Diets comprising hen egg yolk and milk proteins as potential alternatives to shark egg-based diets for larvae of the Japanese eel Anguilla japonica

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ORIGINAL ARTICLE

Aquaculture

Diets comprising hen egg yolk and milk proteins as potential alternatives to shark egg-based diets for larvae of the Japanese eel *Anguilla japonica*

Hirofumi Furuita[1,4] · Tadao Jinbo[2,5] · Masato Higuchi[2,5] · Kazuharu Nomura[1,4] · Ryusuke Sudo[1,6] · Hiroyuki Matsunari[1,4] · Koji Murashita[3,7] · Hiromi Oku[1,4] · Takeshi Yamamoto[3,7] · Hideki Tanaka[1,8]

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Abstract

The only diet that has been capable of rearing eel larvae to the glass eel stage in captivity is the shark egg (SE)-based diet (SE). This study investigated the potential of alternative dietary components, namely hen egg yolk (HEY), milk proteins, and fish protein hydrolysate (FPH), on the growth and survival of eel larvae. In the first experiment, a diet containing HEY and skimmed milk powder (HS) was compared to SE. There were no significant differences in growth and survival rate between the two diets except in the early part of the experiment period. In the second experiment, HS was modified by the addition of FPH and casein (FC), and larval performance was evaluated for three dietary regimens (SE, HS, and FC). The performance of larvae fed HS and FC was found to be comparable or superior to those fed SE. It was observed that larvae fed the alternative diets to SE were able to progress to glass eels. However, more skeletal abnormalities were observed in HS in experiment 1. The results of this study indicate that a combination of HEY and milk proteins is suitable as a larval eel diet, and that it has the potential to replace SE.

Keywords *Anguilla japonica* · Larvae · Diet · Hen egg yolk · Milk protein

Introduction

Japanese eel *Anguilla japonica* is an important species for freshwater aquaculture in East Asia, but culture of the Japanese eel relies heavily on wild-caught glass eels. The problem with this is that catches of glass eels fluctuate wildly from year to year, and resources are in a long-term declining trend. In fact, catches have been extremely poor since 2010 [Fisheries Agency website, https://www.jfa.maff.go.jp/j/saibai/attach/pdf/unagi-17.pdf (in Japanese) accessed 23 Oct 2023]. The decline of eel resources has become a serious problem, not only in Japanese eel, which are the

✉ Hirofumi Furuita
furuita_hirofumi91@fra.go.jp

1 Nansei Station, National Research Institute of Aquaculture, Japan Fisheries Research and Education Agency, Minami-ise, Mie 516-0193, Japan

2 Shibushi Station, National Research Institute of Aquaculture, Japan Fisheries Research and Education Agency, Shibushi, Kagoshima 899-7101, Japan

3 Tamaki Station, National Research Institute of Aquaculture, Japan Fisheries Research and Education Agency, Tamaki, Mie 519-0423, Japan

4 Present Address: Nansei Field Station, Fisheries Technology Institute, Japan Fisheries Research and Education Agency, Minami-ise, Mie 516-0193, Japan

5 Present Address: Shibushi Field Station, Fisheries Technology Institute, Japan Fisheries Research and Education Agency, Shibushi, Kagoshima 899-7101, Japan

6 Present Address: Minami-izu Field Station, Fisheries Technology Institute, Japan Fisheries Research and Education Agency, Minami-izu, Shizuoka 415-0156, Japan

7 Present Address: Tamaki Field Station, Fisheries Technology Institute, Japan Fisheries Research and Education Agency, Tamaki, Mie 519-0423, Japan

8 Present Address: Uragami Station, Aquaculture Research Institute, Kindai University, Nachikatsuura, Wakayama 649-5145, Japan

most intensively cultured and developed in the eel-farming industry, but also in European eel *Anguilla anguilla* and American eel *Anguilla rostrata* (Tanaka 2015). Therefore, the development of artificial production technology for eels is crucial. One of the critical challenges in developing these techniques is the identification of an appropriate diet for eel larvae. Early attempts at feed-based rearing involved the use of rotifers, a common initial feed for many marine fish larvae. Despite numerous feeding trials, no tangible benefits, such as improved growth or extended survival period, were observed when larvae were reared on rotifers (Tanaka 2015). Consequently, alternative feeds were reassessed from a wide range of possibilities (micro-diet, boiled egg yolk, fish eggs, zoo plankton, shrimp, jellyfish, gonad of mussel, etc.), resulting in the development of a diet containing eggs of spiny dogfish *Squalus acanthias,* krill hydrolysate, and soybean peptides for eel larvae (Tanaka et al. 2003). Currently, some facilities use shark egg-based diets for rearing eel larvae.

Several studies have been conducted to improve the quality of shark egg-based diets, suggesting the effectiveness of adding fish protein hydrolysate (FPH) and chitin hydrolysate to the diet (Kim et al. 2014; Okamura et al. 2020). In addition to feed composition, viscosity of the diet has also been shown to be important factor for growth and survival of eel larvae (Okamura et al. 2019; Yamada et al. 2019). Shark egg-based diet is being attempted to be used not only for Japanese eel larvae, but also for rearing European eel larvae (Benini et al. 2023; Bandara et al. 2023). However, growing concerns about the depletion of natural resources of spiny dogfish pose a significant challenge to the sustainability of this approach (Masuda et al. 2012). This makes it increasingly difficult to further develop mass production techniques for glass eels using shark egg-based diets (Masuda et al. 2012). For future mass production, alternative feeds to shark egg-based diet need to be developed.

