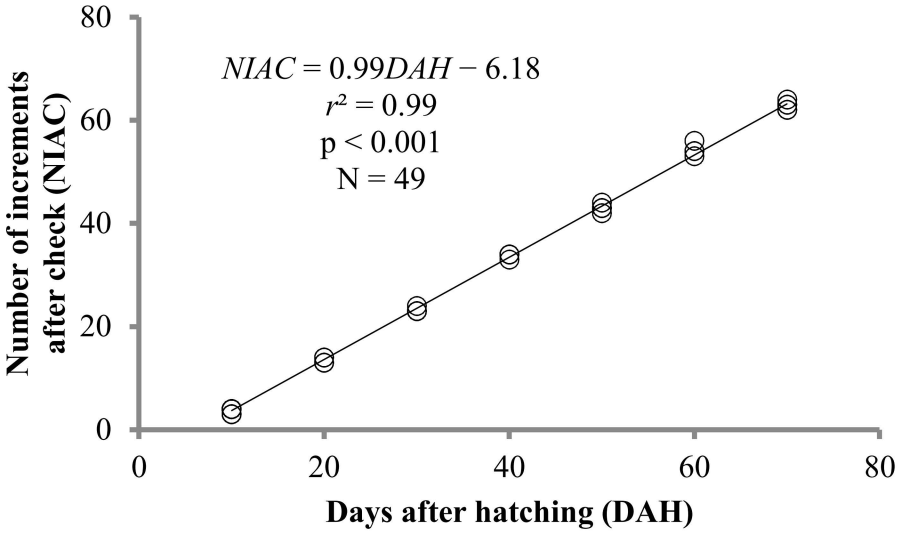

Fig. 6. Marannu *et al.*

Table 1. Temperature and salinity in each rearing tank

Exp.	Rearing condition	Temperature (ºC)	Salinity
1	8ºC	7.9 ± 0.52	33.3 ± 0.92
	10ºC	10.2 ± 0.31	33.1 ± 0.98
	12ºC	12.0 ± 0.21	34.4 ± 0.79
2	6ºC	6.0 ± 0.17	33.5 ± 1.32
	8ºC	7.9 ± 0.24	33.8 ± 0.35
	10ºC	9.9 ± 0.10	34.3 ± 1.06

*Values are shown as mean ± SD.

Table 2. The time required for morphological development in the *P. azonus* embryonic stage in days after fertilization (DAF)

Temperature condition	Otolith formation	Eye pigmentation (range)	Mouth opening	Hatching (range)
8°C	23	32–36	49	58–62
10°C	13	21–30	36	46–51
12°C	11	20–27	35	39–48