Ultrastructures of Oocyte Development and Electrophoretic Patterns of the Yolk Protein Following HCG Treatment in Korean Native Catfish (*Silurus asotus*)[a]

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ABSTRACT : During the rapid phase of gonadal development of the freshwater teleost, the catfish (*Silurus asotus*), the influence of hCG upon the inducement of final oocyte maturation and spawning was investigated electrophoretically and ultrastructurally. The electrophoretic patterns obtained were different in the presence and absence of some of the major or minor zones, because of the hormone level in catfish. The vitellogenin of hormone-treated fish was stained more intensively than that of sham-treated fish. These proteins showed some minor or main bands of egg extracts which migrated at positions corresponding to molecular weights of approximately 90,000. However, the thickness of electrophoretic band in molecular weight for hCG-treated fish was slightly lower than that for saline control. It seemed the plasma protein with molecular weight of approximately 45,000 in hCG-treated fish disappeared. In contrast to the control fish, the ovaries in the catfish treated with hCG shows a marked ultrastructural change under the electron microscope. No dilated profiles were seen in the granulosa cells of the mature oocyte before ovulation. After germinal vesicle breakdown (GVBD), the zona radiata interna (ZRI) becomes more compact, and there is a loss of all the processes from the pore canals. There is a wide space between the vitelline membrane and zona radiata. Also, during final maturation, the microvillar processes from the oocyte are seen no longer to penetrate deeply into the extracellular spaces of the overlying granulosa cells, and the reticulate patterns of the zona radiata interna becomes occluded, giving the zona radiata a more solid appearance. It has been possible to initiate 100% oocyte maturation in yolk granules and follicles *in vivo* by treatment with hCG and a high water temperature (27℃). In hCG-treated fish, the percentages of successful artificial fertilization and hatching were maximal at 15 h after a single injection. It seems clear that a long acting preparation containing hCG can be successfully used in prespawning fish to advance the final events of gonadal maturation and initiate spawning. Further studies are necessary to evaluate the potential of hCG to either stimulate or inhibit the reproductive development of fish at other stages of the seasonal reproductive cycle. *(Asian-Aust. J. Anim. Sci. 2001. Vol. 14, No. 2 : 174-183)*

Key Words : Catfish, hCG, Electrophoretic Pattern, Ultrastructure, Maturation, Ovulation

INTRODUCTION

Vitellogenesis and maturation in teleosts and mammals are generally considered to be dependent upon steroid hormone secretions by the various gonadotropin and pituitary actions (Hirose et al., 1979; Hirose, 1976, 1980; Huat, 1980; Schoonbee et al., 1980; Hunter et al., 1981; Sower et al., 1982; Suzuki, 1983; Chang et al., 1991; Singh and Madan, 2000). In most non-mammalian vertebrates, vitellogenesis ceases once oocytes reach their fully developed size and oocytes eventually undergo maturation and ovulation after an appropriate hormonal stimulation (Wallace and Selman, 1980).

The egg development of various fishes can be studied by various methods including gel electrophoresis (Iwamatsu et al., 1988; Scobbie and Mackie, 1990), egg diameter (Foucher and Beamish, 1980; Howell, 1983; Hoffman and Grau, 1989), light microscopy (Davis, 1977; Howell, 1980; Van Der Merwe et al., 1988; Kjesbu and Kryvi, 1989; Yoon et al., 1991) and electron microscopy (Kjesbu and Kryvi, 1989; Matsuyama et al., 1991; Thiaw and Mattei, 1992; Yoon et al., 1992, 1993).

In an attempt to induce final maturation and spawning, the present experiments were conducted to investigate the influence of hCG upon the reproductive cycles in catfish (*Silurus asotus*) with developing gonads, and to stimulate the reproductive system during the rapid phase of gonadal development.

MATERIALS AND METHODS

Animals, hormone injections and blood collection

Catfish eggs were obtained from the Department of Aquaculture in Kunsan National University. Samples were prepared from individual eggs obtained from the four seasons. The fish weighing from the 100 to 300 g were reared in a freshwater pond (26±1℃). The fish were anaesthetized with MS 222 (1/10,000) and

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were injected intraperitoneally with 5,000 IU/kg human chorionic gonadotropin (hCG, Yuhan Cor, Korea) or 0.8% saline. Blood samples of fish before and after injection were taken from the caudal vein into heparinized vials. The plasma was obtained by centrifugation of the blood at 3,000 rpm for 15 min, and then frozen at -20℃ until use.

