



Title	Otolith microstructure of Arabesque greenling <i>Pleurogrammus azonus</i> : A species with a long embryonic period
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Citation	Fisheries research, 194, 129-134 <a href="https://doi.org/10.1016/j.fishres.2017.06.002">https://doi.org/10.1016/j.fishres.2017.06.002</a>
Issue Date	2017-10
Doc URL	<a href="http://hdl.handle.net/2115/75599">http://hdl.handle.net/2115/75599</a>
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Type	article (author version)
File Information	nakaya.pdf



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

3

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15 **Abstract:**

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

39

40 *Keywords:* Otolith microstructure, Arabesque greenling, *Pleurogrammus azonus*, Long

41 embryonic period, Validation

42

43 **1. INTRODUCTION**

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

56 In general, fish with long embryonic periods start forming increments in the otoliths  
57 before hatching (Geffen, 1983; Tsukamoto and Shima, 1990; Narimatsu and Munehara,  
58 1997; Morales-Nin, 2000; Joh *et al.*, 2008; Nakaya *et al.*, 2008). However there is no  
59 detailed information on the relationship between morphological development during the  
60 embryonic period and the formation of the otolith microstructure.

61 Arabesque greenling *Pleurogrammus azonus* is one of the most important fishery  
62 resources in northern Japan, and since 2010 its population size has decreased markedly  
63 around Hokkaido (Takashima *et al.*, 2016). In the Sea of Japan and Okhotsk Sea  
64 around Hokkaido, Arabesque greenling recruit to the fishery stock before becoming  
65 1-year-old and a high proportion of the fishery catch consists of 0- and 1-year-old fishes  
66 (Hoshino *et al.*, 2009; Takashima *et al.*, 2013). Therefore, the early determination of

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

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

83

## 84 **2. MATERIALS AND METHODS**

### 85 *2.1. Sampling of the wild larvae*

86 *Pleurogrammus azonus* larvae were collected with a plankton net (mouth diameter:  
87 80 cm; mesh aperture: 330 µm) at ca. 1 m depth from the sea surface in the sampling  
88 area off Matsumae (Fig. 1) by the T/S *Ushio-maru*, Hokkaido University during 14–15  
89 January and 15–16 February, 2013. Theilacker (1980) and Fox (1996) reported that  
90 lengths of fish larvae shrunk more when subjected to abrasion in capture nets compared

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

98

## 99 *2.2. Laboratory experiments*

### 100 Exp. 1: Observations of embryonic development and otolith growth

101 We firstly investigated in otoliths of pre-hatch embryos, the relationship between the  
102 occurrence and timing of prominent increments with the morphological development  
103 and otolith size. Broodstock of *P. azonus* were caught with trap net and angling in the  
104 sampling area off Matsumae (Fig. 1). Eggs were obtained by stripping the females.  
105 Sperm were obtained from the testis by dissecting the males. The eggs (diameter:  
106  $2.3 \pm 0.11$  mm (mean  $\pm$  standard deviation (SD)) and sperm were mixed in a bowl, and  
107 then transferred to a hatching jar. Temperature has been considered as one of the  
108 major factors controlling development rate of many species (Heath, 1992). Rearing  
109 temperatures that were representative of the conditions during the spawning period in  
110 the Sea of Japan, coast of Hokkaido (Kambara, 1957; Irie, 1983) were set as constant at  
111 8, 10, and 12 °C. The eggs were incubated in 30 L tanks at each water condition.  
112 The rearing water temperature and salinity were monitored (Table 1) by making daily  
113 measurements using a conductivity meter (YSI Pro30, YSI/Nanotech Ltd.). The light  
114 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 °C until hatching. After hatching, larvae  
125 were reared in 10 L cylindrical tanks with set temperature conditions (6, 8, and 10 °C:  
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  $10.2 \pm 1.12$  °C (mean  $\pm$  SD). The  
138 fish were fed rotifers cultured with Marine Glos EX (Marine Tech, Co. Ltd., Aichi,

