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ORIGINAL ARTICLE

Aquaculture

A new liquid-type diet for leptocephali in mass production of artificial glass eels

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Abstract

Development of artificial production of glass eels still presents many problems to be solved. What and how to feed larvae is one of the most crucial problems for commercial mass production of glass eels. We evaluated a new liquid-type diet and feeding method against the conventional slurry-type diet by comparing the growth and survival of larvae in triplicate for 27 days after hatching. Larvae were fed by immersion in a liquid-type diet that was a 1.5 times dilution of the conventional slurry-type diet based on dogfish yolk spread over the bottom of the rearing tank. Larvae took more and denser liquid-type food in the intestine than the conventional slurry-type diet. Survival rates at 3 weeks after first feeding were about 90% in most experimental groups (5 groups) except one of three slurry-type diet groups (about 70%). Growth rates were 1.35 times higher in liquid-type diet groups (0.27 mm/day at 27 dph) than in conventional slurry-type diet groups (0.20 mm/day). These results suggest that the immersion feeding method in conjunction with the liquid-type diet has the potential to enable large scale production of glass eels by ensuring high growth and survival.

Keywords *Anguilla japonica* · Rearing method · Diet viscosity · Aquaculture · Larval food · Leptocephalus

Introduction

Freshwater eels are one of the most difficult fish for aquaculture from fertilized eggs compared to many other aquaculture species in the world, such as sea bream, flatfishes, and even tuna. Fishery scientists and aquaculture researchers have made a long-lasting effort to achieve artificial production of glass eels from eggs for more than half century (Tanaka et al. 2001; Okamura et al. 2014; Tanaka 2015). Although, as a result of much effort by researchers, the eel life cycle was completed in the laboratory in 2010 (Tanaka 2015), we have not yet succeeded in achieving mass production of artificial glass eels at an industrial scale. Therefore, eel aquaculture at present has totally been based on the seedlings of wild glass eels, not on the artificial seedlings that are raised from eggs, like other fishes.

Eel aquaculture suffers from chronic deficiency of seedlings because the eel resource has been showing rapid decline worldwide for decades, with the northern hemisphere anguillid species being listed as endangered (Jacoby et al. 2015). Thus, artificial production of glass eels has the potential to reduce the impact of glass eel fishing on the wild stock and to facilitate

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the recovery of eel resources by providing artificial glass eels as seedlings for aquaculture, instead of wild glass eels.

There are many problems to be solved before artificial production of glass eels can be used at the commercial scale. Among them, problems of egg quality and finding a suitable diet for rearing of leptocephali are especially important (Tanaka 2015). Low egg quality causes low larval survival, slow growth and deformity, and sometimes results in mass mortality of larvae. This is derived from the artificial reproductive maturation of parent eels by repeated injections of exogenous hormones, in which salmon pituitary extracts are used to cause the gonads of Japanese eels to rapidly develop (Okamura et al. 2014).

As for a diet for larval rearing, a slurry-type diet mainly made of egg yolk of the spiny dogfish, *Squalus sp.*, as well as other materials has been conventionally used after the first success of artificial production of glass eels (Tanaka et al. 2003). Since the resource of the spiny dogfish is decreasing and limited, various efforts have been made to improve the diet components or to replace dogfish eggs with those of other less-threated species such as tiger or gulper shark, salmon, trout or chicken eggs (Masuda et al. 2011, 2013a; Okamura et al. 2013, 2014; Furuita et al. 2014; Tanaka 2015). However, the growth rates of eels fed by these diets remained as low as 0.2–0.3 mm/day (Masuda et al. 2013b; Okamura et al. 2014), compared to growth estimates of 0.4–0.5 mm/day in ocean conditions (Ishikawa et al. 2001; Fukuda et al. 2018).

In the present rearing technique, a paste of slurry-type diet was scattered on the bottom of rearing tanks with a pipette after putting on a strong light from above, causing the larvae with their negative phototaxis (Yamada et al. 2009) to avoid the light by swimming down to the bottom, where they contacted the diet paste and started feeding. However, not all larvae could contact the diet at the bottom, and some larvae without enough swimming ability or insufficient negative phototaxis did not reach the bottom within the 10 min feeding time. These larvae eventually experience starvation while showing no growth after their yolk reserves were exhausted. This tendency is even a bigger problem in larger tanks, when early larvae do not find the location of the food.

