



Title	Feeding effect of selenium enriched rotifers on larval growth and development in red sea bream <i>Pagrus major</i>
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1 **Feeding effect of selenium enriched rotifers on larval growth and development in red**
2 **sea bream *Pagrus major***

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18 **Abstract**

19

20 Feeding trials were conducted to investigate the effect of selenium (Se)-enriched rotifers
21 on growth and development of red sea bream *Pagrus major* larvae. Fish were reared from
22 fertilized eggs (98% hatch rate) to 20 days post hatch (dph) at 19°C with two different food
23 sources; non-enriched S-type rotifers (0.0 µg Se/g D.W., control diet) or Se-enriched rotifers
24 (2.2 µg Se/g D.W., Se-enriched diet) at 10 rotifers/mL, respectively. On the last day of
25 larviculture, the Se-enriched diet accelerated growth and developmental stage of fish larvae.
26 The larvae fed Se-enriched rotifers were advanced in the following parameters compared to
27 those fed control diet: total length (6.06 vs 5.53 mm), standard length (5.74 vs 5.26 mm),
28 head length (1.46 vs 1.28 mm), eye diameter (0.57 vs 0.50 mm), the number of caudal fin
29 rays (5.8 vs 1.9), and the proportion of individuals undergoing notochord flexion (55 vs 3%).
30 Fish larvae of 20 dph showed higher Se concentration (9.5±0.2 µg/g DW) with the Se-
31 enriched diet than with the control diet (1.3±0.3 µg/g DW), but there was no significant
32 differences in the composition of polyunsaturated fatty acids which significantly affect larval
33 growth and development. Therefore, the feeding of Se enriched rotifers enhanced growth
34 and development of the red sea bream *P. major* larvae.

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

37 1. Introduction

38

39 The rotifer is widely used as an initial food source for marine fish larvae with small mouth
40 size in aquaculture, but in the wild copepods are main food source for larvae. The nutrient
41 profiles of rotifer and copepods had been analyzed, and it was found that the rotifer showed
42 considerably lower level of minerals than copepods (Hamre et al., 2008a), also than fish
43 requirements (NRC, 1993). Among deficient minerals, selenium (Se) concentration of
44 rotifers (0.08-0.09mg/kg dry weight, DW) is about 30-fold lower than the level of copepod
45 (2-5 mg/kg DW) and 3 to 8-fold lower than the Se requirements for juvenile fish (Hamre et
46 al., 2008b; Penglase et al., 2011; Ribeiro et al., 2011). Se is the component of the enzyme
47 glutathione peroxidase which has the function of protecting cells from oxidative damage
48 (Rotruck et al., 1973) and is an essential trace element for health of vertebrates including
49 fishes (Doucha et al., 2009). Although Se is the most deficient mineral of rotifers
50 (Penglase et al. 2010), it can be enriched up to copepod levels by fortification of the diet
51 (Bell and Cowey, 1989; Penglase et al., 2011). It has been confirmed that Se-enriched
52 rotifer *Brachionus* sp. by feeding of Se-fortified *Chlorella vulgaris* showed active
53 reproduction such as higher population growth rate and resting egg production (Kim et al.,
54 2014).

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

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

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72 **2. Materials and methods**

73

74 *2.1. Rotifer preparation*

75

76 We employed the euryhaline rotifer *Brachionus rotundiformis* (S-type) as larval feed.
77 Rotifers were cultured with the following two-types of HUFA enriched *Chlorella vulgaris*
78 (Super Fresh Chlorella-V12, Chlorella Industry Co. Ltd., Fukuoka, Japan): 1) non-fortified
79 *Chlorella* (0.0 µg Se/g DW), 2) Selenium (Se)-fortified *Chlorella* (3.2 µg Se/g DW) by
80 adding sodium selenite (Na₂SeO₃) into the phytoplankton culture medium. Each feeding
81 regime was applied to 30-40 L of batch cultures at 17 ppt (artificial sea water) and 25 °C with
82 aeration. The daily amount of *Chlorella* for rotifers was adjusted as 40.5g DW /10⁸ rotifers.
83 On the last day of fish larviculture, remaining rotifers in each tank were sampled by plankton
84 net (45-µm mesh size), rinsed with Milli-Q water (Millipore 0.22 µm) to remove salt, dried
85 from beneath the net using filter paper and were transferred into brown glass screw-capped
86 bottles (20 mL) for chemical analysis. Sampled rotifers were stored at -80°C until chemical

87 analyses.