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

143

### 144 *2.3 Measurements of otolith microstructure and yolk-sac volume estimation*

145 Eggs and larvae preserved in 90% ethanol solution obtained from Exps. 1–3 were  
146 used. We measured the notochord length (NL) of preflexion and flexion larvae of *P.*  
147 *azonus* and the standard length (SL) of postflexion larvae to the nearest 0.1 mm using  
148 an electric slide caliper under a stereo microscope, with both measures being referred to  
149 as body length (BL) in this report. Otoliths were extracted and prepared for the daily  
150 increment validation process. Notochord length (NL), yolk-sac sizes  
151 ( $Volume = 3/4 \pi (L/2 \times H/2 \times W/2)$ , where *L*, *H* and *W* are yolk-sac length, height and  
152 width, respectively) were measured to the nearest 0.1 mm under a stereo-microscope.  
153 Lapillar and sagittal otoliths were removed from each larva and mounted on a glass  
154 slide with epoxy resin. All counts, measurements, and observations of otoliths were  
155 conducted with a binocular microscope connected to a video monitor. Otoliths were  
156 observed with translucent light at 1000 x magnification (100 x objective lens with 10 x  
157 eye-piece lens). Otolith radius (the longest distance from the center of the core to the  
158 edge of the sagitta) and daily increments were counted and measured using otolith  
159 microstructure analysis software (ARP Ver. 5.27, Ratoc System Engineering Co. Ltd.,  
160 Tokyo, Japan). These measurements were repeated three times by different researchers  
161 and adopted when two or more counts of the number of increments agreed. The mean  
162 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  $8.5 \pm 0.96$  mm NL ( $\pm$  standard deviation  
167 (SD)) collected from the sea surface with 9.4–9.7 °C and salinity 33.9–34.0 during  
168 14–15 January, 2013. During 15–16 February, 2013, mean larval size was  
169  $11.4 \pm 1.88$  mm NL with 8.0–9.0 °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  $17 \pm 2.6$   $\mu\text{m}$  (mean  $\pm$  SD in otolith radius (OR)), second was  
177  $36 \pm 1.9$   $\mu\text{m}$  OR, and third was  $38 \pm 1.5$   $\mu\text{m}$  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  $0.8 \pm 0.16$   $\mu\text{m}$  ( $\pm$  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 °C

187 was first completed from 23, 13, and 11 DAF, respectively. The first eye pigmentation  
188 day occurred on 32, 21, and 20 DAF, mouth opening was observed on 49, 36, and  
189 35 DAF, and newly hatched larvae were first observed on 58, 46, and then 39 DAF in 8,  
190 10, and 12 °C, respectively. Newly hatched larvae had eyes with guanine pigmentation  
191 and open mouths. Notochord lengths (mean ± SD) of newly hatched larvae were  
192  $9.6 \pm 0.33$  mm for larvae reared at 8 °C,  $9.6 \pm 0.16$  mm for larvae reared at 10 °C, and  
193  $9.5 \pm 0.48$  mm for larvae reared at 12 °C. There was no significant difference in the  
194 NL among the three rearing temperatures (8, 10, and 12 °C: one way-ANOVA,  
195  $p = 0.42$ ).

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

201 The first prominent increment at approx. 17 µm OR corresponded to the start of the  
202 eye-pigmentation process (8–12 °C, 20–32 DAH, 17–19 µm OR). The second  
203 prominent increment (approx. 36 µm OR) did not correspond to mouth opening  
204 (8–12 °C, 35–49 DAH, 28–32 µm OR) but it corresponded to hatching (8–12 °C,  
205 39–58 DAH, 35–36 µm OR).

