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Changes in Sex Steroid Hormones and Ovarian Development during Artificial Maturation of Female Eel, *Anguilla japonica*

Dae-Jung Kim*, Jun-Young Bae and Eung-Oh Kim[1]

Aquaculture Research Team, Headquarters for Aquaculture, National Fisheries Research and Development Institute (NFRDI), Busan 619-902, Korea; [1]Inland Aquaculture Research Institute, NFRDI, Jinhae 645-806, Korea

Abstract: The present study demonstrates the changes in body weight (BW) and plasma sex steroid hormone profiles during artificial maturation induced by human chorionic gonadotropin (HCG) or salmon pituitary extract (SPE) injections in cultured eel, *Anguilla japonica*, kept in seawater for 3 months. In the weekly SPE-injected female group, BW was relatively stable during vitellogenesis. Following induction of vitellogenesis, females exhibited a rapid increase of BW, and the oocytes were observed to be in the migratory nucleus stage at the end of the experiment. Plasma testosterone (T) and estradiol-17β (E_2) levels increased slightly during vitellogenesis and peaked at an average of 5.82 ng/mL and 4.76 ng/mL, respectively, at the end of the experiment. In the weekly control and HCG-injected female groups, BW slowly decreased during the experimental period, and the oocytes of the two groups were observed to be at the primary yolk globule stage. In the weekly HCG-injected female group, plasma T and E_2 levels increased slightly during vitellogenesis and decreased afterward. In the control female group, however, plasma T and E_2 levels were not altered during the experimental period. Furthermore, plasma 17α,20β-dihydroxy-4-pregnen-3-one (DHP) was not detected in all experimental groups. Fertility and hatching rates of SPE-injected females were significantly higher in those that ovulated 15 h after DHP injection than 18 h. These results indicate that long rearing in seawater increases responsiveness to SPE in ovarian maturation of the Japanese eel, resulting in shortened period from completion of vitellogenesis by sex steroid hormone production.

Key words: female eel, *Anguilla japonica*, sex steroid hormone, ovulation, fertilization, seawater rearing

*To whom correspondence should be addressed.
Tel: 82-51-720-2421; Fax: 82-51-720-2439
E-mail: djkim4128@momaf.go.kr

The life history of catadromous anguillid eels have complex life cycle, and little is known about their reproduction because sexually mature eels or eggs have never been found in the wild (Tsukamoto et al., 2003). At present, the supply of eel larvae for stocking farms is entirely dependent on glass eels captured from the wild. However, eels do not undergo further gonadal development and vitellogenic growth under aquarium conditions because of insufficient gonadotropin synthesis in the pituitary gland (Sato et al., 2003). Therefore, it is important that a reliable supply of larvae is established. In this context, artificial control of gonadal development is very essential for obtaining eel larvae. To solve these problems, hormonal treatment using salmon pituitary extract (SPE) was generally conducted for inducing vitellogenesis in immature eels (Yamamoto and Yamauchi, 1974; Sato et al., 2000; Kim et al., 2006a). A 17α,20β-dihydroxy-4-pregnen-3-one (DHP) injection is effective for the induction of final oocyte maturation and ovulation after the completion of vitellogenesis (Sato et al., 2003). Although vitellogenesis, final oocyte maturation and ovulation were successfully induced by the above-mentioned treatments, the percentage of ovulated females in their studies was very low, and even if ovulated eggs were obtained, these eggs showed low fertility and hatchability (Sugimoto et al., 1976; Satoh et al., 1992), perhaps due to incomplete oocyte maturation and ovulation.

Since the 1970's many studies have been performed with the aim of inducing ovarian growth and maturation of silver eels (Yamamoto and Yamauchi, 1974; Satoh et al., 1992; Sato et al., 2000; Kim et al., 2006a). However, silver eels are not easily obtained at present. Although cultured eels have been used for artificial maturation in some studies, the results were more variable when compared with those from silver eels (Ijiri et al., 1995, 1998; Sato et al., 2000). To

solve these problems, Kagawa et al. (1998) showed that rearing in seawater for 3 months induced vitellogenesis, resulting in oocytes similar to those in natural migrating females (silver stage female). This was the first evidence that vitellogenesis could be induced in cultured eels reared under artificial conditions without any hormone treatment. However, there is no report about the profile of plasma sex steroid hormones for the period of hormonal treatments after rearing in seawater for 3 months.