88

89 2.2. Larviculture

90

91 Fertilized fish eggs of red sea bream *Pagrus major* were obtained from a local fish farmer
92 in this study. Eggs were transferred into 100-L polycarbonate tanks at 10 eggs/L following
93 the procedure of Ruttanapornvareesakul et al., 2010. In each feeding regime, four
94 polycarbonate tanks containing 100 L of 34-ppt artificial sea water with each type of
95 *Chlorella* (non- or Se-fortified one) at 5×10^5 cells/mL, were prepared with aeration at a rate
96 of 50 mL/min. Fish were reared at 19°C with 12-h diurnal photoperiod (900-2100) for 20
97 days. Larvae were fed on two-type rotifers: rotifers fed on non-fortified *Chlorella* (control
98 diet) or those fed on Se-fortified *Chlorella* (enriched diet), at 10 ind/mL from 2 days post
99 hatch (dph) at mouth opening. Every 5 days (1, 5, 10, 15 and 20 dph), 10 fish were
100 randomly sampled from each tank and were anaesthetized with MS 222 followed by 5%
101 formalin fixation. Total and standard length were measured for all sampled larvae using a
102 microscopic measurement system including stereomicroscope (Discovery V8, Zeiss,
103 Germany) equipped with a digital camera (AxioCam, HSm) and an image-analysis software
104 (AxioVision 4.8). Additional measurements such as body depth, head length, eye diameter,
105 notochord flexion and the number of caudal fin rays (Fig. 1) were made on 20-dph samples.
106 On the last day of larviculture (20 dph), the viability and survival rate were estimated. The
107 viability of fish larvae was conducted with air exposure test; the rate of surviving individuals
108 after 10 min from 5-sec air exposure. The survival rate of larvae was calculated from the
109 average number of surviving larvae in four aquaria and these larvae were collected by the
110 same method as rotifer preparation for chemical analyses. To evaluate the quality of
111 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:

$$117 \quad \text{SAI} = \frac{1}{N} \sum_{i=1}^K (N - hi) \times i$$

118 where N is the total number of examined larvae, hi 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% HNO₃ (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

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

139

140 *2.4. Statistical analysis*

141

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

147

148

149 **3. Results**

150

151 *3.1. Nutritional level of rotifers*

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

156

157 *3.2. Larviculture*

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

161 differences in survival rate (87.7 ± 7.8 - $93.2\pm7.0\%$) or viability (70.2 ± 19.4 - $71.6\pm20.1\%$)
162 between two different diet regimes; non (control)- or Se-enriched diet (Table 1). There was
163 no significant difference in dry weight (0.15 ± 0.05 - 0.18 ± 0.05 mg DW/ind., Table 1). Total
164 length and standard length of collected larvae were not significantly different until 15 dph
165 (Fig. 2), but on 20 dph, these parameters and developmental stage (notochord flexion, Fig. 3)
166 were more advanced with Se enrichment (55%) compared to the control group (3%).
167 Morphological parameters of 20 dph including total length (6.06 ± 0.31 mm; the control was
168 5.53 ± 0.12 mm), standard length (5.74 ± 0.29 mm; 5.26 ± 0.11 mm), head length (1.46 ± 0.11 mm;
169 1.28 ± 0.06 mm), eye diameter (0.57 ± 0.04 mm; 0.50 ± 0.02 mm) and the number of caudal fin
170 rays (5.8 ± 3.1 ; 1.9 ± 0.7) were significantly different with Se enrichment (*t*-test, $P<0.05$, Table
171 2).

172 Se concentration of fish larvae (Table 1) was higher with Se-enriched rotifer feeding
173 (9.5 ± 0.2 $\mu\text{g/g}$) than with control feeding (1.3 ± 0.3 $\mu\text{g/g}$, *t*-test, $P<0.0001$). Fatty acid
174 composition (Table 3) of fish larvae was not significantly different between the two diets
175 except in 14:0 (*t*-test, $P=0.0240$), 18:1 ($P=0.0195$), and 18:3 *n*-3 ($P=0.0397$).

176

177

178 **4. Discussion**

179

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

186 1998) of red sea bream larvae. This demonstrates that the tested larvae were of high quality
187 when they were hatched from the eggs. The high quality of hatched larvae and the short
188 rearing period may be reasons that Se effects were not found in the survival and viability of
189 the larvae. Similar results were obtained by Ribeiro et al. (2012), in which they found no
190 effects of Se supplement on the survival rate (94.7-97.7%) of Senegalese sole *Solea*
191 *senegalensis* larvae. Lin and Shiau (2005) also found that Se-enrichment did not affect the
192 survival of juvenile grouper *Epinephelus malabaricus* which had high survival rates from 91
193 to 100%. On the other hand, Hamre et al. (2008) found an increase in survival rate by 32%
194 with multimineral i.e. Se and iodine (I) enrichment in Atlantic cod larvae. It is expected that
195 enriched Se and I had a synergistic effect on the survival of fish larvae even though the effect
196 of single I enrichment on survival should be investigated.

