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EFFECT OF HORMONAL INJECTION ON CONCENTRATION OF SEX STEROID HORMONES AND FINE STRUCTURE OF OVA IN FEMALE *CHRYSICHTHYS RUEPPELLI* DURING SPAWNING SEASON

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Key words: Chrysichthys rueppelli – gonadotropin hormone – pituitary extracts– steroid hormone (estradiol and testosterone) – fine structure.

ABSTRACT

During spawning months "from April to July" the average gonadosomatic index (GSI) of control female *Chrysichthys rueppelli* varied between 37.8 ± 4.3 maximal in May and 12.4 ± 2.5 minimal in July. Both serum estradiol and testosterone concentration positively correlated with GSI. The fish injected by gonadotropin hormone and pituitary extracts as cumulative dose 15500 I.U. + 4 pituitary extracts in first experiment and day by day with cumulative dose 11500 I.U.+ 3 pituitary extracts in second experiment. As a result of injection gradual decrease in the GSI value, serum estradiol and testosterone values were recorded throughout experiment I and II with highly significant correlation at $P< 0.05$. This decreases continued after the end of these two experiments. During the third experiment where the fish injected day by day dose by gonadotropin hormone only, as a result of injection the GSI value, serum estradiol and testosterone decrease progressively with increasing cumulative dose of hormonal induction from 3500 I.U. to 13500 I.U. through 24 days. The fish were injected day by day with GtH & LHRHa, in fourth experiment as a result of injection the GSI value increased gradually from (10.5 ± 6.6) to maximum value (22.5 ± 7.40) with increasing cumulative dose of hormonal induction from 1500 I.U. +250 µm to 1500 I.U. + 1000 µm respectively. While serum estradiol and testosterone decrease progressively with increasing cumulative dose of hormonal induction. The ultrastructural studies of the oocyte wall revealed that there are five different layers in the control ripe oocyte of *Chrysichthys rueppelli* at spawning season. As a result of injection in the third experiment, early atretic oocytes were recorded in the wall of the oocyte after final injection with gonadotropin hormone only. While in fourth experiment late atretic oocyte was recorded after final injection with GtH & LHRHa. In early atretic oocyte there were observed enlargement of theca layer separated from the granulose cells by deformed incomplete basement membrane and the nucleus was de-fragmented in some sites, whereas in late atretic oocytes the cell appeared without any nucleus. The zona radiata becomes more compact without any continuation between granulose cells.

1. INTRODUCTION

In Egypt, fish culture provides a rapid and good source of proteins that necessary to overcome the present deficiency in protein food. Catfish in many species of family Bagridea are in significant commercial and economy important fish inhabiting the inland fresh or some times slightly brackish water of many parts of the world particularly in

tropical and subtropical regions and has characteristics adaptability for culture.

In fact, *Chrysichthys* is considered as one of the ideal examples in pond culture, due to the ability of this fish to survive in a restricted environment, poor oxygen and different treatment of experiment.

For the management of any fishery it is essential to study the length at first sexual maturity and annual maturity stages of the fish.

We found that the first sexual maturity was at length 20 cm and the ripe and spawning stages were observed in April to June and the spent stages were started to appears in four successive months (July, August, September and October) as reported by (Fahmy, 1997).

Many fish exhibit reproductive dysfunctions when reared in captivity. It is most common, that cultured tiger puffer *Takifugu rubripes* females fail to undergo final oocyte maturation, as well as ovulation and spawning, (Matsuyama *et al*., 1997).

Vermeirssen *et al*. (2000) studied the failure of male and female Atlantic halibut fish (*Hippoglossus hippoglossus*) to ovulate, spermiate, and produce only a small volume of viscous milt and fertilized eggs in captivity.

Failure endocrine regulation of fish to release GnRH may be responsible for the lack of final oocyte maturation, ovulation and spawning as reported by (Mylonas and Zohar, 2001).

Successful induced spawning of fish using different substances with direct and indirect gonadotropin action was reported by (Yaron, 1995); (Kucharczyk *et al*., 1997) and (Mikolajczyk *et al.,* 2003).

Duncan *et al.,* (2003) studied the effect of controlled delivery and acute injection of LHRHa *on Sphoeroides annulatus* with increasing doses to induce ovulation of good quality eggs. While Yeh *et al.* (2003) studied effects of exogenous androgens on ovarian development and sex change in female orange-spotted protogynous grouper, *Epinephelus coioides*.

Joy *et al.* (1998) studied the periovulatory changes in hypothalamic and pituitary monoamines following by single injection of GnRHa on the ovulation in the catfish .*Heteropneustes fossilis*. They mentioned that this single dose (0.15 mg/g) could cause a very high rate of ovulation after a latent period.

