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ORIGINAL ARTICLE

Aquaculture

Long-term rearing of Japanese eel larvae using a liquid-type diet: food intake, survival and growth

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Abstract

The efficiency of a new liquid-type diet for long-term rearing of Japanese eel larvae until metamorphosis was examined, as was the effect of diet viscosity on diet intake and on the survival and growth of early larvae. The highest intake of the experimental diet by 6- and 9-day post-hatch (dph) larvae occurred at viscosities of 20–50 mPa·s, much lower than the viscosity of the currently used slurry-type diet (ca. 2900 mPa·s). Long-term feeding trials for 259 days ($n=4$) showed that overall survival rates of larvae (37–59%) fed a liquid-type diet with lower viscosity (40–680 mPa·s) were 2 to 3.4 times higher than the survival rates of larvae fed the slurry-type diet (11–25%). Because nutrients were diluted in the liquid-type diet, the growth of larvae fed this diet was slower after about 200 days and metamorphosis was delayed. However, the yield of glass eels was 1.1 to 3.2 times higher in larvae fed the liquid than the slurry diet. These findings suggest that feeding the liquid-type diet can result in the mass production of glass eels by ensuring high growth, survival and metamorphosis rates.

Keywords Freshwater eel · *Anguilla japonica* · Leptocephalus · Diet viscosity · Diet intake · Glass eel production

Introduction

Recent progress in rearing techniques has enabled leptocephalus larvae of the Japanese eel *Anguilla japonica* to metamorphose into glass eels in the laboratory (Tanaka 2015). To date, however, commercial-scale production of glass eels has not been practical because of the low survival rate and poor growth of eel larvae, especially at the early stage, likely resulting from a relative lack of sources of energy (Okamura et al. 2009b, 2014).

Wild eel larvae feed on particulate organic matter (POM), such as marine snow containing discarded appendicularian houses, gelatinous plankton, and various types of biological residue (Otake et al. 1993; Mochioka and Iwamizu 1996; Riemann et al. 2010; Miller et al. 2012; Feunteun et al. 2015). These materials, however, are difficult to obtain in sufficient quantities for mass rearing of eel larvae. The main food material used to date is the egg yolk of the spiny dogfish *Squalus* sp., enabling eel larvae to grow in captivity (Tanaka 2015). Although hen egg yolk has been suggested as an alternative to dogfish egg, the growth of larvae fed this diet was lower than that of larvae fed a diet containing dogfish egg yolk (Okamura et al. 2013).

Eel larvae are currently reared on a slurry-type diet made from dogfish or chicken egg yolk (Okamura et al. 2014; Tanaka 2015). The slurry is spread on the bottom of the tank like mayonnaise. When a light set above the tank is turned on, the larvae are constrained by negative phototaxis to swim downward (Yamada et al. 2009), enabling them to encounter the diet at the bottom of the tank (Okamura et al. 2009b).

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Many larvae, however, die without any diet intake in their guts, indicating that not all larvae encounter the diet at the bottom and/or have sufficient swimming ability or phototaxis to reach the bottom. Moreover, these slurry-type diets may be too hard for some larvae to bite or swallow, probably because these larvae have poorer ingesting abilities.

These problems may be solved by feeding eel larvae a liquid-type diet (Masuda et al. 2010; Yamada et al. 2019). For example, immersion of eel larvae in cow's milk in a dish enabled these larvae to survive for 26 days post-hatch (dph) (Masuda et al. 2010). This milk-based diet utilizes a different feeding strategy than the slurry-type diet, as all larvae ingest the milk during feeding time. However, survival and growth rates were still problematic. Larvae immersed in a liquid-type diet made from the dogfish egg yolk ingested more than larvae fed the conventional slurry-type diet, resulting in 1.4-fold higher growth rate (Yamada et al. 2019). These results suggested that immersion in liquid-type diets can provide all larvae an equal opportunity to ingest dietary nutrients (Yamada et al. 2019). However, these studies were conducted for only 3 weeks, making it uncertain whether these liquid-type diets are effective for long-term rearing of eel larvae until metamorphosis. Although relatively softer diets may increase dietary intake by younger larvae with low ingesting ability, the relationship between the physical properties of a diet and dietary intake by eel larvae has not yet been evaluated.