197 Se, an essential trace element, being an integral part of glutathione peroxidase (Levander
198 and Burk, 1994) is highly active in cell protection from oxidation by free radicals (Wang et
199 al., 1997), and required for normal growth and physiological function of fishes (Rotruck et al.,
200 1973; Bedwal and Bahuguna, 1994). It was reported that Se deficiency has negative effects
201 on growth and feed efficiency associated with reduced activity of glutathione peroxidase in
202 rainbow trout *Salmo gairdneri* (Bell et al., 1986), *Oncorhynchus mykiss* (Hill et al., 1980), and
203 channel catfish *Ictalurus punctatus* (Gatlin and Wilson, 1984). Our results confirmed the
204 reported function of Se on fish growth. Improved growth of red sea bream larvae was
205 observed significantly in terms of total length, standard length, head length, eye diameter and
206 the number of caudal fin rays with Se enrichment. The Se-enriched fish larvae had 7-fold
207 higher in Se concentration than in the non-enriched control group by feeding of Se-enriched
208 rotifers containing 2.2 µg Se/g DW which is sufficient amount for growth and development
209 (0.25-0.7 µg Se/g DW, NRC 1993). Consequently, it is expected that the advanced growth
210 and development of the larvae were induced, where one of the effects is to increase the

211 activity of glutathione peroxidase.

212 The other evidence supporting Se effect on larval growth and development is fatty acid
213 composition of rotifer and fish in the present study. Essential fatty acids (EFA) such as n-3
214 highly unsaturated fatty acids and arachidonic acid are important for larval growth and
215 development (Izquierdo, 1996). Among these fatty acids, the quantitative level of dietary
216 eicosapentaenoic (EPA, 20:5 *n*-3), docosahexaenoic acids (DHA, 22:6 *n*-3) and other
217 polyunsaturated fatty acid (PUFA) are important for the larval growth and development of
218 red sea bream as well as other marine fish species (Watanabe, 1993; Komilus et al., 2008).
219 Moreover, the ratio of DHA/EPA is regarded as a significant factor for optimal growth and
220 survival of fish larvae and juveniles (Watanabe, 1993). As the fatty acid analysis of rotifers
221 and larvae showed that there were no significant differences in the EFA composition
222 mentioned above while the following fatty acids were varied with Se enrichment. In the
223 case of rotifers (Table 3), 22:1 and unknown fatty acids which are not known to be important
224 for growth and development of red sea bream larvae (Yone and Fujii, 1975; Fujii and Yone,
225 1976) were different between the two diet groups. In the case of fish larvae, the
226 composition of following fatty acids: 14:0, 18:1 and 18:3 *n*-3, were heightened in response to
227 the feeding of Se-enriched rotifers. Nevertheless, there is little information about effects of
228 these three fatty acids on the growth and development of marine fish larvae. The obtained
229 fatty acid data from this study are contrary to the previous study by Hamre et al (2008a).
230 They fed Se and I enriched rotifers to Atlantic cod *Gadus morhua* larvae and found decreased
231 level of larval DHA compared to control groups fed on non-enriched rotifers. Moreover, the
232 DHA/EPA ratio also decreased with Se and I enrichment, and may have been one reason for
233 the lower larval growth of enriched group than control. Our results can account for
234 uncertain effects of Se and I on the larval growth and development as well as fatty acid
235 composition in the previous study (Hamre et al., 2008a): the effects of supplemented Se was

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

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

245

246

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253 **6. References**

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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 ($\mu\text{g/g DW}$)	Survival rate (%)	Viability (%)
Control	0.15 \pm 0.05	1.3 \pm 0.3	87.7 \pm 7.8	71.6 \pm 20.1
Se	0.18 \pm 0.05	9.5 \pm 0.2*	93.2 \pm 7.0	70.2 \pm 19.4

Values are means \pm 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 <i>n</i> -6	24.7±1.8	22.7±2.2	13.6±1.0	14.6±0.9
18:3 <i>n</i> -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 <i>n</i> -6	0.6±0.1	0.3±0.4	0.9±0.1	0.9±0.1
20:5 <i>n</i> -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 <i>n</i> -3	3.0±0.2	3.2±0.2	6.5±0.4	6.8±0.1
22:6 <i>n</i> -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).