Preparation of SDS extracts and electrophoresis

The eggs were frozen individually and stored in a nitrogen tank at -196℃ prior to analysis. Homogenisation of fish eggs was carried out in centrifuge tubes by homogenizer following 3-fold dilution using 2% w/v aqueous sodium dodecyl sulfate. Each homogenate was centrifugated at 10,000 rpm (11,600 g) for 20 min after incubation at 60℃ for 30 min as described by Scobbie and Mackie (1990). The extracts were then heated in a 100℃ water bath for 2 min, cooled, and clarified by centrifugation at 10,000 rpm for 20 min. The extracts were stored frozen at -70℃ until analyzed by electrophoresis.

The extracts were analyzed by SDS-PAGE on 7.5% separating gel and 4.4% stacking gel (Laemmli, 1970). The catfish egg extracts were then diluted 2-fold with sample buffer. Electrophoresis was carried out in a chamber (Hoefer Cor. USA) at 80 V until the tracking dye reached the bottom of the gel. Cooling to approximately 12℃ was performed using water from a constant water temperature circulator (Vision Cor, Korea). Following electrophoresis the gels were stained, washed and destained as described by Laemmli (1970). Standard molecular weight marker proteins were obtained from Sigma (hemocyanin tetramer, 280,000; trimer, 210,000; dimer, 140,000; and monomer, 70,000). The SDS extracts of each oocyte before and after hormone injection were analyzed in the same electrophoretic run and by comparing the electrophoretic patterns obtained with those of eggs.

Light and electron microscopy

For light microscopy, thin slices and pellets were fixed in Bouin's fluid, dehydrated by a series of ethanol solutions, and embedded in paraffin. Thin sections (5 μm) cut by microtome (Reichert-Jung, USA) were stained with hematoxylin and eosin. The various oocytes were observed on the optical microscope (Olympus, Japan). The changes in gonadosomatic index (GSI, gonad weight, g × 100/body weight, g) and hepatosomatic index (HSI, liver weight, g × 100/body weight, g) were employed to monitor gonadal maturation.

For transmission electron microscopy, the living specimens were fixed in 2.5% glutaraldehyde, buffered with 0.1 M PBS, pH 7.2, for 2 h at 4℃, postfixed in 2% osmium tetroxide in the same buffer for 2 h at room temperature, dehydrated by graded series of ethanol, and embedded in Epon 812. Semithin sections of ovary stained with 1% toluidine blue were used to locate the oocytes. Subsequently, ultrathin sections were obtained from the same block by ultramicrotome (No. 2088, LKB, Bromma, Sweden) with a diamond knife. The sections were picked up on copper grids, double-stained with aqueous 5% uranyl acetate and lead citrate solution, and examined in a transmission electron microscope (ISI-LEM 2000, Jeol, Japan) operated at 70 kV.

For SEM observations, 2.5% glutaraldehyde-fixed oocytes were attached to coverslips, washed in 0.1 M phosphate-sucrose buffer, post-fixed in 2% osmium tetroxide, dehydrated by graded series of ethanol and isoamyl acetate. The samples were critical-point dried with CO_2 in a Balzers CPD 030, coated with 25 nm gold-palladium in a ion coater (Hitachi, Japan). The observations were made using a scanning electron microscope (Hitachi, Japan) operated at 20 kV.

RESULTS AND DISCUSSION

Electrophoretic patterns of egg extracts and plasma

The electrophoretic patterns obtained when the SDS extracts of eggs of the two stages (prespawning and spawning stages) in catfish were subjected to electrophoresis (figure 1). The electrophoretic pattern of egg yolk proteins was greatly altered, showing no band that was present in sham-treated fish (MW 210,000). The thickness of electrophoretic band in molecular weight for hCG- treated fish was slightly

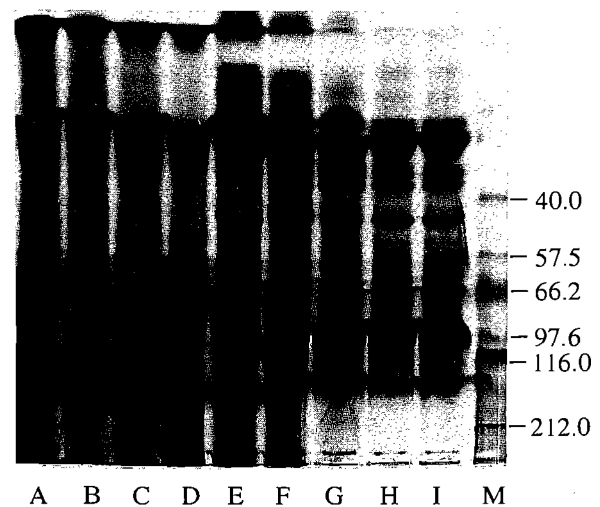


Figure 1. SDS-PAGE patterns of plasma protein (A~D) and oocytes extracts (E~I) in female catfish, *Silurus asotus*. A, B, E, F:sham-treated fish. C, D, G, H, I:hormone-treated fish. M:marker protein (40~212 kda)

higher than that in saline control. These egg yolk polypeptides showed two main bands which migrated at positions corresponding to molecular weights of approximately 45,000 and 90,000, respectively, and some minor bands which migrated faster than the two main bands. The results obtained show very close correspondence in the profiles for all the eggs in the presence and absence of some of the major or minor zones. The vitellogenin of plasma in hormone-treated fish stained more intensively than that of sham-treated fish (MW 15,000, 60,000 and 130,000, respectively). The electrophoretic pattern of plasma proteins in hormone-treated fish showed no bands which were present in sham-treated fish (MW 45,000).

