Feeding effect of selenium enriched rotifers on larval growth and development in red sea bream *Pagrus major*

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Abstract

Feeding trials were conducted to investigate the effect of selenium (Se)-enriched rotifers on growth and development of red sea bream *Pagrus major* larvae. Fish were reared from fertilized eggs (98% hatch rate) to 20 days post hatch (dph) at 19°C with two different food sources; non-enriched S-type rotifers (0.0 μg Se/g D.W., control diet) or Se-enriched rotifers (2.2 μg Se/g D.W., Se-enriched diet) at 10 rotifers/mL, respectively. On the last day of larviculture, the Se-enriched diet accelerated growth and developmental stage of fish larvae. The larvae fed Se-enriched rotifers were advanced in the following parameters compared to those fed control diet: total length (6.06 vs 5.53 mm), standard length (5.74 vs 5.26 mm), head length (1.46 vs 1.28 mm), eye diameter (0.57 vs 0.50 mm), the number of caudal fin rays (5.8 vs 1.9), and the proportion of individuals undergoing notochord flexion (55 vs 3%). Fish larvae of 20 dph showed higher Se concentration (9.5±0.2 μg/g DW) with the Se-enriched diet than with the control diet (1.3±0.3 μg/g DW), but there was no significant differences in the composition of polyunsaturated fatty acids which significantly affect larval growth and development. Therefore, the feeding of Se enriched rotifers enhanced growth and development of the red sea bream *P. major* larvae.

Keywords: Red sea bream; Selenium; Growth; Rotifer; *Chlorella vulgaris*

1. Introduction

The rotifer is widely used as an initial food source for marine fish larvae with small mouth size in aquaculture, but in the wild copepods are main food source for larvae. The nutrient profiles of rotifer and copepods had been analyzed, and it was found that the rotifer showed considerably lower level of minerals than copepods (Hamre et al., 2008a), also than fish requirements (NRC, 1993). Among deficient minerals, selenium (Se) concentration of rotifers (0.08-0.09mg/kg dry weight, DW) is about 30-fold lower than the level of copepod (2-5 mg/kg DW) and 3 to 8-fold lower than the Se requirements for juvenile fish (Hamre et al., 2008b; Penglase et al., 2011; Ribeiro et al., 2011). Se is the component of the enzyme glutathione peroxidase which has the function of protecting cells from oxidative damage (Rotruck et al., 1973) and is an essential trace element for health of vertebrates including fishes (Doucha et al., 2009). Although Se is the most deficient mineral of rotifers (Pengalse et al. 2010), it can be enriched up to copepod levels by fortification of the diet (Bell and Cowey, 1989; Penglase et al., 2011). It has been confirmed that Se-enriched rotifer *Brachionus* sp. by feeding of Se-fortified *Chlorella vulgaris* showed active reproduction such as higher population growth rate and resting egg production (Kim et al., 2014).

Se supplementation of artificial diets is known to enhance growth and development of rainbow trout *Oncorhynchus mykiss* (Hilton et al., 1980) and grouper *Epinephelus malabaricus* (Lin and Shiau, 2005). Selenomethionine (organic Se) is a natural food source of selenium and has higher bioavailability than the sodium selenite (inorganic Se) for Atlantic salmon *Salmo salar* (Lorentzen et al., 1994) and channel catfish *Ictalurus punctatus* (Wang and Lovell, 1997). In addition, it was reported that simultaneous supplementation of Se and I affected the larval fatty acid compositions which are significantly related to

growth and development of Atlantic cod *Gadus morhua* larvae (Hamre et al., 2008). To investigate the effects of supplemented Se associated with fatty acid composition, the present study used rotifers fed with Se-fortified *Chlorella* diet as feed for fish larvae. The red sea bream *Pagrus major* was chosen as experimental organism for Se-enriched rotifers since it is a major finfish species cultured in Japan and effects of fatty acid on growth, survival and viability of larvae were reported (Izquierdo et al., 1989). The final goal of this study was to investigate effects of Se on larval growth and development to promote more effective larviculture.