The present study examined changes in body weight and plasma sex steroid hormone profiles (testosterone; T, estradiol-17β; E_2, DHP), the key factors to determine sexual maturation in female eel, by SPE or human chronic gonadotropin (HCG) treatment after rearing in seawater for 3 months. Fertilization and hatching rates of ovulated eggs were also determined to assess the effects of long-term rearing in the seawater.

MATERIALS AND METHODS

Fish

Cultured female eels (body weight 450-660 g) were purchased from a commercial eel supplier. For acclimation to seawater, they were kept without feeding in 2000 L circulating tanks under a controlled photoperiod of 14 L/ 10D at water temperature of 20°C by the method of Kagawa (1998). They were immersed in to freshwater with a gradual increase in the concentrations of seawater for over 6 days.

Hormone Injection

After 3 months acclimation to seawater, the cultured female eels received weekly intramuscular injections of chum SPE (20 mg/fish/week) or human chorionic gonadotropin (HCG; 2 IU/g body weight/week; Sigma) dissolved in Ringer's solution for Japanese eel (Ohta et al., 1996). The control female eels received weekly intramuscular injections of eel's Ringer solution.

Blood plasma sampling

Blood samples were taken repeatedly from the same individuals at the caudal blood vessels with a heparinized disposable syringe after anesthesia with 2-phenoxyethanol (200 ppm). Plasma was immediately separated by centrifugation (6000 rpm, 15 min, 4°C) and stored at –40°C until radioimmunoassay.

Induction of ovulation

The final treatment was as follows. Two days after the weekly injection, fish which possessed oocytes over 750 μm in diameter at the migratory nucleus stage were processed according to the method described by Ohta et al. (1996) and Kagawa et al. (1998). Briefly, fish were injected with SPE (20 mg/fish) as a priming dose. At 18 : 00 h on the next day following the priming dose, the fish were weighed and given an injection of DHP (2 μg/g body weight; Sigma) intraperitioneally. DHP was dissolved in 50 % ethanol at a concentration of 2 μg/mL. The temperature of the tank was then raised to 22.5°C from 20°C. From 14 h after the DHP injection, the fish were checked for ovulation at 3 h intervals by applying gentle pressure on the abdomen in anterior to posterior direction. Fertility and hatching rates were examined in each ovulated female by the method described by Ohta et al. (1996). Briefly, 2 g of ovulated eggs were inseminated with 1 mL of diluted semen (Kim et al., 2006b). Approximately 100 eggs were incubated at 23°C in a plastic Petri dish filled with 20 mL of filtered seawater. Mean fertility and hatching rates were estimated from 4 replicates.

Fertilization rate (%) =
  100 × no. of fertilized eggs/no. of buoyant eggs
Hatching rate (%) =
  100 × no. of hatched larvae/no. of buoyant eggs

Radioimmunoassay (RIA)

Plasma estradiol-17β (E_2), testosterone (T) and 17α,20β-dihydroxy-4-pregnen-3-one (DHP) levels were measured by radioimmunoassay (RIA) by the methods of Kim et al. (2006a) and Ko et al. (2007).

Histology

Eels were sacrificed by decapitation after deep anesthesia with 2-phenoxyethanol. After measuring body and gonad weights, gonadosomatic index (GSI) was calculated by (gonad weight/body weight) × 100. Small pieces of ovary were fixed in Bouin's solution, embedded in paraplast, sectioned at 5 μm, and stained with hematoxylin-eosin to assess developmental stages of the oocyte. Classification of the developmental stages was made according to the terminology of Yamamoto et al. (1972).

Statistical analysis

All data are presented as mean ± standard errors (SE). Plasma sex steroid hormone levels in each experimental group were analysed by one-way analysis of variance (ANOVA) and Duncan's new multiple range test. Mean fertility and hatching rates were estimated from 4 replicates. Comparison of the two means was made with the Student's *t*-test.

RESULTS

Changes in body weight (BW) and sex steroid hormone levels in female eels

Changes in BW and plasma profiles of sex steroid

hormones in the control group of six female eels are shown in Fig. 1. Most of the fish in the control group showed a slight decrease in BW, which fluctuated until the end of the experiment. A slow decrease in sex steroid hormone (T and E_2) levels in plasma was also observed during the experimental period. The plasma levels of DHP remained low (< 50 pg/mL) or undetectable.