To improve this situation, various ideas were tested. Masuda et al. (2010) reported an immersion method using cow milk as a liquid-type diet in which eel larvae were immersed in a liquid of milk and 'drank' it for 10 h per day; this resulted in a survival of the larvae for 16 days. This suggested a possibility that immersing larvae directly into a liquid-type diet might be useful in improving the growth performance of reared larvae.

The objective of this study was to explore the possibility of a new liquid-type diet for eel larvae, and to test its intake, survival and growth performance by comparing a new liquid-type diet and the conventional slurry-type diet.

Materials and methods

Fishes

Japanese eels were reared from glass eels at the IRAGO Institute (Aichi, Japan) and feminized by being fed a diet containing estradiol-17β (Tachiki and Nakagawa 1993). The feminized eels were matured artificially by administrating a saline suspension of chum salmon *Oncorhynchus keta* pituitary extract (40 mg/kg body weight) by injection once a week following the methods of Horie et al. (2008). Matured females were paired with males that were matured by HCG (human chorionic gonadotropin, Sankyo Yell Co., Ltd., Tokyo, Japan) injection. Fertilized eggs were kept in seawater (25 °C, 35 psu) for a day, and hatched larvae (preleptocephali) were transferred to a cylindrical plastic tank (diameter 45 cm, height 137 cm) and kept in the same seawater for 6 days, during which larvae absorbed their yolk and developed a functional mouth for first feeding on external food. Therefore, no food was given during this period. However, the larvae need to start feeding after yolk absorption on 6 dph since they all die without food at about 18 dph (Okamura et al. 2009). In this study, larvae were fed from 6 dph.

Experimental diet and rearing method

In this study, the diet types were defined by their viscosities as slurry-type (viscosity: 10^3–10^5 mPa·s) and liquid-type (viscosity: $< 10^3$ mPa·s). The standard larval diet for eel leptocephali presently in use is a slurry-type diet (viscosity: 8318.0 ± 111.4 mPa·s, $n = 3$), mainly made of dogfish egg yolk (Tanaka et al. 2003). Composition per 100 g of this diet was 63.5 g dogfish egg yolk, 27.8 g skinned-krill extract, 6.3 g albumen peptide (Kewpie Corporation, Tokyo, Japan), 2.0 g chitin oligosaccharide, 0.5 g vitamin mixture containing vitamins A, B1, B2, B6, D3, E, K, and C, as well as pantothenic acid, niacin, folic acid and inositol (Fish Aid-C, Japan Nutrition, Tokyo, Japan). After mixing all ingredients, the mixture was pasteurized in a 62 °C water bath for 30 min.

The liquid-type diet used for the experiment was prepared by diluting 60 g of the slurry-type diet with 40 g of seawater (viscosity: 26.1 ± 1.7 mPa·s, $n = 3$) for first-feeding larvae (6–9 dph) and 70 g slurry-type diet with 30 g seawater (viscosity: 58.8 ± 7.0 mPa·s, $n = 3$) for 10–27 dph larvae. Because the larvae fed with the liquid-type diet were reared in half sea water and immersed themselves or at least their whole head part with gills into the diet layer at the bottom, we diluted the slurry type diet with seawater

to adjust the osmolarity and to avoid osmotic shock to the epidermis of the gill. Viscosity of diets were determined by a Brookfield viscometer (LV DV-E, AMETEK Brookfield, Massachusetts, USA).

