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Observations on reproduction techniques applicable to the European eel (*Anguilla anguilla* L.)

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SUMMARY- Immature silver male eels *Anguilla anguilla* were injected weekly with a human chorionic gonadotropin hormone (HCG) at a concentration of about 1.5 IU/gBW. The injection increased the gonadosomatic index (GSI) from 0.03 to 9.0. All treated males spermiated after the 5th injection and milt of high quality was obtained: pH amounted to 8.0, spermatocrit values = 75.0%, average sperm swimming speed was 6000μ. Sperm required about five seconds to reach the opposite side of the ripe egg with average diameter of 1.2 mm. Sperm life span reached a maximum duration of 400 seconds at 967.8 mOsmole/kg. Sodium and calcium could be considered as having a direct stimulating effect on sperm motility, while the functional state potassium is essential to activation mechanism of sperm depending on the dose used. A good correlation seems to exist between experimental conditions, ovarian maturation and injection by a combination of (2 mg Carp Pituitary CP / 100 g BW) two times a week within 70 days. GSI increased from 1.66 at the beginning of the experiment to 68.4 on reaching the ripe stage, with increased in body weight from 415 g to 500 g, about 342 g of ripe eggs were obtained. The absolute fecundity amounted to 1.48 X 10^6. The ripe female and male eels were kept in the same tank, at a sea water temperature of 21-23°C. And although spawning behaviour was observed, spawning did not occur. Twenty minutes after insemination, fertilized eggs formed a moderate perivitelline space.

Key words: Artificial maturation, spermiation, ovulation, fertilized egg, European eel.

RESUME *- "Observations sur les techniques de reproduction applicables à l'anguille d'Europe (*Anguilla anguilla *L.)". Des mâles immatures d'anguille argentée* Anguilla anguilla *ont été injectés hebdomadairement avec de l'HCG (gonadotropine chorionique humaine) selon une concentration d'environ 1,5 UI/g de poids corporel. L'injection a fait augmenter l'index gonadosomatique (GSI) de 0,03 à 0,9. Tous les mâles traités ont eu une spermiation après la 5ème injection. Une laitance de haute qualité a été obtenue : le pH s'est élevé à 8,0, les valeurs de spermatocrites = 75,0%, la vitesse de déplacement moyenne du sperme était de 6000μ. Le sperme mettait environ cinq secondes pour atteindre la face opposée de l'oeuf mature qui avait un diamètre moyen de 1,2 mm. La durée de vie du sperme a atteint un maximum de 400 secondes à 967,8 mOsmole/kg. Le sodium et le calcium pourraient être considérés comme ayant un effet stimulant direct sur la motilité du sperme, tandis que le potassium á l'état fonctionnel est essentiel au mécanisme d'activation du sperme en fonction de la dose utilisée. Il semble exister une bonne corrélation entre les conditions expérimentales, la maturation ovarienne et l'injection selon une combinaison de (2 mg Pituitaire de carpe PC / 100 g poids corporel) deux fois par semaine pendant 70 jours. Le GSI a augmenté depuis 1,66 au début de l'expérience jusqu'à 68,4 lors de la phase de maturité, avec une augmentation du poids corporel de 415 g - 500 g et environ 342 g d'eufs ont été obtenus. La fécondité absolue s'est élevée à 1,48 x 10^6. Les anguilles femelles et mâles matures ont été placées dans le même étang, dans de l'eau de mer à une température de 21-23°C. Et bien que le comportement de fraye était observé, la fraye n'avait pas lieu. Vingt minutes après insémination, les oeufs fertilisés ont formé un espace périvitellin modéré.*

Mots-clés : *Maturation artificielle, spermiation, ovulation, oeuf fertilisé, anguille d'Europe.*

Introduction

The European eel (*Anguilla anguilla L.*) constitutes a considerable part of the catch in the Egyptian northern delta lakes fisheries, especially during the period of lake-sea migration.

The migrating European freshwater eels, undergo extensive migration from their feeding grounds in the lakes towards their spawning grounds in the Sargasso Sea. They leave their freshwater feeding grounds at an early stage of sexual maturation (Bertin, 1956; Amin, 1974; 1997 and Kokhnenko *et al.*, 1977). Induced maturation of the European eel in Egypt could be achieved in laboratory by gonadotropic preparation Amin (1986; 1988 a,b; 1990).

