

## 1     **Ingestion by Japanese Eel *Anguilla japonica* Larvae on Various Minute Zooplanktons**

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6     **Abstract:** We observed the feeding incidence of Japanese eel *Anguilla japonica* larvae of 6, 7, 8  
7     and 14 days after hatching (DAH) using various minute zooplanktons such as rotifer (*Proales*  
8     *similis*, *Synchaeta* sp., *Keratella* sp., *Brachionus rotundiformis*, *B. angularis*) and nauplii of  
9     copepod *Paracyclops nana*, and compared those results to slurry type diets (i.e., shark eggs  
10    for control) to evaluate the usability of these planktons as primary food source for the mass  
11    culture of eel larvae. Feeding incidence of the larvae on 6, 7 and 8 DAH was 26.7-100% for  
12    slurry type diet, 20-46.7% for *Proales similis* and 0-6.7% for *Synchaeta* sp. At 14 DAH,  
13    feeding incidence of the larvae on slurry type diet and *Proales similis* reached to 100%,  
14    followed by *B. rotundiformis* (53.3%), *Synchaeta* sp. (20%), *Keratella* sp. (13.3%), and *B.*  
15    *angularis* (6.7%). On this day, slurry type diet (68.9%), *Proales similis* (37.2%) and *Synchaeta*  
16    sp. (1.0%) were detected in mid-hindgut while the other ingested rotifers remained in foregut of  
17    the larvae. These results suggested the possibility of minute illoricate rotifer *Proales similis* as  
18    an initial food source for Japanese eel larvae among the employed zooplanktons.

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20    **Key words:** Japanese eel; Larval rearing; Zooplankton; *Proales similis*

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23 Japanese eel *Anguilla japonica* is esteemed as an important source of protein supply not only  
24 in Japan but also other countries in Asia and Europe (Kagawa et al. 2005). The aquacultural  
25 production of Japanese eel used wild captured glass eels as seedling, but the resources have  
26 been decreasing sharply (Kato and Kobayashi 2001; Kagawa et al. 2005). The transition from  
27 preleptocephali on 8 day after hatching (DHA) to the leptocephalus was artificially succeeded  
28 using slurry type diet made from freeze-dried shark (spiny dogfish; *Squalus acanthias*) eggs  
29 (Tanaka et al. 2001, 2003; Kagawa et al. 2005). Moreover, the efficiency of these shark eggs in  
30 eel larviculture was proven by comparing with other species eggs such as tiger shark  
31 *Galeocerdo cuvier* and gulper shark *Centrophorus atromarginatus* (Masuda et al. 2011). These  
32 food sources made from shark eggs are not available for the mass production of glass eels  
33 because of unstable quantitative-qualitative supply (Baum et al. 2003). Thus, efforts should be  
34 continued to find new dietary sources of eel larvae for the mass production of eel larvae. Earlier  
35 studies suggested that eel larvae actually do not feed, instead directly absorb dissolved organic  
36 matter by epidermal uptake (Kracht and Tesch 1981; Pfeiler 1986). However, by analyzing gut  
37 content of various eel larvae species collected from nature, studies suggest that the larvae feed  
38 on materials identified as dissolved and particulate organic matter (Otake et al. 1993), fine  
39 detrital particles and aggregations (Mochioka 2003) or zooplanktons fecal pellets and discarded  
40 larvacean houses (Mochioka and Iwamizu 1996). Other studies conducted in laboratory  
41 confirmed that eel larvae capable of ingesting food materials including not only slurry type diet  
42 (Tanaka et al. 2001, 2003; Kagawa et al. 2005), also squid paste (Mochioka et al. 1993), S-type  
43 rotifers (Tanaka et al. 1995), hen egg yolk and skinned krill (Okamura et al. 2013).