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

207 Under 6, 8, and 10 °C conditions, the larvae had already absorbed the yolk (with  
208 yolk volume  $< 0.01$  mm<sup>3</sup>) at 9, 8, and 7 DAH, respectively. The first feeding was  
209 initiated 8, 7, and 6 DAH in 6, 8, and 10 °C, respectively. The third prominent  
210 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  $38 \pm 1.0 \mu\text{m OR}$  for  $6^\circ\text{C}$  ( $N = 16$ ),  $39 \pm 1.0 \mu\text{m OR}$  for  
212  $8^\circ\text{C}$  ( $N = 20$ ), and  $38 \pm 1.0 \mu\text{m OR}$  for  $10^\circ\text{C}$  ( $N = 21$ ), and hereafter called the “check”.  
213 There was no significant difference in the check radius among the three rearing  
214 temperatures (6, 8, and  $10^\circ\text{C}$ ) and wild caught larvae ( $38 \pm 1.5 \mu\text{m OR}$ ; 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  $10.3 \pm 0.74 \text{ mm } (\pm \text{SD})$ , and  $16.5 \pm 0.35 \text{ mm}$  at 40 DAH. Mean increment  
219 width in sagittal otoliths was  $0.8 \pm 0.24 \mu\text{m}$  from 6–7 DAH to 40 DAH (Fig. 5b). The  
220 minimum width was  $0.3 \mu\text{m}$ , and  $< 0.35 \mu\text{m}$  was observed in only 0.3% of increments.  
221 After 50 DAH ( $18.1 \pm 0.41 \text{ mm BL}$ ), the growth increased drastically and all  
222 individuals collected at 70 DAH ( $28.3 \pm 1.60 \text{ mm BL}$ ) had already reached the juvenile  
223 stage. Their increment widths increased drastically from 50 DAH ( $2.0 \pm 0.53 \mu\text{m}$ ;  
224 mean  $\pm$  SD) in flexion stage larvae to 70 DAH ( $3.3 \pm 0.84 \mu\text{m}$ ) in juveniles. The third  
225 prominent increments (check;  $37 \pm 1.9 \mu\text{m OR}$ ), 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  $\mu\text{m}$  OR) was coincident with the  
244 time when eye-pigmentation occurred in the embryonic stage. The second prominent  
245 increment (approx. 36  $\mu\text{m}$  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  $\mu\text{m}$  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  $\mu\text{m}$  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 °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  $0.8 \pm 0.24 \mu\text{m}$  ( $\pm$  SD) and also 99.7% of increment measurements were above the  
278 resolution limits of light microscopes ( $0.3 \mu\text{m}$ , Fox *et al.*, 2003;  $0.35 \mu\text{m}$ , 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

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

291 **Acknowledgements**

292 The authors thank Y. Sakurai and T. Nakatani of the Faculty of Fisheries Sciences,  
293 Hokkaido University for valuable comments on the manuscript. We are also grateful  
294 to T. Terada, M. Sato, and H. Horikawa of the Matsumae-Sakura Fisheries Cooperative  
295 Association who provided the broodstock, and crew of T/S *Ushio-maru* of Faculty of  
296 Fisheries, Hokkaido University, who helped with the outdoor sampling. Finally, we  
297 thank the editor and two anonymous reviewers for their critical suggestions.

298

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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<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup>) were  
383  $17 \pm 2.6 \mu\text{m OR}$ ,  $36 \pm 1.9 \mu\text{m OR}$ , and  $38 \pm 1.5 \mu\text{m OR}$  (mean  $\pm$  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 °C:  $OR = 0.65DAF - 1.50$  ( $r^2 = 0.93$ ,  $p < 0.001$ ,  
387  $N = 21$ ), 10 °C:  $OR = 0.72DAF + 1.86$  ( $r^2 = 0.95$ ,  $p < 0.001$ ,  $N = 16$ ), and 12 °C:  
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).

