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Validation of Otolith Increment Formation and the Growth Rate of Fat Greenling Larvae

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Abstract: We validated the daily formation of otolith increments of reared fat greenling *Hexagrammos otakii* larvae and examined the growth patterns of wild larvae. The mean notochord length and otolith radius of newly hatched larvae were 8.31 mm and 27.3 μm, respectively. Newly hatched larvae had some increments on their lapilli and a check was formed at hatching (hatch check). The relationship between age and the number of increments formed outside the hatch check (*NI*) was a linear regression ($NI=0.99Age-0.30$, $n=49$, $r^2=0.98$, $P<0.001$) and its slope did not differ significantly from 1 (*t*-test: $P=0.31$). This indicates that these increments formed daily. Wild fat greenling larvae were collected from the surface layer of Mutsu Bay from February to April in 2003. The relationship between the otolith radius (*OR* in μm) and notochord length (*NL* in mm) was represented by an allometric equation. The mean back-calculated *NL* with the biological intercept method was 9.79 mm at 10 days old, 10.9 mm at 20 days old, 11.6 mm at 30 days old, and 13.3 mm at 40 days old. The daily growth rate was relatively high immediately after hatching (range: 0.15－0.17 mm/day) and gradually decreased. These findings suggest that fat greenling larvae do not have a slow growth period just after hatching.

Key words: Fat greenling larvae; Growth rate; Lapillus; Validation

Among marine fishes, recruitment fluctuation is mainly believed to be caused by variation in survival during their early life history (Houde 1987). Accordingly, various studies have been carried out to clarify the mechanisms that produce this variation (Leggett and DeBlois 1994). One possible mechanism refers to the "match/mismatch hypothesis", which focuses on the temporal overlap between the main hatching season and production cycle of prey organisms (Cushing and Dickson 1976; Cushing 1981), and has been examined in many species (Methot 1983; Wright and Bailey 1996; Narimatsu and Munehara 1999; Sirois and Dodson 2000). The "growth-mortality hypothesis", on the other hand, focuses on the association between the early growth rate and mortality (Anderson 1988; Campana 1996; Meekan and Fortier 1996; Bergenius et al. 2002; Takasuka et al. 2003; 2004; Takahashi and Watanabe 2004); fast growing individuals having the advantage of escape from predators and/or a shortened critical period for survival, and as a consequence, an increased chance of survival. Investigation of these two hypotheses requires estimates of the hatching season and growth trajectories, and otolith microstructure analysis is useful for these estimations.

The formation of otolith daily rings has been reported in many species, and otolith microstructure is a very powerful tool in studies of early life histories (Campana and Neilson 1985). Counting the number of increments provides information on the hatch date or age, and the estimates of the growth trajectory can also be obtained by measuring the increment width.

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However, the daily formation of otolith increments must be validated before otolith microstructure analysis can be applied to field-caught larvae and juveniles (Geffen 1987).

Fat greenling *Hexagrammos otakii* is a coastal rock fish distributed throughout Japan, in the southern part of the Korean Peninsula, and in the Yellow Sea (Amaoka et al. 1995). It is an important species for coastal fishery, and accordingly, the biology and ecology of adult fat greenling have been studied with respect to age and growth (Kobayashi et al. 1990; Tsutsui 1998; Sekigawa et al. 2002), spatial distribution (Kanamoto 1976), feeding habits (Kanamoto 1979; Kwak et al. 2005), parental care of eggs (Munehara and Takenaka 2000), and the spawning season (Akimoto 1991; Sekigawa et al. 2003). Early development and growth have also been examined under rearing conditions (Yusa 1960; Hamai and Kyushin 1966; Fukuhara and Fushimi 1983; Kang et al. 2002), but validations of daily ring formation and otolith microstructure analysis have not been conducted.

In this study, to validate the daily formation of otolith rings and determine the hatching date and growth rate of field-caught larvae of fat greenling, we 1) carried out a rearing experiment and 2) back-calculated the hatch date and growth trajectory of wild larvae.