The two types of diet were compared for their larval growth performances in three replicate rearing experiments. We used six 1.5 l kreisel tanks made of acrylic resin (horizontally placed cylindrical tank;φ15 cm × 7.5 cm, 4.1–4.5 cm/s current along the circular tank wall). About 120 eel larvae were transferred into each tank on 6 dph. After stopping the current to have still water in the tanks and turning the light on, eel larvae in three of the six tanks (control groups) were fed for 10 min with 3 ml conventional slurry-type diet that was linearly laid out (4 cm winding 5 lines) on the deepest part of the bottom of each tank. After the feeding time, the remaining food was washed away with a seawater jet generated by gently pipetting with a special 10 ml komagome pipette (enlarged opening ca. 6 mm) and the water mixing with food was gradually drained out of the rearing system by replacing with new rearing water (0.35 l/min, 23 °C, 17 psu). The circular current was restarted and the light turned back off, keeping the larvae in darkness.

Each of the other three tanks (test groups) were fed with 5 ml of liquid-type diet containing the same amount of food materials as the control group, poured to make a shallow food pool on the bottom of the tank. Maximum depth of the pool was 3 mm, which was enough to immerse the head parts of all larvae in the tank. When the light was turned on, almost all the larvae in the tank moved into the pool to aggregate and stayed there throughout the 10 min feeding time. After feeding, the pool was cleaned with the seawater jet and the lights were turned off. Larvae of both groups were fed five times a day (09:00, 11:00, 13:00, 15:00, 17:00). Feeding behavior was observed in both treatment groups.

Estimation of diet intake and growth performance

Immediately after the first feeding time on 6 dph, 10–17 larvae were sampled randomly from the six tanks and fixed with 5% formaldehyde solution. Fifteen unfed larvae were also sampled as an intestine image control at the same timing. We examined the diet intake only at the beginning of the experiment because the larvae start to have growth variations after the beginning of feeding and the intestine area and thickness increase their variation rapidly, which makes accurate comparisons difficult.

Fixed larvae were photographed by digital camera (WRA YCAM-G900, Wraymer Inc., Osaka, Japan) attached to a microscope. Digital images were taken under the same exposure conditions. Total length was measured from the images (Initial TL) and mean Initial TL (TL_i) at the first feeding was obtained. Diet intake of each individual larva was estimated by the projected area of larval intestine and intestinal optical density (Fig. 1). Net optical density of the intestine was obtained by deducting the optical density of unfed larvae from that of fed ones. These two data were obtained by using image processing software Image J (version 1.5i, National Institutes of Health, Bethesda, USA).

At the end of rearing the larvae for 21 days of the experiment (27 dph), about 30 larvae were sampled randomly from each tank and fixed with 5% formaldehyde solution. The total length (TL_e) of each larva was measured on the digital microscopic image. Individual growth rate (GR: mm/day) during feeding period (21 days) was calculated by the following formula

$$\mathrm{GR} = \left(TL_e - TL_i\right)/21$$

To determine the survival rate of larvae, dead larvae were counted and removed from the tank each day. Survival rates were calculated according to the following formula:

$$\text{Survival rate (\%, at } A \text{ dph)} = (S_e + D_t - D_a) \,/\, (S_e + D_t) \times 100,$$

where S_e is the number of larvae surviving at the end of this experiment (27 dph), D_t is the total number of daily dead larvae from 6 to 27 dph, and D_a is sum of the number of daily dead larvae from 6 to A dph.

Statistical analyses

Differences in the two measurements of diet intake and growth in TL of larvae were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, $p < 0.05$. Difference in distribution of TL between two types of diet were analyzed by chi-square independence test. BellCurve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan) was used for statistical analyses.

Results

Estimation of diet intake

In the control groups, larvae would feed on the slurry-type diet while vertically positioning their bodies head-first into the food, using frequent tail beats. Some part of the diet dispersed like smoke in the water by the active feeding movements of the larvae. The behavior of larvae of the test group was not seen well because of the milky white color of the diet, but the head parts of almost all larvae were immersed in the pool of food while the larvae showing swimming activity.

