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Fig. 1. Yellow phase of the Japanese eel (*Anguilla japonica*), 1st generation, matured and reared in captivity for the first time in 2003. The tube in this picture is a retreat.

# Controlled reproduction in the Japanese eel (*Anguilla japonica*), past and present

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## INTRODUCTORY REMARKS

The Japanese eel, *Anguilla japonica*, is one of the most important species of the aquaculture industry of East Asia. Supply of glass eels for aquaculture is completely dependent on wild catch. However, glass-eel catches in Japan declined linearly from over 200 tons in the early 1960s to 20 tons at present. In recent years an unstable situation has occurred with glass-eel catches not meeting the demand of aquaculture farms. In order to address this rapid decline, a challenging research project for artificial production of glass eels was commenced in the late 1960s. Since then, through a continuing process of trial and error, the production of second-generation larvae was finally achieved in 2010. Throughout the research, a new application of several substances has caused breakthroughs in artificial induction of sexual maturation and larvae rearing. This article reports on a study of controlled eel

reproduction focusing on the finding of substances that accelerated progress. In addition, guideline research on eel reproduction in the wild was conducted aiming at overcoming current bottlenecks that impeded the establishment of large-scale glass-eel production in captivity, by investigating maturing eels and eggs collected in their spawning area in the vicinity of the West Mariana Ridge.

### 1. SALMON PITUITARY

The first finding was salmon pituitary. Japanese eels begin sexual maturation when catadromous migration occurs in autumn or early winter at the age of 5–12 years. At this time, eels become silver eels. Changes also occur in the ovary, which increases in size, and the oocytes develop their early vitellogenic stage. However, in captivity they do not undergo further gonadal development and vitellogenic growth. Furthermore, maturing eels whose ovaries had already developed to a more advanced stage, had never been found in the wild. The first target of the eel research in the 1960s was, therefore, to induce sexual maturation in order to obtain fertilisable eggs.

By this time, in the European eel (*Anguilla anguilla*) it had been possible to induce complete spermatogenesis by injecting urine from pregnant women, human chorionic gonadotropin (HCG), to male eels (Fontaine 1936, Bruun 1949). In contrast, HCG injections into female eels did not induce oocyte development to maturity. The first eggs had been obtained by injections of carp pituitary; however, attempts to fertilize the eggs proved unsuccessful (Fontaine et. al. 1964). Against this research backdrop, Yamamoto and Yamauchi, Hokkaido University, collected salmon pituitaries at salmon hatcheries in Hokkaido, where abundant salmon heads were available after their eggs and sperm had been utilized for the production of salmon (*Oncorhynchus keta*). Maturation could then successfully be induced by weekly injection of salmon pituitary homogenate (SPH), and fertilisable eggs and eel larvae were obtained (Yamamoto and Yamauchi 1974). (Fig. 2) The gonado-somatic index (GSI, gonad weight in % of body weight) reached 40-70 % in mature females and their abdomens swelled into balloons. This peculiar appearance of the females raised the question whether such swelling reflected natural conditions or was a characteristic feature caused by artificial maturation.

In 1976, Yamauchi et al. succeeded in cultivating eel larvae for two weeks, however the larvae did not feed and metamorphosis into leptocephalus larvae did not occur. After this, larvae feeding and production of leptocephali was not successful until 2001 (Tanaka et al. 2001).