44 Esophageal part around pharynx of Japanese eel larvae is narrow without mucus cells  
45 (Yoshimatsu 2011). Due to their characteristics, we hypothesized that initial stage of eel larvae  
46 requires food with small size, smooth and flexible surface and employed following

47 zooplanktons: *Proales similis*, *Synchaeta* sp., *Keratella* sp., SS-type *Brachionus rotundiformis*,  
48 *B. angularis*, nauplii of a copepod *Paracyclops nana*. We observed the feeding incidence and  
49 ingestion of Japanese eel larvae and compared these results with on slurry type diet (i.e., shark  
50 eggs) to estimate their usability as a primary food source of eel larvae.

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## Materials and Methods

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### 54 *Preparation of condensed zooplanktons*

55 The rotifers, *Proales similis* was collected from an estuary in Ishigaki island, Okinawa, Japan  
56 (Wullur et al. 2009), *B. rotundiformis* from brackish water ponds in Manado, North-Sulawesi,  
57 Indonesia (Hagiwara et al. 1995; Rumengan et al. 1998), *B. angularis* from Laos (Ogata et al.  
58 2011), *Keratella* sp., *Synchaeta* sp., from South-Korea (J.C. Park, Kangnung National  
59 University, South-Korea) and a cyclopoid copepod *Paracyclops nana* from Hwajinpo salt  
60 lake, Gangwondo, South-Korea (Lee et al. 2006). Body size of tested zooplanktons was less  
61 than 150  $\mu\text{m}$  and their bodies were characterized as illoricate (soft body without lorica) for  
62 *Proales similis* and *Synchaeta* sp. or loricate (solid body with lorica or carapace exoskeleton)  
63 for *Keratella* sp., *B. rotundiformis*, *B. angularis* and *Paracyclops nana* (Table 1).  
64 Commercial freeze-dried shark egg yolk (Aquaran, BASF Japan) was employed as control  
65 (Tanaka et al. 2001, 2003; Kagawa et al. 2005).

66 Prior to the feeding, the zooplanktons were mass cultured in polycarbonate tanks with 50-120  
67 l of working volume at 25°C. Diluted natural seawater (15 ppt) was used, except for the rotifer  
68 *B. angularis*, which is a freshwater species. Gentle aeration was provided to the cultures at 50  
69 ml/min. Microalgae *Chlorella vulgaris* V-12<sup>®</sup> produced by Chlorella Industry Company  
70 (Fukuoka, Japan), was used as food for batch-culture of following zooplanktons: *Proales similis*,  
71 *B. rotundiformis*, *B. angularis* and *Paracyclops nana*. The batch-culture of *Synchaeta* sp. and  
72 *Keratella* sp. used *Tetraselmis tetrathele* as food. The microalgae were added once or twice a

73 day at  $2 \times 10^6$  cells/ml. Population growth of the zooplanktons was observed twice a day by  
74 counting the number of individuals of each zooplankton species in 1 ml sample (in triplicates)  
75 from each culture tank. The cultures were harvested at exponential growth stage and  
76 concentrated using plankton net with 10 to 45  $\mu\text{m}$  of mesh sizes, depending on the size of the  
77 zooplanktons. When harvesting the copepod nauplii, 150  $\mu\text{m}$  mesh size plankton net was firstly  
78 used to separate the adult stage and then the same procedure as other zooplanktons. All  
79 harvested zooplanktons were soaked in seawater and kept in a refrigerator at temperature 4°C.  
80 From these condensed zooplankton stocks, 100 individuals in each species were measured body  
81 length and width using digital microscope (VH-8000, Keyence Co., Japan) at 450x  
82 magnification. Prior to the measurement, specimens were anesthetized with 0.002% MS 222  
83 (Tricaine; Sigma Chemical Co., USA) to prevent body shrinkage.