Despite more than a decade of research into alternatives, no effective substitutes for shark egg-based diets have been established for eel larvae. Several studies have investigated potential alternatives, including diets based on HEY and skinned krill (Okamura et al. 2013) and FPH-based diets (Masuda et al. 2013b). However, despite such efforts, larvae fed these alternative diets exhibited lower growth and survival than those fed a shark egg-based diet (Okamura et al. 2013). Although an FPH-based diet has successfully produced glass eels (Masuda et al. 2016), it has not yet served as a practical alternative due to its associated low survival rates (Masuda et al. 2013b). HEY has been identified as a potential ingredient for eel larval diets, but concerns have been raised regarding its high lipid and low *n*-3 HUFA levels, which may negatively affect eel larval growth and survival (Okamura et al. 2013). Our preliminary study showed that eel larvae could be raised on diets in which shark eggs were replaced with HEY, although the performance of larvae fed HEY was inferior to those fed shark eggs (Furuita et al. unpublished data 2012). One reason for the poor performance of HEY-based diets relative to shark egg-based diets was thought to be the higher lipid content of HEY than of shark eggs (Fujino 1971; Hayashi and Kishimura 2000). Hence, we examined the effects of defatted HEY on larval eel and found that defatting HEY improved the growth and survival of eel larvae and that the performance of larvae fed a defatted HEY-based diet was similar to that of larvae fed an un-defatted shark egg-based diet (Furuita et al. 2014a). Based on these results, we considered that HEY is an effective feed ingredient for eel larvae. However, the defatting process of HEY is labor-intensive and increases the production costs of diets. A more efficient approach to developing a HEY-based diet is to combine HEY with low-fat ingredients. Masuda et al. (2010) demonstrated that eel larvae could ingest milk colloid added to seawater, and feeding cow's milk prolonged larval survival to 26 days post-hatch (DPH) (Japanese eel larvae typically survive 10–14 DPH without food; Masuda et al. 2010). Furthermore, preliminary experiments indicated that skimmed milk powder could serve as a viable ingredient in a diet for eel larvae (Nagao et al. unpublished data 2012). These results suggest that a combination of milk products and HEY could form a suitable diet for eel larvae, considering that milk products such as skimmed milk powder and casein contain few lipids, and their combination with HEY could reduce the lipid content of the HEY-based diet.

The aim of this study was to evaluate the potential of diets composed of HEY and milk proteins for eel larvae. In the first experiment, the nutritional value of a diet composed of HEY and skimmed milk powder was compared to a shark egg-based diet in a feeding experiment, considering that the use of skimmed milk powder could potentially reduce the cost of larval eel diet. Subsequently, the formulation of the HEY and skimmed milk powder-based diet was modified, and the effects of feed modification were examined by a feeding experiment, as it was suspected that the inclusion of a large amount of skimmed milk powder could be detrimental to eel larvae. Both feeding experiments were conducted until the onset of metamorphosis to glass eels, to investigate the feasibility of replacing the shark egg-based diet with a diet based on HEY and milk proteins.

Materials and methods

Ethics approval and consent to participants

All experimental fish were handled and treated in accordance with the Guidelines for Animal Experimentation at the

National Research Institute of Aquaculture, Japan Fisheries Research and Education Agency.

Fish

Female and male adult eels were induced to maturity using a hormonal treatment at the Shibushi Station of the National Research Institute of Aquaculture (NRIA), Japan Fisheries Research and Education Agency (FRA), Kagoshima, Japan. Glass eels purchased from a commercial dealer were feminized by administration of estradiol-17β mixed with commercial eel feed (10 mg/kg feed) for a period of 6 months (Tachiki et al. 1997). Feminized eels were then fed a standard commercial diet under conventional culture conditions. These eels were injected repeatedly with salmon pituitary extract (20 mg/kg body weight [BW]/week) and finally with 17-hydroxyprogesterone (2.0 mg/kg BW) (Kagawa et al. 1997, 2013; Unuma et al. 2011, 2012). Male eels were purchased from a commercial eel farm in Kagoshima, Japan, and injected repeatedly with Japanese eel recombinant luteinizing hormone (rLH, ARK Resource, Kumamoto, Japan) (500 µg/kg BW/week) (Ohta et al. 2017). Eel larvae hatched from eggs laid by spontaneous spawning from one female and three males (Satoh et al. 1992; Dou et al. 2007) were reared without feeding until the start of the experiment. Only one batch of larvae was used for each experiment.

Diets

The formulation and proximate composition of diets are shown in Table 1. In the present study, milk products, specifically skimmed milk powder and casein, were introduce into the HEY-based diet to reduce its overall fat content. In the first experiment, a diet was designed to evaluate the nutritional value of skimmed milk powder in combination with HEY. The formulation of a shark egg diet (SE) followed

Table 1 Formulation and proximate composition of the experimental diets (%)