Fish eggs so far examined can easily be distinguished by their separation profiles obtained on SDS electrophoresis (Iwamatsu et al., 1988). Approximately ten protein bands are present in a catfish egg. There are slight differences in the relative amounts of minor zones for all the profiles but this degree of variation is unusual in any electrophoretic comparison of proteins from individual specimens of a season (Scobbie and Mackie, 1990). A significant increase in concentrations of alkali-labile protein occurred during the oocyte growth, coincident with the estradiol-17β hormone peak in channel catfish (MacKenzie et al., 1989) and rainbow trout (Yoon et al., 1991). Interestingly, evidence that hCG stimulates various steroid hormone secretions in sexually mature teleost fishes has been presented previously for black porgy (Chang et al., 1991), and ayu (Hirose, 1980). Two additional protein bands are found in the SDS-PAGE separation of hormone-treated fish eggs compared to the sham-treated fish eggs. Of the two proteins, one seems to be vitellogenin based on its relative mobility and molecular weight (Tirumalai and Subramoniam, 1992). Vitellogenin occurs in the plasma during the specific period of embryonic development (Tirumalai and Subramoniam, 1992; Kwon et al., 1993). The vitellogenin seems to have the same electrophoretic mobility in all stages of embryonic development. Its mean level (0.4 mg/ml) showed little seasonal variation (Craik, 1978). The vitellogenin of hormone-treated fish stained more intensively than that of sham-treated fish. On SDS-PAGE, the circulating vitellogenin was resolved into eight polypeptides as has been suggested for the sand crab (Tirumalai and Subramoniam, 1992). In teleosts, as in other non-mammalian vertebrates, it has been demonstrated that vitellogenin, a female-specific serum protein, which is synthesized in the liver in response to circulating estrogen, is released into the bloodstream and then transported to the ovary for vitellogenesis. It is regarded as the immediate precursor of these proteins in egg yolk (Emmersen et al., 1976; Craik, 1978; Ng and Idler, 1983; Guraya, 1986; Petersen and Korsgaard, 1989; Kwon et al., 1993). Among fish, evidence for its presence has been obtained in lesser spotted dogfish (Craik, 1978), eel (Petersen and Korsgaard, 1989), rainbow trout (Hara and Hirai, 1978; Kwon et al., 1993) and sand crab (Tirumalai and Subramoniam, 1992). Two of the low molecular weight subunits of sand crab (Tirumalai and Subramoniam, 1992) and rainbow trout (Hara and Hirai, 1978; Kwon et al., 1993) also showed similar mobility on SDS gels.

Histomorphological changes of fish eggs

Microscopic appearance of oocyte: Development can be divided into two broad phases. In the first, or previtellogenic phase, growth is slow and comparatively few cytoplasmic changes occur (Howell, 1983). The second, or vitellogenic, phase is characterized by rapid growth and the deposition of large amounts of yolk in the cytoplasm. The main oocyte diameter indicated that fish injected by hCG had larger oocytes. The average diameter of oocytes collected from the ovary increased significantly ($p<0.01$) within 15 h after a single injection of hCG. The developmental events observed in catfish oocytes are very similar to those described for most other teleosts reviewed by Shikhshabekov (1972), Bieniarz et al. (1979), Huat (1980), and Howell (1983) and Chang et al. (1991).

As shown in figure 2, the ovaries in the sham-treated fish contained many late maturing oocytes which were characterized by the presence of large quantities of yolk elements that were also observed in late April (middle spring). The ovary at the initiation of this study was mostly composed of oocytes in the last phase of the yolk stage and some in the migratory nucleus stage (figure 2). Ovulation was clearly observed in the fish treated with 5,000 IU hCG per kilogram within 15 h after a single injection (Schoonbee et al., 1980).