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#### 85 *Observation of feeding incidence of Japanese eel larvae*

86 Eel larvae used in the present study were obtained from artificially fertilized eggs (Yamamoto  
87 and Yamauchi 1974; Yamauchi et al. 1976; Tanaka et al. 2001, 2003). These eggs were  
88 incubated in a flow-through hatching container at 23°C and hatched on the two days after  
89 fertilization. By 6 days after hatching (DAH), the pigmentation of the eyes was well developed,  
90 the mouth had moved from abdomen side to the head, and yolk-sack almost exhausted  
91 suggesting that the larvae acquired the ability to take foods. The upper jaw length of the larvae  
92 was measured using a digital microscope (VHX-200, Keyence) at 100x magnifications, and the  
93 mouth size was estimated according to Shirota (1970); upper jaw length times 2<sup>0.5</sup>. Ingestion by  
94 the eel larvae on each zooplankton species was investigated at 6, 7, 8 and 14 DAH. Prior to the  
95 feeding experiment, no food was offered to the larvae of 6, 7, and 8 DAH, but those of 14 DAH  
96 were firstly fed slurry type diet. A well of 6-well microplate (Iwaki, Japan) was filled with 5 ml  
97 of natural seawater (33-34 ppt) and five larvae of Japanese eel were transferred to each well,  
98 followed by the addition of each condensed zooplankton onto the bottom of the wells in

99 triplicates. The amount of each zooplankton added to the wells was equal to 0.19 g of wet  
 100 weight. Those microplates were incubated at 23°C under 300-500 lx of light. Observation on  
 101 the feeding incidence by the larvae was made for 3 to 6 hours with larvae of 6 to 8 DAH and 1  
 102 hour with larvae of 14 DAH. The number of eel larvae ingesting zooplankton was counted to  
 103 obtain feeding incidence of the larvae (percentage of larvae with zooplankton in gut) and the  
 104 percentage of occupied area by the ingested zooplankton in gut of the larvae was measured  
 105 under a digital microscope (VHX-200) at 25-100x magnifications. When measuring the gut  
 106 occupied by the ingested zooplankton (projected area), larval gut was divided into two parts  
 107 (Govoni et al. 1986); foregut (from end of the mouth until end part of the presumptive stomach)  
 108 and mid-hind gut (from end of the presumptive stomach until anus). Data of zooplankton  
 109 feeding incidence, size and food occupied area in gut was analyzed using a one-way ANOVA  
 110 followed by Tukey-Kramer test to examine differences among treatments.

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### Results

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114 The body size of *Proales similis* was the smallest among employed zooplanktons (length  
 115  $91 \pm 11 \mu\text{m}$ , width  $45 \pm 6 \mu\text{m}$ , Tukey-Kramer test,  $P < 0.05$ , Table 1). The calculated mouth size of  
 116 eel larvae of 6 DAH was  $521.2 \pm 27.9 \mu\text{m}$ . Feeding incidence of the eel larvae of 6 to 8 DAH  
 117 was only observed with slurry type diet ( $26.7 \pm 32.1 - 100 \pm 0.0\%$ ) and two illoricate rotifer *Proales*  
 118 *similis* ( $20.0 \pm 20.0 - 46.7 \pm 30.6\%$ ) and *Synchaeta* sp. ( $0.0 - 6.7 \pm 11.6\%$ ). At 6 to 8 DAH, the eel  
 119 larvae gathered the supplied zooplanktons using their mouth soon after the food organisms  
 120 added into the wells and obtained food materials only on the bottom of wells by sucking.  
 121 However, in case of loricate rotifers and nauplii of copepod, the larvae did not excrete these  
 122 food organisms, instead, they stopped sucking activities when the foods blocked the location  
 123 between pharynx and esophageal of the larvae. At 14 DAH, larvae could ingest the loricate  
 124 rotifers; *Keratella* sp. ( $13.3 \pm 11.6\%$ ), *B. rotundiformis* ( $53.3 \pm 11.6\%$ ) and *B. angularis*

Table 1

Fig. 1

Table 2

125 (6.6±11.5%), but no ingestion was observed on nauplii of copepod *Paracyclops nana*.  
126 Feeding incidence was significantly higher with slurry type diet and *Proales similis* than other  
127 diets after 7 DAH (Tukey-Kramer test,  $P<0.05$ ).