Materials and Methods

Validation of daily ring formation

1. Rearing experiment

The rearing experiment was conducted at two times (October and November 2003). Naturally-spawned fat greenling eggs were collected in the sea around the Usujiri Fisheries Laboratory (the Field Science Center for Northern Biosphere, Hokkaido University) by scuba (Fig. 1). The eggs and larvae were reared in 5 *l* cylindrical tanks set in a 150 *l* water bath with a 12/12 h light-dark cycle. To make the rearing conditions uniform between the two experiments, the water temperature was set at 15.6℃ (range: 15.0–16.0℃), which was equivalent to the temperature of the seawater in which the eggs were captured in October. Newly hatched nauplii of *Artemia salina* were fed to larvae from the hatch date once a day at 10:00. The nauplii of *A. salina*, enriched with shark egg powder (Aquarun, BASF Japan Ltd., Tokyo, Japan), were supplied in sufficient amounts that a part of them remained. Larval were sacrificed randomly every day during 0–9 days after hatching from the October sample, and 12, 14, and 17 days after hatching from the November sample (Table 1). Larvae were anesthetized with MS-222 and

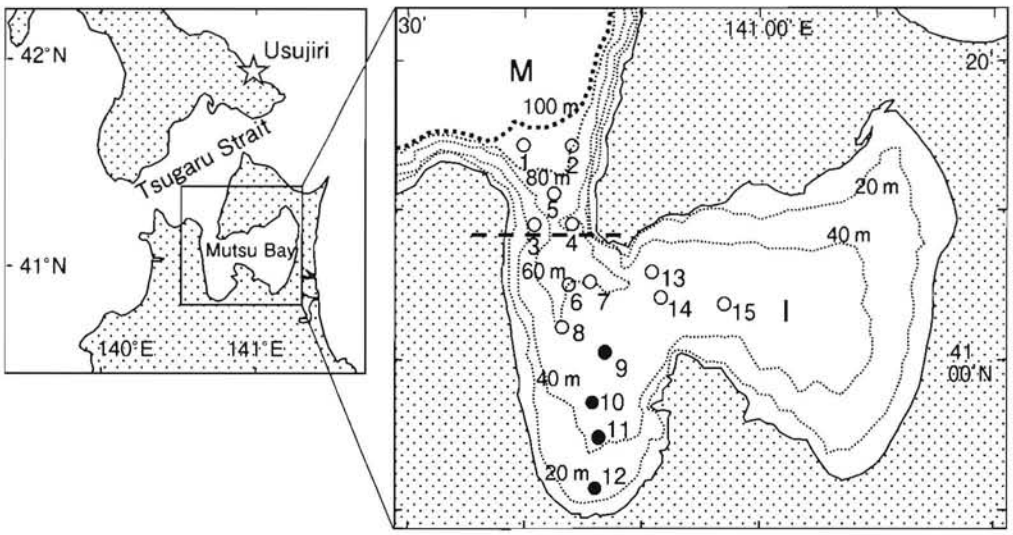


Fig. 1. Locations of *Hexagrammos otakii* egg collection off Usujiri (open star, left panel) for the rearing experiment and sampling stations for wild larvae in Mutsu Bay with isobaths (open and solid circles, right panel). Both open and solid circles show the sampling locations of the training ship *Ushio-maru*, and solid circles show those of the fishing boat *Sada-maru*. Mutsu Bay is divided into two regions, the inner part (I) and the bay mouth (M). Numerals show station numbers.

preserved in 90% ethanol solution. Individuals in which otolith extraction or preparation failed were omitted from the latter analyses.

Table 1. The number of reared *Hexagrammos otakii* larvae sacrified and used for otolith analysis in October and November 2003

Rearing days	The number of larvae sacrificed	The number of larvae for otolith analysis
0	45	7
1	5	0
2	10	6
3	5	5
4	6	4
5	5	4
6	4	4
7	5	3
8	4	3
9	5	5
12	(3)	(2)
14	(4)	(4)
17	(2)	(2)

Numbers in parentheses represent the number of larvae reared in November.

2. Otolith observation and data analysis

Our preliminary observation showed that the sagittal otoliths of reared fat greenling larvae sometimes have 2−4 growth centers (Fig. 2A), and in this case the center of the core as the starting point of otolith-radius measurement could not be identified. The cause of this multi-core phenomenon has not been clarified; on the other hand, the formation of increments on the lapilli was concentric from one growth center (Fig. 2B, C). Thus, lapillar otoliths were used in this study.