In contrast to the control fish, the ovaries in the catfish treated with hCG showed a considerable changes. An increase in oocyte diameter was observed in the stimulated females. The percentage of ripe ova increased abruptly, and then the peak percentage of ripe ova was seen after hCG injection. In some cases the oocytes underwent fluctuations in the number of yolk granules and increases in the size (figure 3). The increase in GSI was significantly greater in the fish hCG-treated group than in the control or sham-treated group. Though this was in late April when ovarian development is not rapid, GSI and HIS rapidly increased thereafter by maxima of 19.9% and 10.7% respectively. The gradual decrease in the number of late maturing oocytes and the increase in the number of ripe ova indicated that active yolk production

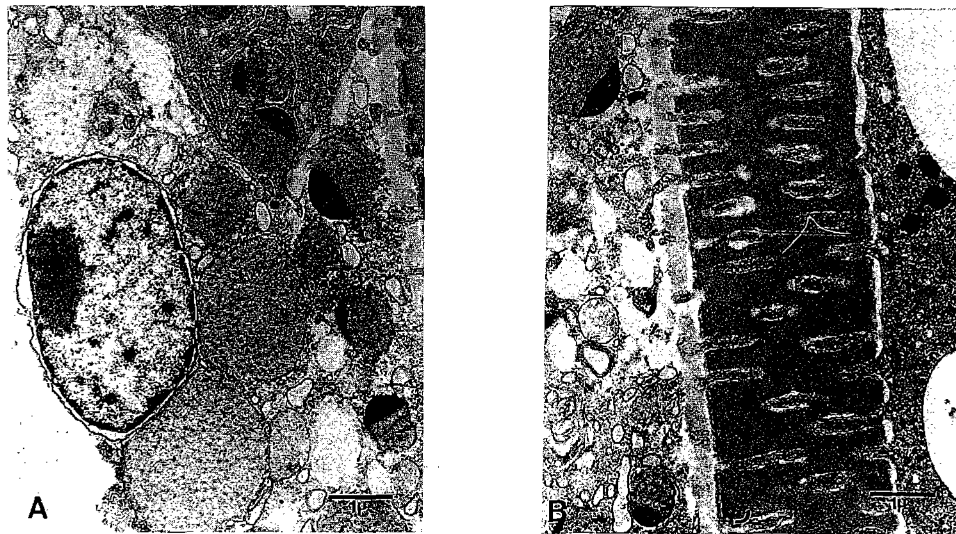


Figure 2. A. Magnification of follicular cell layer of prespawning oocyte showing a large quantity of round collagen fibre and endoplasmic reticulum and follicular nucleus. B. Section of cortical ooplasm, zona radiata and follicular cell layer of sham-treated oocyte just prior to final maturation. Scale bar represents 1 μm

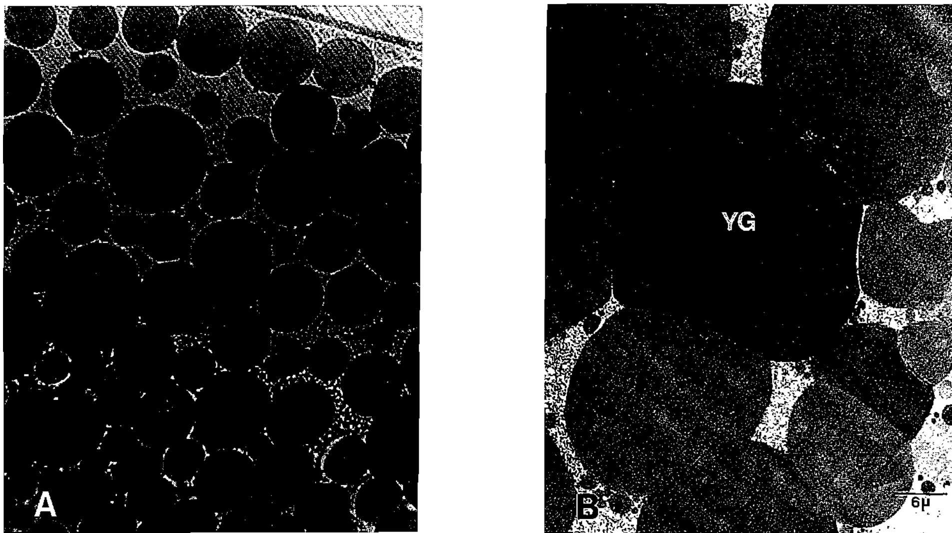


Figure 3. A. 400X. A large number of yolk granules have scattered in outer ooplasm of hand-stripped (spawned) oocyte of hormone-treated catfish. B. Electron micrographs of yolk granules (YG) of mature oocyte. Scale bar represents 6 μm

occurred in late spring and early summer with a resulting further increase in GSI (Davis, 1977; Howell, 1983; Van Der Merwe et al., 1988). The number of mature oocytes as well as the GSI showed a distinct increase while the number of early perinucleolus, late perinucleolus and early maturing oocytes decreased.