128 By dividing gut of the larvae into foregut and mid-hindgut (Table 2), it was observed that the  
129 ingested loricate rotifers; *Keratella* sp., *B. rotundiformis* and *B. angularis* by the larvae on 14  
130 DAH was found only in foregut. The feeding amount was small, and food occupied area in  
131 foregut remained 1.0±0.5% for *Keratella* sp., 3.4±1.6% for *B. rotundiformis* and 0.2±0.3% for *B.*  
132 *angularis*, and food occupied area in foregut was not significantly different among feeding  
133 treatments. The slurry type diet, and two illoricate rotifers; *Proales similis* and *Synchaeta* sp.  
134 only occupied mid-hindgut of the larvae (Fig. 2). The occupied area of mid-hind was  
135 significantly higher on slurry type diet (20.4±18.3 to 68.9±13.1%), followed by on *Proales*  
136 *similis* (1.8±2.7 to 37.2±2.2%) and *Synchaeta* sp. (0±0 to 1.0±1.1%) at 7, 8 and 14 DAH  
137 (Tukey-Kramer test,  $P<0.05$ ).

Fig. 2

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### Discussion

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141 As candidates of novel initial diet for *A. japonica* leptocephali, this study examined the use of  
142 minute rotifers and copepods which are major initial food for marine and freshwater fish species  
143 in nature. These zooplankton species were employed as condensed form (immobile and  
144 nonliving) because eel larvae were successfully reared by slurry diet made from freeze-dried  
145 shark eggs in the previous studies (Tanaka et al. 2001, 2003; Kagawa et al. 2005). As an initial  
146 stage of eel larvae, we compared availability of these zooplanktons by using immobile  
147 condensed form, since morphology of food species is of primary importance comparing to  
148 behavior. Mouth size of eel larvae (521.2±27.9  $\mu\text{m}$ ) is larger than all supplied zooplanktons,  
149 and thus it is possible to ingest all species (Table 1). The larvae on 6 to 8 DAH only had  
150 capability to ingest (feeding incidence) slurry type diet and two smallest illoricate rotifers

151 (*Proales similis* and *Synchaeta* sp.). The slurry diet and two illoricate rotifers were easy to  
152 through esophageal, while the loricate rotifers and nauplii of copepod were not, and  
153 accumulated at the end part of larval mouth on 6 to 8 DAH. It suggests that eel larvae at early  
154 stage require small and soft food despite their large mouth size caused by their histological  
155 characteristics of esophageal part, which is narrow without mucus cells (Yoshimatsu 2011).  
156 Larvae of many teleost species have mucus cells in esophageal (Banglole et al. 1997);  
157 facilitating the larvae being capable of ingesting solid particle such as loricate rotifer  
158 *Brachionus*. The eel larvae of 14 DAH could ingest loricate rotifers (*Keratella* sp., *B.*  
159 *rotundiformis* and *B. angularis*), but none for nauplii of copepod *Paracyclops nana*. The  
160 ingested rotifers were found only in foregut and did not appear in mid-hindgut of the larvae. A  
161 similar occurrence was reported by Tanaka et al. (1995) in which the authors found a larva of 13  
162 DAH has retained one S-type rotifer *B. rotundiformis* in the esophagus and five in the  
163 presumptive stomach area (foregut part) of the larva. These may provide a mechanism of  
164 regulation inbetween foregut and mid-hindgut at the early stage of eel larvae. According to  
165 Ozaki et al. (2006), the foregut of eel larvae may function only for transportation of diet, as well  
166 as physical breakdown of food materials taken orally, and did not support a role of absorption or  
167 digestion. It is suggested that the presence of lorica, as it cannot be digested by eel larvae  
168 (Lubzens et al. 1989), inhibited the larvae to easily break the rotifers. Therefore nutritional  
169 absorption processes that are mainly occurred in mid-hind gut of eel larvae may not occur on the  
170 loricate rotifers unless they could pass through the mid-hindgut.