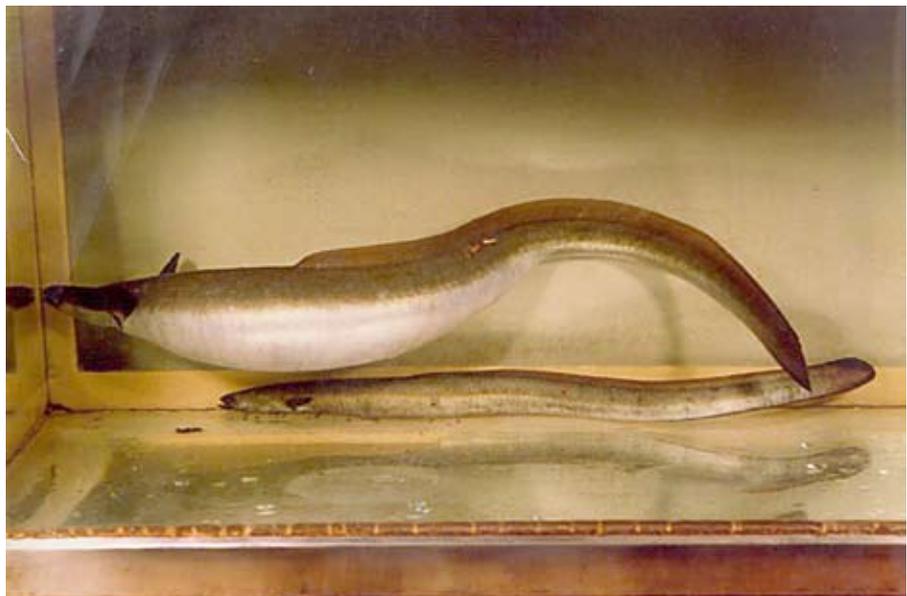


Fig. 2. Artificially matured Japanese eel (*Anguilla japonica*) in the early 1970s. The upper individual is female, the lower male.

### 2. MATURATION INDUCING STEROID

The method of controlled maturation has not been changed basically until now. However, most eels could not complete final maturation and ovulation by SPH injections alone, although most of them could be induced until completion of vitellogenesis. It was still uncontrollable whether fertilized eggs were obtained or not.

In 1985, Nagahama and Adachi, National Institute for Basic Biology, identified a maturation inducing steroid of salmon as  $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one (DHP) for the first time in fish. Thanks to this finding, it became possible that oocyte maturation and ovulation were induced by single injection of DHP to post-vitellogenic eels (Yamauchi 1990). This must be considered the second finding which brought rapid progress in obtaining eggs from many more individuals that had received SPH injections. At present, vitellogenic growth is induced by intraperitoneal injection of salmon pituitary extract (SPE) once a week at 30 mg/kg-body weight in 15°C sea water. After 7-17 injections, if body weight (BW) exceeded 110% of initial body weight, which means the beginning of oocyte hydration, an additional SPE injection is administered to enhance maturational competency, then water temperature is increased until 20°C. Judging by the conflated condition of the oil droplet in oocytes (in many cases at 24 h after the additional SPE injection), DHP is injected intramuscularly at 2 mg/kg-BW, then water temperature is maintained at 20°C or alternatively increased until 23°C, to stimulate final oocyte maturation and ovulation.

At the same time, in male eels, having received a weekly injection of HCG at a dose of 1 U/g-BW for over 6 weeks, spermiation is induced by injection of DHP at 1 mg/kg-BW. Approximately 15 h after DHP injection, eggs were collected by pressing the abdomen, then inseminated with milt, diluted (50 x) by artificial eel seminal fluid (114.5 mM NaCl, 30 mM KCl, 1.6 mM  $MgCl_2$ , 1.3 mM  $CaCl_2$ , 20 mM  $NaHCO_3$ , pH 8.2), and then transferred to filtered seawater at 23°C. A difficult

and important point here is estimating when DHP is to be injected and when eggs are to be collected by squeezing (Fig. 3). In most cases, egg quality, which is evaluated by the fertilisation results, by hatching and 8-day survival rates, is highly variable between eggs of different individuals (Chai et al. 2010). One reason is a difficulty of predicting the appropriate timing for the DHP injection because the time window for the injection that allows the production of high-quality eggs, is narrow. Moreover, insemination soon after ovulation is a prerequisite of success, because egg quality degenerates relatively soon after ovulation (Abe et al. 2010).

### 3. FEMINISING HORMONE

As described above, it had become a little easier to obtain fertilizable eggs and larvae hatching by that time. However, feeding and rearing larvae for more than 2 weeks remained impossible for many years. In the 1990s, an integrated eel reproduction project was started by the Fisheries Agency of Japan. The project team was composed of members of national institutes belonging to the Fisheries Agency, to national Universities and to fishery research stations of local government prefectures. Until this time, female silver eels collected in the wild were commonly used as parent fish for artificial maturation; however, it was not easy to collect them in numbers sufficient for the experiments. The third technical advancement was made possible by Tachiki et al., of the Aichi Fisheries Research Institute, in 1993. They established a method which produced parent females suitable for artificial maturation experiments through feeding estradiol-17 $\beta$  (10 mg/kg-pellet) to glass eels for 5-6 month (Tachiki et al., 1997). This feminising treatment did not only bring about complete feminisation of the eels but it also accelerated oocyte development before the vitellogenic stage. Such feminised eels are capable of inducing vitellogenesis and final maturation after only 2 years from the glass-eel stage (Chai et al. 2010).