The BW of HCG-injected group slowly decreased during the experimental period (Fig. 2). The concentration of T in the plasma increased and peaked 4 weeks after the injection, followed by a gradual decrease until 12th week. The concentration of E_2 in the plasma increased in a cyclic fashion following HCG injection, reached a peak in week 2 after the injection, decreased to 0.74 ± 0.069 ng/mL in week 4, and again increased to 0.85 ± 0.142 ng/mL. The E_2 level was then decreased and returned to approximate pre-treatment level (0.32 ± 0.055 ng/mL). However, plasma levels of DHP remained low (<50 pg/mL) or undetectable throughout the experimental period.

Changes in BW and plasma profiles of sex steroid hormones in the SPE-injected group of 10 female eels are shown in Fig. 3. Most of the female eels did not show any changes in their BW during the first 6 weeks after the SPE injection, but tended to increase in BW rapidly after 8 weeks. The concentrations of plasma T and E_2 increased gradually for 8 weeks after the injection (2.22 ± 0.463 and 2.36 ± 0.443 ng/mL, respectively), then increased rapidly after week 10 (4.24 ± 0.667 and 4.56 ± 0.745 ng/mL, respectively), and reached maximum with mean values of 5.82 ± 0.972 and 4.76 ± 1.181 ng/mL, respectively, on week 12. However, the plasma DHP were remained low (<50 pg/mL) or undetectable throughout the experimental period.

Ovarian development by hormone injection

Figure 4 shows morphological changes of ovary after the 12th injection of exogenous hormone (HCG or SPE). After 3 months of acclimation to seawater, the GSI value in the eels was $1.9 \pm 0.19\%$ (data not shown). After 12th injection of the exogenous hormones, the GSI values of SPE-injected and HCG-injected female eels increased to 47.2% and 3.1%, respectively. The female eels of the control group did not show changes in GSI values (2.1%).

The ovarian development of female eels during the experimental period is shown in Fig. 5. Oocytes before acclimation to seawater were observed to be at the oil droplet stage (Fig. 5A). Five out of 8 females kept in seawater for 3 months had oocytes at the primary yolk globule stage in their ovaries (Fig. 5B); the remaining 3 females possessed oocytes at the oil drop stage. In the control group, the most developed oocytes were under the primary yolk globule stage. In contrast, histological observation of the ovaries in both the HCG-injected and the SPE-injected groups indicated appearance of numerous

