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Morphological Change of Artificially Produced Larvae from wild and Feminized Broodstock of the Eel, *Anguilla japonica*

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자연산과 자성화 유도 뱀장어 친어로부터 생산된 인공 자어의 형태학적 변화

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Abstract

Aquaculture of the commercialized eel, *Anguilla japonica*, relies heavily on wild-caught glass eel. To assess the potential of producing eel larvae artificially, we observed the flotation, fertilization, hatching, and survival rates of artificially fertilized eggs and hatched larvae from wild female and feminized eels. Body shape changes were observed along with the morphological examination of larvae from 30 days after hatching (dah) to approximately 260 dah. The flotation rates were 97.7% and 92.6%, fertilization rates were 68.7% and 67.6%, and hatching rates were 42.3% and 45.5% in eggs from the wild female and feminized eels, respectively, and the survival rates of hatched larvae at 8 dah were 20% and 22%. Eel larvae from the wild female and feminized eels exhibited similar growth rates and change of body shape until metamorphosis. After 120 dah, the growth rates were better in the eel larvae from the feminized eel than in those from the wild female eel. These results indicate that feminized eels are advantageous for the production of eel larvae and might be practical to be used in the artificial production of eel larvae to facilitate and regularize commercial eel harvesting.

Key words : *Anguilla japonica*, Artificial fertilization, Wild female eel, Feminized eel, Artificial production

Ⅰ. Introduction

The eel, *Anguilla japonica* is one of the most commercially important species of aquaculture in several Asian countries including South Korea. In *A. japonica* aquaculture, wild captured glass eels are used as a seed. Thus, the supply of wild captured glass eel is very important in eel culture. However, the unstable supplies and price fluctuations of glass eels represent serious problems in the eel farming industry. Glass eel resources of many species have decreased worldwide every year owing to factors including environmental variation, overfishing, and climate change (Casselmann, 2003;

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Dekker, 2009; Tsukamoto et al, 2009). However, commercial eel culture has not yet been fully developed because of very low survival rates during early larval stages and limited supply because of the protection or import regulations of glass eel resources (Crook and Nakamura, 2013, Luo et al., 2013, Silfvergrip, 2009).

Accordingly, the issue of artificial eel production has been of considerable concern and the subject of advanced research efforts. Artificial eel production methodologies have been studied since the 1960's; Yamamoto and Yamauchi(1974) first succeeded in producing *A. japonica* larvae from artificially fertilized eggs by hormone treatment, followed by successful pre-leptocephalus rearing for 2 weeks by Yamauchi et al.(1976). Finally, in 2005, the first demonstration of glass eel production over the full life cycle was reported (Kagawa et al., 2005).

In Korea, marked improvements in eel artificial production technology have occurred (Kim et al., 2006; 2007), although further study is necessary to obtain high quality and mass production of eel eggs. Furthermore, research to procure abundant female eels has been continued through the process of feminization (Kim et al., 2013), and environmental factors such as temperature have been considered to obtain high egg quality and to produce large amounts of eel larvae (Kim et al., 2013). However, these techniques have not yet been fully optimized.

In the present study, we compared the growth performance of larvae from wild female and feminized eels. In addition, we examined the morphology of eel larvae from 30 to about 260 days after hatching (dah). The results of these analyses will likely be of practical use for facilitating the mass production of eel larvae.

Ⅱ. Materials and Methods

1. Broodstock and hormonal treatment

Two groups of female eels were used in the present study. The first consisted of wild female eels, A. japonica [Silver eel; body weight (BW): 570–840 g] that were caught from the Nakdong River (Busan, Korea) during October and November 2011. The second comprised cultivated female A. japonica (BW: 550–720 g) that were feminized by estradiol-17ß administration (Kim et al., 2013) and were reared at the Inland Aquaculture Research Center for 3 years. Male eels (BW: 250–420 g) that were reared at the Inland Aquaculture Research for 2 years were also transported to the National Institute of Fisheries Science (NIFS) along with the feminized eels as a resource for subsequent experiments. All experimental eels were acclimated to seawater and kept without feeding in 1000 L flow-through tanks under a water temperature of 20°C.