Thus, this study had two purposes. The first was to examine the effect of the physical properties of larval diets on dietary intake, by assessing the effects of diet viscosity on dietary intake by young eel larvae (6 and 9 dph). The second was to assess the effectiveness of a liquid-type diet on the long-term rearing of eel larvae, for over 8 months until metamorphosis, by assessing the survival rate, growth, and production of glass eels.

Materials and methods

Experimental fish

Japanese eel larvae were obtained as described previously (Ohta et al. 1997; Horie et al. 2008). Briefly, fertilized eggs were obtained from parents matured artificially, females by repeated injections of pituitary extracts of chum salmon *Oncorhynchus keta* and males by repeated injections of human chorionic gonadotropin (hCG) (SankyoYell Yakuhin, Tokyo). Hatched larvae were maintained in a 180-l polycarbonate tank supplied with seawater of 34.5 practical salinity units (psu) at 25 °C for 6 days until the completion of mouth opening. Five batches of eel larvae obtained from separate parents were used in the following experiments.

Experiment 1: effect of diet viscosity on diet intake

To examine the effect of diet viscosity on the efficiency of diet intake by eel larvae, an experimental diet containing only 10% (w/w) chicken egg albumen peptide (Kewpie Corp., Tokyo) in seawater (34.5 psu) was prepared. Usual diets for eel larvae contain dogfish eggs and several other ingredients, including various soluble and insoluble contents, as well as viscous materials (ca. 10^4–10^5 mPa·s). To avoid the effects of these viscous materials, only egg peptide solution with lower viscosity (about 2 mPa·s) was used. The viscosity of the experimental diet was adjusted by adding carboxymethyl cellulose (CMC) as a thickener, to attain CMC concentrations of 0, 0.24, 0.36, 0.48, 0.60, 0.72, 0.85, 0.97, 1.10, 1.26, and 1.47% (w/w). The viscosity of each diet was measured using a digital viscometer (DV-E, Brookfield Engineering Laboratories, MA) at room temperature (23 °C). The experimental diets with 0% and 1.47% CMC had viscosities of 2 mPa·s and 2220 mPa·s, respectively. The addition of CMC also altered the moisture content of each experimental diet, from 88.53 to 90%.

Forty eel larvae of 6 dph were directly transferred to 100-ml polyethylene containers containing 100 ml of the experimental diets with 0, 0.24, 0.36, 0.48, 0.60, 0.72, 0.85, 0.97, and 1.10% CMC, as well as seawater alone. Thus, the larvae were completely immersed in the diets and were able to swim there during the experiments. During feeding, each container was maintained at 23 °C for 10 min under room light (about 500 lx). Soon after feeding, all larvae were anesthetized with 150 ppm MS222 (tricaine methanesulfonate) and fixed in 5% formalin-seawater solution for 24 h. Larvae of 9 dph were also fed in the same manner, with the experimental diets containing 0, 0.48, 0.60, 0.72, 0.85, 0.97, 1.10, 1.26, and 1.47% CMC, and seawater.

The amount of diet intake by eel larvae was estimated by measuring the projected area of the gut of all fixed larvae (Fig. 1), based on the assumption that gut expansion is proportional to diet intake. Digital photographs were taken under a microscope, and gut area was measured on the photographs using Image J software (available at https://imagej.nih.gov/ij/, accessed 1 April 2018).

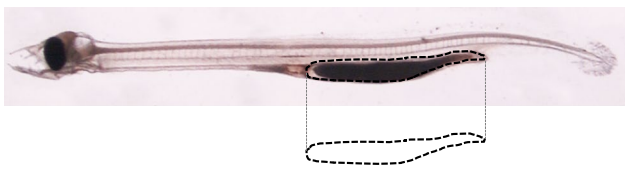

Fig. 1 Projected area of gut (enclosure with broken line) measured to estimate diet intake by *Anguilla japonica* larvae

Experiment 2: effect of diet types on survival, growth and metamorphosis

The long-term effects of slurry-type and liquid-type diets on the survival, growth and metamorphosis of eel larvae were tested in four feeding trials, each lasting 259 days. 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). Each trial tested different batches of 200–500 larvae, each divided into two groups, fed the slurry-type and liquid-type diets (Table 1). Larvae of 6 dph were transferred by ladling with a 500-ml beaker from a 180-l tank to 10-l or 20-l planktonkreizel tanks, made of clear acrylic resin. The 10-l tank was 30 cm in diameter and 15 cm in width, and the 20-l tank was 40 cm in diameter and 15 cm in width. Both tanks were equipped with four inlet tubes (inner diameter, 3 mm), which produced a vertical revolving current, and an outlet mesh screen located on the side wall of the tank. The inlet jets pushed water across this mesh screen. The fast revolving current in the tank kept the leptocephali suspended in the water column (Okamura et al. 2009b). The tanks were filled with 50% seawater, of 17.5 psu maintained at 23 °C. The rates of supply were 1.5 and 2.0 ml min^{-1} for the 10-l and 20-l tanks, respectively. Maintenance in 50% seawater was shown to be advantageous for the growth of eel larvae because of the lower energy cost required for osmoregulation (Okamura et al. 2009a).