The techniques of artificial maturation of the European eel in Egypt has passed through several developmental stages, particularly the achievement of optimal number of hormone injections and dosages giving adequate volumes and high quality milt and eggs. Few studies have been made on this subject.

This study aims to investigate: (i) the developmental changes and their synchronization with morphological changes, during the induced maturation of male silver eels, by the injection of the human chorionic gonadotropin hormone (HCG); (ii) the effect of five weeks of hormone injections on the sperm quality (motility, spermatocrit and pH of the milt); (iii) roles of osmolality and ionic composition to understand the environmental factors which regulate the sperm motility; (iv) the optimal dose of gonadotropic hormones for initiating the ovarian development from immature to full oocyte maturation; and (v) fertility of spermatozoa.

Material and methods

Experiments started on the migrating silver eels *Anguilla anguilla* L. obtained from commercial catch in lake Edku (one of the Egyptian northern delta lakes) during the period of spawning migration that extend from November to February. Experimental fish were gradually acclimatized to seawater over 7 days. During the experiments, the fish were held without feeding in 500 to 700 liter circulating sea water (38.0%) metallic galvanized tightly covered tanks with air compressor in complete darkness. Water temperature ranged from 20 to 24°C oxygen content approximately 80% saturation. Two to three female eels were placed together in each tank, while eight to ten male eels were placed together in each tank.

One hundred and thirty live migrating silver eels were used. Two experiments were carried out. Experiment 1, comprised fifty specimens of male silver eels of 32.0-44.0 cm in length and 80-120 g in weight. Thirty eels were injected intramuscularly once each week with a human chorionic gonadotropin (500 IU HCG). The other twenty eels were used as a control. Four of these were killed each seven days to monitor gonadal development. The effects of weekly intramuscular injections HCG at a concentration of 500 IU/3 fish (1.5 IU/g BW) was currently examined, because in a previous experiment (Amin, 1986) the full testicular ripeness and spermiation were achieved with a GSI of 5.3% after the 7th injection in the period of 45 days through weekly intramuscular injection of HCG at a concentration of 250 IU/fish.

In experiment 2, eighty specimens of female silver eels of 56-80 cm in length and 400-900 g in weight were used. They were divided into seven groups, received intramuscular injections of 500 IU HCG or carp pituitary (CP) suspended in (112.7 mM NaCl + 3.37 mM KCl + 2.35 mM NaHCO3, pH 7.5), in various doses, with or without 500 IU HCG, they were given once a week or twice weekly, and control group. For in vitro experiments, the stage of ovarian development of females injected twice weekly with 500 IU HCG was assessed histologically as early vitellogenic growth, fragments of ovarian tissues (10-50 mg) were isolated, immediately incubated in isotonic solution (ISIM), and supplemented with various concentrations of CP and 100000 U penicillin/l.

One micro litre of the semen was collected after spermiation and immediately diluted with one millilitre of each of the following solutions:

(i) distilled water (DW);
(ii) sea water (SW);
(iii) isotonic solution for in vitro egg maturation (ISIM) (Amin, 1988 b), 123,9 mM NaCl + 5.10 mM KCl + 2.07 mM $CaCl_2$ + 3.41 mM NaH_2PO_4 + 11.76 mM $NaHCO_3$ + 1.91 mM $MgSO_4$ + 5.21 mM glucose at pH 8.05 and 17°C;
(iv) 154,7 mM NaCl + 3.2 mM KCl + 2.3 mM $CaCl_2$ of 322.7 mOsm osmolality;
(v) 309.4 mM NaCl + 6.4 mM KCl + 4.6 mM $CaCl_2$ of 645.2 mOsm osmolality;
(vi) 464.1 mM NaCl + 9.6 mM KCl + 6.9 mM $CaCl_2$ of 967.8 mOsm osmolality;
(vii) 618.8 mM NaCl + 12.8 mM KCl + 9.2 mM $CaCl_2$ of 1290.4 mOsm osmolality;
(viii) K free, 464.1 mM NaCl + 6.9 mM $CaCl_2$;
(ix) Ca free, 464.2 mM NaCl + 9.6 mM KCl; and
(x) Na free, 9.6 mM KCl + 6.9 mM $CaCl_2$.

Test solutions were prepared and kept at room temperature (17 to 22 °C).