The projected area of the intestine of larvae fed with the liquid-type diet (0.177 ± 0.031 mm^2) was 1.17 times larger on the first day of feeding than those fed with the conventional slurry-type diet (0.151 ± 0.024 mm^2) ($p < 0.05$) (Fig. 2). Moreover, the optical density of this area of

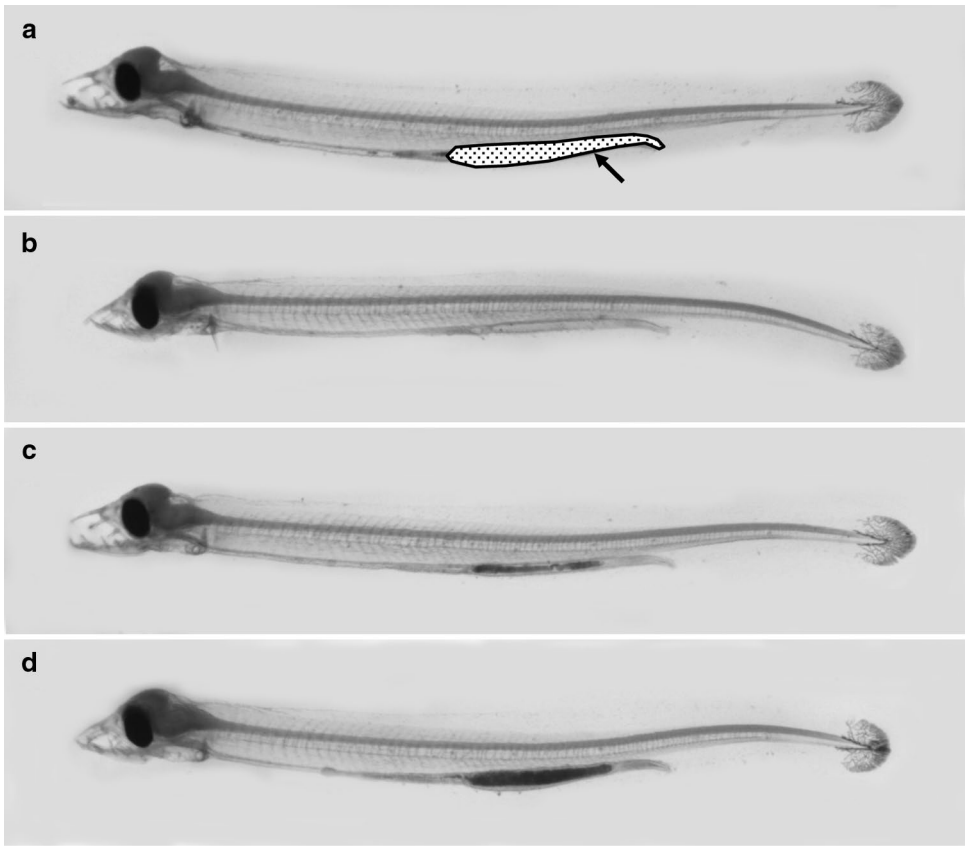

Fig. 1 Amount of ingested diet and gut conditions. **a** Measured area of the gut in shade. **b** Empty gut of unfed larva. **c** Half-filled gut of larva fed with conventional slurry-type diet, and **d** Fully filled gut of larvae fed with a new liquid-type diet

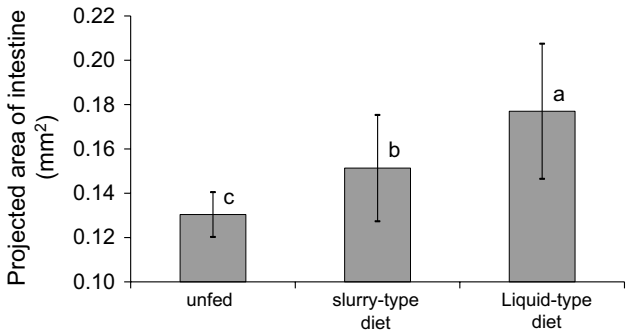

Fig. 2 Difference in projected area of intestine between two types of larval diet. Error bars indicate standard deviation. Letters on the bars indicate significant differences ($p < 0.05$)