2. Materials and methods

2.1. Rotifer preparation

We employed the euryhaline rotifer *Brachionus rotundiformis* (S-type) as larval feed. Rotifers were cultured with the following two-types of HUFA enriched *Chlorella vulgaris* (Super Fresh Chlorella-V12, Chlorella Industry Co. Ltd., Fukuoka, Japan): 1) non-fortified *Chlorella* (0.0 μg Se/g DW), 2) Selenium (Se)-fortified *Chlorella* (3.2 μg Se/g DW) by adding sodium selenite (Na_2SeO_3) into the phytoplankton culture medium. Each feeding regime was applied to 30-40 L of batch cultures at 17 ppt (artificial sea water) and 25 °C with aeration. The daily amount of *Chlorella* for rotifers was adjusted as 40.5g DW /10^8 rotifers. On the last day of fish larviculture, remaining rotifers in each tank were sampled by plankton net (45-μm mesh size), rinsed with Milli-Q water (Millipore 0.22 μm) to remove salt, dried from beneath the net using filter paper and were transferred into brown glass screw-capped bottles (20 mL) for chemical analysis. Sampled rotifers were stored at -80°C until chemical

analyses.

2.2. Larviculture

Fertilized fish eggs of red sea bream *Pagrus major* were obtained from a local fish farmer in this study. Eggs were transferred into 100-L polycarbonate tanks at 10 eggs/L following the procedure of Ruttanapornvareesakul et al., 2010. In each feeding regime, four polycarbonate tanks containing 100 L of 34-ppt artificial sea water with each type of *Chlorella* (non- or Se-fortified one) at 5×10^5 cells/mL, were prepared with aeration at a rate of 50 mL/min. Fish were reared at 19°C with 12-h diurnal photoperiod (900-2100) for 20 days. Larvae were fed on two-type rotifers: rotifers fed on non-fortified *Chlorella* (control diet) or those fed on Se-fortified *Chlorella* (enriched diet), at 10 ind/mL from 2 days post hatch (dph) at mouth opening. Every 5 days (1, 5, 10, 15 and 20 dph), 10 fish were randomly sampled from each tank and were anaesthetized with MS 222 followed by 5% formalin fixation. Total and standard length were measured for all sampled larvae using a microscopic measurement system including stereomicroscope (Discovery V8, Zeiss, Germany) equipped with a digital camera (AxioCam, HSm) and an image-analysis software (AxioVision 4.8). Additional measurements such as body depth, head length, eye diameter, notochord flexion and the number of caudal fin rays (Fig. 1) were made on 20-dph samples. On the last day of larviculture (20 dph), the viability and survival rate were estimated. The viability of fish larvae was conducted with air exposure test; the rate of surviving individuals after 10 min from 5-sec air exposure. The survival rate of larvae was calculated from the average number of surviving larvae in four aquaria and these larvae were collected by the same method as rotifer preparation for chemical analyses. To evaluate the quality of employed fish eggs and hatched larvae, hatching rate and survival activity index (SAI,

112 Shimma and Tsujigado, 1981) of hatched larvae was calculated. We placed 30 fertilized
113 eggs in a 500-mL beaker containing 500-mL same saline water as the larviculture at 19°C in
114 total darkness without aeration. Dead larvae were counted and removed every 24 h until
115 total larval mortality to estimate survival and resistance to starvation. Triplicate observation
116 was used to calculate SAI using the following equation:

117 $$\mathbf{SAI} = \frac{1}{\mathrm{N}} \sum_{i=1}^{\mathrm{K}} (\mathrm{N} - \mathrm{h}i) \times i$$

118 where N is the total number of examined larvae, h*i* is the cumulated mortality by *i*-th day, K
119 is the number of days elapsed until all larvae died due to starvation.
120
121 *2.3. Selenium and fatty acid analysis*
122
123 Se and lipid compositions of cultured rotifers and fish larvae were performed by Chlorella
124 Industry Co., Fukuoka, Japan. To analyze Se concentration, four freeze-dried samples (each
125 100 mg of rotifers or 20 mg of fish larvae) were digested with 60% HNO_3 (0.5 mL for rotifers
126 or 1 mL for fish larvae) at 190 W for four minutes using microwave oven followed by one-
127 minute cooling (Homma-Takeda et al., 2013). This procedure was repeated six times. The
128 digested samples were diluted by ultrapure water and were analyzed for Se by Agilent
129 technologies 7700x series ICP-MS system (Agilent Technologies, Tokyo, Japan) with 0.05
130 (for rotifers) or 0.125 (fish larvae) μg/g of detection limit.
131 Total lipid and fatty acid composition were analyzed after the extraction following Folch et
132 al. (1957). The sample methanolysates were prepared at 100°C for two hours after the
133 addition of 2M hydrogen chloride methanol. Fatty acid methyl esters (FAME) were
134 extracted by n-hexane. Gas chromatography analysis was performed using a GC-2010
135 (Shimadzu Scientific Instruments, Inc.) equipped with a HR-SS-10 column (Shinwa

Chemical Industries, Ltd.). The column temperature was regulated at 150 to 220°C. Individual fatty acids were quantified by means of the response factor to 15:0 fatty acid as the internal standard.