171 Euryhaline rotifer *B. plicatilis* species complex has been speculatively used for larval rearing  
172 of marine fishes (Hagiwara et al. 2001), there are more than 2,000 rotifer species in the phylum  
173 Rotifera, which include smaller sized species comparing to SS-type rotifers (*B. rotundiformis*).  
174 Such trials have been reported by Wullur et al. (2009, 2011) and Hirai et al. (2012), which used  
175 minute rotifer *Proales similis* as initial food for seven band grouper and Napoleon wrasse,  
176 respectively. Results of this study demonstrated a significantly higher ingestion of eel larvae on

177 *Proales similis* than on other supplied zooplanktons from 6 to 14 DAH (Fig. 1). Feeding  
178 incidence of the eel larvae on *Proales similis* was comparable with slurry type diet and it  
179 similarly passed to larval mid-hind gut (Fig. 2). Sustainable supply of *Proales similis* can be  
180 ensured because this species can be mass propagated and enriched using the same method as  
181 *Brachionus* (Wullur et al. 2009). The tested eel larvae obtained supplied zooplanktons only on  
182 the bottom of wells by sucking, instead of capturing food available in water column as have  
183 been seen in most teleost fish species. The rotifer *Proales similis* is a benthic species distributed  
184 at the sediment surface (Schmid-Araya 1993), and thus condensing process to harvest cultured  
185 rotifers with filtration should not be needed on this species. The heavy mortality with the slurry  
186 diet occurred by a failure in first feeding between 10 and 15 DAH by water exchange to prevent  
187 bacterial proliferation (i.e., too short feeding time as 5 h/day, Tanaka et al. 2001). Employment  
188 of *Proales similis* as live food should induce lower mortality of eel larvae by lower-frequency  
189 water exchange, namely by feeding for sufficient time. Future studies will be focused on the  
190 digestion and nutritional absorption as well as on the survival and growth of eel larvae with  
191 *Proales similis* to evaluate the usability of this zooplankton species as the first food source in  
192 eel larviculture.

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## 290 微小動物プランクトンに対するウナギ仔魚の初期摂餌

291

292 Stenly WULLUR ・ 吉松隆夫 ・ 田中秀樹 ・ 大谷諒敬 ・ 阪倉良孝 ・ 金 禧珍 ・ 萩原篤志

293

294 ウナギ *Anguilla japonica* の仔魚飼育にはアブラツノザメ *Squalus acanthias* の卵を原料

295 とする懸濁態飼料が用いられている。しかし、これをウナギ種苗を量産するために十

296 分量確保できる見込みはなく、大量に確保可能な代替飼料を探す必要がある。本研究

297 では微小動物プランクトン (*Proales similis*, *Synchaeta* sp., *Keratella* sp., *Brachionus*298 *rotundiformis*, *B. angularis*) とカイアシ類 (*Paracyclops nana*) のノープリウス幼生、懸濁

299 態飼料 (対照区) を用い、ウナギ仔魚の摂餌行動観察を通じて餌料としての可能性を

300 検討した。孵化後 6, 7, 8 日目の仔魚の摂餌率はサメ卵ベースの飼料で 26.7-100%、

301 *Proales similis* で 20-46.7%, *Synchaeta* sp. で 0-6.7% となった。孵化後 14 日目の仔魚では302 サメ卵飼料と *Proales similis* で 100% と増加し、*B. rotundiformis* では 53.3%、*Synchaeta*303 sp. で 20%、*Keratella* sp. で 13.3%、*B. angularis* で 6.7% となった。このとき、68.9%304 のサメ卵飼料、37.2% の *Proales similis*、1.0% の *Synchaeta* sp. が中後腸に達していたが、

305 他のワムシ類は前腸部のみに見られた。以上の結果から、今回用いた微小動物プラン

306 クトンの中では *Proales similis* が、ウナギ仔魚飼育の餌料生物として最も有望であるこ

307 とが示された。

308 **Tables**

309

310 **Table 1.** Body length and width (mean  $\pm$  standard deviation) of the zooplanktons used in the present study