Sperm motility was determined by measuring the time until spermatozoa ceased forward movement when observed with light microscope. Milt pH was measured with a portable pH meter, one drop of milt from the genital pore was placed quickly on the sensor of the pH meter. Spermatocrit (packed cell volume/ total milt volume x 100) of each male was determined by collecting semen in nonheparinized tubes and spinning in a centrifuge for 10 min at 12.000 rpm. Sperm speed was calculated by dividing the sperm travel distance by the travelling time in seconds. To trace the movement of sperm, we utilized the grid pattern of the haemocytometer (200 x 200 μ m) according to Trippel and Neilson (1992).

The gonads were removed and weighed, a part of them was fixed in Bouins fixative and processed for histological examination using standard techniques. The gonadosomatic index GSI or gonad weight represented as a percentage of gutted body weight was determined.

Milt from one (500 IU HCG) injected eel was combined by the dry method, with about five grams ripe eggs from (8 mg CP + 500 IU HCG) injected female, and the eggs were incubated in static normal seawater at 23°C.

Results and discussion

Induced maturation of the male silver eel

At the beginning of hormonal treatment, testes of silver males were quite immature and the most advanced stage was ascribed to spermatogonia (Fig. 1). Several changes in the morphology of the germ cells as a function of gonadotropin and steroid production was obtained. Soon, after the first injection, the seminiferous tubules and central lumen became differentiated, the spermatogonia were singly found settled around the lumen. After the second injection (15 days), the number of cysts of spermatogonia decreased and cysts of primary spermatocytes were noticed surrounding the narrow empty lumen; clusters of Sertoli and Leydig cells could be detected. After the third injection (3 weeks), sexual cells belonging to all stages of development were visible and completely formed spermatozoa were observed in the lumen of testis, many Sertoli and Leydig cells increase in size and number (Fig. 2). Active testicular development was observed after 27 days of treatment (four injections). A large numbers of spermatozoa, could be noted in the lumen of tubules and were distinguished by their elongated crescent-shaped bodies and dark staining chromatin without any particular arrangement (Fig. 3). All treated males spermiated after the 5th injection, (32 days), the GSI values increased from 0.03 + 0.005 % at the time of capture coinciding with the onset of spawning migration to 9.0 + 1.10 % at the time of spermiation (Fig. 4).

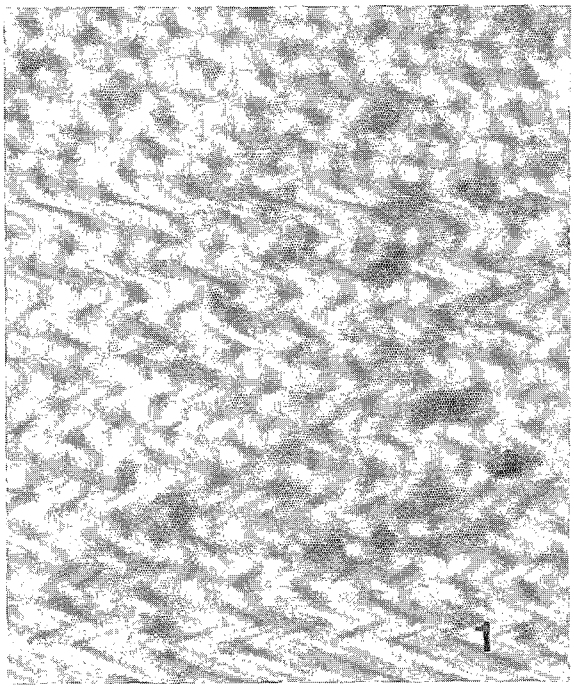

Fig. 1. Testis of an immature European silver eel; spermatogonia (SG)-x 1,000.