	Shark egg (SE)	Hen egg yolk–skimmed milk (HS)	Fish hydrolysate–casein (FC)
Frozen shark egg	83.9	–	–
Krill hydrolysate[a]	10.5	–	–
Soybean peptide[b]	5.2	14.8	5.4
Hen egg yolk[c]	–	24.7	16.1
Skimmed milk powder[d]	–	49.4	16.1
Casein[e]	–	–	21.5
Fish protein hydrolysate[f]	–	–	32.2
Pollack visceral oil[g]	–	7.4	–
Salmon roe oil[h]	–	–	5.4
Vitamin mix[i]	0.4	1.2	1.1
Taurine[j]	–	2.5	2.2
Proximate composition (dry matter %)			
Dry matter	38.8	40.2	28.6
Crude protein	54.7	40.9	64.1
Crude lipids	44.5	24.8	23.6
Neutral lipids	31.0	20.2	17.5
Polar lipids	13.5	4.6	6.1
Crude ash	3.7	6.5	5.2
Crude sugar	2.1	27.3	8.1

[a]YOP-C (Nippon Suisan Kaisha, Tokyo, Japan)

[b]Hi-Nute HK (Fuji Oil, Osaka Japan)

[c]Dried egg yolk no. 1 (Kewpie Egg, Tokyo, Japan)

[d]Hokkaido skim milk (Yotsuba Milk Products, Sapporo, Japan)

[e]Casein sodium salt (Sigma-Aldrich, St. Louis, MO, USA)

[f]CPSP Special G (Sopropêche, Wimille, France)

[g]Kanematsu Shintoa Foods (Tokyo, Japan)

[h]Sujiko oil (Marinetech, Tahara, Japan)

[i]Furuita et al. (2014a)

[j]Wako Pure Chemical Industry (Osaka, Japan)

Furuita et al. (2014a), with some modifications: Frozen shark eggs were used in this study instead of lyophilized shark eggs. The HEY-skimmed milk powder (HS) diet also contained soybean peptide and pollack visceral oil.

In the second experiment, fish protein hydrolysate (FPH)-casein diet (FC) diet was formulated to address the drawbacks of HS, specifically to reduce the lactose content and increase the protein and *n*-3 highly unsaturated fatty acid (*n*-3 HUFA) levels. FC contained FPH, casein, and salmon roe oil in addition to the ingredients used in the HS diet. The nutritional value of FC was compared with SE and HS. All ingredients were mixed with distilled water and homogenized using a blender (Panasonic, Osaka, Japan) to produce slurry-type diets. These diets were dispensed into a 50-mL tube and stored in a freezer at −20 °C until use.

Fish husbandry

Experiment 1

Experiment 1 was conducted at the Shibushi Station. Larvae, at 6 DPH with an average total length (TL) of 7.4 ± 0.1 mm and body depth (BD) of 0.6 ± 0.0 mm (means ± SE), were transferred into 10-L round-bottom circular tanks (Masuda et al. 2013a) with an initial density of 250 larvae/tank in triplicate. Filtered seawater (23 °C) was supplied at a flow rate of 0.6 L/min. Experimental diets (SE and HS) were provided from 7 to 370 DPH. Feeding was conducted by pipetting 7–15 mL of each diet, as fish growth, onto the tank bottom five times daily at 2-h intervals (from 7:00 to 15:00). The water supply was briefly interrupted just before each feeding and resumed 15 min later to flush out the uneaten food. After the last feeding and subsequent flush of the day, surviving larvae were transferred to a clean tank using a vinyl chloride tube as a siphon. Larvae were anesthetized with 400 ppm phenoxyethanol, and their TL and BD were measured (n = 4–25 per tank, n = 19–60 per treatment) using a profile projector (Nikon, Tokyo, Japan) at 20 DPH and every 20 days thereafter. The fish were projected onto the projector and measured with a ruler. After measurement, the fish were returned to the tank. Skeletal abnormalities after metamorphosis were classified as abnormal only in individuals with severe abnormalities, with reference to Kuroki et al. (2016).

Experiment 2

Experiment 2 was conducted at the Nansei Station of NRIA, FRA, Mie, Japan. Five DPH larvae were transferred to the Nansei Station from the Shibushi Station. The larvae, at 6 DPH with an average total length (TL) of 6.8 ± 0.1 mm and body depth (BD) of 0.5 ± 0.0 mm (means ± SE), were transferred into 10-L round-bottom circular tanks (Masuda et al. 2013a) with an initial density of 500 larvae/tank in triplicate. Filtered seawater (23 °C) was supplied at a flow rate of 0.6 L/min. Experimental diets (SE, HS, and FC) were provided from 7 to 340 DPH. Feeding and larval transfer procedures were identical to those of experiment 1, except that feeding was conducted between 9:00 and 17:00. Larvae were anesthetized with 400 ppm phenoxyethanol and photographed to measure the growth rate (n = 10–23 per tank, n = 30–67 per treatment) with a binocular microscope (SZH10, Olympus, Tokyo, Japan) equipped with a digital camera (DP71, Olympus) before reaching 21 DPH and with a digital camera (EOS Kiss, Canon, Tokyo, Japan) after reaching 40 DPH and every 20 days thereafter. After the photos were taken, the fish were returned to the tank. The TL and BD of the photographed larvae were measured using ImageJ 1.49 software (National Institutes of Health, USA). Skeletal abnormalities after metamorphosis were classified as in experiment 1.