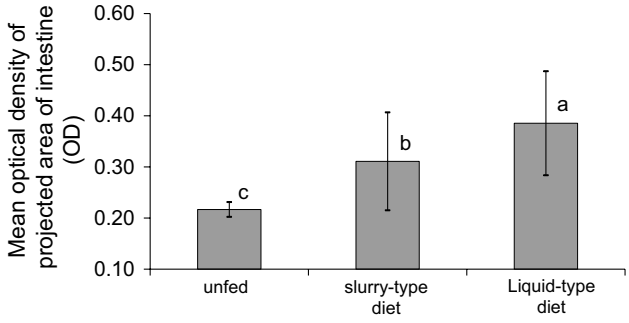

Fig. 3 Difference in concentration of diet ingested into intestine between two types of larval diet. Error bars indicate standard deviation. Letters on the bars indicate significant differences ($p < 0.05$)

larvae fed with liquid-type diet (OD: 0.385 ± 0.102) was 1.24 times higher than those fed with conventional diet (OD: 0.311 ± 0.096) (Fig. 3). These data suggest that larvae fed with the liquid-type diet ingested more food than those fed on the conventional diet, although the feeding behavior could not be observed very well.

Survival rate

Larvae fed with liquid-type diet in the 3 tanks all showed high survival rates (89, 91, 94%) at the end of the experiment (27 dph) (Fig. 4). Larvae in two tanks fed with conventional slurry-type diet showed similarly high survival rates (94, 94%), while those in one tank (No. 3) showed a period of increased mortality at 9–11 dph, but their survival became stable afterward at 68% at the end of the experiment. Although the cause of the sudden decrease in

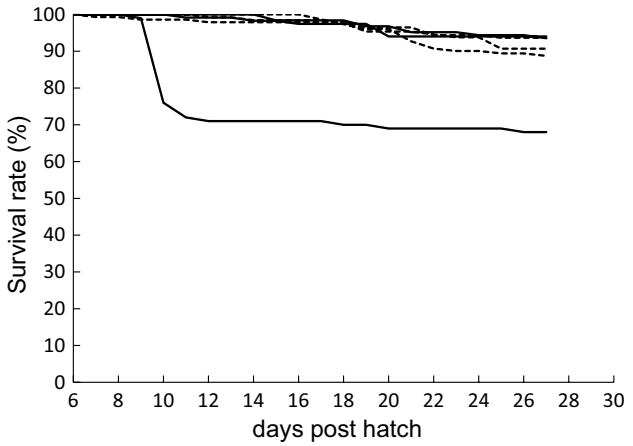

Fig. 4 Survival rate of eel larvae fed with conventional slurry-type (solid line) and newly developed liquid-type diets (dashed line). One of the tanks fed with conventional slurry-type (No. 3) showed a period of increased mortality at 9–11 dph, but their survival became stable afterward at 68% at the end of the experiment

survival rate was unclear, we speculate that a low feeding incidence at the first feeding period (6 dph, 60%) might have influenced this.

Growth

Initial TL was 7.08 ± 0.13 mm (mean ± SD, $n = 86$) for all fish groups. The TLs of larvae on 27 dph fed with the liquid-type diet were significantly larger ($p < 0.001$) in all 3 triplicates (mean ±SD; 12.68 ± 0.99 mm, $n = 86$) than those with the conventional slurry-type diet (11.19 ± 0.89 mm, $n = 98$) (Fig. 5a). Mean growth rates of the former groups in three tanks were 1.35 times higher (0.27 ± 0.05 mm/day) than those of the latter (0.20 ± 0.04 mm/day) ($p < 0.001$). There was a significant difference in TL distribution between the two types of diet (Fig. 5b, c, chi-square independence test, $p < 0.001$). The mode of conventional diet was 10.00 mm while that of the liquid-type was 12.00 mm. No significant difference was detected in the dispersion of TL distribution between the two diets ($p = 0.34$).