2.4. Statistical analysis

The effect of Se enrichment on larval growth, development, and fatty acid composition were analyzed by *t*-test. Tukey-Kramer *post hoc* test was performed after repeated measures ANOVA to test dietary effect on the growth of fish larvae associated with age. All of the statistical analysis was carried out using Statview version 5.0 software (SAS Institute, Inc., USA).

3. Results

3.1. Nutritional level of rotifers

Se enriched rotifers contained 2.2 μg/g DW of Se, whereas Se was not detected in non-fortified *Chlorella vulgaris*. The fatty acid composition of rotifers from the two dietary regimes was similar (Table 3), except for 22:1 (*t*-test, P=0.0152) and the sum of unknown fatty acid (P=0.0368).

3.2. Larviculture

Red sea bream eggs showed 98.9±1.9% of hatching rate and hatched larvae from these eggs survived 9 days of starvation. Calculated survival activity index (SAI) of employed larvae was 13.9±0.5. After 20 days of rearing, the fish larvae showed no significant

differences in survival rate (87.7±7.8 - 93.2±7.0%) or viability (70.2±19.4 - 71.6±20.1%) between two different diet regimes; non (control)- or Se-enriched diet (Table 1). There was no significant difference in dry weight (0.15±0.05 - 0.18±0.05 mg DW/ind., Table 1). Total length and standard length of collected larvae were not significantly different until 15 dph (Fig. 2), but on 20 dph, these parameters and developmental stage (notochord flexion, Fig. 3) were more advanced with Se enrichment (55%) compared to the control group (3%). Morphological parameters of 20 dph including total length (6.06±0.31 mm; the control was 5.53±0.12 mm), standard length (5.74±0.29 mm; 5.26±0.11 mm), head length (1.46±0.11 mm; 1.28±0.06 mm), eye diameter (0.57±0.04 mm; 0.50±0.02 mm) and the number of caudal fin rays (5.8±3.1; 1.9±0.7) were significantly different with Se enrichment (*t*-test, $P<0.05$, Table 2).

Se concentration of fish larvae (Table 1) was higher with Se-enriched rotifer feeding (9.5±0.2 μg/g) than with control feeding (1.3±0.3 μg/g, *t*-test, $P<0.0001$). Fatty acid composition (Table 3) of fish larvae was not significantly different between the two diets except in 14:0 (*t*-test, $P=0.0240$), 18:1 ($P=0.0195$), and 18:3 *n*-3 ($P=0.0397$).

4. Discussion

This study showed that a selenium (Se)-enhanced diet promoted growth and development of red sea bream larvae (Table 2), but not survival and viability (Table 1). Fish larvae hatched from high quality eggs with 98.9% of hatching rate showing a higher level of survival activity index (SAI) which reflects the activity of larvae (Mushiake et al., 1993) than of other fishes (striped jack and yellowtail, Vassallo-Agius et al., 2001). Moreover, longevity under continuous starvation was longer (9 days) than in another report (8 days by Takeuchi et al.,

1998) of red sea bream larvae. This demonstrates that the tested larvae were of high quality when they were hatched from the eggs. The high quality of hatched larvae and the short rearing period may be reasons that Se effects were not found in the survival and viability of the larvae. Similar results were obtained by Ribeiro et al. (2012), in which they found no effects of Se supplement on the survival rate (94.7-97.7%) of Senegalese sole *Solea senegalensis* larvae. Lin and Shiau (2005) also found that Se-enrichment did not affect the survival of juvenile grouper *Epinephelus malabaricus* which had high survival rates from 91 to 100%. On the other hand, Hamre et al. (2008) found an increase in survival rate by 32% with multimineral i.e. Se and iodine (I) enrichment in Atlantic cod larvae. It is expected that enriched Se and I had a synergistic effect on the survival of fish larvae even though the effect of single I enrichment on survival should be investigated.