Chemical analysis

Moisture was determined by drying at 105 °C for 10 h. Crude ash was determined by combustion in a muffle oven at 600 °C for 5 h. Crude protein content ($N \times 6.25$) of diets was determined by semi-micro Kjeldahl method. Crude sugar was measured by phenol–sulfuric acid method using glucose as a standard (Dubois et al. 1956). Crude lipids were extracted with chloroform/methanol (2:1 v/v) according to the method reported by Folch et al. (1957). Total lipids were separated into polar and neutral lipids with a silica cartridge (Sep-Pak Plus; Waters, Milford, CT, USA) as described by Juaneda and Rocquelin (1985). Fatty acid methyl esters (FAME) were prepared according to the procedure reported by Miyashita et al. (1999) and subsequently purified using the Sep-Pak cartridge and diethyl ether/hexane (5:95). FAME were analyzed by gas/liquid chromatography (GLC; GC-2010, Shimadzu, Kyoto, Japan) equipped with a hydrogen flame ionization detector and an Omegawax 320 fused silica capillary column (30 m × 0.32 mm i.d., 0.25 µm film thickness; Sigma-Aldrich, St. Louis, MO, USA). The analytical conditions for FAME by GLC were described in Furuita et al. (2014a). Amino acid composition of diets was analyzed by the Food Analysis Technology Center SUNATEC (Mie Japan).

The determination of water-soluble and insoluble proteins in the diets was performed as follows. Each diet was placed into a tube with filtered (0.45 µm) seawater and shaken by hand. The tubes were then centrifuged at 15,000×g for 30 min. The water-soluble fraction was removed by pipette, and the precipitant was resuspended in distilled water. The protein content of the diet and the precipitant were measured using a commercial assay kit (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Rockford, IL, USA). The amount

of water-soluble protein was calculated by subtracting the insoluble protein from the total protein content of the diet.

Statistical analysis

Data from experiment 1 were analyzed using Student's *t* test. In the case where the standard deviation showed a significant difference between treatments, the Wilcoxon test was applied. Data from experiment 2 were subjected to one-way analysis of variance followed by the Tukey–Kramer multiple range test for further analysis. When the standard deviation showed significant differences between treatments, the Dunnett test was applied. For survival rates, data were arcsine-transformed prior to analysis. A probability value threshold of 0.05 was set to indicate a significant difference. All data are presented as mean ± standard error. All statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA).

Results

Diet

The proximate and fatty acid composition of the diets are shown in Tables 1 and 2. The lowest protein content was observed in HS, followed by SE and FC. The lipid content was comparable between HS and FC, which was lower than that in SE. SE had a higher level of *n*-3 HUFA and lower levels of 18:1n-9 and 16:0 than HS and FC. The fatty acid composition of HS and FC was similar, although FC was higher in *n*-3 HUFA and lower in 20:1 and 22:1 than HS. The amino acid composition of the diet is shown in Table 3. There was no significant difference in proportion of essential amino acids between the experimental diets. The proportions of water-soluble and insoluble proteins are shown in Fig. 1. Variations in the proportions of these proteins were noted between the diets. Water-insoluble proteins were most abundant in SE, followed by HS and FC.

Larval performance

Experiment 1

The survival rate and growth of larvae fed SE and HS are shown in Fig. 2, and detailed data are given in Online Resource, Table S1. The survival rate of larvae fed HS was lower than that of larvae fed SE at 20 DPH. At 40 DPH, there was no significant difference in survival rate between the SE and HS groups. Although the growth of larvae fed HS was significantly less than that of larvae fed SE up to 80 DPH, no significant difference was observed between the two groups after 100 DPH. Larval growth was not evaluated after 200 DPH because larvae from both groups began metamorphosis before 200 DPH. Table 4 provides a summary of the metamorphosis of eel larvae at the end of the

Table 2 Major fatty acid composition of total lipids in the experimental diets (% of total fatty acids)

Fatty acid	Shark egg (SE)	Hen egg yolk–skimmed milk (HS)	Fish hydrolysate–casein (FC)
16:0	15.5	22.2	20.1
16:1*n*-7	3.8	3.5	3.3
18:0	2.5	7.0	6.6
18:1*n*-9	13.6	30.8	31.8
18:2*n*-6	1.1	10.4	10.2
20:1	5.9	3.9	1.7
20:4*n*-6	3.7	1.3	1.4
20:5*n*-3	8.8	3.1	5.4
22:1	4.2	4.3	1.1
22:5*n*-3	4.1	0.5	1.2
22:6*n*-3	18.4	3.5	6.8
∑Saturates	21.3	31.6	28.6
∑Monoenes	32.6	44.5	40.6
∑*n*-6	7.3	12.7	12.9
∑*n*-3	33.0	8.0	15.3
∑*n*-3HUFA	32.1	7.4	14.2
20:4*n*-6/20:5*n*-3	0.42	0.42	0.26
22:6*n*-3/20:5*n*-3	2.1	1.1	1.3
n-3 HUFA in diet (DW%)	14.3	1.8	3.4

Table 3 Amino acid composition of the experimental diets (g/kg diet DW)

Amino acids	Diets		
	SE	HS	FC
Essential			
Arginine	37	20	34
Histidine	12	10	15
Isoleucine	25	16	26
Leucine	46	33	51
Lysine	34	28	47
Methionine	12	9	17
Phenylalanine	19	18	27
Threonine	23	17	27
Tryptophan	6	5	6
Valine	22	19	32
Nonessential			
Alanine	24	15	30
Aspartic acid	45	36	53
Glutamic acid	71	75	113
Glycine	19	10	33
Cystine	6	5	6
Proline	22	27	47
Serine	30	24	36
Tyrosine	17	17	27
Taurine	7	27	26
Total	477	410	652