Discussion

This study is the first report to validate the possible viability of a liquid-type diet for actual rearing of eel leptocephali for a substantial period after hatching. We found that eel larvae immersed in the liquid-type diet grew significantly faster than those fed a conventional slurry-type diet, despite the concentration of nutrition in the liquid-type diet being only 60–70% of the slurry-type diet (Fig. 5). Larvae seemed to ingest the liquid diet by drinking it in the food pool, rather than the head-first feeding on the conventional diet. We were

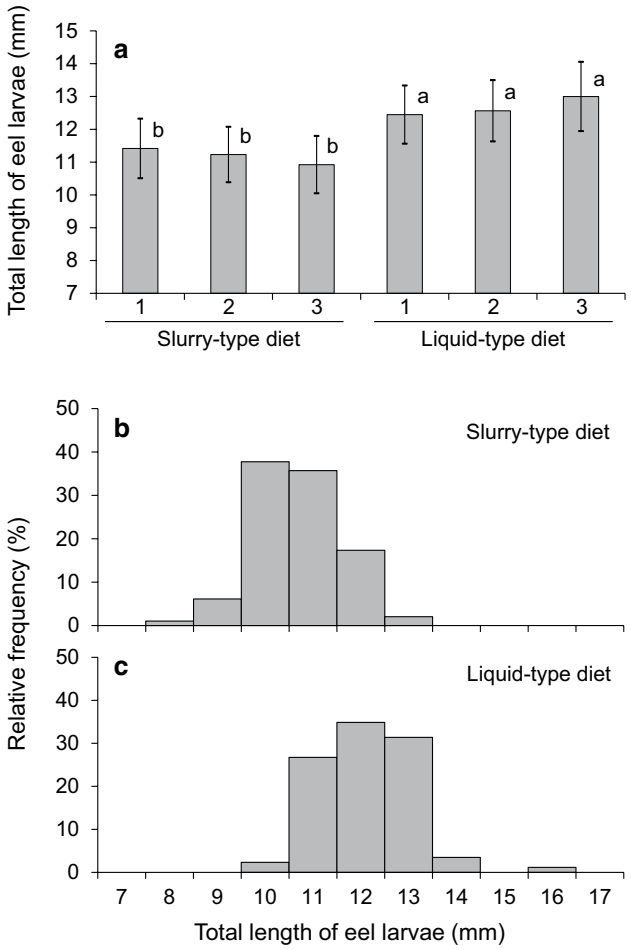

Fig. 5 Growth of eel larvae fed with two different types of diets 27th days post hatch. Difference in total length of larvae among six rearing tanks fed with two different types of diets (**a**), distribution of total length of larvae fed with conventional slurry-type (**b**) and newly developed liquid-type diets (**c**). Error bars indicate standard deviation. Letters on the bars indicate significant differences ($p < 0.05$)

able to confirm in a transparent experimental chamber that the eel larvae frequently swallowed drops of diet as well as seawater through their thin esophagus (see the movie: Online Resource 1). The larvae ingested the diet by plunging into the diet mass, taking diet into the mouth and opening their buccal cavity to suck the diet into the esophageal inlet. During this type of ingestion sequence, diet with inappropriately high viscosity must be disadvantageous for efficient diet uptake. Therefore, it appears possible that the larvae of the test groups that were immersed in the liquid-type diet swallowed much diet in the food pool at the bottom of tanks, and it was more difficult for the control larvae to ingest the higher viscosity slurry-type diet.

There seemed to be two reasons for the higher growth rate of larvae fed with the liquid-type diet. One is that the head parts of the larvae were completely dipped into liquid-type diet during most of feeding period as observed in the

experiment, while in control groups some larvae could not meet with diet at all. This is especially the case for the early stage of larvae, like those in this experiment, since small eel larvae, with low swimming ability at the first feeding period, appear to have difficulty accessing and ingesting the harder slurry-type diet located on the bottom of rearing tank. In fact, the feeding incidences of the three tanks fed with liquid-type diet (number of larvae with diet in their alimentary tract/number of observed larvae ×100) at age 6 were 84.6−93.3%, while those of three tanks fed with slurry-type diet were 60.0−82.3% (Yamada et al. unpublished).

The second reason is that early eel larvae may not be good at biting off the hard paste-like material and swallowing it through their undeveloped jaws and extremely narrow esophagus (Yoshimatsu 2011). The larvae in the liquid diet appeared to ingest more food compared to the thicker paste material, even though the concentration of food materials in the liquid diet was lower. The larvae of control groups might have fed on only partially dissolved food materials created by the agitation of the larvae, which has a lower concentration of food compared to the material ingested by the test group larvae, and thus they might have obtained a smaller amount of diet in both volume and density. This may account for the larvae provided with the liquid-type diet having ingested more food than those fed with the conventional slurry-type diet on the first day of feeding (Fig. 2, 3).