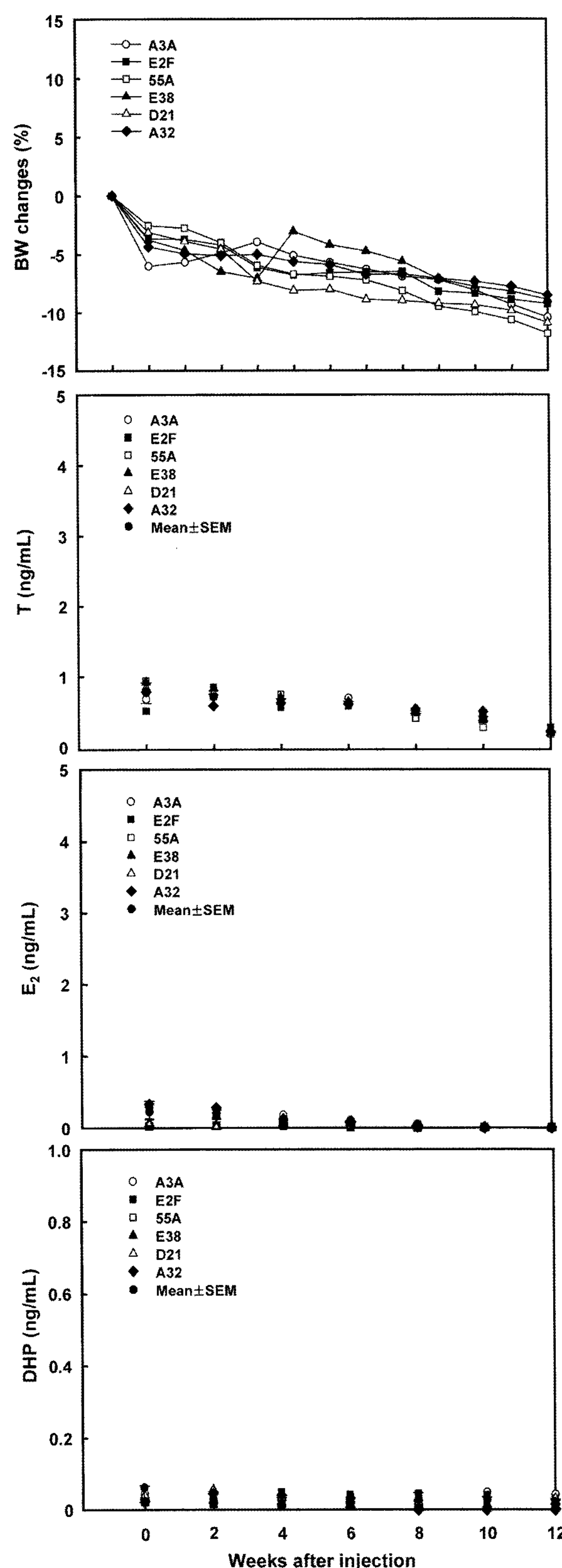

Fig. 1. Changes in body weight (BW) and plasma levels of sex steroid hormones (testosterone, T; estradiol-17β, E_2; 17α,20β-dihydroxy-4-pregnen-3-one, DHP) in cultured female eel, *Anguilla japonica* injected with eel's Ringer (control). Different symbols indicate individual fish. Closed circles (●) represent mean ± SE in each point. A significant difference was observed between points indicated by different letters ($p < 0.05$).

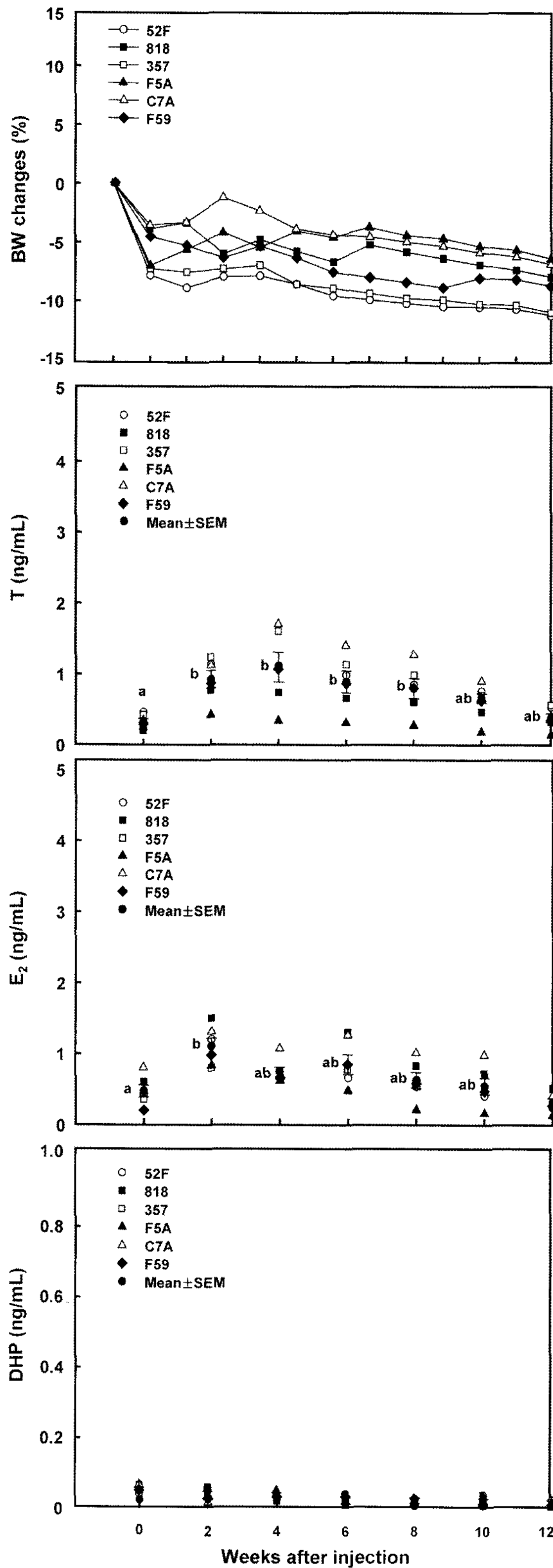

Fig. 2. Changes in body weight (BW) and plasma levels of sex steroid hormones (testosterone, T; estradiol-17β, E_2; 17α,20β-dihydroxy-4-pregnen-3-one, DHP) in cultured female eel, *Anguilla japonica* injected with human chorionic gonadotropin (HCG). Different symbols indicate individual fish. Closed circles (●) represent mean ± SE in each point. A significant difference was observed between points indicated by different letters ($p < 0.05$).