SE shark egg, *HS* hen egg yolk–skimmed milk, *FC* fish hydrolysate–casein

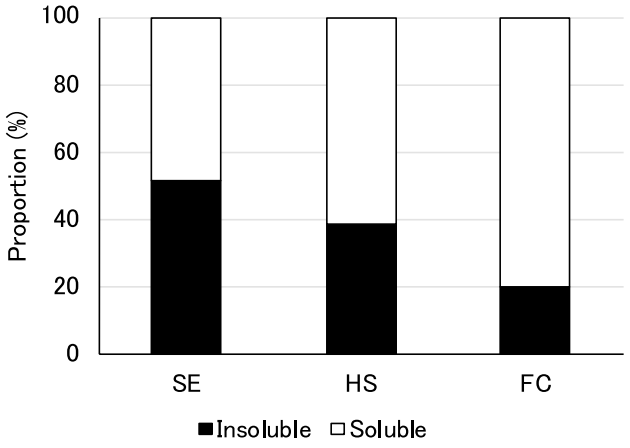

Fig. 1 Proportion of water-soluble and water-insoluble proteins in the experimental diets

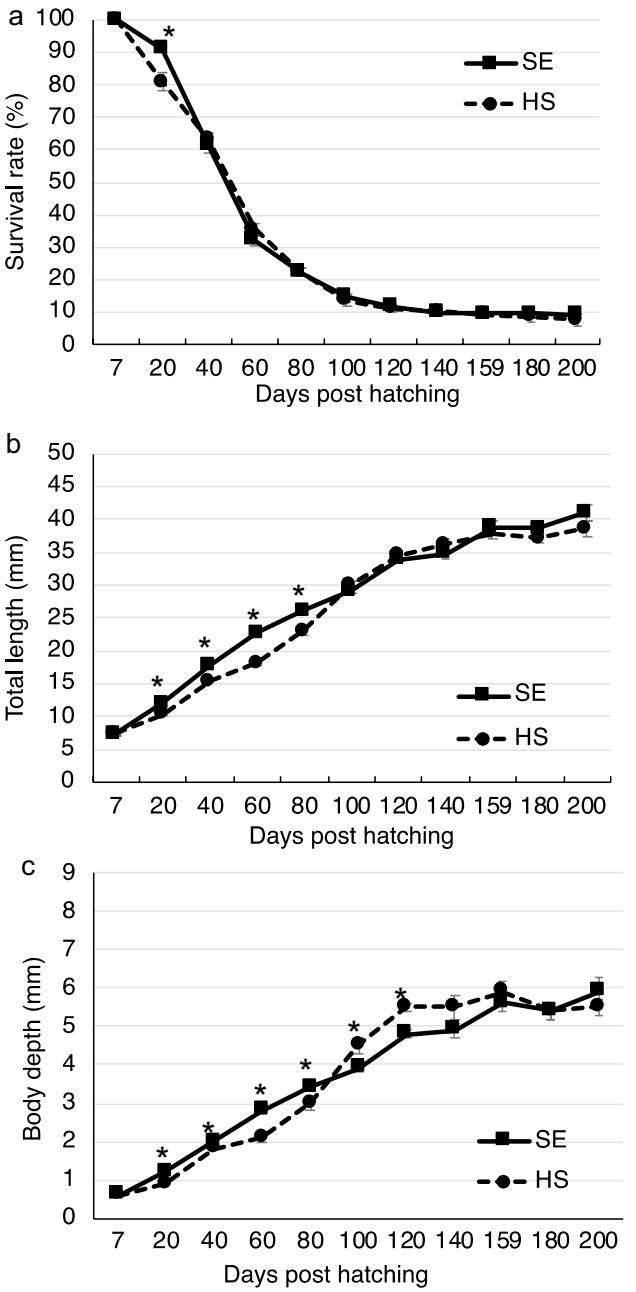

Fig. 2 Changes in survival rate (**a**), total length (**b**), and body depth (**c**) of eel larvae fed the experimental diets in experiment 1. *SE* shark egg, *HS* hen egg yolk–skimmed milk. Asterisk indicates significant differences were found between groups. See Table S1 for details

experiment (370 DPH). The average age of onset to metamorphosis was 270 DPH for SE and 239 DPH for HS, with HS being earlier. The number of metamorphosed larvae was 40 for SE and 27 for HS. The percentage of normal glass eel was 85% for SE and 22.2% for HS. Mortality of fish fed HS during metamorphosis was 29.6%, whereas no mortality was observed in fish fed SE.

Experiment 2

The survival and growth of larvae are shown in Fig. 3, and detailed data are given in Online Resource, Table S2. The survival rate of the FC group was significantly higher than

Table 4 Summary of metamorphosis of eel larvae fed the experimental diets

	Experiment 1		Experiment 2		
	SE	HS	SE	HS	FC
Age of onset to metamorphosis (DPH)	270 ± 42	239 ± 52	281 ± 12	254 ± 26	254 ± 30
Number of larvae onset to metamorphosis	40	27	3	30	49
Normality of glass eel (%)					
Normal	85.0	22.2	33.3	36.7	32.6
Deformity	15.0	48.1	33.3	50.0	40.8
Death during metamorphosis	0	29.6	33.3	13.3	26.5