Se, an essential trace element, being an integral part of glutathione peroxidase (Levander and Burk, 1994) is highly active in cell protection from oxidation by free radicals (Wang et al., 1997), and required for normal growth and physiological function of fishes (Rotruck et al., 1973; Bedwal and Bahuguna, 1994). It was reported that Se deficiency has negative effects on growth and feed efficiency associated with reduced activity of glutathione peroxidase in rainbow trout *Salmo gairdneri* (Bell et al., 1986), *Oncorhyncus mykiss* (Hill et al., 1980), and channel catfish *Ictalarus punctatus* (Gatlin and Wilson, 1984). Our results confirmed the reported function of Se on fish growth. Improved growth of red sea bream larvae was observed significantly in terms of total length, standard length, head length, eye diameter and the number of caudal fin rays with Se enrichment. The Se-enriched fish larvae had 7-fold higher in Se concentration than in the non-enriched control group by feeding of Se-enriched rotifers containing 2.2 μg Se/g DW which is sufficient amount for growth and development (0.25-0.7 μg Se/g DW, NRC 1993). Consequently, it is expected that the advanced growth and development of the larvae were induced, where one of the effects is to increase the

activity of glutathione peroxidase.

The other evidence supporting Se effect on larval growth and development is fatty acid composition of rotifer and fish in the present study. Essential fatty acids (EFA) such as n-3 highly unsaturated fatty acids and arachidonic acid are important for larval growth and development (Izquierdo, 1996). Among these fatty acids, the quantitative level of dietary eicosapentaenoic (EPA, 20:5 *n*-3), docosahexaenoic acids (DHA, 22:6 *n*-3) and other polyunsaturated fatty acid (PUFA) are important for the larval growth and development of red sea bream as well as other marine fish species (Watanabe, 1993; Komilus et al., 2008). Moreover, the ratio of DHA/EPA is regarded as a significant factor for optimal growth and survival of fish larvae and juveniles (Watanabe, 1993). As the fatty acid analysis of rotifers and larvae showed that there were no significant differences in the EFA composition mentioned above while the following fatty acids were varied with Se enrichment. In the case of rotifers (Table 3), 22:1 and unknown fatty acids which are not known to be important for growth and development of red sea bream larvae (Yone and Fujii, 1975; Fujii and Yone, 1976) were different between the two diet groups. In the case of fish larvae, the composition of following fatty acids: 14:0, 18:1 and 18:3 *n*-3, were heightened in response to the feeding of Se-enriched rotifers. Nevertheless, there is little information about effects of these three fatty acids on the growth and development of marine fish larvae. The obtained fatty acid data from this study are contrary to the previous study by Hamre et al (2008a). They fed Se and I enriched rotifers to Atlantic cod *Gadus morhua* larvae and found decreased level of larval DHA compared to control groups fed on non-enriched rotifers. Moreover, the DHA/EPA ratio also decreased with Se and I enrichment, and may have been one reason for the lower larval growth of enriched group than control. Our results can account for uncertain effects of Se and I on the larval growth and development as well as fatty acid composition in the previous study (Hamre et al., 2008a): the effects of supplemented Se was

proven to improve the larval growth and development accompanied by no significant changes in fatty acid composition, and thus simultaneously enriched I may be lead to those changes even though the effects of mono-enriched I should be investigated associated with these issues.

The present study approached Se effects on the growth and development of red sea bream larvae by the feeding of Se-enriched rotifers cultured with Se-fortified *Chlorella*. The obtained results demonstrated that supplemented Se enhances the larval growth and development with no changes in EFA composition. It showed a possibility to heighten efficiency of larviculture using the Se-enriched rotifers.

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Tables

Table 1
Larval characteristics: dry weight, selenium concentration, survival rate and viability, with different food sources (i.e., control or selenium enriched rotifers) on the last day of larviculture (20 dph)

Diet	Dry weight (mg/ind)	Se concentration (μg/g DW)	Survival rate (%)	Viability (%)
Control	0.15±0.05	1.3±0.3	87.7±7.8	71.6±20.1
Se	0.18±0.05	9.5±0.2*	93.2±7.0	70.2±19.4

Values are means±SD of tetraplicate observations (*n*=4). Asterisk in the column presents significant differences between different feeding groups.