Before this discovery, attempts at using cultivated females, reared with normal food as parent females for artificial maturation, showed poor results in obtaining eggs. Most of the cultivated female eels were not induced until their oocytes entered the maturation phase (Ijiri et al. 1995). A later study revealed that ovarian follicles over approximately 180  $\mu\text{m}$  could respond to SPE stimulation

for their complete maturation. However, ovarian follicles of most cultivated eels reared for at least 7 years were smaller. By contrast, the follicles of the majority of feminised eels reared only 2 years have already reached this size (Ijiri et al. 1998). Recently, it was reported that feminised eels with follicle diameters >160  $\mu\text{m}$  could successfully induce final maturation, if SPE injection was begun before mid winter (Chai et al., 2010). By this finding, it turned out possible to prepare plenty of parent females for artificially induced maturation.

### 4. SHARK EGG

On the other side, efforts at rearing larvae had been extremely challenging. After having absorbed their yolk and oil droplet stores, they could not survive and died in about 2 weeks without showing signs of metamorphosis into leptocephali. Tanaka et al., of the National Research Institute of Aquaculture, succeeded feeding rotifers, the most common initial food for marine fry, as well as microdiet and boiled chicken-egg yolk. However, such feeding did not enable eel larvae to survive for

a longer period (Tanaka et al. 1995). After much trial and error, it was found that shark eggs strongly attracted active feeding of larvae. Basic nutrition was, therefore, supplemented with shark egg, and finally larvae rearing was successful for over 100 days (Tanaka et al. 2001). Soon after this record was established, the same authors reported a remarkable progress in rearing larvae until the glass-eel stage and even further to the yellow-eel stage in 2003 (Fig. 1, page 13).

The finding of shark egg as initial food for eel larvae is considered as the fourth breakthrough. The artificially produced glass eels could be grown and were artificially matured. Thereafter, a second generation of larvae was produced in 2010. However, the quality of eggs obtained through controlled maturation is still highly variable, and the survival rates of the larvae are usually extremely low. In addition, growth of the larvae is slower in captivity than in the wild. Under controlled conditions, metamorphosis into glass eels occurred about 250 days after hatching, in contrast to 100-140 days estimated for wild larvae. Moreover, body height was notably lower in captive leptocephali than in wild ones. Characteristic conditions of artificially produced eel larvae may be their low egg quality and the composition of the diet. To address these issues, we began seeking answers in their spawning area.

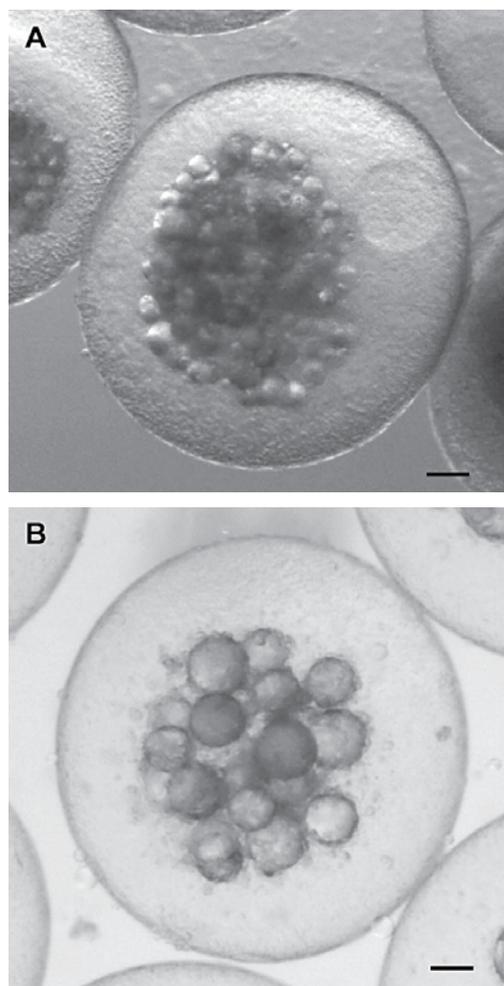


Fig. 3. Ovarian follicle of Japanese eel (*Anguilla japonica*). (A) Prior to DHP injection to induce oocyte maturation and ovulation. Germinal vesicle is visible close to peripheral region of oocyte. (B) Oocyte after ovulation. Bar = 100  $\mu\text{m}$ .



Fig. 4 The first female Japanese eel (*Anguilla japonica*) captured at its spawning area in 2008. The body looked seriously exhausted by spawning and their gonads appeared to be degenerated vestigial ovaries.