Hormonal treatment was carried out for artificial maturation as described previously (Kim et al., 2006, Kim et al., 2007). Female eels were repeatedly injected with salmon pituitary extract (20 mg acetone-dried pituitary powder/individual), followed by injection with 17α, 20ß-dihydroxy -4-pregnene-3-one (2 μg/g BW, Sigma, St. Louis, MO, USA), and male eels were repeatedly injected with human chorionic gonadotropin (hCG; 1 IU/g BW/week, Dae-Seung Microbiology Co. Ltd., Seoul, Korea). After 8–10 hormone injections, eggs and semen were obtained from matured female and male eels, respectively.

2. Determination of flotation, fertilization, hatching, survival, and metamorphosis

rates

The eggs used in the present study were ovulated from a single wild female eel (ID:1072) and a single feminized eel (ID:5444) that had been matured at the same time. From these, 2 g of ovulated eggs was inseminated with 1 mL pre-diluted milt (sperm motility >80 %) for the determination of egg fertility and hatchability in micro-plates; the majority of ovulated eggs were inseminated with enough volume of pre-diluted milt for the mass production of larvae (Ohta et al., 1997).

The flotation, hatching, and survival rates of the inseminated eggs were determined using a micro-plate method (Unuma et al., 2004). Just after insemination in sea water, 3 mL seawater containing over 300 eggs was transferred to a dish and the number of floating and sinking eggs was counted for flotation rate count. Approximately 100 floating eggs were transferred individually into the wells of 48-well micro-plates (SPL, Seoul, Korea) that were filled with 1 mL filtered (pore size, 0.2-μm) seawater containing 100,000 IU/L penicillin G (Janssen Pharmaceutical, Geel, Belgium), 0.1 g/L streptomycin sulfate (USB Corporation, Cleveland, OH, USA), and 1 μg/mL poly-ethylene glycol 6,000 (PEG 6,000; Calbiochem, Darmstadt, Germany). The plates were maintained at 23°C in an incubator with humidity maintained at 100% to avoid evaporation of the rearing water. Fertilization rate was checked at 6 h after insemination. The flotation rates of the fertilized eggs obtained from the wild female and feminized eels were 97.7% and 92.6%, respectively. The fertilization rates at 6 h after insemination were 68.7% and 67.6% respectively(<Table 1>). The number of hatched larvae at 3 days after insemination and surviving larvae at 8 dah in each 48-well plate were counted. The hatching rates at 3 days after artificial insemination in the two broodstock groups were 42.3% in the wild female and 45.5% in the feminized eel. The survival rates at 8 days after hatching in were 20% and 22%, respectively. The number of final metamorphosed larvae was compared between the two separate 20 L aquariums (about 1500 larvae/aquarium, 4 aquariums for each group). The final metamorphosis rate was converted into percentages; the assessment period lasted until 14 months from hatching. After 14 months following hatching, the final metamorphosed rates were 0.1% in the wild female aquarium and 0.15% in the feminized aquarium.

<Table. 1> Measured data regarding flotation, fertilization, hatching and metamorphosed rates

	Flotation rate (%)	Fertilization rate (%)	Hatching rate (%)	Survival rate (8 dah; %)	Metamorphosed rate (about 14 months post-hatching; %)
Eggs from a wild female eel	97.7	68.7	42.3	20	0.1
Eggs from a feminized eel	92.6	67.6	45.5	22	0.15

Flotation rate (%) = (Number of floating eggs/Number of total eggs) × 100; Fertilization rate (%) = (Number of floating eggs in micro-plates/Number of total eggs in micro-plates) × Flotation rate; Hatching rate (%) = (Number of hatched larvae in micro-plates/Number of total eggs in micro-plates) × Flotation rate; Survival rate (%) = (Number of surviving larvae in micro-plates/Number of total eggs in micro-plates) × Flotation rate; Metamorphosed rate (%) = (Number of metamorphosed individual /1500*4) × 100