Utoh, *et al.* (2005) studied the annual changes in ovarian development and plasma estradiol level in reared female common Japanese conger, *Conger myriaster.* They reported a decline in plasma estradiol level correlated with ovarian atresia. They also mentioned that the environmental stress is the main cause of follicular atresia.

Nagahama (1987) studied gonadotropin action on gametogenesis and steroidogenesis in teleost gonads. He reported that testosterone is produced by special theca cells which are considered as precursor of estradiol.

In teleosts fish the ovarian follicle cells synthesize vitellogenin as reported by authors: (EL-Gharabawy *et al.,* 2003) in *Trachinotus ovatus* and (Prisco *et al*., 2004) in *Torpedo marmorata* .

Ultrastructure changes in oocytes of teleost fish were studied by several authors during several experimental conditions including induces spawning, (Takaaki *et al.,* 2001) in Japanese eel.

The aim of the present study was to give detailed study of sex steroid hormone (estradiol and testosterone) for both control and injected *C. rueppelli* during spawning season after treatment with several doses of hormones, and study fine structure of the oocyte wall of control and injected female to give information about ultrastructure deformation in the wall after treatment with several doses of hormones during spawning season.

2- MATERIAL AND METHODS

2.1. Collection of sample:

Specimens of *C. rueppelli* (200 fish) were obtained alive at ripe and spawning stage from fishermen in the Nozha hydrodrome and Abou- El Kheer Bridge at the high way of Alexandria – Cairo. Fish samples were kept in fiberglass tanks (1000L volume) for 10 days for acclimation at fresh water, natural photoperiod and water temperature of about 27^{o}C. Fish broad were selected with an average weight between 150 gm and 280 gm, and average length between 20 cm and 28 cm. The sex ratio was two females to one male. Artificial pelleted diet with 35% crud protein and worms (1:1) was used for feeding at a rate of 5% of the total fish weight.

2.2. Experimental design and treatment:

a) Chemicals: Human chorionic gonadotropin hormone was used from Serono S.P.A. Roma Italy chemical under the commercial name (Profassi ampule) each containing 5000 I.U. of HCG, dry carp pituitary extract and LH-RH from Argent chemical laboratories Lot # LH 2508R manufactured Redmond, WA98052 under title of 8702 152nd AVE.N.E, were used also for injection.

b) Treatment: Nine fiberglass tanks (1000L) were used for the experiment. The water was renewed daily and the tanks were cleaned with gentle aeration. One tank was used as control, the fish were injected with saline solution (0.9% Nacl), while the remaining eight tanks were used for the four experiments as follows:

- Experiment I: The injection was carried out daily by chronic gonadotropin hormone (HCG) and pituitary extract as reported in table 1.

- Experiment II: The fish was injected day by day by (day after day) by HCG and pituitary extract as indicated in table 1.

- Experiment III: The female was injected by HCG only as indicated in table1.

- Experiment IV: Luteinizing releasing hormone (LHRHa) in combination with HCG was used for injected as in table (1).

2.3. Injection of fish:

Females were anesthetized using phenoxyethanol (3 ppm), intramuscular injection beside the dorsal fin.

2.4. Determination of sex steroid hormones:

Samples of fish were taken during and at the end of each experiment to determine the gonadosomatic index (GSI) value and collect blood to detect the quantitative determination of steroid hormones "testosterone and estradiol" by the Radioimmunoassay Iodine 125 Testo-Ct2 kit of hormone was used for detection of testosterone hormone, whereas Coat –A-Count estradiol was used for determination of estradiol under catalog number TKE21.

2.5. Blood sampling:

The blood samples were collected from caudal artery the fish was wrapped in a towel leaving the tail and anal fin free by smooth cutting before caudal peduncle until reaches to vertebral bones. The blood samples were collected in clean test tube and kept in refrigerator for 18-24 h supernatant serum were pipetted in vials and stored at - 20^{o}C until required.

Statistical analysis was done by Microsoft windows 2000 Excel program.

Table (1): Doses of injection by gonadotropin hormone and pituitary extract on female *C. rueppelli* daily (experiment I), day by day dose (experiment II), gonadotropin hormone (I.U.)only (Exp.III) and gonadotropin with Luteinizing releasing hormones (experiment IV) during spawning season.