Zooplankton species	Body dimension ( $\mu\text{m}$ )	
	Length	Width
<i>Proales similis</i>	91 $\pm$ 11 <sup>d</sup>	45 $\pm$ 6 <sup>f</sup>
<i>Synchaeta</i> sp.	101 $\pm$ 9 <sup>cd</sup>	56 $\pm$ 6 <sup>e</sup>
<i>Keratella</i> sp.	118 $\pm$ 9 <sup>b</sup>	63 $\pm$ 9 <sup>d</sup>
<i>Brachionus rotundiformis</i>	136 $\pm$ 15 <sup>a</sup>	107 $\pm$ 14 <sup>a</sup>
<i>Brachionus angularis</i>	108 $\pm$ 8 <sup>bc</sup>	70 $\pm$ 8 <sup>c</sup>
<i>Paracyclopsina nana</i>	142 $\pm$ 82 <sup>a</sup>	76 $\pm$ 20 <sup>b</sup>

311 Different alphabetical letters on the right side of the presented data indicate significant differences among zooplankton species in  
 312 each parameter (a>b>c>d>e>f, Tukey-Kramer test,  $P<0.05$ ,  $n=100$ ).

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317 **Table 2.** Proportion of occupied area (mean  $\pm$  standard deviation) by the ingested food in foregut (FG) and mid-hind gut (MHG) of  
 318 Japanese eel *Anguilla japonica* larvae on 6, 7, 8 and 14 DAH

Tested diet	Occupied area by food in larval gut (%)							
	6 DAH		7 DAH		8 DAH		14 DAH	
	FG	MHG	FG	MHG	FG	MHG	FG	MHG
<i>Proales similis</i>	0 $\pm$ 0	1.8 $\pm$ 2.7 <sup>ab</sup>	10.2 $\pm$ 17.8	16.4 $\pm$ 9.6 <sup>b</sup>	0 $\pm$ 0	8.3 $\pm$ 8.9 <sup>b</sup>	2.2 $\pm$ 2.1	37.2 $\pm$ 2.2 <sup>a</sup>
<i>Synchaeta</i> sp.	0 $\pm$ 0	0.5 $\pm$ 0.9 <sup>ab</sup>	0 $\pm$ 0	0 $\pm$ 0 <sup>c</sup>	0 $\pm$ 0	0.6 $\pm$ 1.0 <sup>c</sup>	0 $\pm$ 0	1.0 $\pm$ 1.1 <sup>b</sup>
<i>Keratella</i> sp.	0	0 <sup>b</sup>	0	0 <sup>c</sup>	0	0 <sup>c</sup>	1.0 $\pm$ 0.5	0 $\pm$ 0 <sup>c</sup>
<i>Brachionus rotundiformis</i>	0	0 <sup>b</sup>	0	0 <sup>c</sup>	0	0 <sup>c</sup>	3.4 $\pm$ 1.6	0 $\pm$ 0 <sup>c</sup>
<i>Brachionus angularis</i>	0	0 <sup>b</sup>	0	0 <sup>c</sup>	0	0 <sup>c</sup>	0.2 $\pm$ 0.3	0 $\pm$ 0 <sup>c</sup>
<i>Paracyclopsina nana</i>	0	0 <sup>b</sup>	0	0 <sup>c</sup>	0	0 <sup>c</sup>	0	0 <sup>c</sup>
Slurry type diet	0 $\pm$ 0	20.4 $\pm$ 18.3 <sup>a</sup>	0 $\pm$ 0	51.8 $\pm$ 12.9 <sup>a</sup>	0 $\pm$ 0	57.0 $\pm$ 7.8 <sup>a</sup>	2.3 $\pm$ 4.1	68.9 $\pm$ 13.1 <sup>a</sup>

319 Different alphabetical letters on the right side of the presented data indicate significant differences among tested diets (a>b>c, Tukey-  
 320 Kramer test,  $P<0.05$ ,  $n=3$ ).