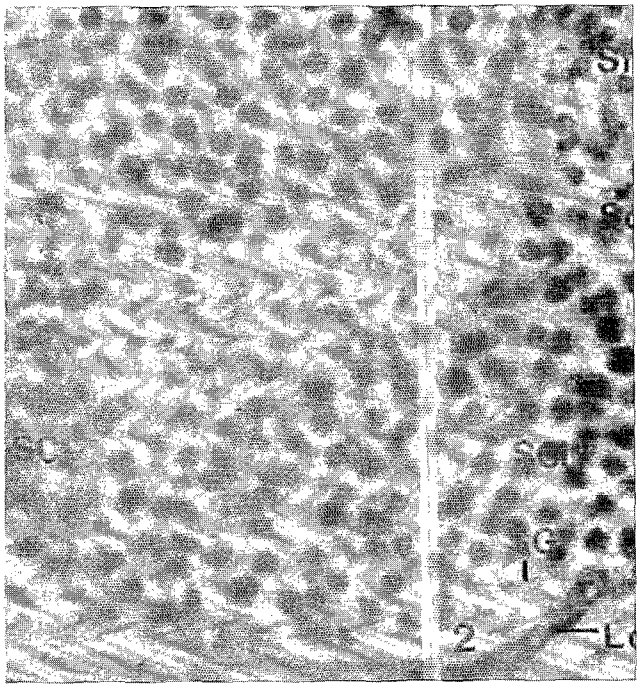

Fig. 2. Testis at the end of the third week of HCG treatment of the European silver eel, containing spermatogonia (SG), primary and secondary spermatocytes (SCI, SCII), spermatids (ST), spermatozoa (S), Sertoli (Sc) and Leydig (Lc) cells-x 1,000.

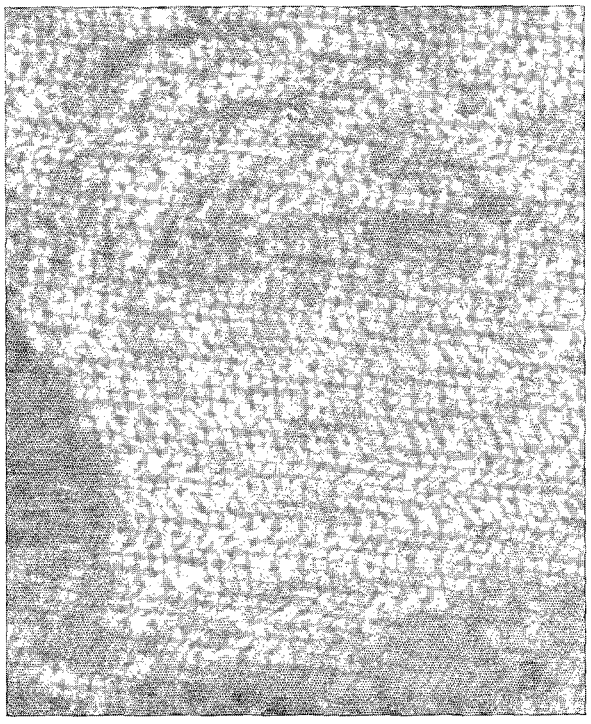

Fig. 3. Lumen packed with spermatozoa in the testis of the European silver eel-x 1,000.

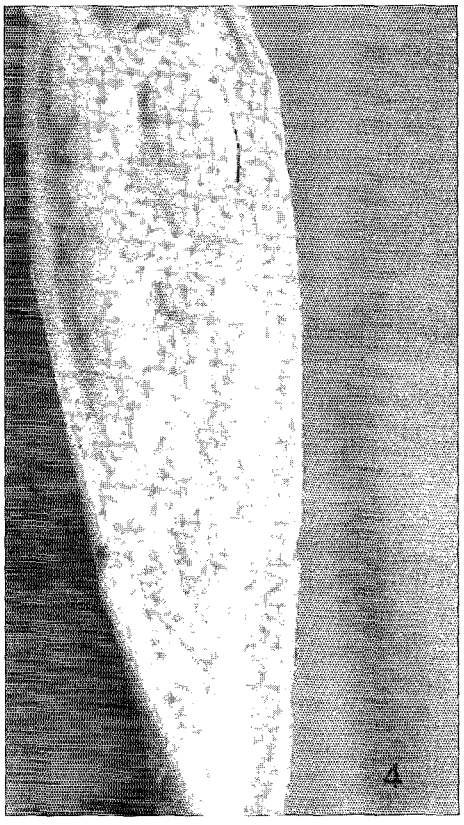

Fig. 4. Maximum development of the testes of a ripe European silver eel.

The spermatozoa was about 50 microns long, the average elongated head length was 5.0μ. The pH value ranged from 7.85 to 8.10 with average of 8.0 + 0.3. Spermatocrit ranged from 70.0 to 80.0 with average of 75.0 + 2.5 %. The swimming speed of motile sperm was constant as it passed through the 200μ m field of view, the average swimming speed was 6000+200 μ.