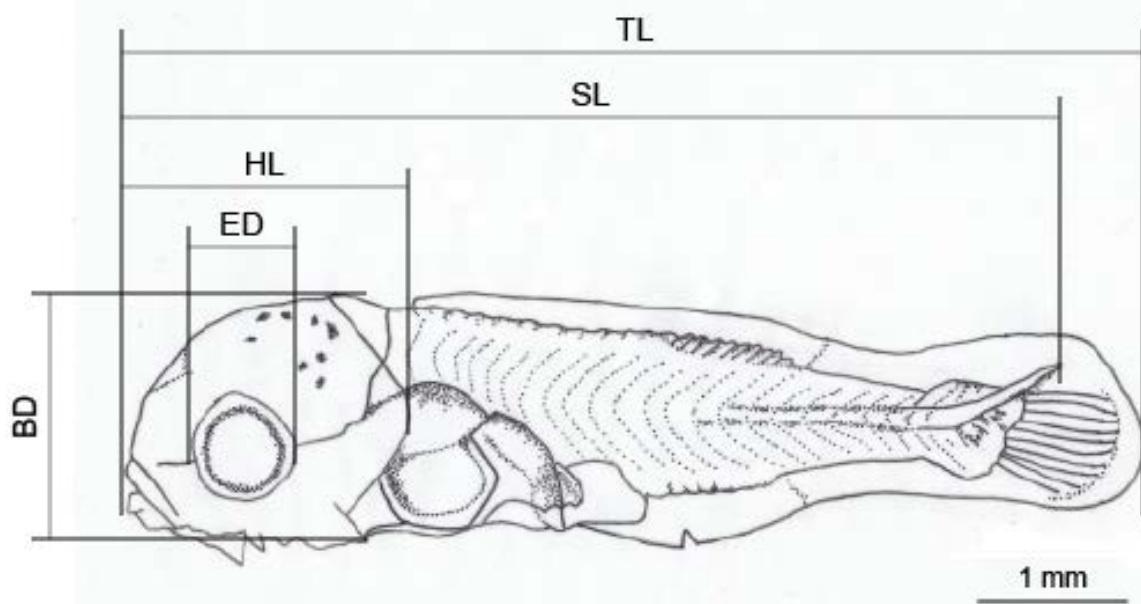


Fig. 1.