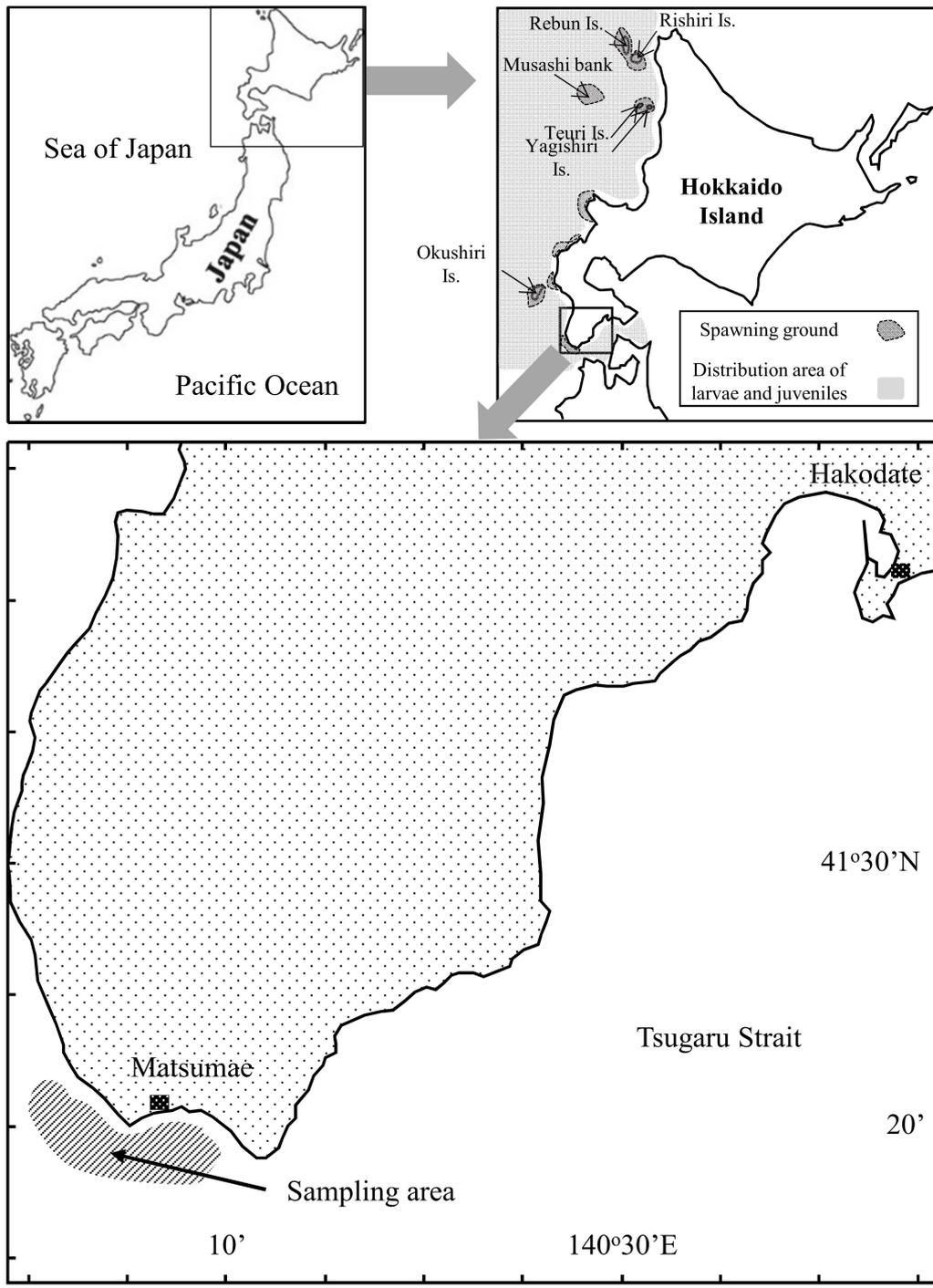


Fig. 1. Marannu *et al.*

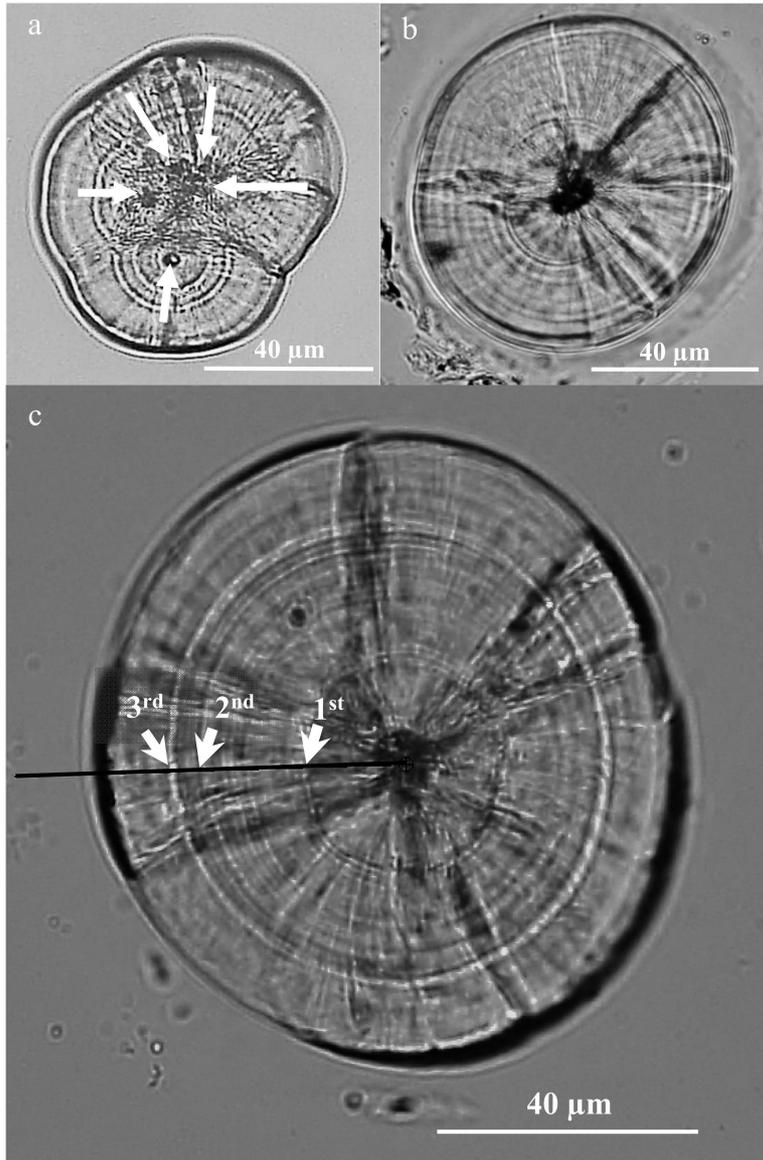


Fig. 2. Marannu *et al.*