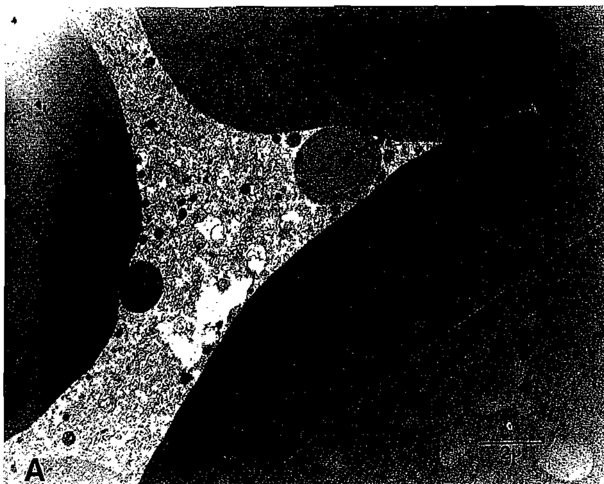
The GSI values employed in this study proved to be useful and sensitive parameters to monitor gonad maturation. These results were similar to the results of Lambert et al. (1978) and Crim and Idler (1978) who reported that at late time of vitellogenesis stage, GSI levels significantly increased and reached at maximum value. These results also showed a similar tendency to the results of MacKenzie et al. (1989) who found that oocyte diameter and GSI of American channel catfish (*Ictalurus punctatus*) began to develop during autumn, in the previtellogenic phase, and showed a tendency to increase during September, in the vitellogenic phase.

This result also agreed with the observations of Clemens and Reed (1964) who found that mean GSI reached maximum values (17.6%) during the breeding season, along with the completion of oogenesis. This result also agreed with the results of Bieniarz et al. (1979) who reported that the mean GSI and number of oocytes of carp (*Cyprinus carpio*) reached maximum values during the full vitellogenesis, along with the completion of the oogenesis.

Based upon the changes in GSI and egg diameter, the hCG accelerated vitellogenic development in the ovaries. A clear demonstration of the effectiveness of hCG treatment on spawning following handstripping in the catfish was provided by the present series of experiments. Evidence that hCG stimulates spawning in sexually mature teleost fishes has been presented previously for cyprinid loach (Suzuki, 1983), Japanese flounder (Hirose et al., 1979), milkfish (Kuo et al., 1979), goldfish and aruan (Huat, 1980), sharptooth catfish (Schoonbee et al., 1980), and ayu (Hirose, 1976, 1980). There is evidence that hCG may also play a role in stimulating rapid ovulation and spawning in the prespawning fish. Hirose et al. (1979) noted maximal fertilization and hatching rate one day following ovulation. High doses of hCG also induced ovulation in milkfish (Kuo et al., 1979), ayu (Hirose et al., 1977; Hirose, 1980), killifish (Wallace and Selman, 1980), and cyprinid loach (Suzuki, 1983). These results indicated that ovulation would be induced by corticosteroids derived from the mature ovary of fish when activated by injection of gonadotropin (Hirose, 1976; Wallace and Selman, 1980).

In general the percentages of late maturing oocytes from 820 to 850 μm in major diameter showed a tendency to increase fairly steadily from late May to early June. These yolk elements seem to be the main reason for the increasing size and consequent increase in mass of the oocyte. The number of late maturing oocytes in gonads can therefore influence the GSI. In the later stages of vitellogenesis, the yolk vesicles are displaced to the periphery of the oocyte and give rise to the cortical alveoli which, after fertilization, contribute to water hardening of the egg. In late May late maturing oocytes began to be transformed into ripe ova. At this time the yolk granules began to break open allowing the yolk to coalesce. There was an accompanying increase in oocyte size, presumably due to the absorption of fluid which caused the zona radiata to become thicker. The diameter of a ripe egg in catfish, was 1,500 μm in comparison to the ripe egg diameter of 2,900 μm found in freshwater catfish, *Tandanus tandanus* (Davis, 1977), 600 μm in Pacific hake, *Merluccius productus* (Foucher and Beamish, 1980), 400 μm in yellowtail flounder, *Limanda*

Figure 4. Changes in thickness (μm) of the zona radiata in sham-treated fish and hormone-treated