3. Artificial rearing of eel larvae

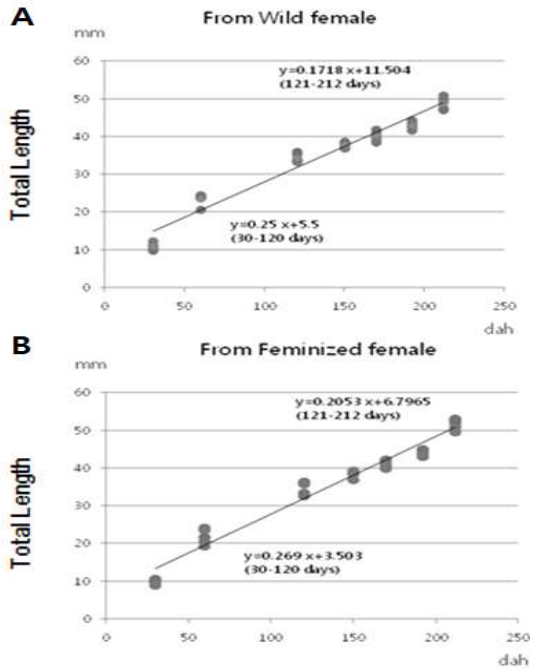
Mass fertilized and hatching larvae were first kept in 500 L polycarbonate tanks supplied with filtered seawater (Cartridge filter pore size: 10-μm) at 23°C for 8 dah until functional mouth development occurred. Afterward, approximately 1500 larvae at initial densities were transferred to one round-type 20 L aquarium using a silicon tube as a siphon. Filtered seawater was supplied at a rate of 0.9–1.0 L/min. The larvae were fed a shark egg-based slurry-type diet that was modified from Tanaka et al.(2003) five times a day at 2 h intervals from 9 dah (Kim et al., 2014). The water supply to the rearing tanks was stopped when the diet was added. After feeding (for 20 min), the water supply was restarted to flush out any uneaten diet. After the final flush, each rearing tank was connected to a clean tank with a polycarbonate tube to transfer the living eel larvae by siphoning. In this study, two separate aquariums were observed. One aquarium contained larvae from the wild female eel and the other held larvae from the feminized eel.

4. Sampling and measurements

To assess larva size, 5–10 larvae were randomly sampled from each aquarium on Days 30 and 60 after hatching and then fixed in 5% formalin. However, as the remaining populations were below 80 to 10 larvae in each aquarium by 120–257 dah, respectively, the mean sizes estimated for these days might be biased. The total length (TL) was measured under a binocular microscope after anesthetized with 100 ppm 3-aminobenzoic -acid ethyl ester (MS-222; Sigma). Photographs of the larvae were taken using an Axio Imager A1 microscope (Carl Zeiss, Oberkochen, Germany) and images were captured using an Axio Cam MRC5 camera (Carl Zeiss) and AxioVision software (Axiovs40, v 4.6.3.0).

Ⅲ. Results

1. Comparisons of growth performance between the two groups prior to the metamorphosis stage



[Fig. 1] Growth in total length of eel larvae from wild female (A) and feminized (B) eels. TL: total length (mm).

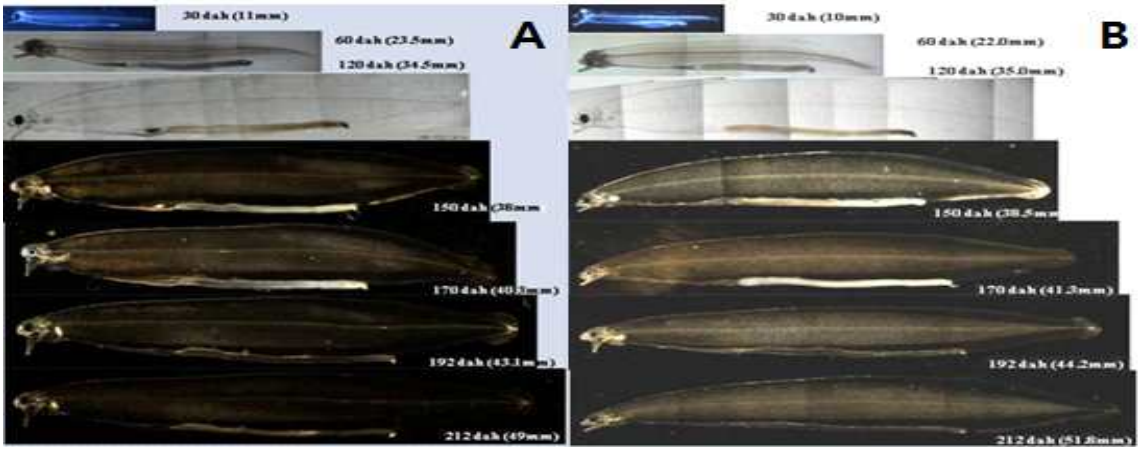
Eel larvae from the wild female and feminized eels were photographed at 30, 60, 120, 150, 170, 192, and 212 dah. The total body length gradually increased prior to metamorphosis; quantification of the daily growth is displayed in [Fig. 1] as divided between 0–120 and 121–212 dah. The growth rates of the eel larvae from the wild female eel were