SE shark egg, *HS* hen egg yolk–skimmed milk, *FC* fish hydrolysate–casein

that of the other two groups throughout the experiment. The survival rate of larvae fed HS was significantly lower than that of larvae fed SE at 21 and 40 DPH, but no significant differences were observed between the HS and SE groups after 40 DPH. The total length and body depth of larvae fed FC were significantly greater than those of the other two groups throughout the experiment, except for body depth of larvae fed HS at 200 DPH, which was not significantly different from those fed FC. Comparing the growth rate between experiments 1 and 2, larvae fed both SE and HS showed slower growth in experiment 2 until 160 DPH. After 160 DPH, the growth of larvae fed HS was similar. Metamorphosis of larvae fed FC, HS, and SE began at 205 DPH, 215 DPH, and 273 DPH, respectively, in experiment 2. Therefore, the measurement of larval growth was stopped at 200 DPH. Table 4 presents a summary of the metamorphosis at the end of the experiment (340 DPH). The average age of onset to metamorphosis was 281 DPH for SE, 254 DPH for HS, and 254 DPH for FC. The number of metamorphosed larvae was 3 for SE, 30 for HS, and 49 for FC. The percentage of normal glass eel was 33.3% for SE, 36.7% for HS, and 32.6% for FC. Mortality of fish during metamorphosis was 33.3% for SE, 13.3 for HS, and 26.5% for FC. There were no noticeable differences between the experimental groups with respect to abnormalities of fish.

Discussion

Our current study suggests that a diet combining HEY and milk proteins could be a viable alternative. Both growth and survival of larvae fed this diet were comparable to those of larvae fed a shark egg-based diet, and it was possible to raise larvae on this diet until they metamorphosed into glass eels. Consequently, these results demonstrate that a diet combining HEY and milk proteins can effectively serve as a substitute for a shark egg-based diet in the rearing of eel larvae.

In our present study, milk products were introduced into the HEY-based diet to reduce its overall fat content, since HEY has the disadvantage of being high in fat (Fujino 1971; Okamura et al. 2013). In addition, fish oil was added to increase the *n*-3 HUFA level of the diet because HEY is low in *n*-3HUFA (Fujino 1971; Okamura et al. 2013). In the HS, skimmed milk powder was the only milk product used. A large amount of skimmed milk powder inclusion, however, resulted in a decrease in protein and an increase in sugar content in HS relative to SE, as skimmed milk powder contains more than 50% lactose (Doi et al. 1991). The protein requirement for juvenile Japanese eels has been reported to be approximately 45% (Nose and Arai 1973; Okorie et al. 2007). This is similar to the level found in HS, although the optimal protein requirement for eel larvae remains unclear. In general, fish require more protein in early life stages than as adults, due to their rapid growth rate and high protein utilization for energy during the larval stage (Cahu and Zambonino Infante 2001). Péres et al. (1996) investigated the optimal level of dietary protein for sea bass *Dicentrarchus labrax* larvae using isoenergetic compound diets. Their results showed optimal growth with a 50% protein diet, slightly slower growth with 60% protein, and poor growth with 30% and 40% protein diets. Similarly, the protein requirement for rockfish *Sebastes schlegelii* larvae was estimated at 54.0% using granulated micro-diets (Jang et al. 2022). Considering the developmental stage of eel and the findings for other fish larvae, the protein content of HS may have been suboptimal for eel larvae. In addition, the high crude sugar content of HS (27%), together with its low protein content, could potentially have affected eel performance. Carnivorous fish typically have limited ability to utilize carbohydrates, and excess dietary carbohydrates have been shown to result in reduced feed intake and growth (Hemre et al. 2002). The optimal level of carbohydrates in diets for marine fish is considered to be 10–20% (Takeuchi 2009), which are significantly lower than HS. Furthermore, the type of carbohydrate may also influence fish development. For instance, juvenile tilapia *Oreochromis niloticus* × *O. aureus* fed lactose showed less weight gain than those fed starch, maltose, and sucrose when the fish fed diets contained 44% carbohydrate (Shiau and Chuang 1995). Therefore, the high lactose content in HS, both in terms of amount and type, could potentially have an adverse effect on the growth and survival of eel larvae.