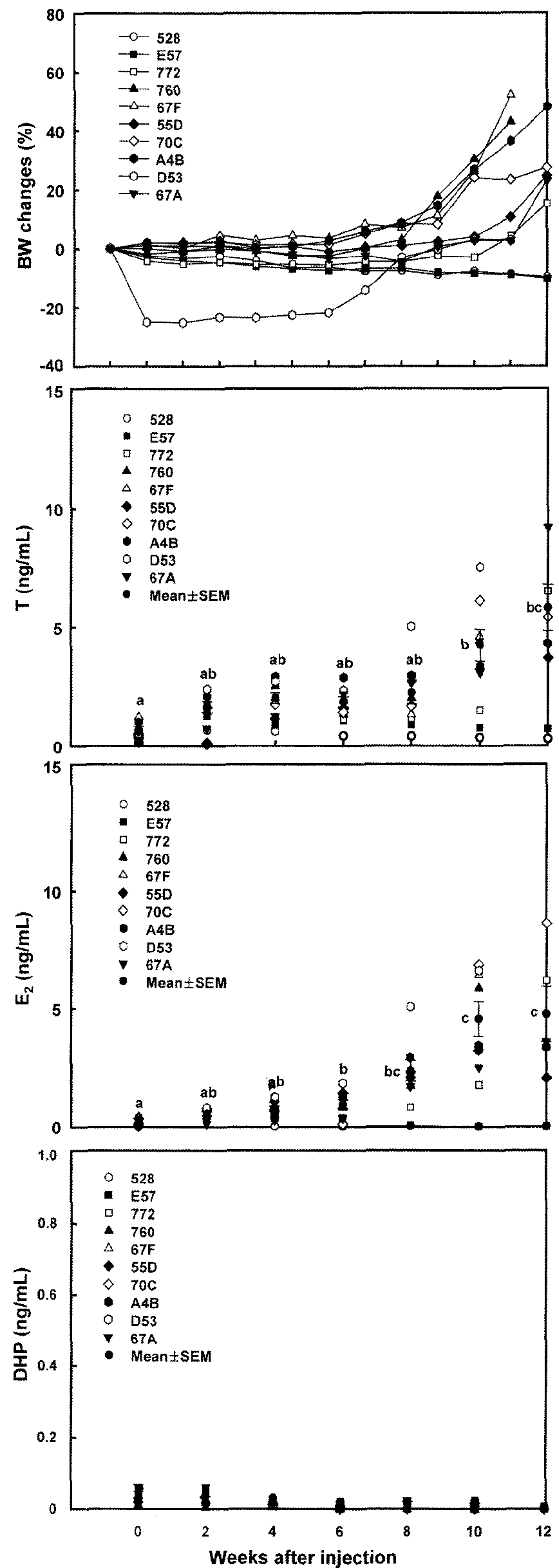

Fig. 3. Changes in body weight (BW) and plasma levels of sex steroid hormones (testosterone, T; estradiol-17β, E_2; 17α,20β-dihydroxy-4-pregnen-3-one, DHP) in cultured female eel, *Anguilla japonica* injected with salmon pituitary extraction (SPE). Different symbols indicate individual fish. Closed circles (●) represent mean±SE in each point. A significant difference was observed between points indicated by different letters ($p < 0.05$).