The liquid-type diet appears to be a better fit for eel larvae with a narrow esophagus. In the wild, eel larvae appear to feed on the type of particulate organic matter (POM) called marine snow, consisting materials originating from phytoplankton and other organisms (Alldredge and Silver 1988), based on their trophic position determined from analysis of their amino-acid nitrogen isotopic ratios (Miller et al. 2012) and observations of their gut contents. Except for some observable objects, such as discarded appendicularian houses and zooplankton fecal pellets, the gut contents of eel larvae consist of liquid-like amorphous substances, suggesting that they feed on detritus or marine snow in the ocean (Otake et al. 1993; Mochioka and Iwamizu 1996; Miller et al. 2011; Tomoda et al. 2018). Eel larvae have been speculated to feed on phytoplankton-derived marine snow as one food source that includes polysaccharide-containing transparent exopolymer particles (TEP), proteinaceous Coomassie stainable particles (CSP) and pico/nano-planktonic particles of 2 μm in diameter (Tomoda et al. 2018), which cultured larvae have been observed to consume (Tomoda et al. 2015). All these considerations suggest that eel larvae in the natural environment feed on soft semi-liquid food containing nutritious particles and other objects.

Besides the growth rate, the survival rates and growth variation of the larvae are important in larval rearing. In this study larvae fed with a conventional slurry-type diet showed almost similar survival and growth variation to the larvae fed with the liquid-type diet. This result may be caused by the small-scale experimental tanks (1.5 l) and the short rearing period (21 days). However, it is noteworthy to mention that the new diet developed in this study has potential to greatly decrease the growth variation, since all the larvae have a chance to meet with food equally in a liquid-type diet, in relation to the same amount of food materials being provided. In fact, it was confirmed using 20 l rearing tanks that the growth variation was greatly diminished when using a liquid-type diet, and high density rearing (e.g. 1000 fish/l) was realized at high survival rates (e.g. 92%) up to around 37 dph (IRAGO Institute, unpublished).

Immersing larvae in a liquid with high molecule proteins has been tested in physiological experiments to examine a hypothesis that leptocephalus can absorb dissolved organic matter in the sea water as a possible food source not only from the intestine but directly from the body surface (Hulet 1978; Pfeiler 1986; Otake et al. 1993). Otake et al. (1995) examined the hypothesis by immersing pike eel larvae *Muraenesox cinereus* into sea water containing horseradish peroxidase, and revealed that leptocephali could not directly uptake large nutrient-like molecules such as horseradish peroxidase across the body surface, but ingested it from the epidermis of the intestine like other standard fish larvae. Masuda et al. (2010) also immersed Japanese eel larvae into a 50% cow milk solution as a possible feeding method of leptocephali, and found that eel larvae could ingest the liquid-type diet and survive for 16 days. However, survival rate decreased to 10% within 5 days and a substantial advantage in growth was not obtained.

Low growth rate is a serious problem because the long rearing period increases all kinds of costs (manpower, electricity, diet, etc) for production of glass eels. There are various factors that likely decrease larval growth. Diet quality (Okamura et al. 2014; Furuita et al. 2014) and quantity (Masuda et al. 2013b; Okamura et al. 2018) are factors directly responsible for these problems. In addition to low growth rate, extremely large growth variation is another important problem. For example, the larval age for metamorphosis ranged from 180 to 671 days post hatching (dph) within the same rearing lot (Masuda et al. 2013b). Not only dietary deficiency but rearing conditions such as temperature (Masuda et al. 2013b; Okamura et al. 2018), salinity (Okamura et al. 2009) and larval density (Masuda et al. 2013b) are also important factors.

In the present study, advantages in survival and growth performance by feeding Japanese eel larvae with a liquid-type diet was demonstrated. The results obtained here can hopefully be a breakthrough to increase the efficiency of the rearing method of leptocephali for future success of artificial mass production of glass eels.

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