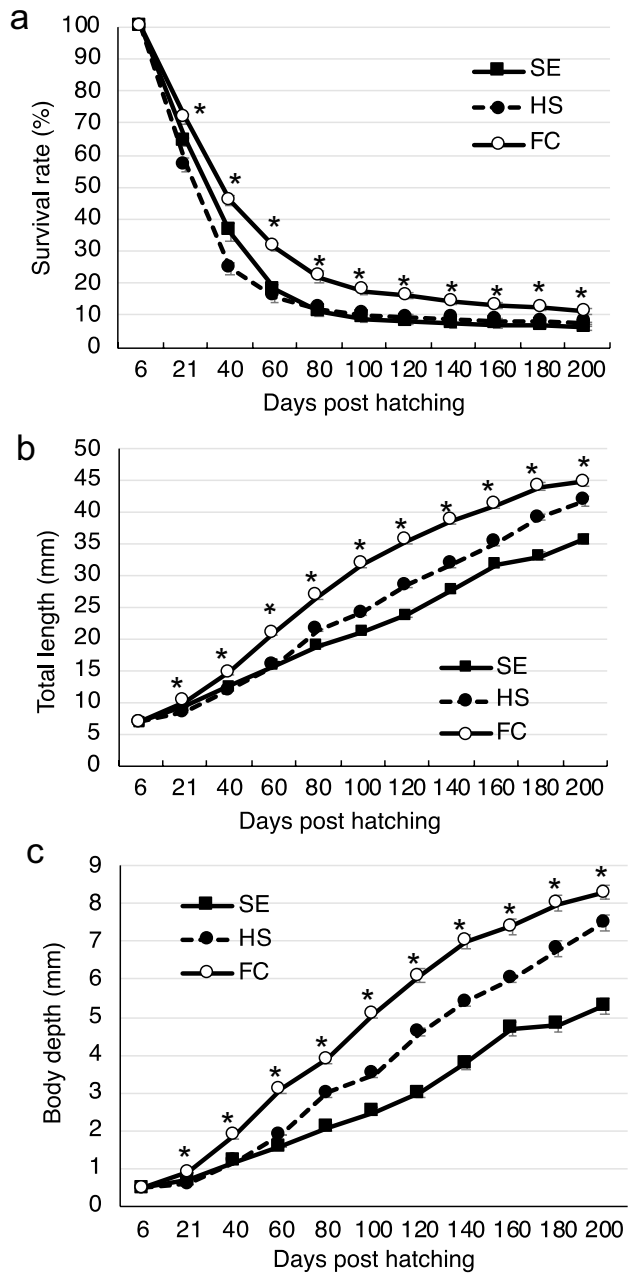

Fig. 3 Changes in survival rate (**a**), total length (**b**), and body depth (**c**) of eel larvae fed the experimental diets in experiment 2. *SE* shark egg, *HS* hen egg yolk–skimmed milk, *FC* fish protein hydrolysate–casein. Asterisk indicates that significant differences were found between groups. See Table S2 for details

Despite the lower protein and higher sugar contents of HS, the performance of larvae fed HS was comparable to that fed SE in our first experiment. Furuita et al. (2014a) indicated that defatting HEY and shark eggs improved the performance of early-stage (7–27 dph) eel larvae, with growth and survival of larvae fed a defatted HEY-based diet approaching those of larvae fed a shark egg-based diet. The lipid levels of the defatted HEY-based and the shark egg-based diets were approximately 30% and 45%, respectively (Furuita et al. 2014a). In the present study, we reduced the lipid level of HS to 26%, in contrast to SE, which maintained a lipid level similar to that of the un-defatted shark eggs-based diet in the previous study. This reduction in the lipid level in HS appeared to have a beneficial effect on the larvae.

Larval performance is influenced not only by the amount of protein but also by the type of protein in the diet. Water-soluble proteins are generally digested and utilized more efficiently by stomach-less fish larvae than water-insoluble proteins (Tonheim et al. 2007). The proportion of water-soluble proteins in SE was approximately 40%, whereas it was about 80% in HS and FC. These results suggest that eel larvae were able to utilize the proteins in HS more efficiently than those in SE, despite the lower protein content of HS. The combination of higher digestible protein and lower lipid levels in HS probably had a positive effect on the larvae, resulting in the similar larval performance with SE.

n-3 HUFA are essential fatty acids (EFA) of marine fishes, and the requirement for *n*-3 HUFA is also high during the larval stage, similar to protein. A deficiency in *n*-3 HUFA typically results in decreased growth and survival. The *n*-3 HUFA requirement for larvae fed *Artemia nauplii* has been documented as 3.0% for red seabream *Pagrus major* (Izquierdo et al. 1989), 3.9% for yellowtail *Seriola quinqueradiata* (Furuita et al. 1996), and 3.5% for Japanese flounder *Paralichthys olivaceus* (Izquierdo et al. 1992). In this study, the *n*-3 HUFA level in HS was only 1.2%, lower than the requirements for other fish larvae. In contrast, the levels in SE and FC were 14.3% and 3.4%, respectively. EFA requirements in fish are influenced by dietary lipid levels as well as dietary EFA levels. The optimal levels of *n*-3 HUFA in the total fatty acids are 10–20% for red seabream fingerlings (Takeuchi et al. 1992a) and 15–20% for yellowtail fingerlings (Takeuchi et al. 1992b). The *n*-3 HUFA level in the total fatty acids in HS was 7.4%, which was lower than the optimal levels for red seabream and yellowtail. The specific requirement for *n*-3 HUFA in eel larvae is currently unclear, but it is suggested to be lower than that for other fish larvae. This hypothesis is based on the lack of a rapid decrease in survival rate observed in the HS group. However, it remains uncertain whether the *n*-3 HUFA level in HS was sufficient for maximum growth and survival of eel larvae. The *n*-3 HUFA deficiency in HS may have contributed to the lower growth and survival observed in the early stages of this study. Conversely, the *n*-3 HUFA level in SE was much higher than that in the other two diets, as well as the *n*-3 HUFA requirements for larvae of other species (Izquierdo et al. 1989, 1992; Furuita et al. 1996). Excessive *n*-3 HUFA in the diet could have detrimental effects on fish development (Rodriguez et al. 1994; Takeuchi et al. 1992a, b). An increase in deformity was observed in cod larvae fed

rotifers enriched with purified DHA oil, in contrast to those fed rotifers enriched with shark egg powder, even though both rotifers contained similar levels of DHA (Takeuchi et al. 1994). This suggests that the balance of DHA and vitamin E (VE) in rotifers is critical for the development of cod larvae, since purified DHA oil-enriched rotifers contained a trace level of VE, whereas the VE content in rotifers enriched with shark eggs was 10.7 µg/g DW (Takeuchi et al. 1994). In the present study, it is uncertain whether the larvae fed SE were influenced by the excess *n*-3 HUFA. However, the effects of excess *n*-3 HUFA in the SE might have been mitigated, because most dietary lipids are excreted rather than absorbed when eel larvae are fed a shark egg-based diet (Furuita et al. 2014b). Furthermore, the high level of VE found in shark eggs (Furuita et al. unpublished data 2016) may have counteracted the potential negative effects of its excessive *n*-3 HUFA. Nevertheless, further investigation is warranted, as establishing a clear understanding of the EFA requirement in eel larvae is crucial in order to develop optimal diets for their successful cultivation.