Notochord length (*NL*: the tip from the upper jaw to the end of the notochord) was measured with an electronic caliper and larvae were classified into two developmental stages (preflexion and flexion) by the state of the notochord end. No postflexion larvae were collected in this study. Lapilli were extracted under a dissecting microscope and mounted on a glass slide

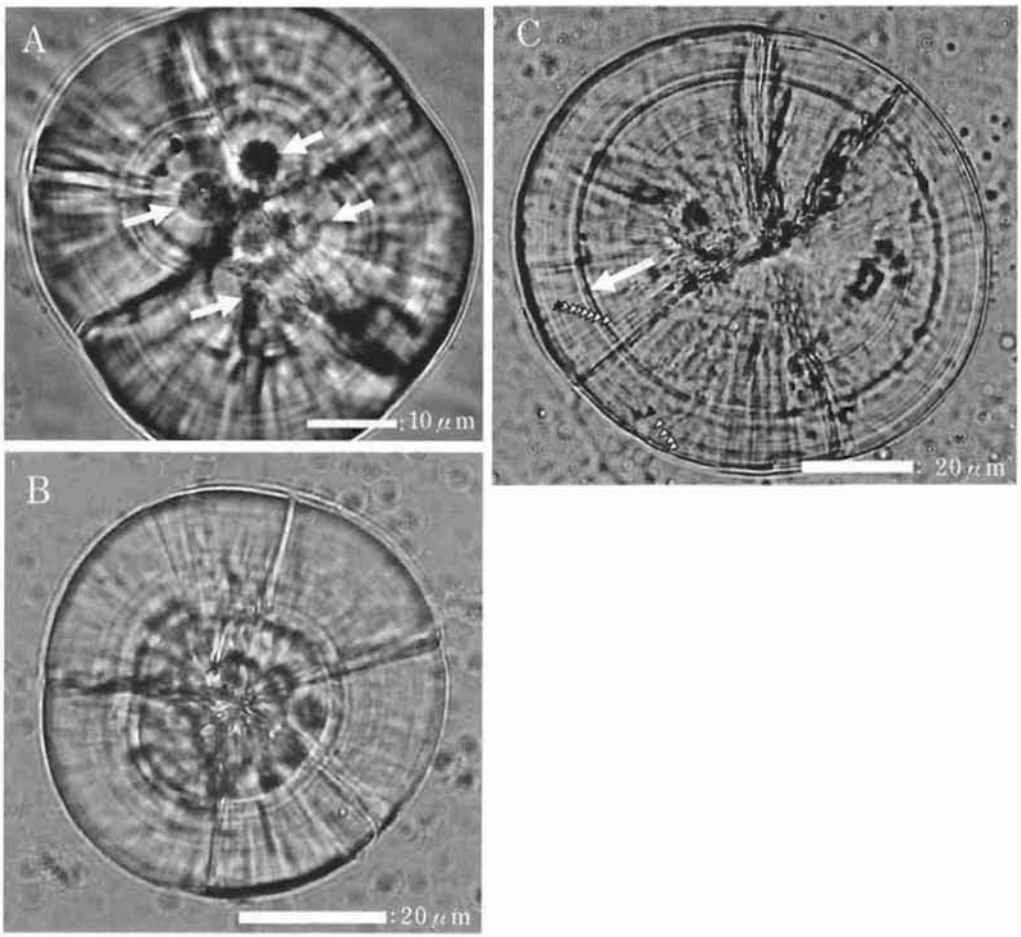


Fig. 2. Microphotographs of otoliths from reared *H. otakii* larvae. A: sagitta from a three-day-old larva, B: lapillus from a larva just after hatching, C: lapillus from a 12-day-old larva. Four arrows in A indicate four growth centers on otolith. In C, an arrow shows the hatch check, blank wedges show increments, and two solid wedges indicate the same increment (12 increments were observed outside the hatch check).

with epoxy resin. Mounted lapilli were ground almost to the core with lapping film (9 μm particles) before observation. Otolith images were magnified under a light microscope with translucent light under a magnification of ×1000 (oil-immersed ×100 objective lens, NA 1.30, with a ×10 eye-piece lens) or ×400 (×40 objective lens, NA 0.75). After otolith images were captured with a CCD digital camera mounted on the microscope, the otolith rings were counted and measured with otolith microstructure analysis software (ARP Ver. 5.00, RATOC System Engineering Co., Tokyo, Japan). Otolith radius (*OR*) was measured as the shortest distance from the center of the core to the edge of the lapillus. We consider that a growth increment is a minute bipartite structure consisting of incremental and discontinuous zones (terminology of Mugiya et al. 1981).