The standard ingredients of the artificial diet used in this experiment included 63.5 g dogfish egg yolk, 27.8 g skinned-krill extract, 6.3 g chicken egg 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), along with sufficient seawater (10–20 ml) to achieve the required viscosity. After mixing all ingredients, the mixture was pasteurized in a 62 °C water bath for 30 min.

The standard diet was used to prepare two types of diet with different viscosities (slurry-type and liquid-type) and different feeding strategies (Fig. 2). The total dry weight per day of diet fed to larvae was dependent on larval age, but was the same in the two groups throughout the feeding trials, regardless of tank volume and the number of larvae (Fig. 2a). To provide a sufficient amount of food to each larva, the amount of food in this experiment was much larger than that given in the previous study (Okamura et al. 2009b). The viscosity of each diet was adjusted by adding seawater, with the liquid-type diet having a higher moisture content and larger volume (Fig. 2b, c). The viscosity of the slurry-type diet was set at about 2900 mPa·s, corresponding to the slurry-type diet conventionally used (Okamura et al. 2013), and was constant throughout the feeding trials. Based on the results of Experiment 1 in this study (above), the viscosity of the liquid-type diet was set at about 40 mPa·s for the first 24 days. Based on the assumption that larval ability to ingest diet increases with age, liquid diet viscosity was increased stepwise during the trials (Fig. 2d), being about 180 mPa·s at 30 dph, 380 mPa·s at 60 dph and 680 mPa·s at 100 dph (Fig. 2d). The viscosity of each diet was measured at room temperature (23 °C) using a viscometer. However, these viscosities fluctuated about ± 5%, due to differences in humidity and the conditions of the ingredients.

Batches of larvae were fed each diet five times per day, at 0900, 1100, 1300, 1500 and 1700. The feeding operation commenced by stopping the water supply and turning on a fluorescent lamp placed above the tank, so that about 200 lx reached the bottom of the tank. In the second step, a certain amount of diet (Fig. 2) was slowly spread on the bottom of the tank with a glass pipette (opening, about 6 mm) and the larvae were fed for 10 min. In the third step, the water supply was restarted and the remnants of uneaten diet were removed by a separate jet of water with a handheld nozzle (Okamura et al. 2009b). During feeding of the slurry-type

Table 1 Performance of *Anguilla japonica* larvae fed slurry-type and liquid-type diets in four rearing trials

Trials	Tank volume (l)	Diet viscosity (high/low)	Initial number of larvae	Final survival rate (%)	Number of glass eels	Metamorphosis rate (%)
1	10	High	210	24	34	16.2
		Low	188	48*	44	23.4*
2	20	High	357	11	12	3.4
		Low	380	37*	37	9.7*
3	10	High	263	25	30	11.4
		Low	318	52*	33	10.4
4	10	High	569	24	85	14.9
		Low	499	59*	153	30.6*