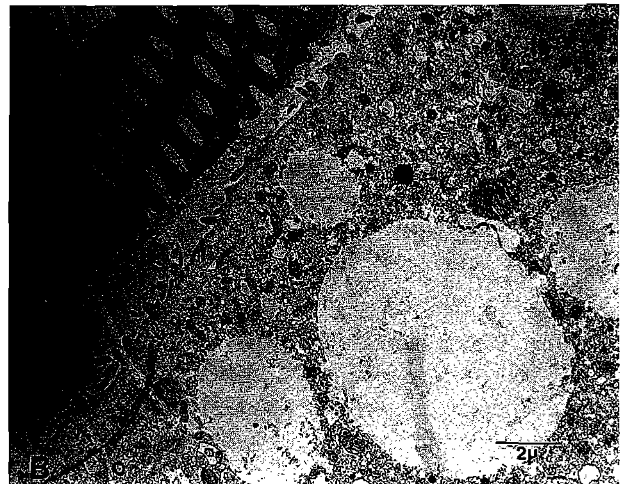


Figure 5. A. Many yolk granules are located in the ooplasm of the spawned oocyte of hormone-treated catfish. B. Part of the distinct banding of the zona radiata in a mature oocyte. Scale bar represents 2 μm

ferruginea (Howell, 1983), 730 μm in red sea bream, *Pagrus major* (Matsuyama et al., 1991), 320 μm in cod, *Gadus morhua* L (Kjesbu and Kryvi, 1989), 1,400 μm in euryhaline killifish, *Fundulus heteroclitus* (Wallace and Selman, 1980), 2,100 μm in Gulf killifish, *Fundulus grandis* (Greeley et al., 1988), 1,200 μm in freshwater Korean loach, *Misgurnus anguillicaudatus* (Yoon et al., 1992), and 3,200 μm