Within the testicular tubules the spermatozoa were still alive but motionless. The sperm motility occurred when the semen was diluted with distilled water. With ISIM solution (308.8 mOsm/kg) the duration of motility was about 2.5 time longer than that in distilled water (Fig. 5). With increasing osmolality the duration increased, reaching a maximum motility in solution (vi) at 967.8 mOsm/kg. Meanwhile, in seawater (1000 mOsm/kg) the motility decreased. By increasing ion concentration (1290.4 mOsm/kg) the sperm motility was inhibited. Sodium and calcium could be considered as having a direct stimulating effect on sperm motility (Fig. 6), in K free solution, duration of sperm motility extended 2.0-3.5 times longer than that in Ca and Na free solutions, and was shorter than that in solution (vi). In the medium consisting of NaCl and $CaCl_2$ (Fig. 7), the maximum duration of 600 + 30 seconds was obtained for values of NaCl: 309.4-464.1 mM and $CaCl_2$: 60-70 mM.

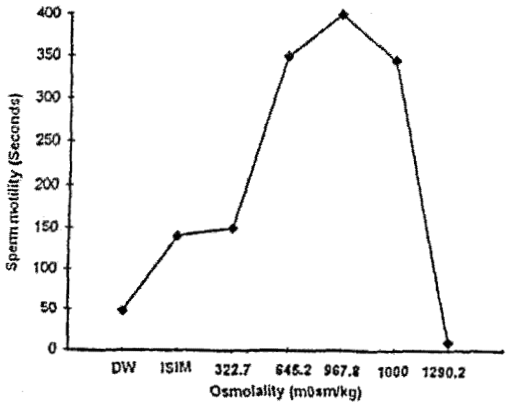

Fig. 5. The effect of osmolality on sperm motility of the European eel.

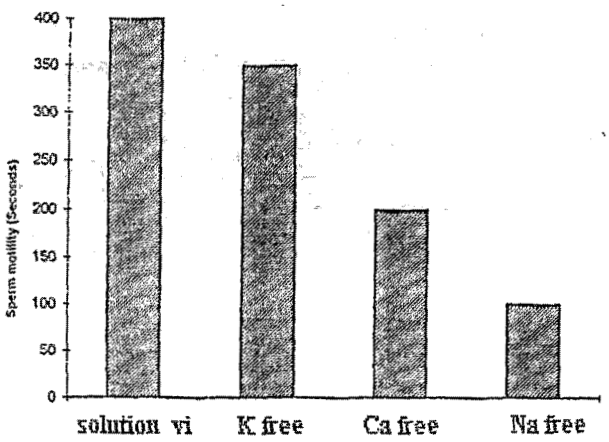

Fig. 6. The effect of NaCl, KCl, $CaCl_2$ on sperm motility of the European silver eel.

The spermiation induction of silver eel with weekly HCG dose (1.5 IU/g BW) giving rise to good sperm quantity and quality and produced fertile spermatozoa after the 5^{th} injection (32 days), confirm the suitability of such injection for steroids secretion from Leydig cells and elevation of pH value.

Similar results obtained by (Ohta *et al.*, 1996) in Japanese eel, that sperm motility was attained during the 7^{th}-9^{th} injections by HCG (250 IU) and percent motility at 15 sec after dilution with 450 mM NaCl was 70%. Miura *et al.* (1991) concluded that the gonadotropin stimulates the secretion of the fish androgen from Leydig cells, which in turn, activates Sertoli cells to stimulate premitotic spermatogonia and consequently the complete process of spermatogenesis. The spermatozoa have a longer life span at an osmolality between 645.2 and 1000.0 mOsm/kg, and significant

correlation was noted between duration of motility and Na, K and Ca ions which suggest that sodium and calcium are involved in the acquisition of sperm motility; complete lack of potassium did not affect the motility. Baynes *et al.* (1981), reported that divalent ions especially Ca are potent stimulators of such motility in sperm of rainbow trout. Furthermore, Morisawa (1985) stated that K concentration was not involved in inhibiting sperm motility in seawater species.