*$p < 0.05$

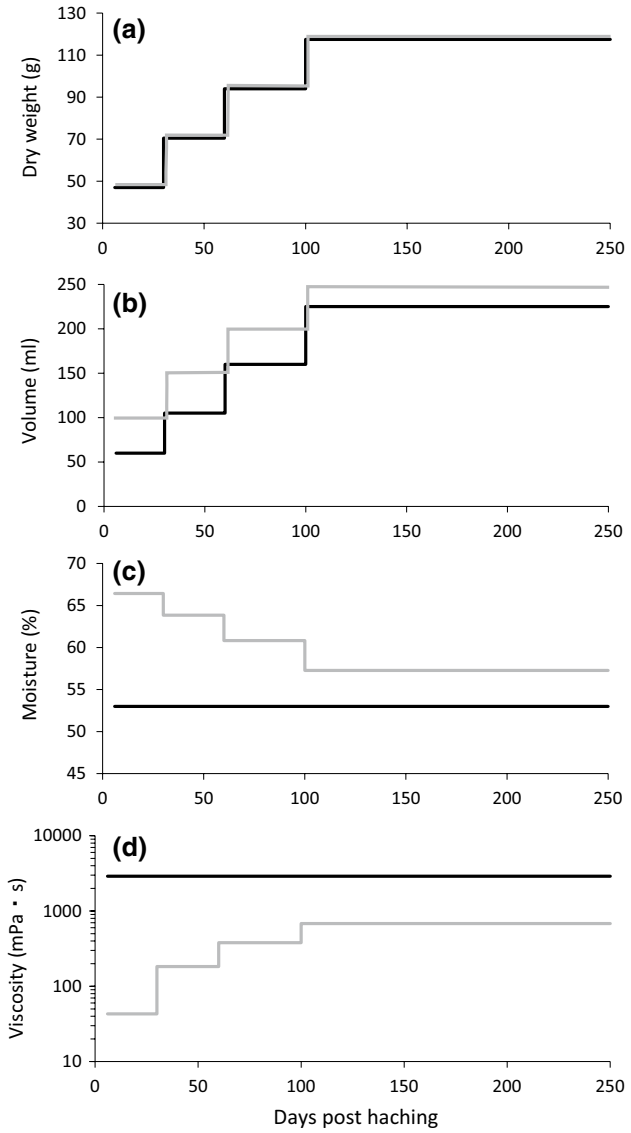

Fig. 2 Feeding regimes and diet profiles for rearing trials of *Anguilla japonica* larvae. Black lines: slurry-type diet; gray lines: liquid-type diet. **a** Total amount (dry weight) of each diet supplied to each tank per day; **b** total volume of each diet supplied to each tank per day; **c** changes in moisture content of each diet; **d** changes in viscosity of each diet

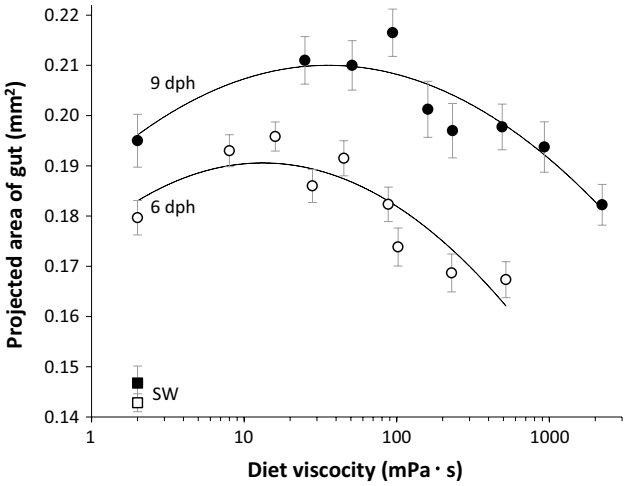

Fig. 3 Effect of diet viscosity on diet intake by *Anguilla japonica* larvae estimated from changes in the projected area of the gut region

diet, the latter was spread linearly like mayonnaise (5–10 winding lines, each about 7 mm in diameter) on the bottom of the tank. In contrast, the liquid-type diet formed a shallow food pool on the bottom of the tank. The maximum depth of the pool was 10–20 mm, depending on the amount of the diet, an amount sufficient for immersion of the entire body of all young larvae or the heads of all grown larvae in the tank. Because the density of the liquid-type diet was higher than that of 50% seawater, the two liquids formed a stable liquid–liquid interface. Feeding operations were started on 6 dph and stopped at 250 dph; after this time, the larvae were maintained without feeding for 14 days to induce metamorphosis (Okamura et al. 2012).

Dead larvae were counted and removed from the tanks at each feeding session. Glass eels that completed metamorphosis were also removed from the tanks. The completion of metamorphosis was defined as a ratio of pre-anal length (PAL) to total length (TL) of < 50% and a ratio of body depth (BD) to TL of < 10% (Kuroki et al. 2010). In trial 3, larvae at the leptocephalus stage, excluding glass eels, were randomly sampled at 96 dph (slurry-type: $n = 46$; liquid-type: $n = 40$) and 215 dph (slurry-type: $n = 33$; liquid-type: $n = 37$). These larvae were anesthetized with 150 ppm MS222 and their TL was measured. At 264 dph, all fish in four trials were sampled and the numbers of leptocephali and glass eels were counted. The cumulative survival rate at each day was calculated using the formula: survival rate at i dph (%) = (initial number of fish − cumulative number of dead fish at i dph)/initial number of fish × 100. The metamorphosis rate was calculated as cumulative number of glass eels at 264 dph/initial number of fish × 100. The initial number of fish in each trial was calculated as the cumulative number of dead fish removed during the experiment plus the number of surviving fish.