Table 2
Morphological parameters of red sea bream larvae with different food sources (i.e., control or selenium enriched rotifers) on the last day of larviculture (20 dph)

Diet	Total length (mm)	Standard length (mm)	Body depth (mm)	Head length (mm)	Eye diameter (mm)	No. of caudal fin rays
Control	5.53±0.12	5.26±0.11	1.23±0.07	1.28±0.06	0.50±0.02	1.9±0.7
Se	6.06±0.31*	5.74±0.29*	1.40±0.13	1.46±0.11*	0.57±0.04*	5.8±3.1*

Values and asterisk in each column respectively present means±SD of tetraplicate observation (n=4) with 10 larvae per an observation and significant differences (P<0.05) between different feeding groups.

Table 3

Total fatty acids (Total, mg/g dry weight) and fatty acid composition (% of total fatty acids) of rotifers and red sea bream larvae associated with selenium treatment; none for control or selenium enrichment (Se)

Fatty acid	Rotifer		Red sea bream larvae	
	Control	Se	Control	Se
Total	70.3±5.6	64.5±13.1	72.1±14.1	87.9±9.5
14:0	1.4±0.0	1.4±0.1	0.4±0.0	0.5±0.0*
14:1	1.0±0.4	1.4±0.5	0.1±0.1	0.1±0.1
16:0	15.4±0.5	15.0±0.7	17.4±0.6	17.1±0.4
16:1	1.1±0.1	0.9±0.2	0.3±0.1	0.3±0.0
16:2	5.6±0.5	5.6±0.3	0.3±0.1	0.3±0.0
18:0	3.1±0.3	3.1±0.2	10.1±0.3	9.6±0.4
18:1	3.7±0.2	3.3±0.4	4.1±0.1	4.4±0.1*
18:2 *n*-6	24.7±1.8	22.7±2.2	13.6±1.0	14.6±0.9
18:3 *n*-3	5.4±0.8	5.3±1.0	1.0±0.2	1.4±0.2*
20:0	nd	nd	nd	nd
20:1	1.1±0.1	1.0±0.1	0.5±0.3	0.8±0.1
20:4 *n*-6	0.6±0.1	0.3±0.4	0.9±0.1	0.9±0.1
20:5 *n*-3	4.8±0.4	4.7±0.3	4.5±0.4	4.9±0.1
22:0	nd	nd	0.6±0.7	0.9±0.2
22:1	1.0±0.3*	0.3±0.3	0.5±0.3	0.7±0.1
24:0	0.1±0.2	0.1±0.2	nd	nd
24:1	0.8±0.0	0.8±0.1	0.8±0.5	1.1±0.1
22:5 *n*-3	3.0±0.2	3.2±0.2	6.5±0.4	6.8±0.1
22:6 *n*-3	8.2±1.7	9.3±2.5	13.5±1.0	13.3±0.7
UNK	19.3±0.4	21.9±1.8*	24.8±2.7	22.6±1.1
Σ PUFA	46.6±0.7	45.4±1.3	40.0±1.4	41.8±0.7
DHA/EPA	1.7±0.3	2.0±0.5	3.0±0.4	2.7±0.2

Values and asterisks in each column respectively present means±SD and significant differences (P<0.05) between two different diet regimes by tetraplicate tests for rotifer and fish larvae (n=4). Abbreviations: nd=not detected, UNK= unknowns, PUFA=polyunsaturates, DHA=docosahexaenoic acid (22:6 *n*-3), EPA=eicosapentaenoic acid (20:5 *n*-3)

Figures

Fig. 1. Five morphological characteristics to estimate larval growth and development. Abbreviations are defined as followings: TL, total length; SL, standard length; HL, head length; ED, eye diameter; BD, body depth.

Fig. 2. Variation of total length of red sea bream larvae fed on non-fortified (control) or selenium (Se) fortified S-type rotifer for 20 days. Each plot and error bar represents the mean and standard deviation of four replicates.

Fig. 3. Largest individuals of 20 dph among collected specimens on different feeding regime: (a) non-enriched larva (6.1 mm), (b) selenium enriched larva (6.8 mm).