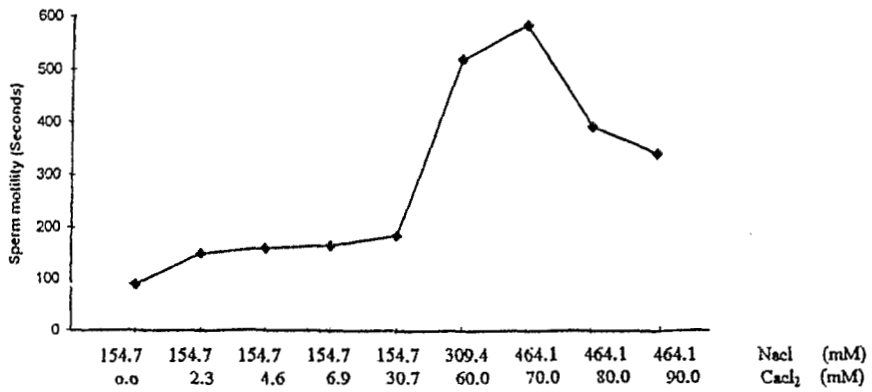

Fig. 7. The effect of Na/Ca ratio constituting solution on sperm motility of the European silver eel.

Induced maturation of the female and breeding of the eel

At the beginning of the treatment, or at the onset of the spawning migration, ovaries were quite immature (Fig. 8), the GSI averaged 1.66 + 0.50. The different practices revealed that:

(i) Females injected twice weekly with 500 IU HCG did not demonstrate ovarian maturation. The ovary was still in early vitellogenic growth. Primary yolk stages were visible in the group injected 15 times.

(ii) Ovarian tissues from previous responder females were used for in vitro experiments. After 36 hours of incubation with a CP concentration of 0.1 mg/ml, the oocytes became fully ripe and contained small or large oil droplets.

(iii) Females weekly injected with 4 mg CP or 4 mg CP + 500 IU HCG did not respond for a period of 80 days.

(iv) Females weekly injected with 8 mg CP for 78 days, followed by two times a week injection, attained a nearly ripe ovary condition after 180 days.

(v) Females weekly injected with 12 mg CP + 500 IU HCG showed a significant gonadal enlargement (GSI 12.0) after 47 to 72 days.

(vi) Two injections per week with 12 mg CP + 500 IU HCG gave positive results after a period of 40 to 47 days with a GSI of 38.8%. About 25% of the total egg number attained full ripeness.

(vii) Two injections per week with 8 mg CP + 500 IU HCG for 70 days injections) produced ripeness whole ovaries, about 342 g of ripe eggs were obtained with a GSI of 68.4% (Fig. 9). On the other hand the body weight increased from 415 g at the start of the treatment to 500 g at dissection (about 120%). Transparent ripe eggs with average diameter of 1.2+0.06 mm, were obtained (Fig. 10), they contained a large oil globules. The absolute fecundity amounted to 1.48 X 10^6.

The ripe female and male eels were kept in the same tank at a sea water temperature of 21-23°C. Mating began with the males which gently touched the females very often, followed by moving together with bodies in contact near the surface. Spawning did not occur. By the dry method; eggs and milt were stripped from the fish into glass dishes; spermatozoa had about 85% fertilized eggs, 20 minutes after insemination, fertilized eggs formed a moderate perivitelline space (Fig. 11). Cleavage and development of the eggs in normal seawater at 23°C were not obtained.

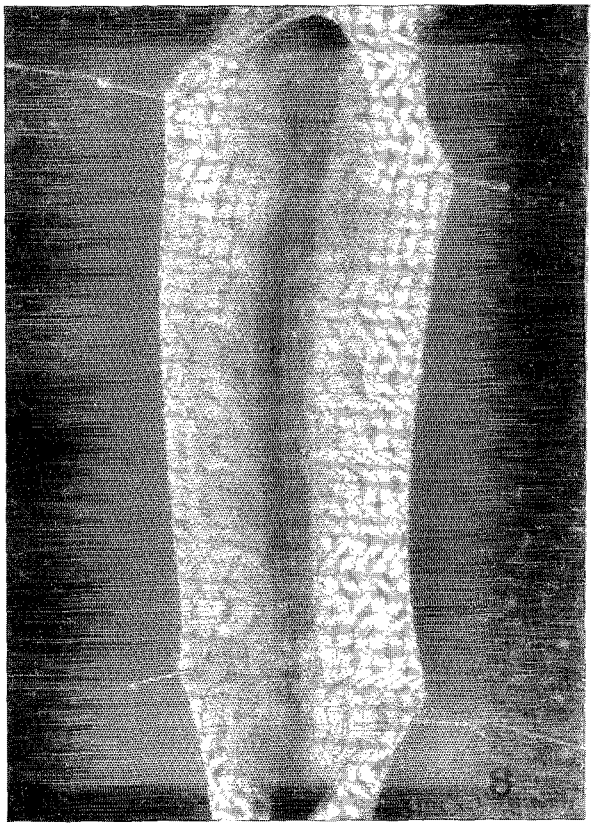

Fig. 8. Ovaries of an immature European silver eel at the start of the experiment.