No. of doses	Experiment I				Experiment II				Experiment III				Experiment IV			
	No. of fish	Date of injection	Daily dose/fish	cumulative dose	No. of fish	Date of injection	Daily dose/fish	cumulative dose	No. of fish	Date of injection	Daily dose	cumulative dose	No. of fish	Date of injection	Daily dose/fish	cumulative dose
1	28	24-Apr	500 I.U.	500 I.U.	28	24-Apr	500 I.U.	500 I.U.	28	04-May	1500 I.U.	1500 I.U.	20	27-May	500 I.U.	500 I.U.
2	28	27-Apr	1000 I.U.	1500 I.U.	28	27-Apr	1000 I.U.	1500 I.U.	28	06-May	1000 I.U.	2500 I.U.	20	29-May	500 I.U.	1000 I.U.
3	28	28-Apr	1 Pit	1500 I.U. + 1 Pit	28	29-Apr	1 Pit	1500 I.U. + 1 Pit	28	08-May	1000 I.U.	3500 I.U.	20	31-May	500 I.U.	1500 I.U.
4	28	29-Apr	1000 I.U.	2500 I.U. + 1 Pit	28	01-May	1000 I.U.	2500 I.U. + 1 Pit	24	10-May	1000 I.U.	4500 I.U.	20	02-Jun	250mm	1500 I.U.+ 250mm
5	28	30-Apr	1000 I.U.	3500 I.U. + 1 Pit	24	04-May	1000 I.U.	3500 I.U. + 1 Pit	24	13-May	1500 I.U.	6000 I.U.	15	04-Jun	250mm	1500 I.U.+ 500mm
6	28	01-May	1 Pit	3500 I.U. + 2 Pit	24	06-May	1 Pit	3500 I.U. + 2 Pit	24	15-May	1500 I.U.	7500 I.U.	15	06-Jun	250mm	1500 I.U.+ 750mm
7	24	02-May	1500 I.U.	5000 I.U. + 2 Pit	24	08-May	2000 I.U.	5500 I.U. + 2 Pit	20	17-May	1500 I.U.	9000 I.U.	10	09-Jun	250mm	1500 I.U.+ 1000mm
8	24	04-May	1500 I.U.	6500 I.U. + 3 Pit	20	13-May	1000 I.U.	6500 I.U. + 2 Pit	20	21-May	1500 I.U.	10500 I.U.				
9	24	05-May	1 Pit	6500 I.U. + 3 Pit	20	15-May	1 Pit	6500 I.U. + 3 Pit	16	26-May	3000 I.U.	13500 I.U.				
10	24	06-May	1500 I.U.	8000 I.U. + 3 Pit	20	17-May	2000 I.U.	8500 I.U. + 3 Pit								
11	20	08-May	1500 I.U.	9500 I.U. + 3 Pit	16	19-May	3000 I.U.	11500 I.U. + 3 Pit								
12	20	13-May	1 Pit	9500 I.U. + 4 Pit												
13	20	15-May	1500 I.U.	11000 I.U. + 4 Pit												
14	20	17-May	1500 I.U.	12500 I.U. + 4 Pit												
15	16	19-May	3000 I.U.	15500 I.U. + 4 Pit												

2.6. Ultrastructure examination:

At the end of the experiments, four small blocks (2 mm x 2mm) of ovary specimens after three days of second injection (9000 I.U.) of gonadotropin hormone only at 20th of May (third experiment) and after 14th days of last injection (1500 I.U. and 500µg) of LHRHa and gonadotropin hormone (fourth experiment) were fixed overnight at 4°C in 4% buffered glutaraldehyde and then in 1% osmium tetroxide for one hour at room temperature, rinsed twice in cacodylate buffer, dehydrated through a graded ethanol series, cleared in propylene oxide and embedded in polarbed 812 (polaron) epoxy resin. Ultra thin sections of one micron thick were prepared using glass and diamond knives, and stained with Uranyl acetate and lead citrate, sections were examined using a transmission electron microscope.

3-RESULTS AND DISCUSSION

During spawning months "from April to July" the average GSI of female *C. rueppelli* varied between 37.8 ± 4.3 maximal in May and 12.4 ± 2.5 minimal in July, (Table 2). The average concentration of serum estradiol ranged between 0.9 ng/ml ± 1.22 maximal in April and 0.55 ng/ml ± 0.15 minimal in July. Highly significant correlation was recorded in serum estradiol concentration during April at $P< 0.05$. The maximum concentration of the average serum testosterone were recorded in April and May, whereas, the minimum value was recorded in July 0.06 ng/ml ± 0.02. Highly significant correlation was detected in serum testosterone concentration during April and May at $P < 0.05$, (Table 2).

Both serum estradiol -17 β and testosterone levels were positively correlated with GSI and oocytes diameter, as reported by Assem (1995) in *Solea* sp.; Zaki *et al*. (1994) in *liza ramada*; Abdo (1996) in sea bass; and Cornish (1998) in tilapia, Sisneros**,** *et al.* (2004) for the vocal plainfin midshipnan, as referred to our results as control.