0.2500 mm/day during 0–120 dah and 0.1718 mm/day during 121–212 dah. The body shape became elongated and compressed and the body depth gradually increased. Upon reaching approximately 30 mm in TL, the body shape appeared as a leaf shape. This tendency progressed until metamorphosis ([Fig. 1, 2]).

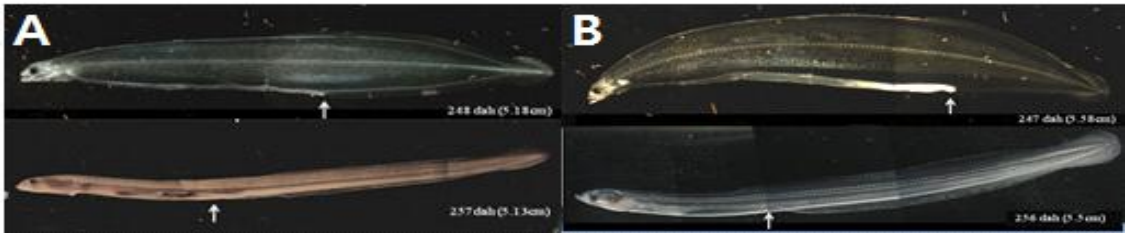
In comparison, the growth rates of the eel larvae from the feminized eels were 0.2690 mm/day during 0–120 dah and 0.2053 mm/day during 121–212 dah. The leptocephali from both the wild female and feminized eels grew in body length and width until metamorphosis, with a similar tendency of change in body shape between both groups ([Fig. 1, 2]).

2. Morphological changes in eel post-larvae during the metamorphosis stage

Metamorphosis in the eel post-larvae occurred after approximately 245 dah (from 245–260 dah); the patterns of morphological changes were similar between the two groups. The head and tail regions were slenderized, the body changed to a cylindrical shape, and the membranous caudal fin was elongated toward the anterior ([Fig. 2]). The position of the anus gradually moved to the anterior region and the body length began to shorten somewhat as the individual began the process of metamorphosis ([Fig. 3]).

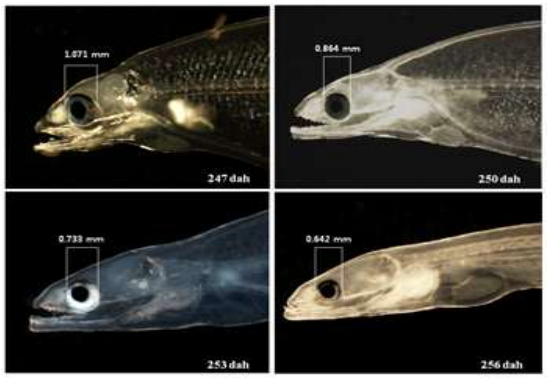


[Fig. 2] Morphological changes in larvae from the wild female (A) and feminized (B) eels from 30–212 dah.



[Fig. 3] Morphological changes in post-larvae from the wild female (A) and feminized (B) eel during metamorphosis (wild eel, 248 and 256 dah; feminized eel, 247 and 256 dah). Arrows: position of the anus.

As the first step of metamorphosis, the width of the eye shortened ([Fig. 4]). The teeth had degenerated by the completion of metamorphosis and gill development was predominant, such that the increase of length and density of the gill filaments could be readily observed([Fig. 4]).