## 5. REFERRING TO THE WILD

The question where the Japanese eels spawn, had remained a mystery for many years. In 2005, Tsukamoto had identified a precise spawning location by collecting eel larvae that had hatched 2 to 5 days previously at 14°N, 142°E in the southern part of the West Mariana Ridge (Tsukamoto 2006). On receiving the news, the Fisheries Agency decided to send a research trawler, the *R/V Kaiyo Maru* (of the Fisheries Agency), to that area. On this cruise, in summer 2008, Chow and Kurogi, of the National Research Institute of Fisheries Science, succeeded in capturing four adult Japanese eels by a large mid-water trawl. Two of them were post-spawning females; however, their bodies looked seriously exhausted by spawning and their gonads appeared to be degenerated ovaries (Chow et al. 2009, Kurogi et al. 2011) (Fig. 4). In the following spring to summer, four research vessels gathered again in the spawning area.

In May 2009 around the new moon period, thirty-one eggs of Japanese eel were collected with a large plankton net of the *R/V Hakuho Maru* (JAMSTEC, Atmosphere and Ocean Research Institute). Immediately after egg collection, *Kaiyo Maru* and the *R/V Tenyo Maru* (of the National Fisheries University) rushed to the point and conducted a trawling survey; however, no adult eel was caught, but *Kaiyo Maru* successfully collected many preleptocephali that appeared to be from the same spawning. Next June, *Kaiyo Maru* shipped to the egg sampling point again and started a trawling survey around new moon. During several days before new moon, east of the egg sampling point, remarkable catches of male Japanese eels with running testes were made (Fig. 5). Another research vessel, the *Hokko Maru* (of the Fisheries Research Agency), which was carrying out a trawling survey in the northern part of the Western Mariana Ridge, made a hasty cruise to the point, arrived within one day and trawled in parallel with *Kaiyo Maru*.

Finally, three female Japanese eels with functional ovaries were captured in the night of new moon. Two of them seemed to be in a condition just after spawning. However, they had maintained a large, healthy bright red liver and ovaries in spawning-condition. The ovaries possessed clear post-ovulated follicles (like empty shells of oocytes) and oocytes in mid-vitellogenic stage. The physiological conditions of females captured in 2008 and 2009 suggest that eel ovaries are polycyclic having the potential of spawning more than once. The third female captured by the same net had still ovulated eggs in its

body. Unfortunately, the eggs seemed to be over-ripened ovulated eggs judging from the over-conflated oil droplet and suggesting that this female did not seem to have spawned normally during the last spawning period like the other two females. Notably, this female looked like having a swollen abdomen with abundant eggs inside, and the GSI value was close to 50% as in artificially matured females (Fig. 6). The diameter of ovulated oocytes was approximately 950 µm, thus also equal to that of the artificially obtained ones (Tsukamoto et al., 2011).

During a 2009 trawling survey four males and four females of the Japanese eel and two males and one female of the giant mottled eel (*Anguilla marmorata*) in spawning condition were caught. Trawling surveys were also carried out in 2010 by *Kaiyo Maru* and *R/V Oshoro Maru* (Hokkaido University). However, female Japanese eels were not captured on this cruise despite considerable sampling effort. At present, eggs (ovulated oocytes) before spawning as a precious basis for comparison with artificially produced ones, exist only from these cruises. The eggs are now subjected to large-scale RNA sequencing. Hopefully, the RNA pattern accumulated in the eggs will reveal characteristic features of high-quality eggs. During the above research cruises, 753 preleptocephalus larvae were collected and analyses of contents of the digestive canal are in progress. We hope that novel information from these analyses may lead to further breakthroughs, allowing stable production of high-quality eggs as well as an improvement of early-larval-stage survival.

## CLOSING REMARKS

Over the past 50 years of research, more than one full life-cycle of the Japanese eel could be accomplished in the laboratory, and the natural conditions of maturation, egg production and early larvae life were revealed by marine field studies. Understanding the environmental conditions in the spawning area and the physiological conditions of wild maturing eels and their larvae may help advance the establishment of commercial glass-eel production, which could become another fantastic story in the future.

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Fig. 5. Mature male Japanese eel (*Anguilla japonica*) captured at its spawning area. The colour was silvery black and eyes were greatly enlarged.



Fig. 6. Mature female Japanese eel (*Anguilla japonica*) captured in its spawning area. The body was scarred by mesh of the trawl. Eggs were emanating from the genital pore.

**Concluding remark by M.B.:** It should be realised how difficult it was to put this article together under the circumstances of the tsunami/earthquake/atomic-energy disaster hitting also some of the laboratories involved in the above research.

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