Experiment I was started at 24 April each fish was injected daily by chronic gonadotropin hormone (HCG) and pituitary extract as a cumulative dose of hormone, (Table 1). Samples of blood serum female were examined and gonadosomatic index (GSI) value calculated in 2, 8, 19, 20, 22, 24 and 26 of May, (Table 3). The GSI value began to decrease at 2 May after eight days of injection when females receiving total cumulative dose of gonadotropin 3500 I.U. and 2 dry pituitaries extract to reach 18.3 ± 3.9 compared to control fish, (Table 2). Gradual decrease in the GSI values were recorded throughout the experiment and continued after the last days of the injection to reach 3.5 ± 2.01 as a minimum value after six days of last injection with 15500 I.U. and 4pituitaries of a cumulative dose of hormone, at 25th of May .

Although the results revealed gradual decrease in the average concentration of serum estradiol and testosterone during the first experiment and continued to decrease from 0.84 ± 0.85 ng/ml for estradiol and 2.09 ± 2.3 ng/ml for testosterone after eight days of injection when females receiving total cumulative dose of gonadotropin 3500 I.U. and 2 dry pituitaries extract, at 2 May to reach 0.3 ng/ml ± 0.12 for estradiol and 0.009 ng/ml ± 0.008 for testosterone, after six days of last injection with 15500 I.U. and 4pituitaries of a cumulative dose of hormone, at 25th of May.

In the present work, anesthetic "phenoxyethanol with 3ppm" were used during the injection of the fish to decrease the effect of stress on endocrine and ovulatory response during handling. This result agreement with Cleary *et al*. (2002) who stated that capture and handling stress affects the endocrine and ovulatory response and may cause the formation of atretic follicle in response to exogenous hormone treatments in *Pagrus auratus.*

Highly significant correlation was recorded in serum estradiol of female *C. rueppelli* at 2, 8 and 19 May at $P < 0.05$, and highly significant correlation was noticed in serum testosterone for female *C. rueppelli* at 2 and 8 May at $P < 0.05$, (Table 3).

The second experiment was conducted through the period extend from 24th April to 19th May.

Blood serum of each female was examined for estradiol and testosterone hormone at 4, 13, 19, 20, 22, 24 and 26 May. The mean GSI value revealed gradual decrease from 17.8 ± 2.5 maximal after ten days of injection with cumulative dose of 2500 I.U. + 1 pituitary extract to minimum GSI value (9.7 ± 1.2) was recorded after six days of last injection with (11500 I.U. and 3 pituitaries) of a cumulative dose of hormone, at 25th of May.

The average concentration of serum estradiol and testosterone gradually decreased from 0.8 ± 0.52 ng/ml and 2.05 ± 1.5 ng/ml at 4th of May after injection with 2500 I.U. + one dry pituitary gland extract to reach the minimum value (0.42 ng/ml ± 0.25 for estradiol and 0.5 ng/ml ± 0.5 for testosterone), after six days of last injection with (11500 I.U. and 3 pituitaries) of a cumulative dose of hormone, at 25th of May.

In female *C. rueppelli* after injection with GTH and pituitary extract in first and second experiment, the estradiol and testosterone levels were declined from the end of the injections and continued to decrease after the end of the experiment. This decline in estradiol and testosterone will affect on the gonadotropin cell activity and causes atretic and degenerated cells. In normal fish high level of germinal vesicle break down and were ovulate. This was characterized by low estradiol levels and rise in testosterone as discussed by (Barry *et al.*, 1995).

High significant correlation was recorded for estradiol and testosterone concentration after ten and nineteen days of injection (4th and 13th of May) at $P < 0.05$, (Table 3).

Third experiment revealed that the GSI value reached a maximum value after seven days from the beginning of the experiment, when female receiving cumulative dose 3500 I.U. was 14.6 ± 3.5, while after seven days (2nd of June) of last injection with 13500 I.U. of a cumulative dose of hormone, the GSI value was 11 ± 4.16 (Table 4).

Although gradual decrease in concentration of serum estradiol to from 0.7 ng/ml ± 0.2 after seven days from the beginning of the experiment (at 10th May) to 0.56ng/ ml ± 1.36 after seven days of last injection with 13500 I.U. of a cumulative dose of hormone, at 2nd of June.

High significant correlation was recorded in 10 and 17 May at $P < 0.05$. The concentration of serum testosterone in female *C. rueppelli* was recorded at 10 May (2 ng/ ml ± 0.32) and then gradually decreased to reach (1.05 ng/ml ± 1.48), after three days of last injection with 13500 I.U. of a cumulative dose of hormone, at 29 May as indicated in Table (4).