[Fig. 4] Morphological changes from the wild female in the head region in post-larvae during metamorphosis. From 247-256 dah are shown.

Ⅳ. Discussion

In the present study, no significant differences were observed in the results of fertilization and hatching rates between eggs from wild female or feminized eels. In addition, the growth rates and morphological changes during the metamorphosis stage of the larvae from the wild female eel were equivalent to the results of larvae from the feminized eel.

In our previous study, we examined the fertilization (75%) and hatching (55%) rates in cultured *A. japonica* female eels (Kim et al., 2007). Kagawa et al.(1998) similarly reported fertilization and hatching rates of approximately 65% and 58%, respectively. In recent years, success has also been obtained in artificially producing eggs and larvae from the European eel, *A. Anguilla*, American eel, *A. rostrata*, Australian short-finned eel, *A. australis*, and other eel species; however, these only survived for a few days (Lokman and Young, 2000; Oliveira and Hable, 2010; Palstra et al., 2005). In comparison, the first successful larvae and glass eel production in the world was obtained using *A. japonica*; furthermore, a second generation (F2) was finally produced in 2010 (Ijiri et al., 2011).

Specifically, hatched eel larvae in the aquarium for about 8 dah during which time the larvae absorb their yolks. After 8 dah, the eel larvae obtain nourishment from the environment. Body length and body depth increase until 240-250 dah and then along with metamorphosis, the position of the anus moves forward, the head and tail become narrow, and then the body form changes to the long cylindrical shape of the glass eel (Tanaka, 2003).

For mass production, stable supplies of healthy parental eels are necessary. However, it is difficult to collect these eels; in addition, they are obtainable only during very limited seasons. Furthermore, most cultured eels become males such that the percentage of females is very low (Satoh et al., 1992). Recently, feminizing technologies of eels associated with estradiol-17β have been studied (Chiba et al., 1993; Kim et al., 2013; Tachiki et al., 1997). These feminized eels provide an opportunity for using females throughout the year. It has been considered that eel larvae from wild female eels would be healthier and exhibit preferential growth patterns; however, we observed no significant differences between the wild female eel and feminized eel in our study.

Tanaka et al.(2001) also performed a comparison between reared and wild specimens among 0 to 100 dah larvae. The developmental processes of

artificially reared eel larvae were generally similar with those of the wild eel. However, the morphologic patterns of TL and the proportions of body dimension, in particular the ratio of body depth to TL and the number of pre-anal myomeres during 0 to 100 dah were higher in wild than in reared specimens of similar TL. Mochioka(1996) did not observe these patterns in fish over 50 mm TL.

Anguillid leptocephali possess several useful characteristics for morphological studies including the total number of myomeres, ano-dorsal myomeres, origin of the dorsal, fin position of the anus, and vertical blood vessels. However, pre-leptocephalus stage larvae are difficult to identify because the total number of myomeres and the origin of the dorsal fin are not fully developed (Aoyama et al., 1999; Castle, 1963). Although these morphological details were not analyzed in the present study, obvious differences in growth and viability rates were not observed between the larvae from the wild female and feminized eels.

During the process of metamorphosis, no marked differences in the transformational course or in the time required were found between the two groups of metamorphosed leptocephali. The development of the head region and the movement of the anus position were very impressive in all metamorphosed animals. Some leptocephali exhibited a worn snout, but this deformity occurred because of the intake behavior of leptocephalus in eating a slurry type diet that squeezed at bottom of rearing tank rather than in the leptocephali of a particular group.

Substantial differences relating to the growth mode were not found between larvae from the wild female eel and those from the feminized eel, and the growth rate of eel larvae from the feminized eel was better than the rate from the wild female in the present study. This demonstrates that feminized eels would likely be useful for the production of eel larvae. The process of feminization is directly related to the mass production of maturated female eels; in turn, healthy maturated female eels are directly associated with good quality eggs.

Ⅴ. Conclusions

The fertilization, hatching rates, growth rates and morphological changes during the metamorphosis stage of larvae from a feminized eel and a wild female eel did not exhibit any substantial differences. We thus concluded that feminized eels might be useful for the artificial production of larvae. To further develop this potential, additional research is necessary to improve the egg quality in feminized eels.

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