The regression line that represented the relationship between the age (X) and number of increments formed outside the hatch check (Y; a prominent increment; see Results) was compared with $Y=X$ using the t-test. The radius of the hatch check was compared with the otolith radius of hatching larvae by one-way ANOVA. The larvae from rearing experiments in October and November were treated as one sample, because scarcely any morphological change in the body and the otolith was observed between 9-day-old larvae in October and 12-day-old larvae in November.

Collection and otolith analysis of wild larvae

1. Field sampling

Field sampling was conducted from 25 – 26 February to 24 April 2003 in Mutsu Bay during the daytime (Fig. 1 and Table 2). Wide-area sampling was conducted aboard T/S *Ushio-maru* (179 t) on 25 – 26 February and 8 – 9 April. Larvae were collected using horizontal tows at depths of 1, 10, 20, 30, 40 and 50 m at 2.0 kt for 5 – 10 minutes with MTD nets (0.56 m diameter and 0.33 mm mesh aperture; Motoda 1971) and oblique tows with a ring net (0.8 m diameter and 0.33 mm mesh aperture) from the sea bottom to the surface at a wire-retrieve-speed of 0.5 m/s and a ship speed of 2.0 kt. On 16 March, 24 March and 24 April, sampling at three or four stations along a transect (depth range: 25 – 53 m) was conducted aboard the fishing boat *Sada-maru*. Larvae were collected using horizontal tows at a depth of 1 m and oblique tows with a ring net from the sea bottom to the surface. Flow meters were attached to all nets to estimate the volume of water filtered. Collected larvae were preserved in 90% ethanol solution. The water temperature was measured with a CTD (conductivity-temperature-depth profiler, SBE-19, Sea-Bird Electronics, Bellevue, WA) at each sampling station.

The density of larvae in each horizontal haul and oblique haul was calculated from the filtered water volume as the number of individuals/1000 m^3 and the number of individuals/m^2, respectively.

Horizontal haul: *Density* (inds./1000 m^3)=

$$N_i/(A \cdot F_a/F_c \cdot 1000)$$

Oblique haul: *Density* (inds./m^2)=

$$N_i/(A \cdot F_a/(F_c \cdot W))$$

where N_i is the number of larvae collected, A is the effective mouth area of the ring net (m^2), F_a

Table 2. The number of casts and *H. otakii* larvae collected with plankton nets

Sampling date	Ship†	MTD nets		A ring net				Temp.††
		Horizontal haul		Horizontal haul		Oblique haul		
		Casts	Larvae	Casts	Larvae	Casts	Larvae	
25 – 26 Feb.	T/S	24 (4)	34 (34)	–	–	13	78	5.6℃
16 Mar.	F/B	–	–	4 (4)	7 (7)	5	0	5.2℃
24 Mar.	F/B	–	–	3 (3)	0 (0)	9	0	4.9℃
8 – 9 Apr.	T/S	40 (8)	6 (6)	–	–	9	4	6.1℃
24 Apr.	F/B	–	–	3 (3)	0 (0)	9	0	8.4℃

† T/S: training ship *Ushio-maru*; F/B: fishing boat *Sada-maru*; –, no data. Numbers in parentheses represent the number of horizontal casts at 1 m depth and number of larvae collected from this layer. Horizontal hauls were carried out at depths of 1, 10, 20, 30, 40, and 50 m with MTD nets and at 1 m with the ring net.

†† : Mean water temperature at 1 m depth on stations 10 and 11.

is the rotation of the flow meter during collection (rot.), F_c is the mean rotation of the flow meter per 1 m tow calculated from triplicate 20 m tows without net cloth (rot./m), W is the warp out length (m).

2. Otolith analysis

From all cruises, 129 larvae of fat greenling were collected and of these 85 individuals were used for otolith analysis. These larvae were chosen to include all sampling stations from which fat greenling larvae were collected, excluding a larva collected at Stn. 9 on 16 March due to otolith preparation failure (Table 3).

