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

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

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

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

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

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

Materials and Methods

Preparation of condensed zooplanktons

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

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

day at $2x10^6$ cells/*ml*. Population growth of the zooplanktons was observed twice a day by counting the number of individuals of each zooplankton species in 1 *ml* sample (in triplicates) from each culture tank. The cultures were harvested at exponential growth stage and concentrated using plankton net with 10 to 45 μm of mesh sizes, depending on the size of the zooplanktons. When harvesting the copepod nauplii, 150 μm mesh size plankton net was firstly used to separate the adult stage and then the same procedure as other zooplanktons. All harvested zooplanktons were soaked in seawater and kept in a refrigerator at temperature 4°C. From these condensed zooplankton stocks, 100 individuals in each species were measured body length and width using digital microscope (VH-8000, Keyence Co., Japan) at 450x magnification. Prior to the measurement, specimens were anesthetized with 0.002% MS 222 (Tricaine; Sigma Chemical Co., USA) to prevent body shrinkage.

Observation of feeding incidence of Japanese eel larvae

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

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

Results

The body size of *Proales similis* was the smallest among employed zooplanktons (length 91±11 μm, width 45±6 μm, Tukey-Kramer test, $P<0.05$, Table 1). The calculated mouth size of eel larvae of 6 DAH was 521.2±27.9 μm. Feeding incidence of the eel larvae of 6 to 8 DAH was only observed with slurry type diet (26.7±32.1-100±0.0%) and two illoricate rotifer *Proales similis* (20.0±20.0-46.7±30.6%) and *Synchaeta* sp. (0.0-6.7±11.6%). At 6 to 8 DAH, the eel larvae gathered the supplied zooplanktons using their mouth soon after the food organisms added into the wells and obtained food materials only on the bottom of wells by sucking. However, in case of loricate rotifers and nauplii of copepod, the larvae did not excrete these food organisms, instead, they stopped sucking activities when the foods blocked the location between pharynx and esophageal of the larvae. At 14 DAH, larvae could ingest the loricate rotifers; *Keratella* sp. (13.3±11.6%), *B. rotundiformis* (53.3±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 *Paracyclopina 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 Fig. 2
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, $P<0.05$).
138
139 **Discussion**
140
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 μm) 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

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

Euryhaline rotifer *B. plicatilis* species complex has been speculatively used for larval rearing of marine fishes (Hagiwara et al. 2001), there are more than 2,000 rotifer species in the phylum Rotifera, which include smaller sized species comparing to SS-type rotifers (*B. rotundiformis*). Such trials have been reported by Wullur et al. (2009, 2011) and Hirai et al. (2012), which used minute rotifer *Proales similis* as initial food for seven band grouper and Napoleon wrasse, 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.
193
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195
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微小動物プランクトンに対するウナギ仔魚の初期摂餌

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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。本研究では微小動物プランクトン (*Proales similis, Synchaeta* sp., *Keratella* sp., *Brachionus rotundiformis, B. angularis*)とカイアシ類 (*Paracyclopina nana*) のノープリウス幼生、懸濁態飼料（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%、*Proales similis* で 20-46.7%, *Synchaeta* sp.で 0-6.7%となった。孵化後 14 日目の仔魚ではサメ卵飼料と *Proales similis* で 100%と増加し、*B. rotundiformis* では 53.3%、 *Synchaeta* sp.で 20%、 *Keratella* sp.で 13.3%、 *B. angularis* で 6.7%となった。このとき、 68.9%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、他のワムシ類は前腸部のみにみたれた。以上の結果から、今回用いた微小動物プランクトンの中では *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とが示された。

Tables

Table 1. Body length and width (mean ± standard deviation) of the zooplanktons used in the present study

Zooplankton species	Body dimension (μm)	
	Length	Width
Proales similis	91±11 [d]	45±6 [f]
Synchaeta sp.	101±9 [cd]	56±6 [e]
Keratella sp.	118±9 [b]	63±9 [d]
Brachionus rotundiformis	136±15 [a]	107±14 [a]
Brachionus angularis	108±8 [bc]	70±8 [c]
Paracyclopina nana	142±82 [a]	76±20 [b]

