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

Stenly WULLUR ¹, Takao YOSHIMATSU ², Hideki TANAKA ³, Masataka OHTANI ⁴, Yoshitaka SAKAKURA ⁵, Hee-Jin KIM ⁵ and Atsushi HAGIWARA ⁵*¹

**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 *Paracyclops 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 *Paracyclopinna 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 *Paracyclopinna 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 *Paracyclopinna 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 *Paracyclopinna 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 $2 \times 10^6$ 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 $\mu$m of mesh sizes, depending on the size of the zooplanktons. When harvesting the copepod nauplii, 150 $\mu$m 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*
(6.6±11.5%), but no ingestion was observed on nauplii of copepod *Paracyclopus nana*. Feeding incidence was significantly higher with slurry type diet and *Proales similis* than other diets after 7 DAH (Tukey-Kramer test, *P*<0.05).

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

**Discussion**

As candidates of novel initial diet for *A. japonica* leptocephali, this study examined the use of minute rotifers and copepods which are major initial food for marine and freshwater fish species in nature. These zooplankton species were employed as condensed form (immobile and nonliving) because eel larvae were successfully reared by slurry diet made from freeze-dried shark eggs in the previous studies (Tanaka et al. 2001, 2003; Kagawa et al. 2005). As an initial stage of eel larvae, we compared availability of these zooplanktons by using immobile condensed form, since morphology of food species is of primary importance comparing to behavior. Mouth size of eel larval (521.2±27.9 μm) is larger than all supplied zooplanktons, and thus it is possible to ingest all species (Table 1). The larvae on 6 to 8 DAH only had 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 Paracyclopsina 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
Proales similis than on other supplied zooplanktons from 6 to 14 DAH (Fig. 1). Feeding incidence of the eel larvae on Proales similis was comparable with slurry type diet and it similarly passed to larval mid-hind gut (Fig. 2). Sustainable supply of Proales similis can be ensured because this species can be mass propagated and enriched using the same method as Brachionus (Wullur et al. 2009). The tested eel larvae obtained supplied zooplanktons only on the bottom of wells by sucking, instead of capturing food available in water column as have been seen in most teleost fish species. The rotifer Proales similis is a benthic species distributed at the sediment surface (Schmid-Araya 1993), and thus condensing process to harvest cultured rotifers with filtration should not be needed on this species. The heavy mortality with the slurry diet occurred by a failure in first feeding between 10 and 15 DAH by water exchange to prevent bacterial proliferation (i.e., too short feeding time as 5 h/day, Tanaka et al. 2001). Employment of Proales similis as live food should induce lower mortality of eel larvae by lower-frequency water exchange, namely by feeding for sufficient time. Future studies will be focused on the digestion and nutritional absorption as well as on the survival and growth of eel larvae with Proales similis to evaluate the usability of this zooplankton species as the first food source in eel larviculture.

Acknowledgements

The rotifer Proales similis was collected during the 189th cruise of Kakuyo-maru, Faculty of Fisheries, Nagasaki University in July, 2004. The authors express thanks to Russ Shiel for identification of rotifer species, Kim Shin-Kwon for his help in providing eel larvae. The Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan is gratefully acknowledged for the scholarship awarded to S.W. This research was partially supported by the MEXT, Grant in Aid for Scientific Research (B), 2009-2011, No. 21380125 and 2012-2014, No.
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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

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

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

<table>
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<tr>
<th>Zooplankton species</th>
<th>Body dimension (µm)</th>
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<tbody>
<tr>
<td></td>
<td>Length</td>
<td>Width</td>
<td></td>
</tr>
<tr>
<td>Proales similis</td>
<td>91±11 $^d$</td>
<td>45±6 $^f$</td>
<td></td>
</tr>
<tr>
<td>Synchaeta sp.</td>
<td>101±9 $^{cd}$</td>
<td>56±6 $^e$</td>
<td></td>
</tr>
<tr>
<td>Keratella sp.</td>
<td>118±9 $^b$</td>
<td>63±9 $^d$</td>
<td></td>
</tr>
<tr>
<td>Brachionus rotundiformis</td>
<td>136±15 $^a$</td>
<td>107±14 $^a$</td>
<td></td>
</tr>
<tr>
<td>Brachionus angularis</td>
<td>108±8 $^{bc}$</td>
<td>70±8 $^c$</td>
<td></td>
</tr>
<tr>
<td>Paracyclopsina nana</td>
<td>142±82 $^a$</td>
<td>76±20 $^b$</td>
<td></td>
</tr>
</tbody>
</table>

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, $P<0.05$, $n=100$).
Table 2. Proportion of occupied area (mean ± standard deviation) by the ingested food in foregut (FG) and mid-hind gut (MHG) of Japanese eel *Anguilla japonica* larvae on 6, 7, 8 and 14 DAH

<table>
<thead>
<tr>
<th>Tested diet</th>
<th>Occupied area by food in larval gut (%)</th>
<th>6 DAH</th>
<th>7 DAH</th>
<th>8 DAH</th>
<th>14 DAH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FG</td>
<td>MHG</td>
<td>FG</td>
<td>MHG</td>
<td>FG</td>
</tr>
<tr>
<td><em>Proales similis</em></td>
<td>0±0</td>
<td>1.8±2.7ab</td>
<td>10.2±17.8</td>
<td>16.4±9.6b</td>
<td>0±0</td>
</tr>
<tr>
<td><em>Synchaeta sp.</em></td>
<td>0±0</td>
<td>0.5±0.9ab</td>
<td>0±0</td>
<td>0±0c</td>
<td>0±0</td>
</tr>
<tr>
<td><em>Keratella sp.</em></td>
<td>0</td>
<td>0b</td>
<td>0</td>
<td>0c</td>
<td>0</td>
</tr>
<tr>
<td><em>Brachionus rotundiformis</em></td>
<td>0</td>
<td>0b</td>
<td>0</td>
<td>0c</td>
<td>0</td>
</tr>
<tr>
<td><em>Brachionus angularis</em></td>
<td>0</td>
<td>0b</td>
<td>0</td>
<td>0c</td>
<td>0</td>
</tr>
<tr>
<td><em>Paracyclopina nana</em></td>
<td>0</td>
<td>0b</td>
<td>0</td>
<td>0c</td>
<td>0</td>
</tr>
<tr>
<td>Slurry type diet</td>
<td>0±0</td>
<td>20.4±18.3a</td>
<td>0±0</td>
<td>51.8±12.9a</td>
<td>0±0</td>
</tr>
</tbody>
</table>

Different alphabetical letters on the right side of the presented data indicate significant differences among tested diets (a>b>c, Tukey-Kramer test, P<0.05, n=3).
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).
Fig. 1.
Fig. 2.

WULLUR et al. (60%)