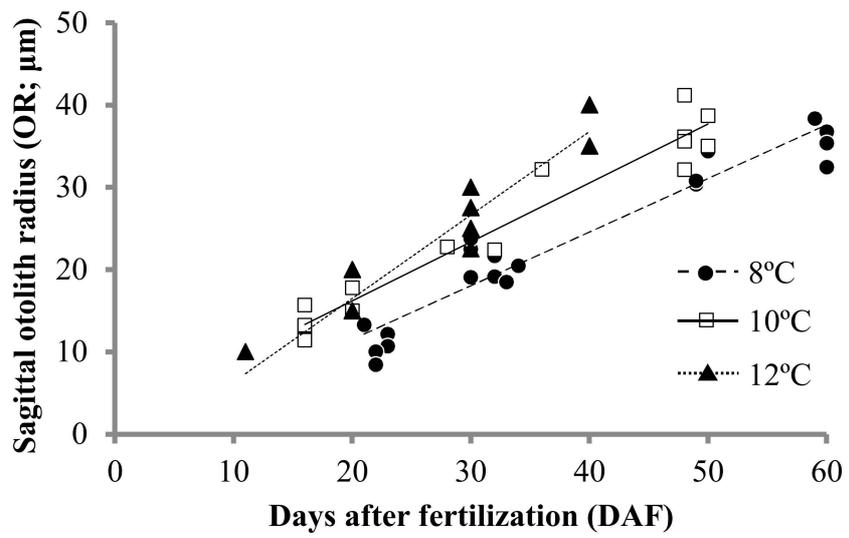


Fig. 3. Marannu *et al.*

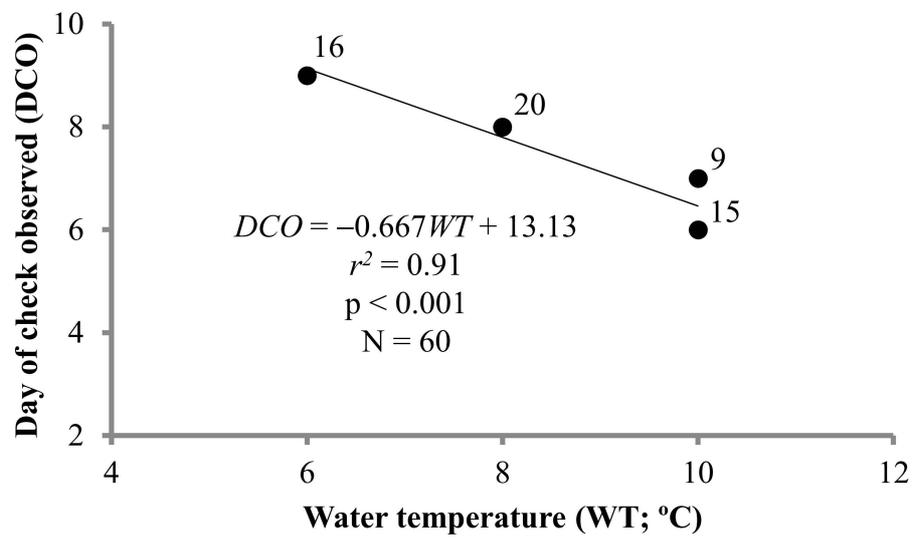