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

317 **Table 2.** Proportion of occupied area (mean ± 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
Proales similis	0±0	1.8±2.7 ^{ab}	10.2±17.8	16.4±9.6 ^{b}	0±0	8.3±8.9 ^{b}	2.2±2.1	37.2±2.2 ^{a}
Synchaeta sp.	0±0	0.5±0.9 ^{ab}	0±0	0±0 ^{c}	0±0	0.6±1.0 ^{c}	0±0	1.0±1.1 ^{b}
Keratella sp.	0	0 ^{b}	0	0 ^{c}	0	0 ^{c}	1.0±0.5	0±0 ^{c}
Brachionus rotundiformis	0	0 ^{b}	0	0 ^{c}	0	0 ^{c}	3.4±1.6	0±0 ^{c}
Brachionus angularis	0	0 ^{b}	0	0 ^{c}	0	0 ^{c}	0.2±0.3	0±0 ^{c}
Paracyclopina nana	0	0 ^{b}	0	0 ^{c}	0	0 ^{c}	0	0 ^{c}
Slurry type diet	0±0	20.4±18.3 ^{a}	0±0	51.8±12.9 ^{a}	0±0	57.0±7.8 ^{a}	2.3±4.1	68.9±13.1 ^{a}

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

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

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

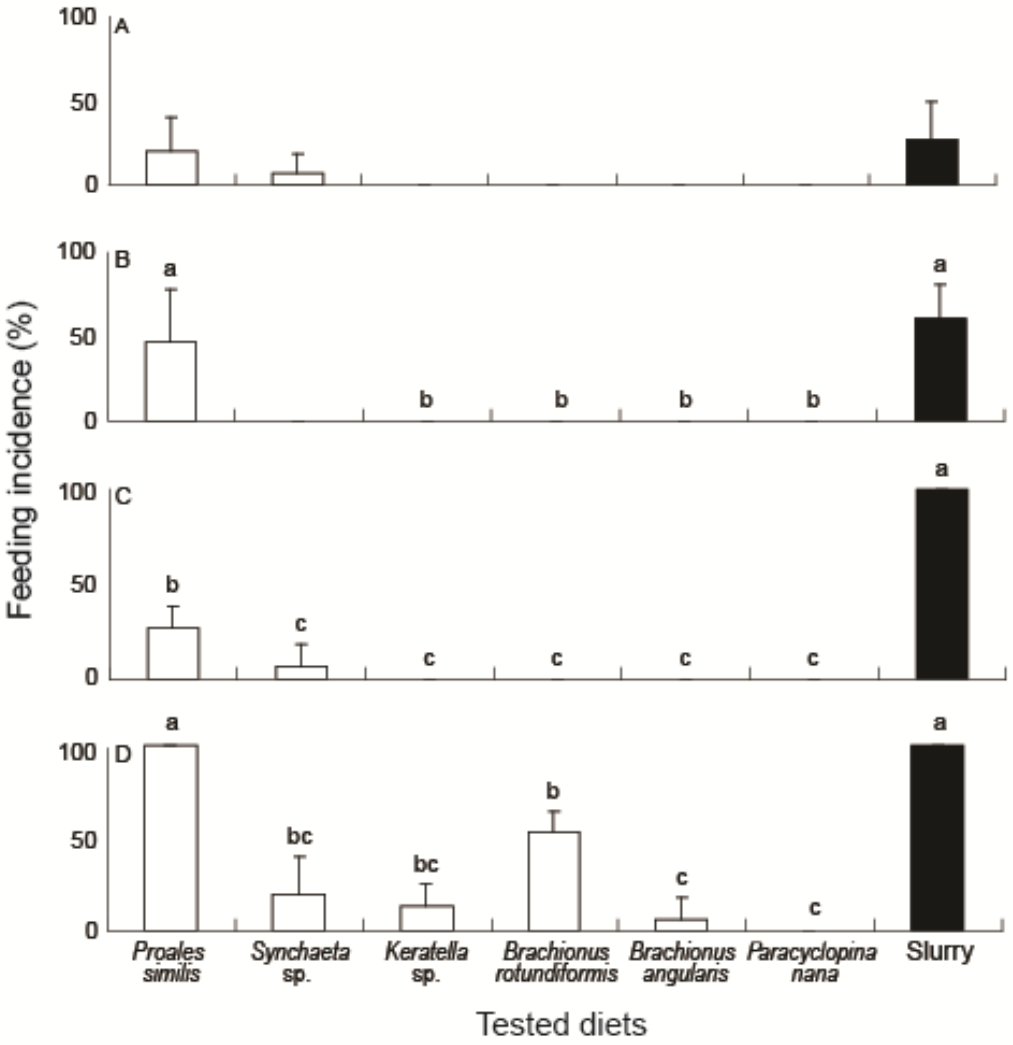
A
B
C
D
100
50
0
Feeding incidence (%)
a
b
c
bc
Proales similis
Synchaeta sp.
Keratella sp.
Brachionus rotundiformis
Brachionus angularis
Paracyclopina nana
Slurry
Tested diets

Fig. 1.