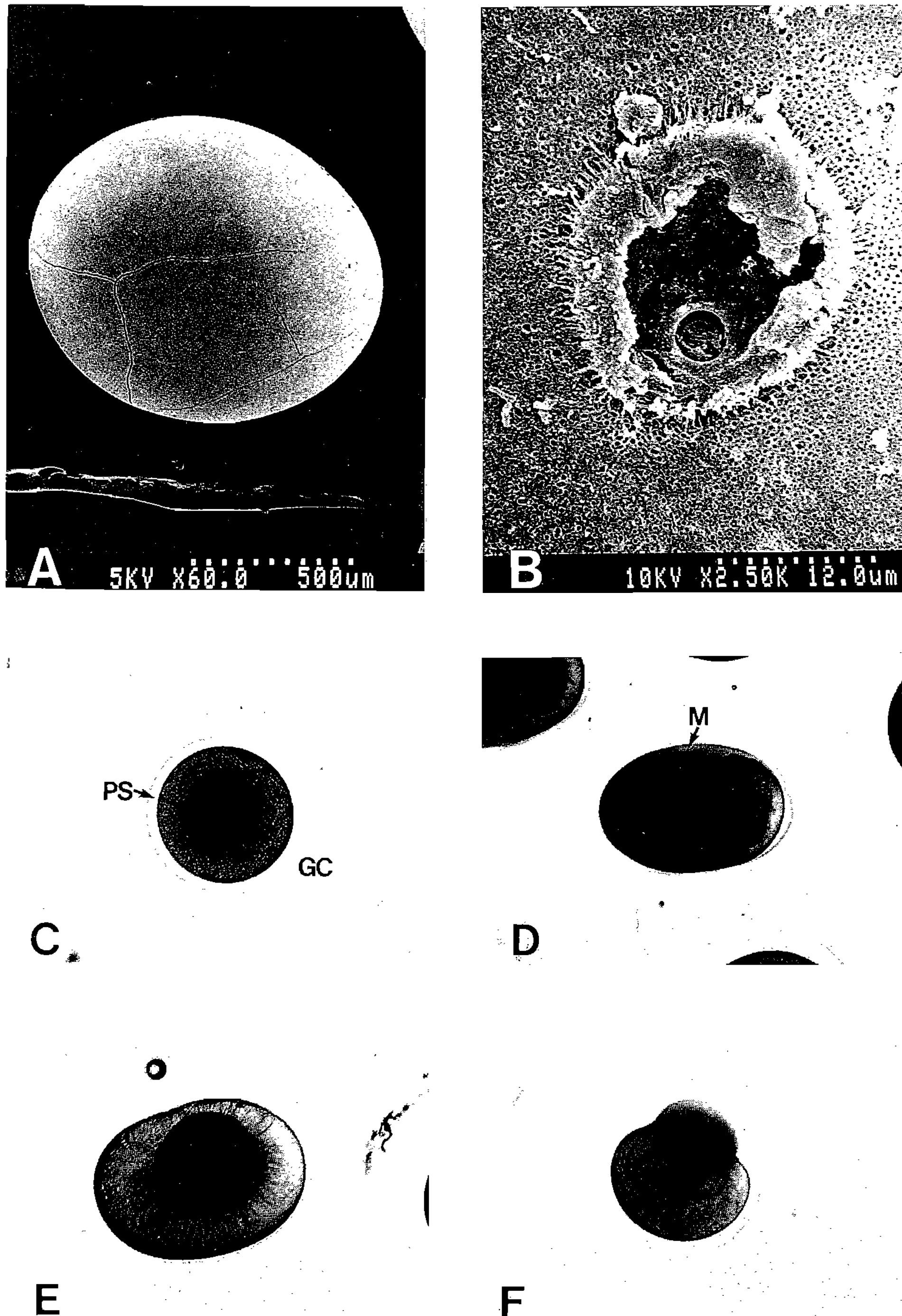


Figure 6. A. Scanning electron microscophs of the micropyle in egg envelope of catfish. B. The surface surrounding the outer opening has many small pores. M: micropyle. Scale bar=500 μm (A) and 12 μm (B). C. 12X. External views of fertilized eggs 10 min after insemination. PS: perivitelline space, GC: gelatin coat. D. 12X. Egg 50 min after insemination. M: micropyle. E. 18X. Egg 1 h after artificial insemination. F. 12X. Egg 2 h after insemination.

found in rainbow trout, *Oncorhynchus mykiss* (Yoon et al., 1993).

Ultramicroscopic appearance of oocyte membrane: In contrast to the control fish, the ovaries in the catfish treated with hCG showed a considerable change under the electron microscope. The thickness differences of zona radiata in the developmental stages of oocytes are shown in figure 4. The thickness of zona radiata in hCG-treated fish was significantly greater than that in sham-treated fish. After germinal vesicle breakdown (GVBD), the zona radiata interna (ZRI) becomes more compact, and there is a loss of all the processes from the pore canals (Kjesbu and Kryvi, 1989). Prior to ovulation, the zona radiata becomes more dense, striated and without pore canals, and there is a wide space between the granulosa cells and the zona radiata (figure 5). Also, during final maturation, the microvillar processes from the oocyte are seen no longer to penetrate deeply into the extracellular spaces of the overlying granulosa cells, and the reticulate pattern of the zona radiata interna becomes occuluded, giving the zona radiata a more solid appearance.

Ultrastructurally, the previously dilated rough endoplasmic reticulum shrinks, and no dilated profiles are seen in the granulosa cells of the mature oocyte before ovulation (Matsuyama et al., 1991). Numerous oval and rod-shaped mitochondria with tubular cristae, and smooth endoplasmic reticulum, are a remarkable feature in the cytoplasm of the special thecal cells during oocyte maturation (Matsuyama et al., 1991; Thiaw and Mattei, 1992). A number of mitochondria with lamellar cristae of variable shape, and a Golgi complex, are apparent in the granulosa cell cytoplasm, and a few lysosomes are also found during final maturation and ovulation in hCG-treated fish. The closure of the pore canals during final maturation has also been described in various fish (Guraya, 1986; Matsuyama et al., 1991; Yoon et al., 1991).

The highest level of plasma estradiol-17β was observed in fish with an ovary in which the largest oocytes undergo GVBD. It is reasonable to assume that the highest level of plasma estradiol-17β in a fish with a mature ovary was due to the largest oocytes (Matsuyama et al., 1991). In all of the teleost species studied so far, plasma concentrations of estradiol-17β have been reported to be highest during final maturation of the ovarian cycle (MacKenzie et al., 1989; Yoon et al., 1991). Also, estradiol-17β exhibited distinct seasonal variation, peaking very early in the breeding season, decreasing gradually thereafter with the cessation of spawning in the seasonal regression of ovaries. Increased plasma estradiol was accompanied by oocyte growth and preceded increases in plasma alkali-labile protein phosphorus, an indicator of circulating vitellogenin, supporting a role for estradiol in regulating vitellogenesis in flounder (Emmersen and Emmersen, 1976), gulf killifish (Greeley et al., 1988), channel catfish (MacKenzie et