Data analyses

The relationship between diet viscosity and diet intake by larvae was evaluated by assessing the mean ± standard error of the projected area of the gut and fitting the data to a quadratic curve (Fig. 3). Survival rates of the diet groups in long-term feeding trials were compared by log-rank tests (Fig. 4). Between-group differences in metamorphosis rates were compared by two-proportion Z-tests (Table 1). Between group differences in mean TL were evaluated by Student's

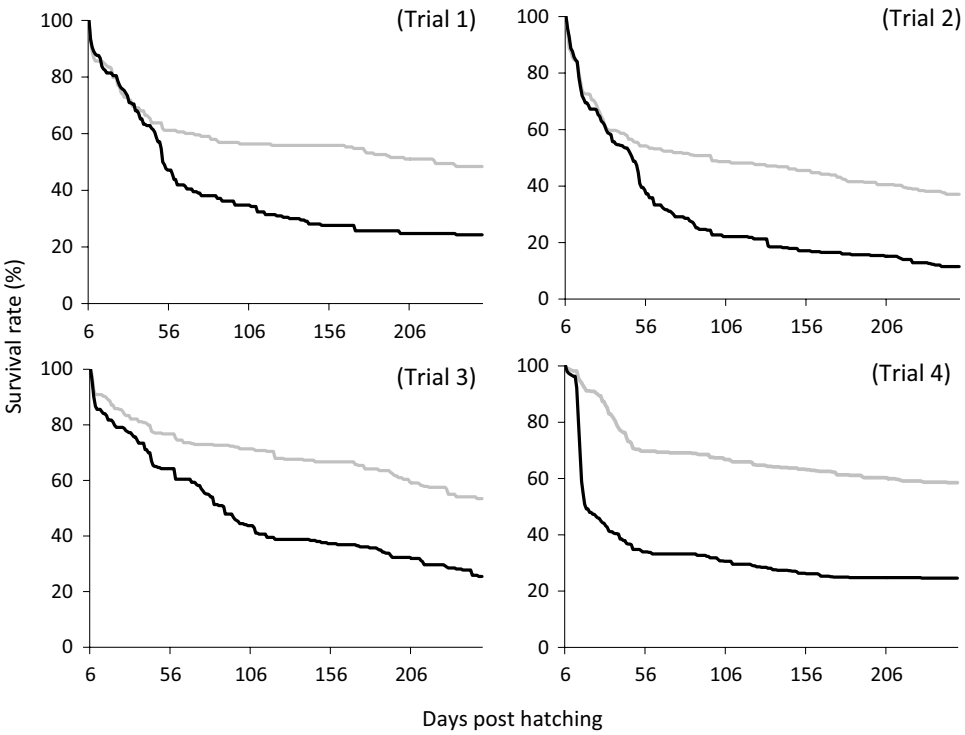

Fig. 4 Cumulative survival rates of four feeding trials of *Anguilla japonica* larvae. Black lines: slurry-type diet; gray lines: liquid-type diet