The relationship between the otolith radius (OR) and notochord length (NL) of the wild larvae was regressed as an allometric relationship ($NL=a\cdot OR^b$) according to the least-square method (see Results) using the Solver program in Excel (Microsoft, Redmond, WA, USA). Therefore, we assumed that the relationship between OR and NL of wild larva can be expressed as the allometric relationship individually, and the growth trajectory of the wild fat greenling larvae was estimated using the biological intercept method (Campana 1990). In this study, notochord length (NL_0) and otolith radius (OR_0) at hatching were determined as 8.31 mm and 27.3 μm, respectively, in the rearing experiment. Thus, two parameters (a and b) were estimated from NL_0, OR_0, $NL_{capture}$, and $O_{Rcapture}$ for each individual. $NL_{capture}$ and $OR_{capture}$ represent the notochord length and otolith radius of each individual at capture. Lastly, we back-calculated the individual NL at age i (NL_i) by substituting OR_i for the individual allometric relationship. The growth rate at age i (GR_i) was calculated by subtracting NL_{i-1} from NL_i.

The relationship between larval growth and water temperature was investigated in wild larvae collected on 25–26 February, because a relatively large number of larvae was collected (Table 2). Pearson's correlation coefficient (r) was estimated between the mean width of five increments formed most recently and the sea-surface temperature at the sampling station.

Results

Rearing experiment

The mean notochord lengths of the fat greenling larvae were 8.31 mm at hatching (range: 7.85–8.80 mm; CV=3.1%), 9.29 mm at 9 days old (3.3%), 10.3 mm at 14 days old (5.3%), and 11.9 mm at 17 days old (0.8%).

At hatching, some obscure increments were already present on the lapillus (Fig. 2B), and after several days, a prominent check was observed, with clear regular increments formed outside it (Fig. 2C). The mean radius of the check was 27.3 μm, which did not differ from the mean otolith radius at hatching (one-way ANOVA; $P=0.95$).

The relationship between rearing days after hatching (i.e. age) and the number of increments (NI) formed outside the prominent check regressed as a linear line ($NI=0.99Age-0.30$, $n=49$, $r^2=0.98$, $P<0.001$, Fig. 3), with the slope and the intercept not significantly different from 1 and 0, respectively ($P=0.31$ and 0.05, respectively).

Temporal and spatial distribution of wild larvae

Wild fat greenling larvae were collected in Mutsu Bay from 25–26 February to 8–9 April, but not on 24 April, 2003 (Table 2). In the bay

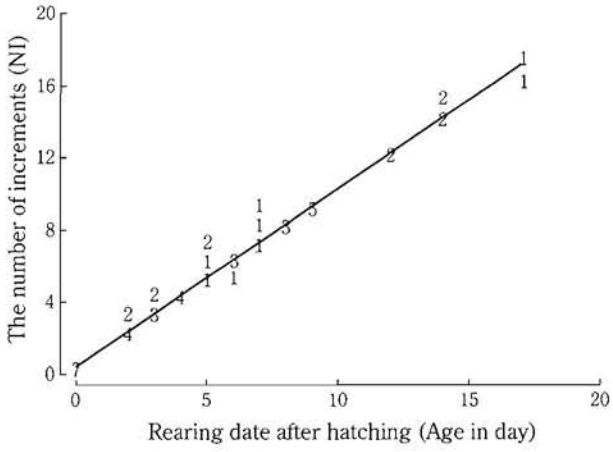


Fig. 3. Relationship between rearing date after hatching (age in days) and the number of increments (NI) outside the hatch check in reared *H. otakii* larvae. Numerals indicate the number of individuals on each coordinate. The solid line is the regression line: $NI=0.99Age+0.30$ ($n=49$, $r^2=0.98$, $P<0.001$).