321

322

**Figures**

323

324

325 **Fig. 1.** Feeding incidence (mean±SD) of Japanese eel larvae on six minute zooplanktons  
326 and slurry type diet on 6 (A), 7(B), 8 (C) and 14 (D) days after hatching. Alphabetical  
327 letters indicate significant differences in each treatment at the same age group (a>b>c,  
328 Tukey-Kramer test,  $P<0.05$ ,  $n=3$ ).

329

330 **Fig. 2.** Japanese eel *Anguilla japonica* larvae of 14 DAH with the rotifer *Proales similis*  
331 in gut (A) and without food in gut (B).

332

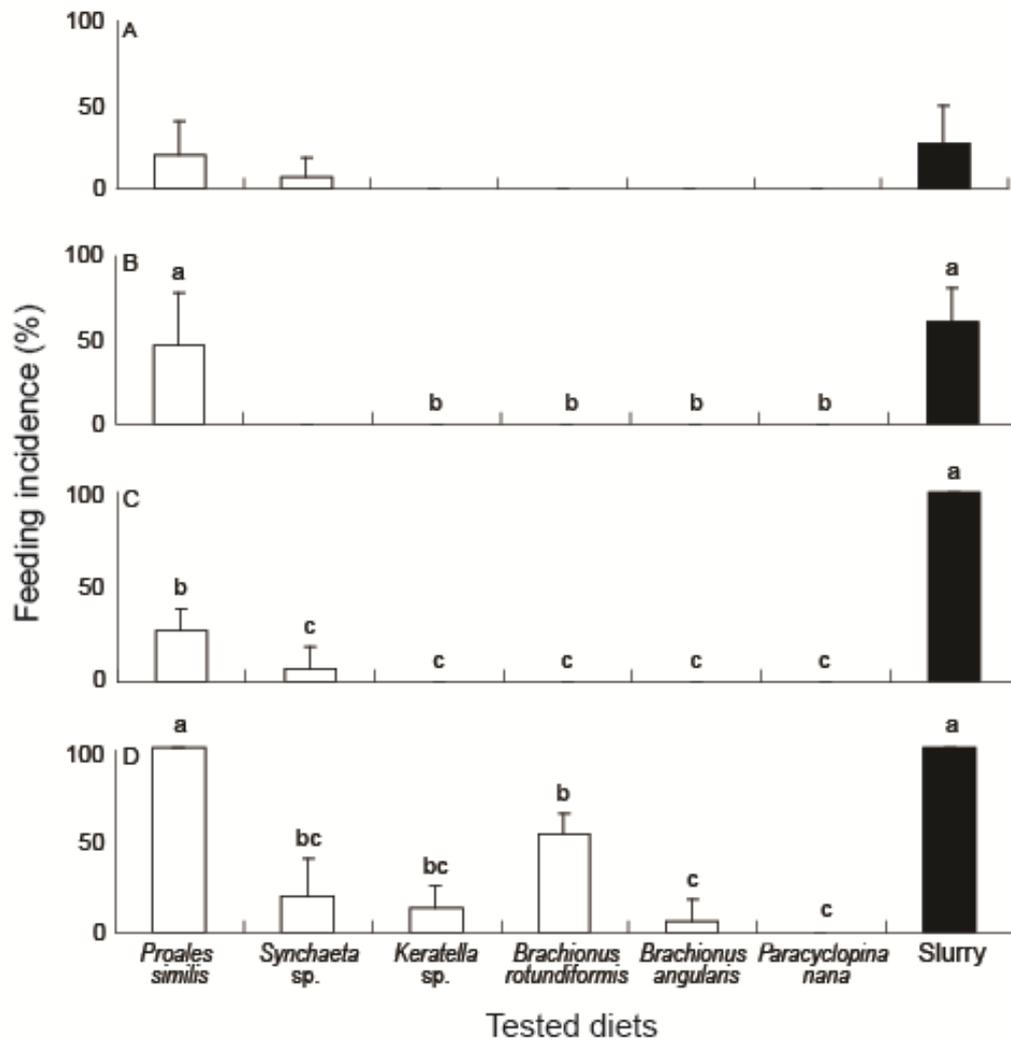


Fig. 1.