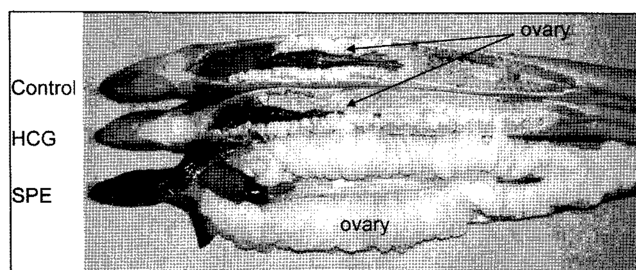

Fig. 4. Representative photographs of the ovaries after 12 weeks of exogenous hormone injection of cultured female eel, *Anguilla japonica* reared in seawater for 3 months. Note that the ovary of SPE-injected female is bigger than that of control or HCG-injected female.

yolk globules in ooplasm after the 2nd injection (Fig. 5C). After the 12th injection, almost all of the eels in the HCG-injected group were still in the yolk globule stage. Eight fish in the SPE-injected group completed vitellogenesis, and the germinal vesicles of their oocytes were located at the periphery of the ooplasm near the animal pole (migratory nucleous stage, Fig. 5D).

Ovulation time, fertility and hatching rates

In the SPE-injected female eels, ovulation was observed in 5 out of 8 females at 09 : 00-10 : 00 h (15-16 h after DHP injection), and in 3 females at 12 : 00-13 : 00 h (18-19 h after DHP injection). In the SPE-injected female eels, the fertility rates of eels which ovulated 15 h and 18 h after DHP injection were 75.2 ± 9.3% and 15.1 ± 4.3%, respectively, and hatching rates 55.2 ± 12.3% and 0%, respectively. Both fertility and hatching rates were significantly ($p < 0.01$) higher in eggs ovulated at 15 h than at 18 h after DHP injection (Table 1). In the control and HCG-injected female eels, however, ovulation was not observed.

DISCUSSION

The present study demonstrates changes in body weight and plasma sex steroid hormone profiles during artificial maturation of cultured eels kept in seawater for 3 months. Most of the females had oocytes of the primary yolk globule stage in their ovaries. These histological observations are similar to those of the silver forms of wild female eels,