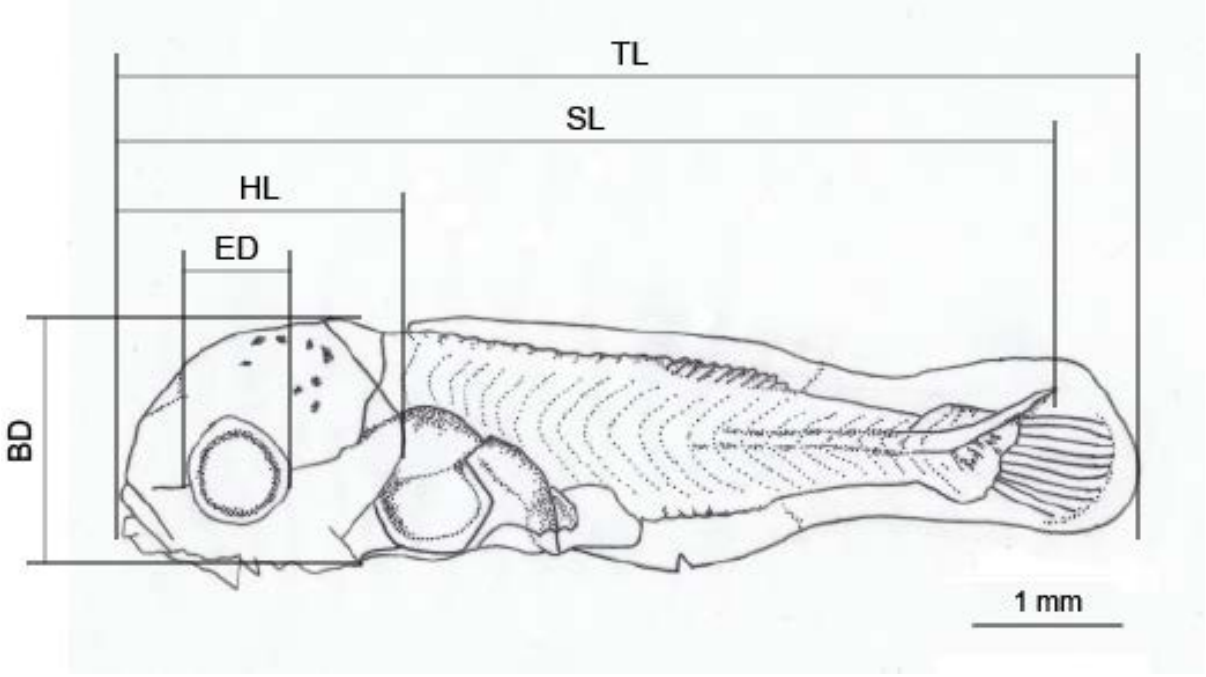

Fig. 1.