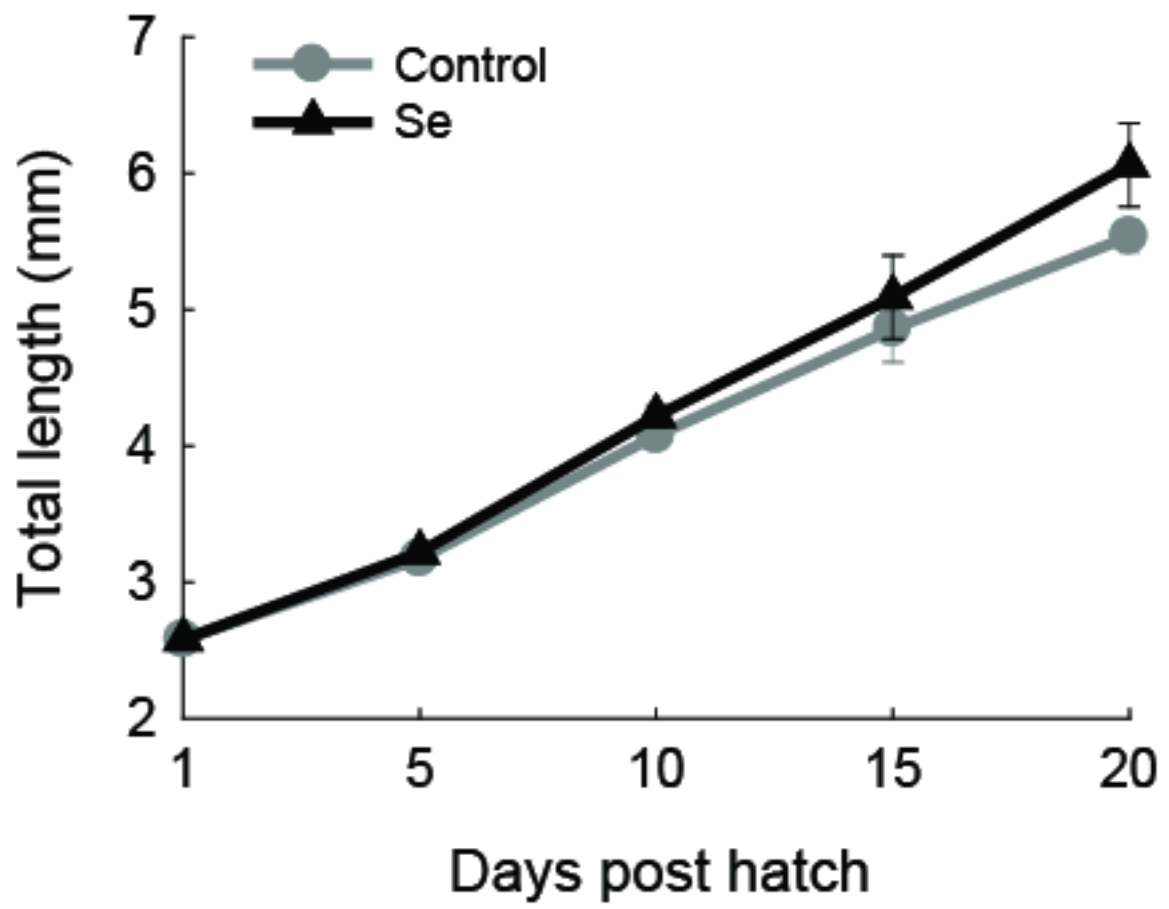


Fig. 2.

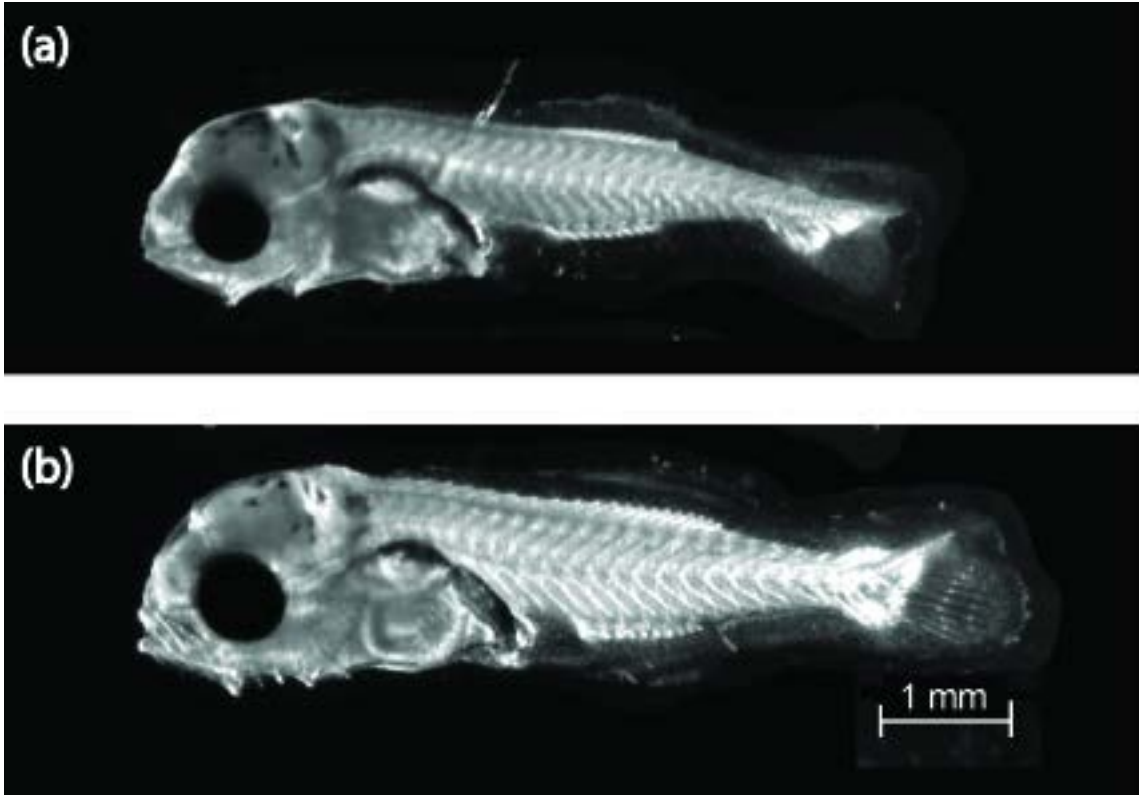


Fig. 3.