Table 3. Densities of *H. otakii* larvae collected by oblique hauls with a ring net (inds./m^2; upper panel) and by horizontal hauls at 1 m depth with MTD nets and a ring net (inds./1000 m^3; bottom panel)

	Bay mouth					Inner part									
Stn.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
25–26 Feb.	0	0	0	0	3.8	4.1	–	1.0	11.3	1.1	0	–	11.9	1.6	43.1
16 Mar.	–	–	–	–	–	–	–	–	0	0	0	0	–	–	–
24 Mar.	–	–	–	–	–	–	–	–	–	0	0	0	–	–	–
8–9 Apr.	–	–	0	0	–	0	2.1	–	0.9	0	0	0	–	1.0	–
24 Apr.	–	–	–	–	–	–	–	–	–	0	0	0	–	–	–

	Bay mouth					Inner part									
Stn.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
25–26 Feb.	0	–	–	–	–	279.9	–	–	–	71.7	0	–	–	–	–
16 Mar.	–	–	–	–	–	–	–	–	10.4	30.8	6.8	0	–	–	–
24 Mar.	–	–	–	–	–	–	–	–	–	0	0	0	–	–	–
8–9 Apr.	–	–	–	–	–	–	–	–	–	–	–	–	–	3.0	–
24 Apr.	–	–	–	–	–	–	–	–	–	0	0	0	–	–	–

–, no data.

mouth area, larvae were scarce (Table 3) and no larvae were collected at the shallowest station (Stn. 12) at a depth of 25 m. On 25–26 February and 8–9 April, we carried out simultaneous-horizontal tows using MTD nets at depths of 1, 10, 20, 30, 40 and 50 m, and fat greenling larvae were only collected from the 1 m layer (Table 2), and the neustonic appearance of fat greenling larvae was similar to those previously reported in some literature (Horiki 1979; Akimoto 1991).

The notochord lengths of larvae used for otolith analysis ranged from 8.2–15.4 mm and the mean (±SD) was 10.9±1.31 mm. Developmental stages of wild fat greenling larvae were preflexion (81%) and flexion (19%) larvae.

Hatching period and growth trajectory

The maximum number of increments formed outside the prominent check was 43 (Fig. 4A). The number of <17 days old larvae was 68% of all specimens. There was no morphological change in the body and the otolith between reared larvae and wild ones, except for heavy pigmentation on the body surface of the reared larvae. The youngest flexion larvae and the oldest preflexion larvae were 14 days old (11.7 mm NL) and 31 days old (11.5 mm NL), respectively. The hatch dates of wild fat greenling larvae ranged from 7 January to 18 March 2003. The relationship between the otolith radius (*OR*) and the notochord length (*NL*) was fitted

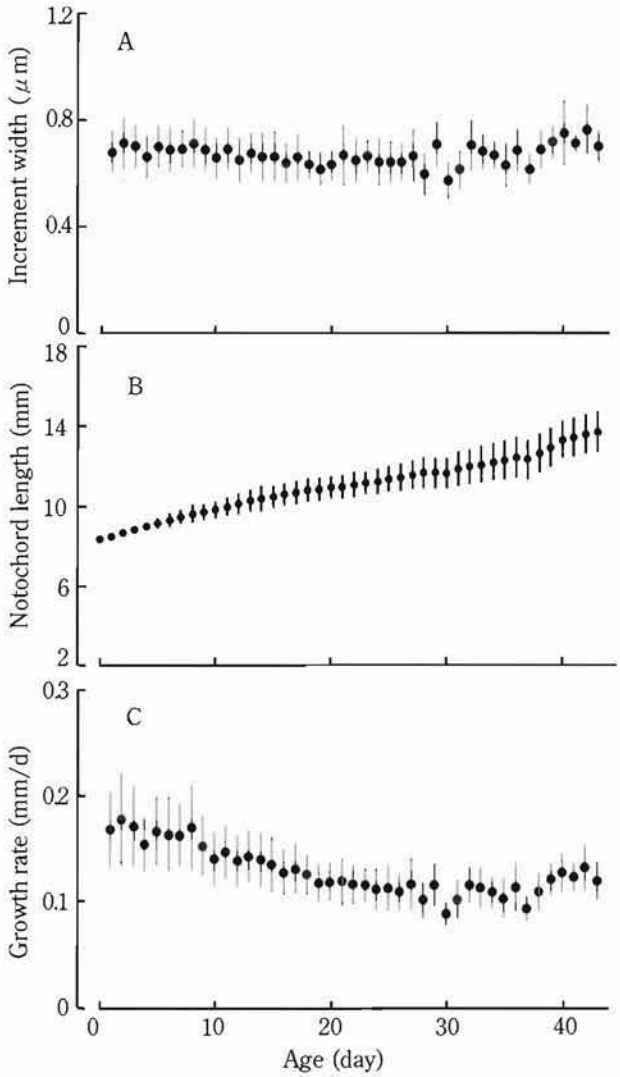


Fig. 4. Change in daily increment width in the lapillar otolith (A), the back-calculated notochord length (B), and growth rate (C) of wild *H. otakii* larvae collected from February 25 to April 9, 2003. Circles and vertical bars indicate mean values and standard deviations, respectively.