The drop of estradiol in female *C. rueppelli* after injection of different four experiments probably reflects the rapid decline in aromatase activity of the granulose cells, and the ability of follicles to convert testosterone to estradiol by enzyme aromatase, as indicated by Young, *et al.,* (1983) in *Oncorhynchus rhodurus*; Shigeho *et al.* (1995) in *Anguilla japonica* and Lee *et al.* (2002) in *Acamthopargrus schlegeli*.

While fourth experiment revealed that the GSI value gradually increased from 10.5 ± 6.6 after nine days from the beginning of the experiment where females received cumulative dose 1500 I.U. + 250 µm of LHRHa to maximum value 27.5 ± 7.4 after four days of last injection of cumulative dose 1500 I.U. + 1000 µm of LHRHa, as shown in Table (4).

While serum estradiol and testosterone gradual decrease from 0.6 ng/ml ± 0.1 to 0.5 ng/ ml ± 0.14 respectively after nine days from the beginning of the experiment where females received cumulative dose 1500 I.U. + 250 µm of LHRHa to 0.18 ng/ml ± 0.13 and 0.04 ng/ml ± 0.01 respectively, after four days of last injection of cumulative dose 1500

I.U. + 1000 µm of LHRHa, as shown in table (4).

Serum levels of estradiol and testosterone hormone in *C. rueppelli*, after administration of hormones (LHRH and HCG) gradually decreased at spawning season this decreased to minimum value may be due to behavioral response for ovulation.

Drori *et al.,* (1994) on common carp, reported that the latency (initial egg release) was always longer in treated carp with GnRH that carp pituitary extract (CPE) and the GTH product elevated in response to the administration of hormone, to perform a final oocyte maturation.

Haddy and pankhurst (2000) studies the effect of exogenous hormone (LHRH or HCG) on stimulating changes on plasma steroids and ovulation in wild black bream *Acanthapagrus butcheri* which improved by treatment at capture. These authors fined that treatment with LHRHa at capture resulted in a better ovulatory response and the handling stress reduced the responsiveness of fish to exogenous hormone treatment, and the best results are obtained if hormonal treatment is administered at the time of capture.

High significant correlation was recorded in 4th of June in serum estradiol and testosterone at $P < 0.05$.

For induce spawning of female *C. rueppelli* at first, second experiment with GTH and pituitary extract and third experiment with GTH only the GSI decreased to reach minimum value therefore we must be controlled by a surge of gonadotropin that controlling the induced spawning as discussed by Mikolegezyk, *et al.* (2003). The use of luteinizing releasing hormone (LHRH) or gonadotropin releasing hormone for induced pituitary GTH release and initiation of final oocyte maturation (FOM) or spawning was reported in other fish species by several authors: Breton *et al.* (1990) in *Oncorhynchus mykiss*; Mylonus *et al.* (1998) in *Morone saxatilis*; Carlosfeld *et al.* (1998) in *Piaractus mesopotamians*. This result in agreement with our results when GSI increased from 10.5 ± 6.6 to 27.5 ± 7.4 after four days of last injection of cumulative dose 1500 I.U. + 1000 µm of LHRHa.

As reported in the present results, levels of estradiol and testosterone decreased with increasing of cumulative dose of hormonal induction as results of early and late atretic oocyte observed. These atretic stages may be due to the influence of hypothalamus – pituitary gonad – axis (pituitary control by feed back mechanism), for controlling further follicular development. Seasonal cycles of plasma estrogens have been correlated with ovarian development and vitellogenesis, (Mac-Kenzie *et al*. 1989) in *Ictalurus punctatus*, and acts at the level of the pituitary to regulate gonadotropin hormone (GTH) secretion, (Yen *et al*. 2002) and Rodolfo *et al*. (2003).

3.1. Ultrastructures examination of the normal ripe ova in female *C. rueppelli*:

There are five different layers in the normal ripe oocyte of *C. rueppelli* at spawning season in which the outer most layer is theca layer, while the second is follicular epithelium or the granulose layer. The basement membrane separates the first layer from the second. Then the third and fourth layers are zona radiata, which in turn characterized with two layers zona radiata externa and interna. The fifth layer was the cortical alveoli, which is considered as the inner most layer, of the follicular envelope, f ig 1a&b.

3.1.1. The Theca layer:

The theca layer is the outer most layer in the oocyte envelope is composed of theca cells which contain large flattened nuclei with irregular outline. The nucleus has condensation of chromatin close to the nuclear envelope and few cytoplasmic organelles. As oogenesis proceeds, theca cells increase in number, but their shape are elongated and flattened, f ig 1a&b.