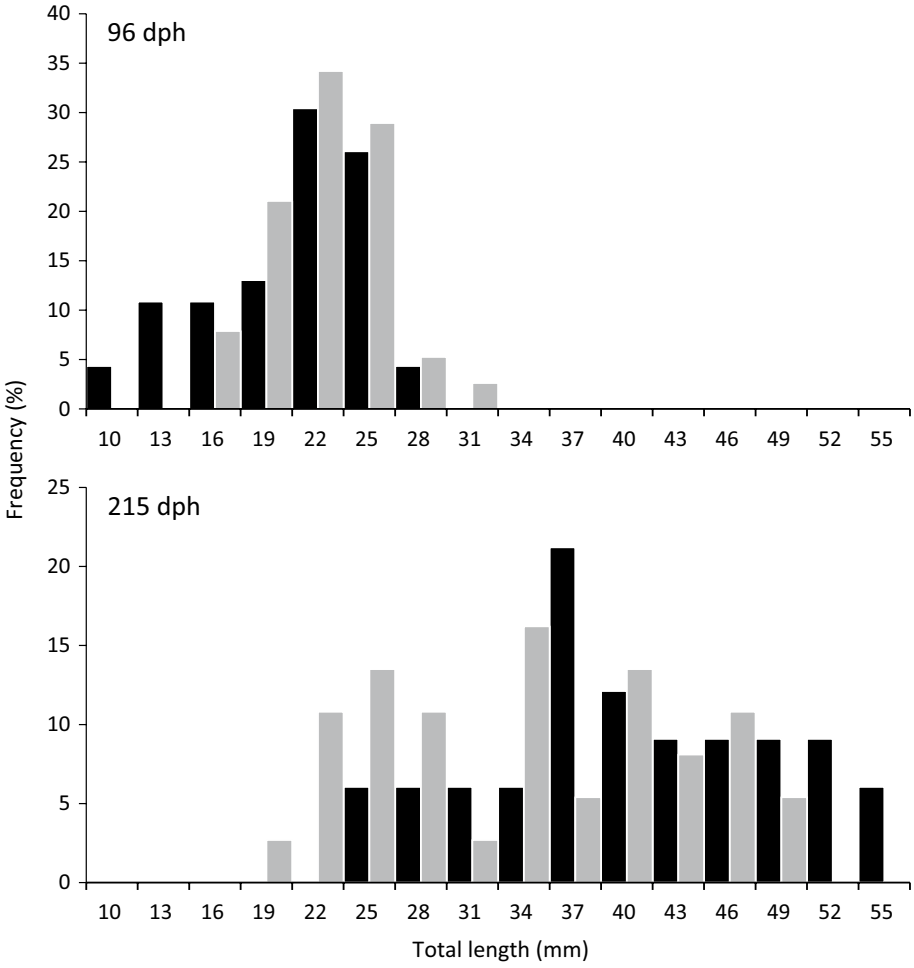

Fig. 5 Length frequency distributions of *Anguilla japonica* larvae at 96 and 215 days post hatching in Trial 3. Black bars: slurry-type diet; gray bars: liquid-type diet

t-tests and their variances were evaluated by *F*-test (Fig. 5). All statistical analyses were performed using BellCurve for Excel (Version 3.00, SSRI, Tokyo, Japan). Differences were considered significant at $p < 0.05$.

Results

Diet intake

For the 10-min feeding experiments, each larva swam freely in the containers and migrated downwards when the light was turned on. Thus, most of the larvae were distributed at the bottom of the containers. When eel larvae were given only seawater, the projected areas of the gut at 6 and 9 dph were 0.14–0.15 mm^2, indicating an absence of food their guts (Fig. 3). After being fed the experimental diet, their projected gut areas were 1.2- to 1.5-fold larger, indicating they had ingested substantial amounts of food, with gut areas being greater at 9 dph than at 6 dph. Peak ingestion was observed when larvae at 6 dph were fed diets of viscosity between 10 and 20 mPa·s, whereas peak ingestion at 9 dph was observed when larvae were fed diets of viscosity of about 50 mPa·s. As diet viscosity increased over their peaks, however, gut volume decreased in both age groups.

Survival, growth and metamorphosis

Larvae fed slurry-type diets began to swim vertically and reached the bottom soon after the light was turned on. These larvae always maintained their bodies vertically head-first at the bottom of the tank, using frequent tail beats, whereas they seemed not to be attracted by the food placed at the bottom of the tank. Thus, approximately 80% of these larvae encountered the diet placed on the bottom of the tank by chance. Larvae fed liquid-type diets entered directly into the diet pool when reaching the bottom of the tank. The whole bodies of larvae of < 20 mm TL were immersed in the diet pool during feeding time, whereas only the anterior portions, including the head, of larger larvae (> 20 mm TL) were immersed.

Survival rates were significantly higher in larvae fed liquid-type than slurry-type diets (log-rank test, $p < 0.05$, each) (Fig. 4). Between-group survival differences were greater during the first 60 days. The final survival rates in all the liquid-type groups were 2- to 3.4-fold higher than those in the slurry-type groups (Table 1).