by an allometric curve ($NL=1.36OR^{0.57}$, $n=86$, $r^2=0.99$, $P<0.001$). The mean increment width ranged from 0.57 to 0.76 μm and was nearly stable, increasing slightly in larvae older than 38 days (Fig. 4A). Mean back-calculated notochord lengths were 9.79 mm (CV=4.1%) at 10 days old, 10.9 mm (5.4%) at 20 days old, 11.6 mm (6.7%) at 30 days old, and 13.3 mm (6.9%) at 40 days old (Fig. 4B). Mean growth rates were between 0.15 and 0.17 mm/day from hatching to 8 days old and thereafter gradually decreased, and after 28 days old, the rates were between 0.09 and 0.13 mm/day (Fig. 4C).

On 25–26 February, the range of sea-surface temperatures at eight sampling stations (Table 3) from which fat greenling larvae were collected was 3.1–5.5℃ (mean: 4.4℃). The larvae collected in this cruise were divided into 4 groups according to their age (≤10 days old, 11–20 days old, 21–30 days old, ≥31 days old). Between age groups, the mean widths of five increments formed most recently did not differ significantly (one-way ANOVA: $P=0.61$). Moreover, the mean widths did not differ between three sampling stations in which ≥4 fat greenling larvae were collected (Stn. 5, 6, and 15; one-way ANOVA; $P=0.58$). Therefore, the mean widths of all individual were pooled. There was no significant correlation between increment width and sea-surface temperature ($r=0.04$, $P=0.76$).

Discussion

Validation

We concluded that increments outside the hatch check on the lapillus of fat greenling larvae formed daily until they were 17 days old, and larval age could be estimated by counting these increments (Fig. 3). Increment deposition was found on the lapillus before hatching (Fig. 2B) and a prominent check was formed at hatching (hatch check; Fig. 2C). Check formation is considered to occur in relation to important events during early life history (Victor 1982; Penny and Evans 1985; Joh et al. 2005). Although first check formation was observed at hatching for fat greenling larvae, for many other species it is formed at the timing of transition from endogenous to exogenous nutrition (Brothers et al. 1976; Hayashi et al. 1989; Joh et al. 2005). The incubation period of fat greenling is relatively long (Tange and Takeda 1969) and hatching larvae have a large body size. Furthermore, their eyes are already pigmented and their jaws already functional at hatching (Fukuhara and Fushimi 1983), suggesting that fat greenling larvae are ontogenetically well-developed at hatching among demersal marine fishes. Moreover, larvae at hatching were available prey items. These facts are possibly a part of the reason for increment formation before hatching and check formation at hatching.

The growth of wild larvae

Although we validated the daily formation of otolith increments in larvae from hatching to 17 days old in the rearing experiment, we carried out the otolith analysis of wild larvae >17 days old. Field-caught larvae >17 days old continuously had clear and regular otolith increments formed concentrically. Moreover, no obvious change in the relationships between age and body length was observed between ≤17 days old and the older larvae (Fig. 5). Therefore, we assumed that daily formation of otolith increments were continued within the range of larval ages that was used for otolith microstructure analysis in the field (maximum age: 43 days old, see Results). The occurrence of sub-daily rings, however, was reported in some species (Miller and Storck 1982; Palomera et al. 1988; Powell and Laban 2000). To investigate the occurrence