Kobayashi *et al*. (2005), reported that ultrastructure steroid-producing cells, such as endoplasmic reticulum mitochondria with tubular cristae and large number of free

ribosome in cytoplasm were identified in the oocyte wall of gobiid fish (*Trimma okinawae*), that agrees with the present results for normal female *C. rueppelli*, the number of organelles including mitochondria and Golgi bodies, dramatically increased during development at the tertiary yolk stage.

In the amago salmon, it has been demonstrated that under the influence of gonadotropin, the theca layer of the follicle produces testosterone which then serves as a precursor for the production of estradiol - 17 β in the granulose layer (Kagawa *et al*. 1982; Nagahama, 1987 and Sisneros**,** *et al.* 2004). In addition to being used as a precursor, testosterone may be important in regulating gonadotropin secretion through the feedback mechanism, Crim *et al.* (1981).

3.1.2. The follicular epithelium layer (granulose):

Follicle cells consist of the regular squamous or cuboidal epithelial layer around the oocytes nuclear envelope and few cytoplasmic organelles. Granulose layer is distinguishing by large number of amoeboid nucleus, with an irregular outline exhibit condensation of chromatin material and characterized with the presence of few cytoplasmic organelles of the steroid producing cells; numerous mitochondria, endoplasmic reticulum and the moderately developed Golgi complex is close to the nucleus, f ig 1a&b.

In normal condition of female *C. rueppelli*, the number of organelles in follicular epithelium including mitochondria and Golgi bodies dramatically increased during development at the tertiary yolk stage.

The same results was reported by Takaaki *et al.* (2001) when he studied ultrastructure of the oocytes in Japanese eel *Anguilla japonica* during artificially induced.

3.1.3. Zona radiata (vitelline envelope):

The zona radiata is acellular layer, which appears coincident with cortical alveoli. Zona radiata consists of an outer layer, zona radiata externa (ZRE) and inner layer, zona radiata interna (ZRI). ZRE appears electron-dense, compact and acquires an architecturally complex structure of horizontal strata. ZRI consist of irregular membranous reticular network lamellae. With growth of the oocyte the zona radiata increase in thickness and become transverses perpendicularly by pore canals containing microvillar processes from both the granulose cells and the oocyte, fig 1a&b.

3.2.1. Fine structure of early atretic oocyte in ovary of female *C. rueppelli* after three days of second injection (9000 I.U.) of gonadotropin hormone only during spawning season:

Ultrastructure changes in early atretic oocyte of *Chrysichthys rueppelli* in enlargement of theca layer, which is composed of cells with small flattened irregular nucleus with dense chromatin material, with a very few and deformed organelle, like shrinkage cluster of deformed mitochondria without tubular cristae, large amount of tubular smooth endoplasmic reticulum and vacuoles. Fig. (2a&b). This epithelial layer, surrounding the oocyte, separated from the granulose cells by deformed incomplete basement membrane, while the follicular layer (granulose) was characterized by deformed irregular cells with electron dense (dark in appearance) vacuole and nucleus with dense chromatin material.

Moreover, the nucleus of these deformed granulose cells was defragmenter in some sites into two pieces, whereas other cells are without any nucleus. The granulose layer was observed in some sites with two rows of cells, while in other places recognized as a large elongated irregular cell extended from the theca layer to zona radiata layer Figs. (2 a&b).

In the present results after several injection of female *C. rueppelli* the absence of theca layer with hypertrophy of granulose with loose cytoplasmic organelle and zona radiata layers were considered a good sign of infertility of the fish due to absence of steroid

hormones which are used for reproduction, these results are in agreement with El-Zaeem and Assem (2004).

3.2.2. Fine structure of late atretic oocyte in ovary of female *C. rueppelli* after 14th days of last injection (1500 I.U. and 500µg) of LHRHa and gonadotropin hormone during spawning season:

The ultrastructure change of late atretic oocyte after treatment with hormone was characteristics with enlargement theca layer composed of more or less circular nucleus with of different size and dense chromatin material, (Figs. 3 a&b). Also, this deformed layer was observed with very deformed cytoplasmic organelle (mitochondria and large numbers of endoplasmic reticulum).

Deformed and ruptured basement membrane was distinguished and separating the theca layer and follicular layer, Fig. 3 a&b.

Dramatic alterations in the fine structure become apparent in the granulose cells during the late atretic oocyte this layer was characterized with loss of any cytoplasmic organelle, and distinguished with large vacuoles in between cells. The zona radiata becomes more compact without any connection between granulose cells. There was beginning of rupturing of zona radiata layer that faces the granulose layer (Figs. 3 a&b).