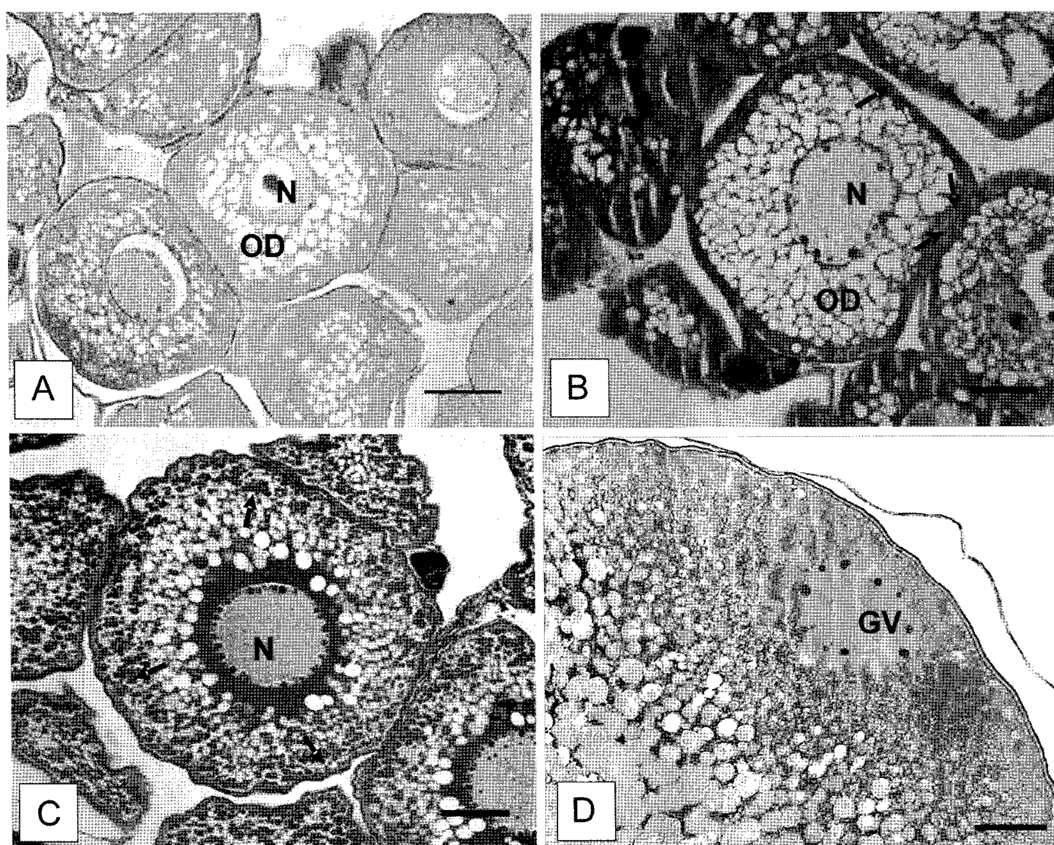

Fig. 5. Light micrographs of the ovaries in the cultured female eel, *Anguilla japonica*. A, Oocytes of female eels without acclimation to seawater were observed to be at the oil droplet stage; B, Oocytes at the primary yolk globule stage were observed in the ovaries of cultured female eels acclimated to seawater for 3 month (initial experimental group); C, Oocytes of SPE injected cultured female eels 2 weeks after the beginning of the experiment were observed to be at the secondary yolk globule stage; D, Oocytes of SPE injected cultured female eels 12 weeks after the start of the experiment were observed to be at the migratory nucleus stage. Abbreviations: N, nucleus; Arrow (in B,C), yolk globule; OD, oil droplet; GV, germinal vesicle. Bars indicate 50 μm.

Table 1. Fertility and hatching rate of SPE-injected female eel, *Anguilla japonica* reared in seawater for 3 months

Ovulation time (Time after DHP Injection)	09 : 00-10 : 00 (15 h-16 h)	12 : 00-13 : 00 (18 h-19 h)
Fertility (%)	75.2 ± 9.3*	15.1 ± 4.3
Hatching rate (%)	55.2 ± 12.3*	-

*Each value represents the mean ± SE ($p < 0.01$)

reported in previous studies (Yamamoto et al., 1974; Kim et al., 2006a). Kagawa et al. (1998) observed vitellogenic oocytes in the ovary and also recorded a reduction in the period needed for the final treatment of fish kept in seawater for 3 months. These results suggest that rearing of females in seawater for 3 months induces vitellogenesis, similar to that in natural migrating females. More detailed experiments should be done in the future to determine whether 3 months is the minimum period needed for completion of oocyte growth.

Artificial propagation of eels by hormonal treatments has long been attempted, because eels have immature ovaries which do not enter vitellogenesis due to environmental factors. In Japan, hormonal treatments have been intensively applied to induce maturation in Japanese eels since the 1960's. Oocytes of 1 mm in diameter were obtained by repeated injections of a high dose synahorin (a mixture of mammalian pituitary extracts and HCG) and synthetic estrogen (Satoh et al., 1992; Ochiai et al., 1972), although the success rates in obtaining full-grown oocytes were very low. Yamamoto et al. (1974) successfully induced oocyte growth and obtained full-grown oocytes by repeated injection of SPE. Finally, by this method, Yamamoto and his colleagues succeeded in production of eel larvae using oocytes from a mature female (Yamamoto and Yamauchi, 1974; Yamauchi et al., 1976). Since then, repeated injections of SPE have been routinely used for induction of vitellogenesis in female eels.

It is well known that the BW of female teleosts increases rapidly prior to ovulation (Shehadeh and Ellis, 1970; Ishida et al., 1972). The increase is directly related to hydration of pre-ovulatory oocytes (Hirose et al., 1974; Hirose, 1976). In the Japanese eel, the change in female BW is also a reliable indicator of the last phase of ovarian maturation (Yamamoto et al., 1974; Sugimoto et al., 1976; Oka, 1979; Satoh et al., 1992). In the present study, BW gain indices of SPE-treated females were about 30-40%, and histological observation showed that these females had oocytes of migratory nucleus stage. These results indicate that an increase in BW gain index to about 30-40% at the priming dose is a reliable sign that treatment for induction of last phase of maturation and ovulation can be started.

Plasma T and E_2 levels of fish in our experiments gradually increased with each SPE (20 mg/fish/week) injection. In contrast, the levels of steroid hormones (T and E_2) gradually increased until 6 weeks after onset of HCG (2 IU/g BW/week) injection and then decreased gradually until the 12th week. Sato et al. (2003) reported that the final maturation and fertilization rate in immature Japanase silver eels treated with salmon GTH or a combination of salmon FSH and salmon LH were higher than those in immature Japanase silver eels treated with salmon FSH or salmon LH only. In this study, therefore, the higher levels of steroid hormone in cultured female eels injected with SPE compared with those injected with HCG may be an indication that SPE (maybe a combination of FSH and LH) is necessary to maintain vitellogenesis and to induce final oocyte maturation.