Given the shortcomings of both the SE and the HS diets for larval eel, a new formulation, FC, was developed in the second experiment. FC was designed with an increased protein level and decreased sugar level to address the shortcomings of HS. In addition, the *n*-3 HUFA content was also increased to meet the known requirements of other marine fish larvae (Izquierdo et al. 1989, 1992; Furuita et al. 1996). Three ingredients were included in the FC formulation because of their beneficial properties: FPH, casein, and salmon roe oil. While FPH has been shown to have potential as an ingredient in eel larval diets, and glass eel have been produced with FPH-based diets, excessive amounts of hydrolysates of fish protein and casein have been shown to have negative effects on the growth of the larvae of European sea bass (Cahu et al. 1999) and common carp *Cyprinus carpio* (Carvalho et al. 2004), respectively. Therefore, the FC formulation contains approximately 30% FPH with a reduced level of soy peptides relative to HS. Casein, a major component of milk proteins, has been used in purified and semi-purified diets for fish nutritional studies. A semi-purified casein-based (37% fishmeal and 28.5% casein mixture, which contained 35% casein, 35% casein hydrolysate, and 28% casein salt) diet was found to promote significant growth in European seabass larvae, whereas a diet consisting of a 57% casein mixture, induced poor growth (Cahu and Zambonino Infante 1995). However, casein is not suitable for use in eel diets due to its insolubility in water. In contrast to casein is water-soluble (Carvalho et al. 2004). Therefore, casein was incorporated into FC to partially replace skimmed milk powder. Salmon roe oil, rich in *n*-3 HUFA and phospholipids, was also added to FC. Phospholipids play a vital role in fish development during the larval stage (Coutteau et al. 1997). Geurden et al. (1995) suggested that larvae may not be able to synthesize phospholipids at a rate sufficient to meet the requirements during the periods of high cell multiplication. It has also been suggested that phospholipids are more efficient than neutral lipids as a source of *n*-3 HUFA (Gisbert et al. 2005). The inclusion of salmon roe oil instead of pollack visceral oil in FC was expected to improve larval performance. These modifications resulted in positive effects on the larvae, and both the growth and survival of larvae fed FC were superior to those of the other two diets. However, it is difficult to directly compare the performance of SE and FC because the growth of the larvae fed SE in the second experiment was lower than that in the first experiment. This may have been due to the reduced efficacy of the frozen shark eggs during storage, as shark eggs are rich in lipids and *n*-3 HUFA, which are prone to oxidation. However, it is not clear whether the decline in SE performance is due to oxidation or to other factors, since there are no data on changes in the peroxide value of shark eggs with storage. Comparing the growth rate of larvae fed SE in the first experiment, the growth performance of larvae fed FC appears to be comparable to that of larvae fed SE. Further studies are needed to directly compare the nutritional performance of SE and FC.

Skeletal abnormalities of glass eel were not noticeably different between groups in experiment 2, but were more common in HS than SE in experiment 1. Skeletal abnormalities in larvae and juveniles are known to be caused by an excess or deficiency of various nutrients in feeds (Cahu et al. 2003). Deficiencies in amino acids, peptides, and vitamin C cause skeletal abnormalities in larvae (Cahu et al. 2003), but these components of HS and FC are not expected to differ significantly from those of SE (Tables 1 and 3). On the other hand, it is known that an excess of vitamin A given during the larval period can cause vertebral abnormalities in flatfish after metamorphosis (Fernández and Gisbert 2011). However, it is unlikely that skeletal deformity in larvae fed HS and FC was caused by excess vitamin A, since shark eggs contain more vitamin A than HEY (Furuita et al. unpublished data, 2016). The cause of the high incidence of vertebral abnormalities in fish fed alternative diets to SE is unclear at this time. Thus, clarification is needed as to the nutritional factors causing these abnormalities in glass eel, to enable the use of alternative diets to shark eggs for the production of glass eel.

In conclusion, the results of this study demonstrate that HEY and milk protein are effective ingredients in diets for eel larvae, as evidenced by the growth and survival of eel larvae fed a diet based on HEY and milk protein that was comparable to larvae fed a diet based on shark eggs. This suggests that a diet based on HEY and milk protein could serve as a viable substitute for a diet based on shark eggs. However, the growth of larvae in captivity still lags behind that observed in the wild (Tanaka 2015). The growth rate

of wild leptocephalus is estimated to be 0.3–0.5 mm/day, whereas the growth rate of captive larvae is 0.1–0.3 mm/day (Okamura et al. 2014). In addition, fish fed the alternative diet showed more skeletal abnormalities than those fed SE. Therefore, dietary improvements in FC are needed to enhance larval growth and to reduce skeletal abnormalities, and investigating the specific nutritional requirements of eel larvae is crucial in order to develop appropriate diets.

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Data availability The data presented in this study are contained within the article.

Declarations

Conflict of interest This study was funded by the Ministry of Agriculture, Forestry and Fisheries (MAFF) of Japan.

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