After several injections of female *C. rueppelli* the zona radiata lost the reticular network structure and assumed a layered structure of uniform electron density at the migratory nucleus stage, this result agree with Takaaki *et al.* (2001) and Assem (2003).

Through out all the experiments done in the present study, the structure of zona radiata in *Chrysichthys rueppelli* changes dramatically and the reticular network disappeared as the oocyte undergo over-ripening and going to atretic stage, as a result of injection. This is in general agreement with Burzawa *et al.* (1994) stated that the structural change was observed in zona radiata of European eel in which vitellogenesis was induced by treatment with carp gonadotropin.

The oocyte cytoplasm were recognized by a large liquified part of yolk granules as a deformed character due to treatment with several doses of hormones, as sign of atretic follicle. The granulose layer was characterized with lacking of any organelle.

Ultrastructure studies largely suggested that the granulose cells and special theca cells are the major cellular sites of steroid synthesis in the ovaries of fish,as reported by Matsuyama *et al*. (1991) in *Pagrus major*,; Balubid (2003) in *Oreochromis spilurus*, Gaber (2003) *in Bagrus bayad* and Kobayashi *et al*. (2005) in *Trimma okinawa*e.

Table (2): Concentration of serum estradiol (ng/ml) and testosterone (ng/ml) hormone in control female *C. rueppelli* during spawning season in relation to gonadosomatic index.

No. of fish	Months	Average GSI ± SD	Con. Of estradiol ± SD ng/ml	Con. Of testosterone ± SD ng/ml
6	April	20.5 ± 5.5	0.9 ± 1.22**	2.04 ± 1.7 **
6	May	37.8 ± 4.3**	0.6 ± 0.33	2.06 ± 1.8 **
6	June	30.5 ± 7.7	0.65 ± 0.22	0.7 ± 0.02
6	July	12.4 ± 2.5	0.55 ± 0.15	0.06 ± 0.02

**** Highly significant correlation at P< 0.05**

Table (3): Relationship between cumulative dose of "Pituitary extract and gonadotropin hormone/fish" and average concentration of serum estradiol and testosterone (ng/ ml) and gonadosomatic index in female *C. rueppelli* during spawning season (Exp. I, II)

Explanation	Experiment I (cumulative dose)							Experiment II (day by day dose)						
No. of fish	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Date	2-May	8-May	19-May	20-May	22-May	24-May	26-May	4 May	13 May	19 May	20 May	22 May	24 May	25 May
GSI value	18.3 ± 3.9	15.6 ± 2.6	11.3 ± 1.5	10.2 ±2.72	4.7 ± 1.09	4.2 ± 1.2	3.5 ± 2.01	17.8 ±2.5	15.3± 1.9	11.9±1.5	10.7 ±2.08	10.4 ±2.6	10± 2.01	9.7± 1.2
Total cumulative dose of hormone/fish	3500 I.U. + 2 Pit	8000 I.U. + 3 Pit	12500 I.U. + 4 Pit	15500 I.U. + 4 Pit-	15500 I.U. + 4 Pit	15500 I.U. + 4 Pit -	2500 I.U. + 1 pit	5500 I.U. + 2 pit	8500 I.U. + 3 pit	11500 I.U. + 3 pit	11500 I.U. + 3 pit	11500 I.U. + 3 pit-	-11500 I.U. + 3 pit	
Conc. of estradiol ± SD ng/ml	0.84 ± 0.85 **	0.77± 0.8**	0.7 ± 0.81**	0.56 ±0.74	0.35± 0.25	0.33 ± 0.15	0.3 ±0.12	0.8 ± 0.52**	0.72± 0.6**	0.65 ± 0.35	0.56 ±0.12	0.53 ±0.92	0.5± 0.78	0.42± 0.25
Conc. of Testosterone ± SD ng/ml	2.09 ± 2.3 **	1.98 ± 1.2**	1.7 ± 0.59	1.57± 1.25	0.012± 0.005	0.009 ± 0.008	0.009 ± 0.008	2.05 ± 1.5 **	1.87 ± 1.2**	1.56 ± 0.95	1.5± 0.82	1.2 ±1.73	0.56 ± 0.68	0.5± 0.5

** Highly significant correlation P< 0.05
SD: Standard Deviation

Table (4): Relationship between cumulative of gonadotropin hormone (Exp. III) or gonadotropin and LHRH (Exp.IV) in relation to the average Concentration of serum estradiol (ng/ml) and testosterone (ng/ml) and gonadosomatic index in female *C. rueppelli* during spawning season (Exp. III & IV).