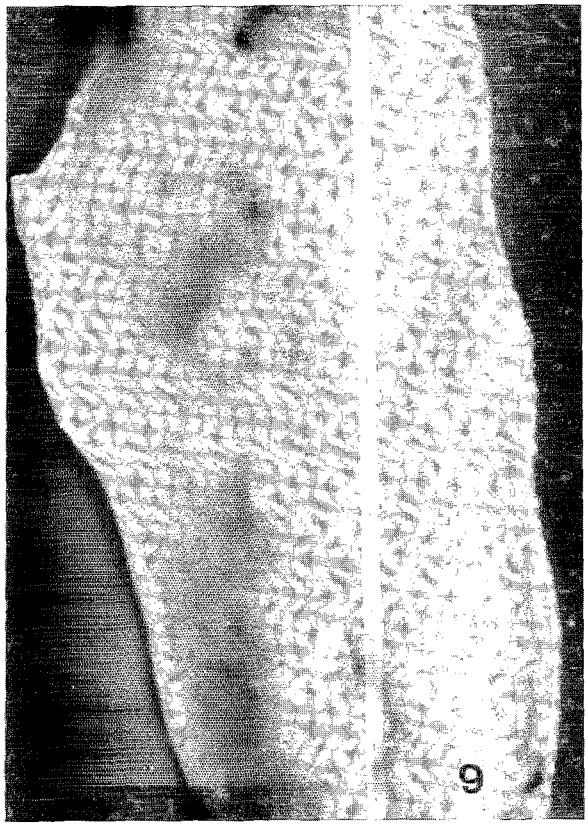

Fig. 9. Maximum development of the ovaries of a ripe European silver eel.

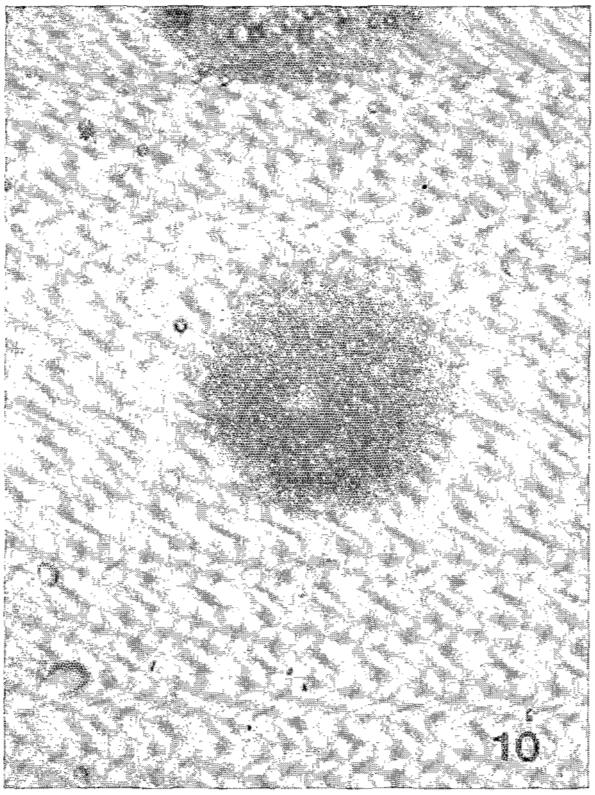

Fig. 10. Transparent ripe egg of the European silver eel with diameter of 1.2 mm.