Fig. 4. Marannu *et al.*

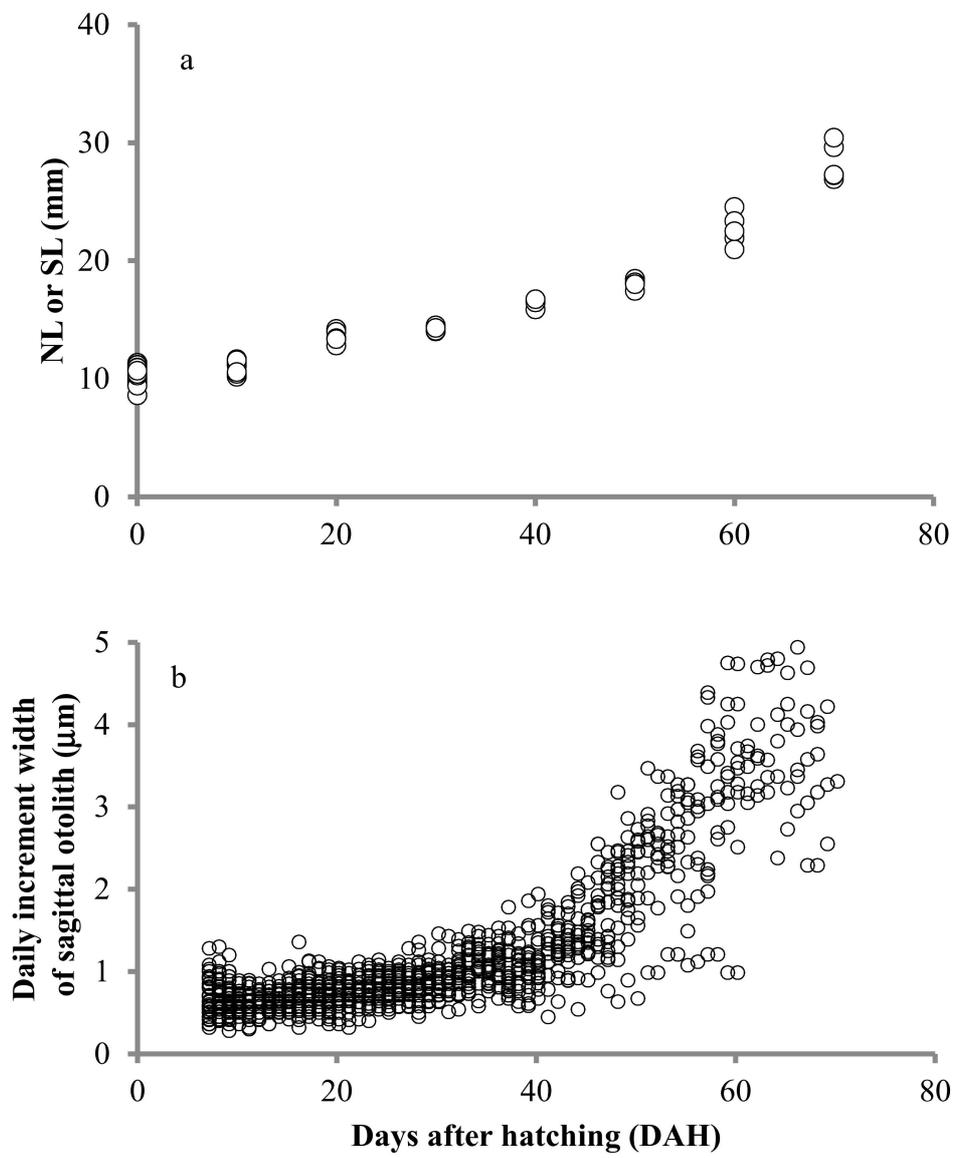


Fig. 5 Marannu *et al.*

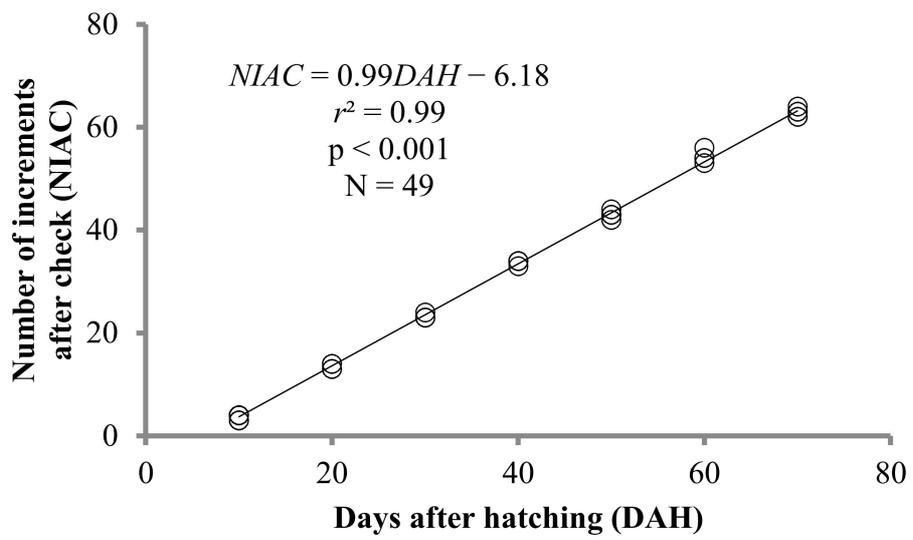


Fig. 6. Marannu *et al.*

Table 1. Temperature and salinity in each rearing tank

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