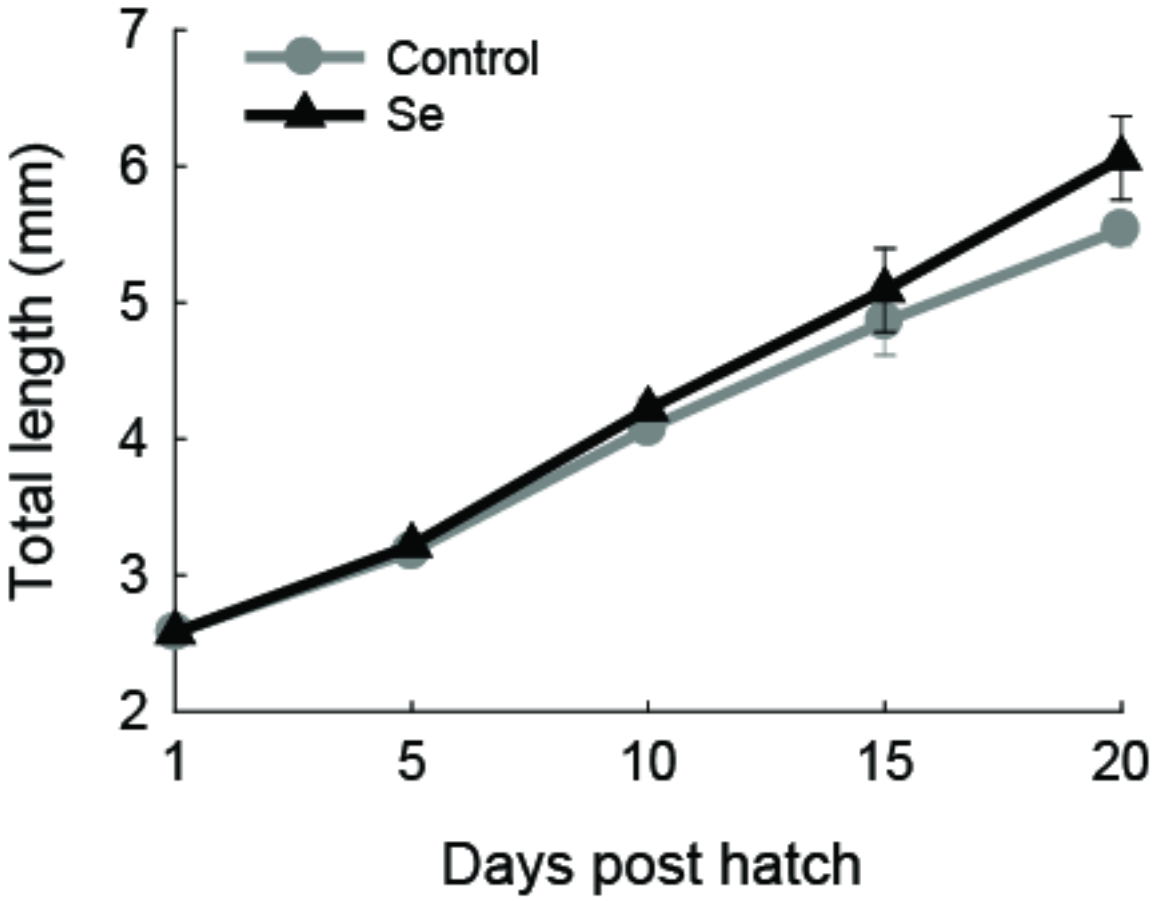

Fig. 2.

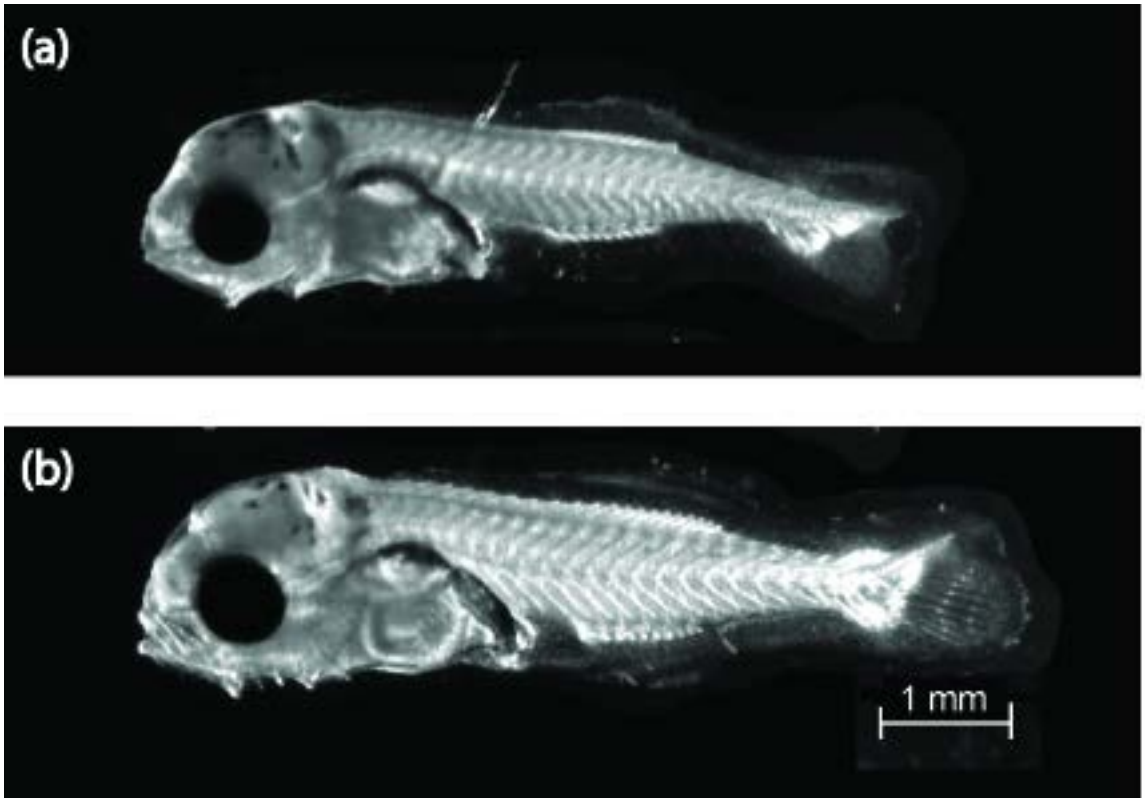

Fig. 3.