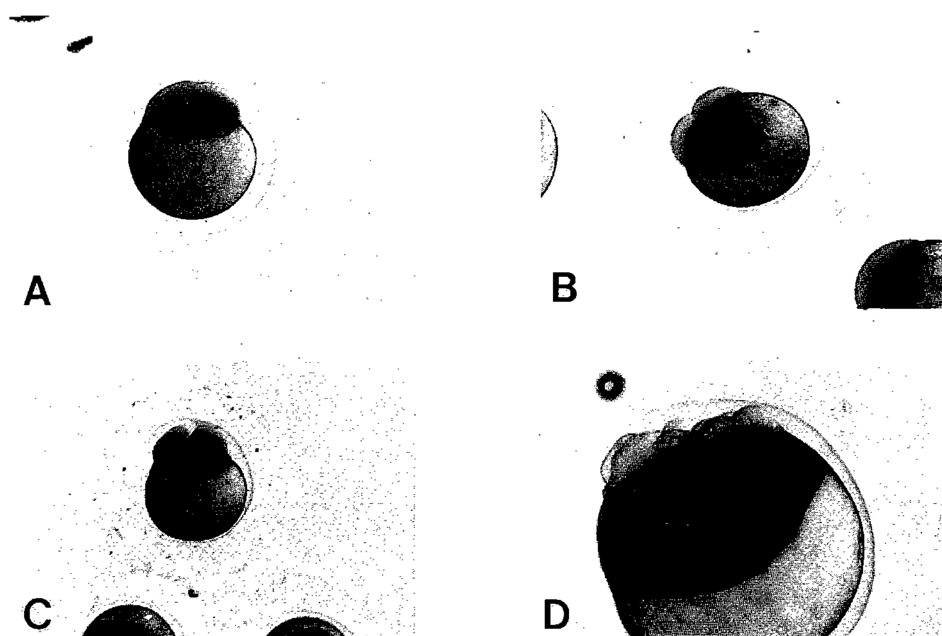


Figure 7. External views of fertilized eggs which are almost transparent in ooplasm and blastomere. A. 2 h 20 min after fertilization. 12X; B. 2 h 30 min after fertilization, 12X; C. 2 h 40 min after fertilization. 12X; D. 2 h 50 min after fertilization. 12X.

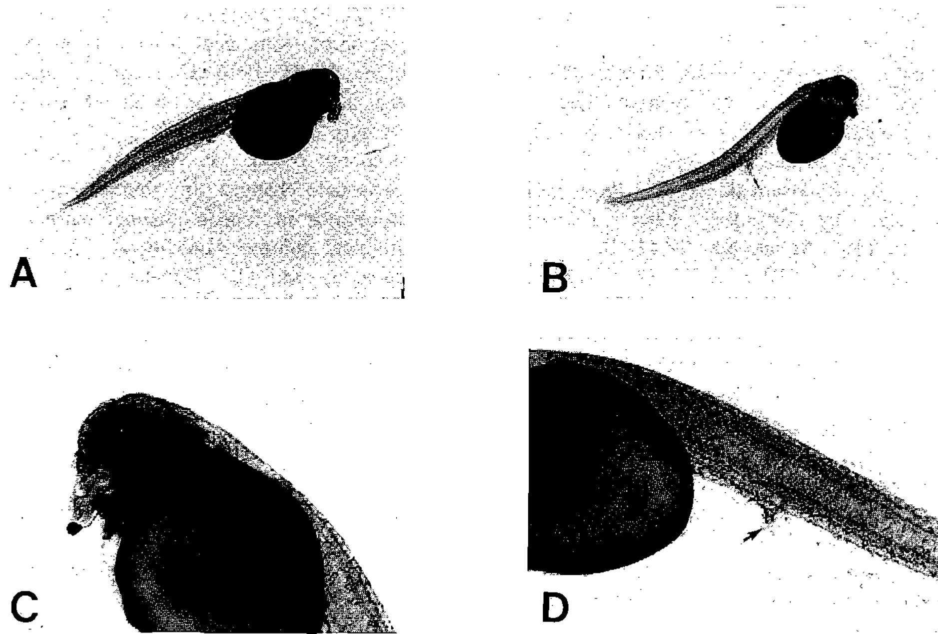


Figure 8. Stages of development of the catfish, *Silurus asotus*. A and B. 51 h 30 min after fertilization. 7.2X; C and D. 1 day after hatching. 15X.

al., 1989), and rainbow trout (Yoon et al., 1991).

Evidence that hCG stimulates various steroid hormone secretions in sexually mature teleost fishes has been presented previously for black porgy (Chang et al., 1991), and ayu (Hirose, 1980). These observations provide further confirmation of the role of hCG in final maturation and ovulation in fish. More detailed studies involving *in vitro* experimentation will be necessary in order to determine the exact role of the ovarian follicles throughout oogenesis, and such studies are now in progress in our laboratory.

Vitellogenesis and maturation in teleosts are generally considered to be dependent upon gonadotropin secretion by the pituitary. The presence of asynchronous ovaries which continuously produce eggs is presumptive evidence that the circulating titer of gonadotropin remains relatively constant throughout the breeding season. An important feature of the transition from vitellogenesis into maturation in catfish is the gain of the ability by the follicular cells to respond to endogenous levels of hormone and to begin the process leading to oocyte maturation.

The fertilization and hatching rates of eggs from hCG-treated catfish, were maximal from one day to two after ovulation (figures 6, 7 and 8). This result indicates that the eggs are of satisfactory quality for artificial fertilization, early embryonic development and normal hatching of larvae (Kuo et al., 1979; Hirose, 1980). However, the use of repeated injections of hCG results in lower fertilization and hatching rates (Hirose et al., 1977; Hirose, 1980). In contrast to their results, Suzuki (1983) reported that the mean fertilizability and hatchability per spawning were not correlated with repeated injections of hCG or with the number of spawning experiments of the females.

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