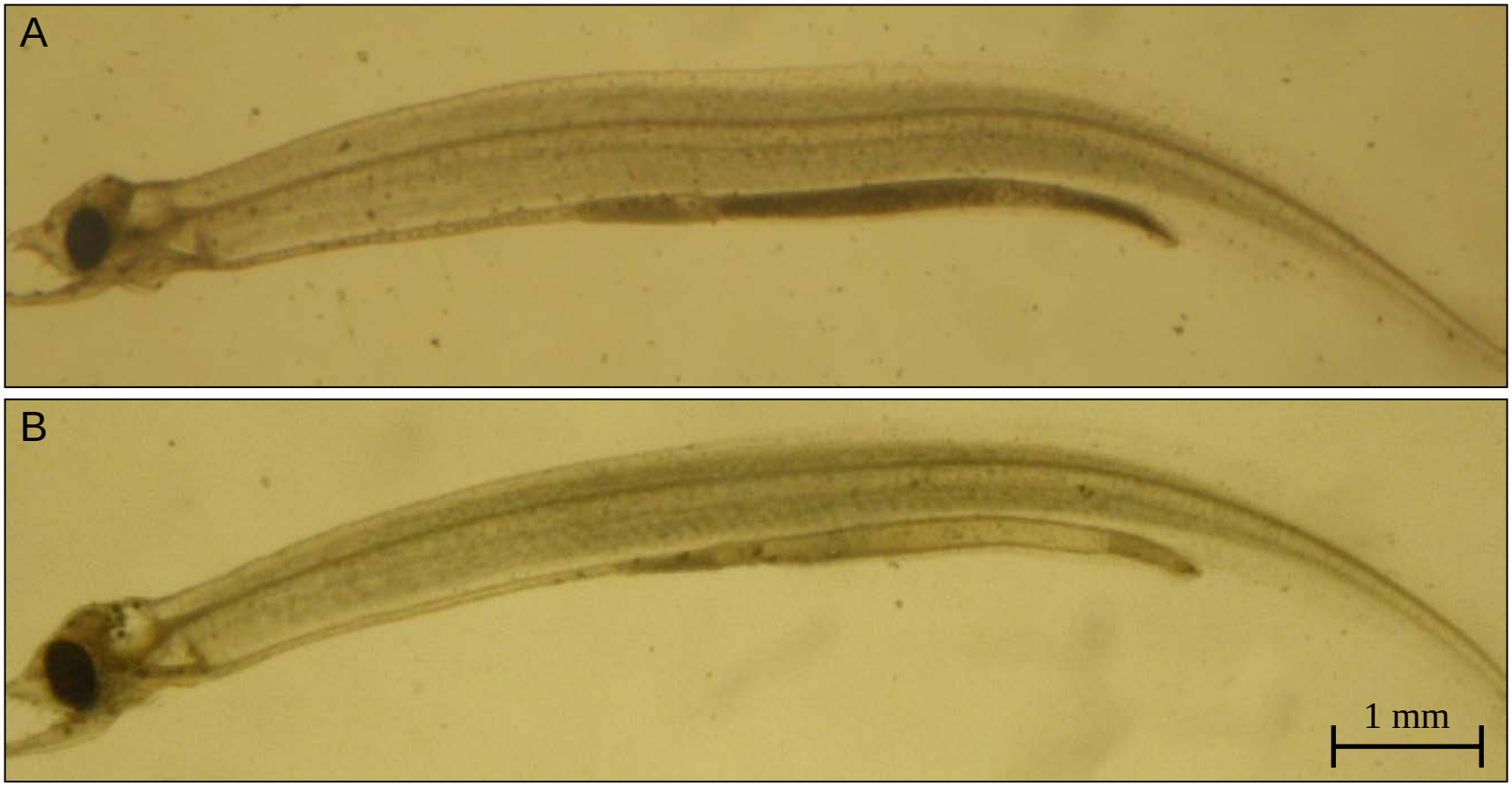

Fig. 2.