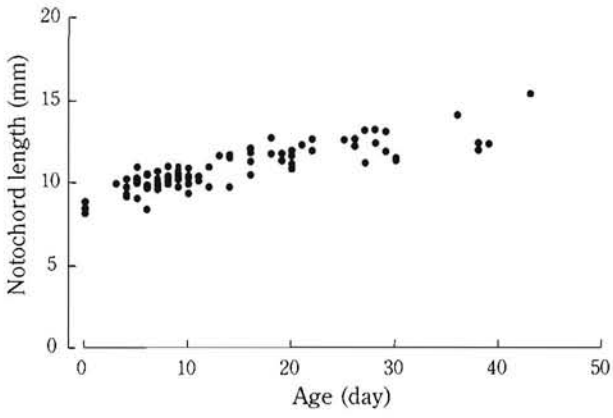


Fig. 5. The relationship between the age (day) and notochord length (mm) of wild larvae at capture.

of sub-daily rings in the lapilli of >17-day-old larvae and to observe the otolith microstructure of older larvae, another rearing experiment is needed on larvae >17 days old and juveniles.

The growth rate of wild fat greenling larvae was fast from hatching to 8 days old (0.15–0.17 mm/day) and then decreased (Fig. 4C). Growth patterns at larval stages are often represented by a sigmoid curve for many fishes; that is, the growth rate is slow after hatching, thereafter rapidly increasing, and becoming slow again near to metamorphosis (Jones 2002). For example, Meekan and Fortier (1996) report that the daily growth rate of Atlantic cod *Gadus morhua* was 0.15 mm/day at 10 days old and increased to 0.25 mm/day at 40 days old, and thereafter the growth rate decreased. The relationship between the age and body length of Japanese sandlance *Ammodytes personatus* larvae was fitted to the logistic model (Tomiyama and Komatsu 2006) and this means that the growth rate is relatively low immediately after hatching and then gradually increased. The newly hatched larvae of fat greenling were large with a well-developed body, and larvae could feed on prey at the hatch date. These characteristics were probably the reason for their high growth performance immediately after hatching.

On 25–26 February, there is no correlation between the recent growth rate and the environmental water temperature. Although a temperature-dependent growth rate has not been reported for fat greenling larvae, both reared and wild specimens, water temperature is commonly considered as one of the most important environmental factors influencing larval growth (Jones 2002). On 25–26 February, the range of sea-surface temperatures where fat greenling larvae collected was relatively narrow (3.1–5.5℃), and consequently, the growth dependence on temperature might be unclear. Further comparisons between the larval growth rate and water temperature are required under a wide range of water temperatures.

Wild fat greenling larvae were collected only in the surface layer in Mutsu Bay (Table 2). Some literature reports that fat greenling larvae concentrate at the surface (Akimoto 1991; Horiki 1979). Doyle et al. (1995) report the neustonic appearance of some hexagrammid larvae (*Hexagrammos decagrammus*, *H. lagocephalus*, *H. octogrammus* etc.) in the western Alaska Bay and suggest that they are likely to be tolerant to diverse and poor prey abundance in the surface layer because of their relatively large body size. Fat greenling larvae also have a large body size and feeding ability at hatching. Thus, we supposed that fat greenling larvae also have the advantage of a neustonic existence.

Fat greenling has a relatively long spawning season during late autumn-winter (Akimoto 1991; Sekigawa et al. 2003), when water temperatures rapidly decrease. Consequently, larvae are exposed to various environmental conditions depending on the timing of hatching, and thus, their growth and survival are also likely to fluctuate. In the future, we will examine annual and seasonal changes in the diet and growth rate of fat greenling to clarify their survival mechanisms and environmental adaptations.

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アイナメ仔魚の耳石日周輪形成の確認と成長率

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アイナメ飼育仔魚の耳石日周輪形成の確認と，天然仔魚の成長様式を検討した。礫石上には孵化時に明瞭な輪紋（孵化チェック）が形成された。日齢（*Age*）と孵化チェックの外側の輪紋数（*NI*）の関係は直線で示され（$NI=0.99Age-0.30$, $n=49$, $r^2=0.98$, $P<0.001$），傾きは1とは有意に異ならなかったことから（t検定：$P=0.31$），これらの輪紋は日周輪と判断された。陸奥湾では2003年2月から4月まで天然仔魚が表層で採集された。耳石半径と脊索長の関係はアロメトリー式で表され，バイオロジカル・インターセプト法による平均逆算体長は10日齢で9.79 mm，40日齢で13.3 mm と推定された。成長率は孵化直後に比較的高く（範囲：0.15－0.17 mm/日），その後次第に低下したことから，アイナメでは孵化直後の低成長期はみられないものと考えられた。