At 96 dph in trial 3, the mean TL was significantly greater in larvae fed liquid-type (24.0 ± 3.8 mm) than slurry-type (22.0 ± 4.5 mm) diets (*t*-test, $p < 0.05$). The variance in TL proportions within each group at 96 dph differed significantly (*F*-test, $p < 0.05$), indicating a greater variation in size in larvae fed the slurry-type than liquid-type diets (Fig. 5). At later times, however, the larvae fed slurry-type diets grew faster than those fed liquid-type diets, and started metamorphosis earlier. In trial 3, metamorphosis started at 194 dph in the slurry-type group, compared with 220 dph in the liquid-type group. At 215 dph, the mean TL of leptocephali, except for glass eels and metamorphosing larvae, was significantly larger in larvae fed slurry-type (41.9 ± 8.7 mm) than liquid-type (35.6 ± 8.7 mm) diets (*t*-test, $p < 0.01$) (Fig. 5). The variance in TL proportions at 215 dph was similar in the two groups (*F*-test, $p = 0.99$). Other trials showed a similar trend. Metamorphosis started at 163–201 dph in larvae fed slurry-type diets, and at 185–221 dph in larvae fed liquid-type diets (Fig. 6). In three of the four trials, however, the metamorphosis rate was significantly higher in larvae fed liquid-type diets than slurry-type diets (*Z*-test, $p < 0.05$) (Table 1).

Discussion

The present data suggested that the viscosity of diet affected the efficiency of diet intake by eel larvae. The amount of diet ingested was higher in larvae fed diets of low viscosity, with these diets having much lower viscosity than currently used slurry-type diets (Tanaka et al. 2001; Okamura et al. 2009b). However, the experimental diets of different viscosities in Experiment 1 also showed differences in moisture content through the addition of CMC. Thus, diet ingestion by eel larvae may be influenced by differences in moisture content. However, the differences in moisture of these experimental diets were within 1.47%, whereas diet viscosity ranged between 2 and 2220 mPa·s, a greater than 1000-fold difference. These findings suggest that the effect of viscosity was much greater than that of moisture in Experiment 1.

Based on this finding, we performed four long-term, 259-day feeding trials in Experiment 2 comparing liquid-type diets differing in viscosity. We found that survival rates and the yield of glass eels were much improved by feeding low-viscosity diets, suggesting that these diets enhance food intake by eel larvae irrespective of diet ingredients. Again, the effects of moisture in diets remain unclear. The liquid-type diets were adjusted by adding seawater, indicating the impossibility of distinguishing between the effects of moisture and viscosity. However, the effect of viscosity is likely greater than that of moisture, as in Experiment 1. At least high moisture and/or low viscosity diets were clearly found to be beneficial for rearing eel larvae. In addition, because the diet used in Experiment 2 was richer in nutrients than the diet in Experiment 1, diet intake by eel larvae in Experiment 2 was likely greater, although the effect of diet ingredients on diet intake has been not tested in eel larvae.

These findings also indicate the importance of nutritional status during the early stage in eel larvae. Survival rates of larvae fed slurry-type diets decreased during the first