In the present study, plasma levels of T in cultured female eels increased after the start of SPE injection, and the levels were relatively high compared to those of E_2 during vitellogenesis, as in the study of Chiba et al. (1994) on European eels. Sufficient T is therefore available as a precursor of E_2. Moreover, plasma levels of E_2 also increased dramatically at the end of vitellogenesis and during the migratory nucleus stage, as in cultured European eel (Chiba et al., 1994) and Japanase eel (Ijiri et al., 1995). This increase in E_2 is a peculiar feature of artificially-matured eels and may be a consequence of artificial maturation. DHP has been known as maturation-inducing hormone (MIH) in Japanase eel (Ohta et al., 1996), although the details are still unknown because naturally matured eels have not been captured from the wild. In the present study, DHP was undetected in the plasma throughout the experimental period, even in the migratory nucleus stage. Until now, whether increased E_2 level in the late stage of oocyte development inhibits the production of DHP and whether DHP is not involved in the hydration and germinal vesicle migration of oocytes of artificially matured eels were unknown. A recent study has demonstrated the physiological importance of 11-ketotestosterone (11-KT; a specific sex steroid hormone in male fish) in Japanase female eel (Matsubara et al., 2005). According to the study, changes in the profile of serum 11-KT showed similarities with those of E_2. Therefore, it is probable that a relatively high level of 11-KT in eels plays important roles in sexual maturation including oocyte growth.

In the case of cultured eels, it will take a long time to promote complete ovarian development compared with silver eel. Eight to ten weekly injections of a combination of HCG (100 IU/fish) and carp pituitary extract (30 mg/fish) were required to complete vitellogenesis in the silver European eel (Boetius and Boetius, 1980). In contrast, 10 to 15 weekly injections of SPE (50 mg/kg BW) in cultured European eel and 15 to 19 weekly injections in cultured Japanese eels (Hirose, 1992) were required, although the hormone doses were not the same in the two cases. In the

present study, 10 to 12 weekly injections of SPE (20 mg/fish) led to attainment of the migratory nucleus stage and to production of ovulated eggs from 8 out of 10 females after DHP (2 μg/g BW) injection in cultured eels kept in seawater for 3 months.

Although many trials to induce ovulation in the eel have been made, there is little information on the fertility and hatching rates of the eggs. The scarcity of information is caused by the lack of reliable methods to induce maturation and ovulation. Notably, fertilization and hatching rates are affected by the duration that eggs are retained in the body cavity of the eel (Ohta et al., 1996). In our study, 5 of 8 ovulated females produced hatchable eggs. Furthermore, fertilization and hatching rates were relatively high; those of eggs that ovulated 15-16 h after DHP injection were about 75% and 55%, respectively, and hatched larvae were obtained (data not shown). Previously, Sugimoto et al. (1976) reported that only 1 of 5 ovulated females produced hatchable eggs, and Satoh et al. (1992) only produced hatchable eggs from 2 of 15 ovulated females. Kagawa et al. (1998) reported fertility and hatching rate of eggs that ovulated 15 h after DHP injection were about 65% and 58%, respectively, although those of females that ovulated 18 or 21 h after DHP injection were less than 30% and 20%, respectively. Furthermore, both fertilization and hatching rates of ovulated female eels decreased rapidly, to almost 0% by 6 h after ovulation (Ohta et al., 1996). Therefore, these results indicate that artificial fertilization must be carried out immediately after ovulation to obtain good-quality eggs.

In summary, the present study indicates that long rearing in seawater increases responsiveness to SPE in ovarian maturation of the Japanese eel, resulting in shortened period for completion of vitellogenesis by sex steroid hormones (T and E_2) production. Therefore, this strongly suggests that cultured eels can be used in ovarian maturation experiments as silver eels, not only to obtain good-quality eggs but also to study the mechanism of endocrine regulation of gonadal development in eels. Further studies are necessary to clarify the environmental conditions required for completion of vitellogenesis and oocyte maturation.

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