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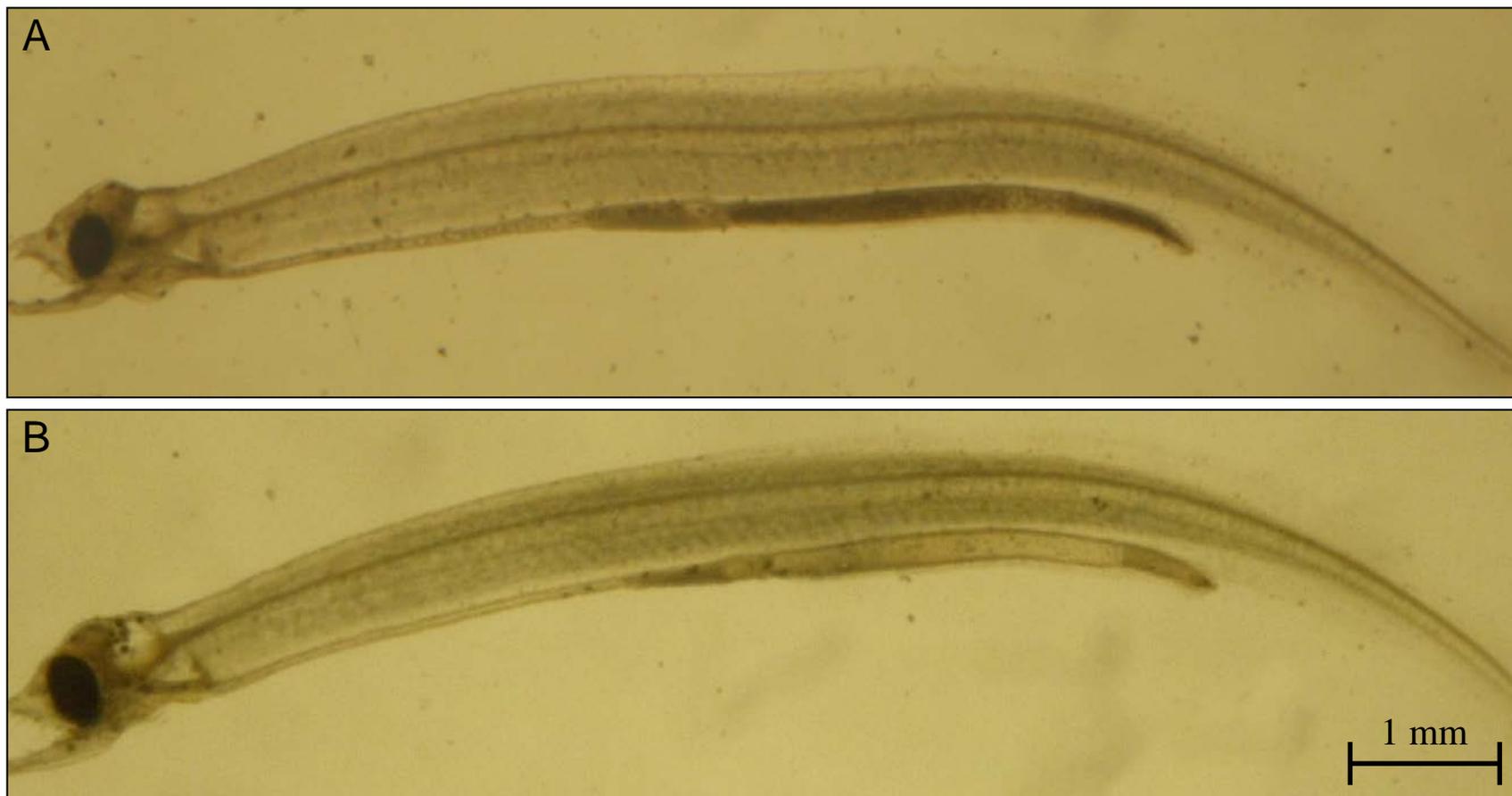
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**Fig. 2.**

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