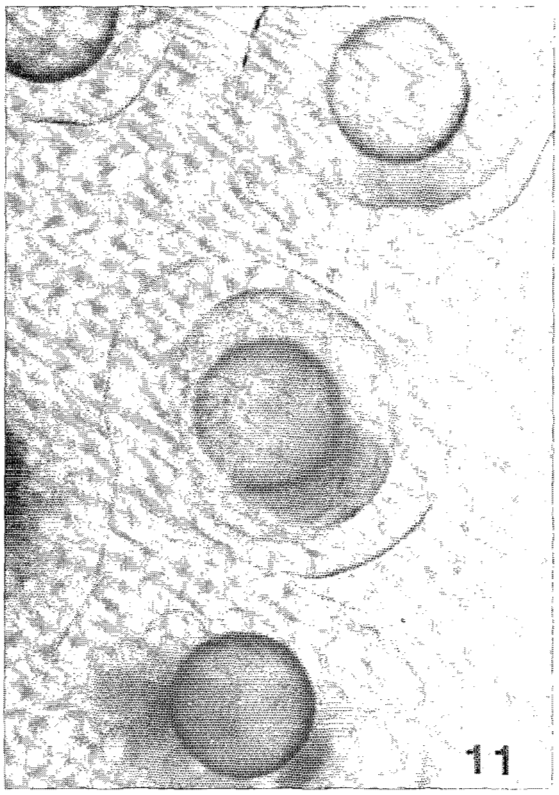

Fig. 11. Fertilized eggs of the European silver eel.

Water quality, water temperature, time of injection, state of gonads prior to treatment, stocking density and methodology of hormone administration are all important factors in spawning induction.

Our results suggest that vitellogenic growth was not stimulated by (HCG), perhaps due to the inability of the gonadotropin (GTH) receptor on the follicular membrane to recognize HCG. When female eels were treated by the administration of only pituitary gland in various dosages, none of the eels matured. Sugimoto *et al.* (1976) reported that injection of salmon pituitary extract can often induce final maturation of oocytes and ovulation, but success rates are low and even if ovulated eggs are obtained, the eggs show low fertility and hatching rates. Ijiri *et al.* (1995) reported that chum salmon pituitary homogenate (SPH) administration over 25 weeks did not induce vitellogenic growth of ovarian follicle to the migratory nucleus stage in all cultivated Japanese eels. On the other hand, injection of 17,20 B-Dihydroxy-4-pregnen-3-one (DHP) induced final maturation and ovulation in artificially matured Japanese eel after injection with salmon pituitary extract as a priming dose Kagawa *et al.* (1997).

The administration of 8 mg CP and HCG gave positive results under our experimental conditions and good relationship between the increase in the body

weight of the female eel and maturation. The concentration of milt from one eel with artificial seminal plasma added to the five grams of ripe eggs must be sufficient for obtaining good fertilization rate. A similar high value of gonadosomatic index (60.7) was obtained by Boetius and Boetius (1980) when female European eels were injected with 15 mg carp pituitary + 500 IU HCG twice a week. Satoh *et al.* (1992) reported that the normal body weight of female Japanese eels ranged between 110-130% (about 120%) of that at the start of the experiment , and mature female eel with a body weight of more then 130% always gave rise to denatured eggs.

Amin (1991) determined, the energy consumed during gonadal development, migration, spawning and spent stage by silver female and male European eels *Anguilla anguilla*. She found that in almost all cases, females consume more energy to build up the ovaries than the energy needed to build up the male testes. The ovaries consume 25.08% of the total energy, while the testes consume 20.28%. Migration and simultaneous routine metabolism (E_R) account for about 35.7 and 34.2% of the total energy was utilized by female and male silver eels, respectively. The energy of routine metabolism was higher in male (9.6%) than in female (6.0%) silver eel. Approximately 17.1% for ovulation and 15.8% for spermiation, of the total energy was utilized.

Conclusion

Injection of HCG was used to induce maturation of silver male *Anguilla anguilla*, and injection of a combination of 2 mg CP/100 g BW + 500 IU HCG two times a week has been used to induce maturation in silver female and produced about 342 g of ripe eggs. Milt and eggs with high quality were obtained. Fertilization rates of 85% were achieved.

Although artificial breeding of eel in several countries is still not completely successful, we will adopt more advanced methodology for treating and incubation of fertilized eggs. The last experiments carried out by Ijiri *et al.* (1995) stressed on the identification of the deficiencies in the process of vitellogenesis and final maturation of the eggs of Japanese eel. Such deficiencies were thought to affect the hatched larvae which did not metamorphose into leptocephali (Yamauchi *et al.*, 1976).

Obtaining of fertilized eggs with early embryonic development was a result of continuous and ongoing research through several years, the anticipated hatching did not take place.

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