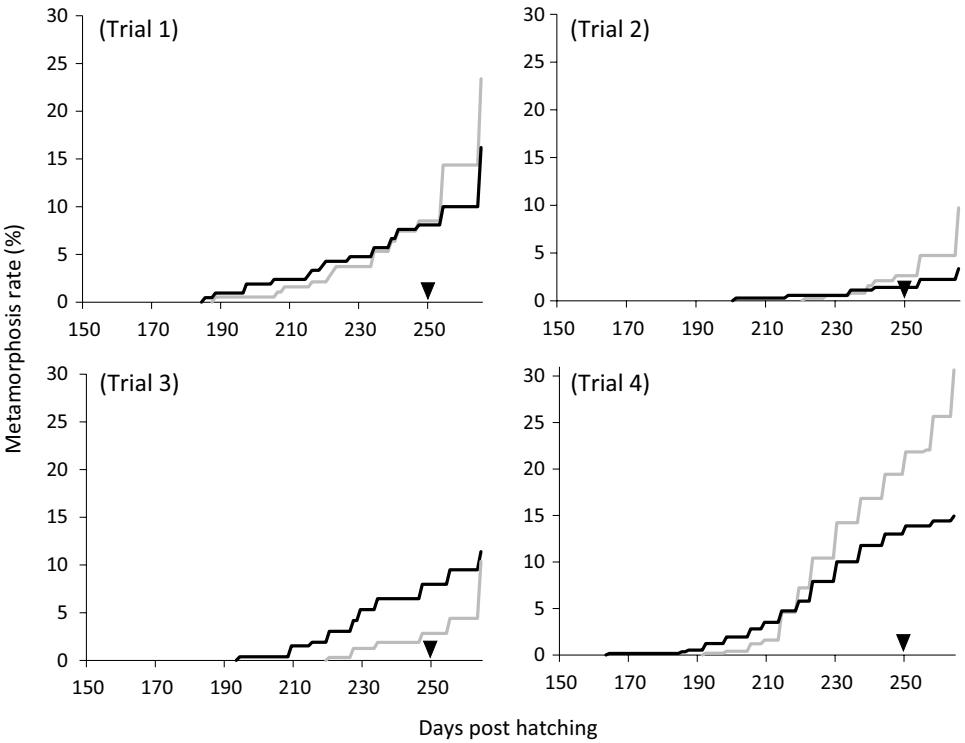

Fig. 6 Metamorphosis rate of *Anguilla japonica* in four feeding trials. Black lines: slurry-type diet; gray lines: liquid-type diet; *black arrows*: end of feeding

60 days, indicating their inability to ingest sufficient amounts of these diets. We previously reported that over 90% of larvae fed slurry-type diets died within 50 days (Okamura et al. 2009b). However, the survival rate of early stage larvae fed a liquid-type diet was higher, probably due to efficient diet intake. Generally, the period of transition from endogenous nutrition to exogenous feeding is critical for later stage survival (May 1974). Thus, efficient early stage dietary intake may be important for subsequent survival of eel larvae.

The ability of eel larvae to ingest the diet may increase as they grow, as diet intake was greater at 9 dph than at 6 dph, clearly indicating an increase in swallowing ability. Although growth of early stage larvae was higher in those fed a liquid-type than a slurry-type diet, this relationship was reversed in later stage larvae, perhaps owing to an increase in ingesting ability. Because nutritional components in the liquid-type diet are more diluted, feeding a liquid-type diet may be disadvantageous to grown larvae, which require more energy to maintain their basal metabolism. Therefore, a more efficient rearing strategy may be to increase diet viscosity as ingesting ability increases. Establishment of a more appropriate feeding strategy, however, requires assessment the effects of diet viscosity on diet intake in grown larvae.

The present results suggest that the ability of eel larvae to ingest food items is lower than previously thought. Histological observations on young eel larvae (6–10 dph) showed that the esophagus does not contain the developed mucous cells that facilitate food swallowing (Yoshimatsu 2011), suggesting that eel larvae have difficulty ingesting particulate materials. Furthermore, assessment of musculoskeletal anatomy suggested that the maximum bite force of first feeding eel larvae was 65 μN, allowing these larvae to feed only on soft food materials, such as gelatinous prey (Bouilliart et al. 2015). Therefore, the ability of eel larvae to bite and/or swallow food items may be insufficient for intake of currently used slurry-type diets.

Liquid-type diets have another possible advantage. Feeding eel larvae with a slurry-type diet with a mayonnaise-like appearance, placed on the bottom of tanks, results in a percentage of the larvae being unable to encounter the diet because of the limited area of the diet spread on the bottom. However, the volume of liquid-type diets is greater, due to the addition of moisture, with these diets forming a "food pool" on the bottom of the tank, enabling more eel larvae to encounter the diet. Further, the diet with a viscosity of < 680 mPa·s used in this study had a milk-like form, allowing eel larvae to enter freely and swim in the liquid diet, and swallow it directly. Therefore, a liquid-type diet can provide an equal opportunity for most larvae to eat efficiently, resulting in a variance in TL proportion at 96 dph being smaller in larvae fed liquid-type than slurry-type diets. Our preliminary experiments suggest that a low-viscosity diet made from chicken egg yolk, instead of dogfish eggs, was efficient in feeding eel larvae (Okamura et al. unpublished data). Thus, the components of a diet may not be key to efficient diet intake by eel larvae, although minimum nutrition is required for growth.

In conclusion, the viscosity of a diet is likely to be an important factor for the ability of eel larvae to successfully ingest food materials, although effects of moisture are pending. The ability of eel larvae to bite and swallow is likely insufficient for ingesting the currently used slurry-type diet. Liquid-type diets with low viscosity can allow most larvae to eat efficiently, enhancing survival and metamorphosis rates. Using this liquid-type diet, we have consistently and successfully produced 2500–3000 glass eels in a 300-l tank (IRAGO Institute unpublished data), a much greater production than in our previous report (5 glass eels in a 19-l tank) (Okamura et al. 2009b). The techniques described here are likely required for the mass production of artificial glass eels.

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