Explanation	Experiment III (gonadotropin hormone)							Experiment IV (gonadotropin and LHRH)			
No. of fish	4	4	4	4	4	4	4	5	5	5	5
Date	10 May	17 May	26 May	27 May	29 May	31 May	2 June	4 June	9 June	10 June	13 June
GSI value	14.6 ± 3.5	13.5 ± 1.9	12.3 ± 1.5	11 ±2.9	11 ± .64	11 ± 2.1	11 ± 4.16	10.5 ± 6.6	20.8 ± 10.4	22.3 ± 9.8	27.5 ± 7.4
Total cumulative dose of hormone/fish	3500 I.U.	7500 I.U.	10500 I.U.	13500 .U.	13500 I.U.	13500 I.U.	13500 I.U.	1500 I.U. + 250 µm	1500 I.U. + 750µm	1500 I.U. +1000µm	1500 I.U. +1000µm
Conc. of esradiol ± SD ng/ml	0.7 ± 0.2 **	0.67± 0.7**	0.65 ± 0.2	0.62 ± 0.32	0.6 ± 0.1	0.6± 0.5	0.56 ± 0.36	0.6 ± 0.1**	0.38 ± 0.2	0.26 ± 0.2	0.18 ± 0.13
Conc. of Testosterone ± SD ng/ml	2 ± 0.32 **	1.84 ± 0.5 **	1.5 ± 0.25	1.35± 0.18	1.05 ± 0.48	1.25 ± 0.48	1.4 ± 0.12	0.5 ± 0.14 **	0.2 ± 0.05	0.06 ± 0.025	0.04 ± 0.01

** Highly significant correlation P< 0.05

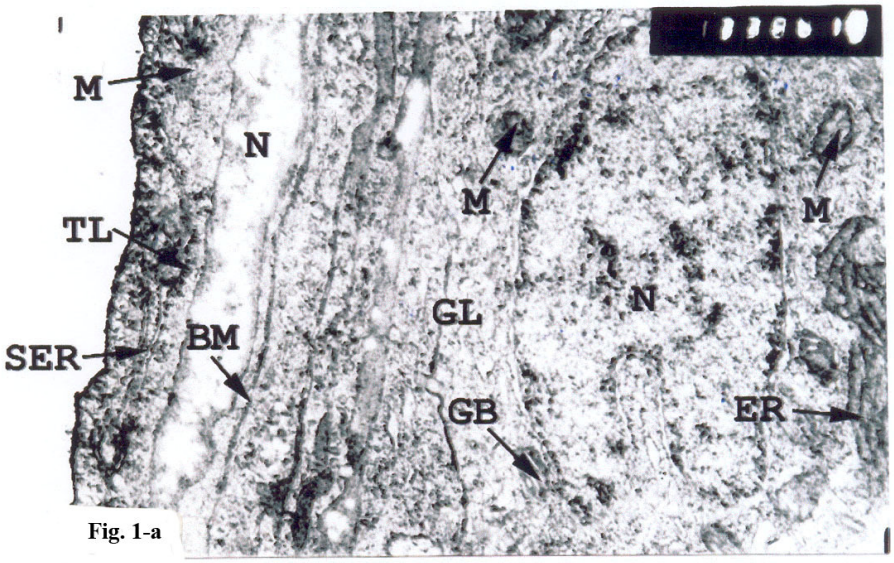

Fig. 1-a

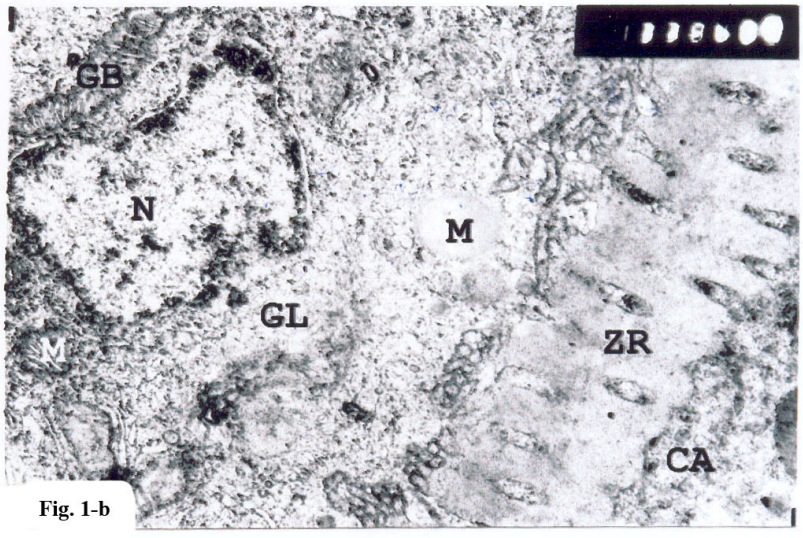

Fig. 1-b

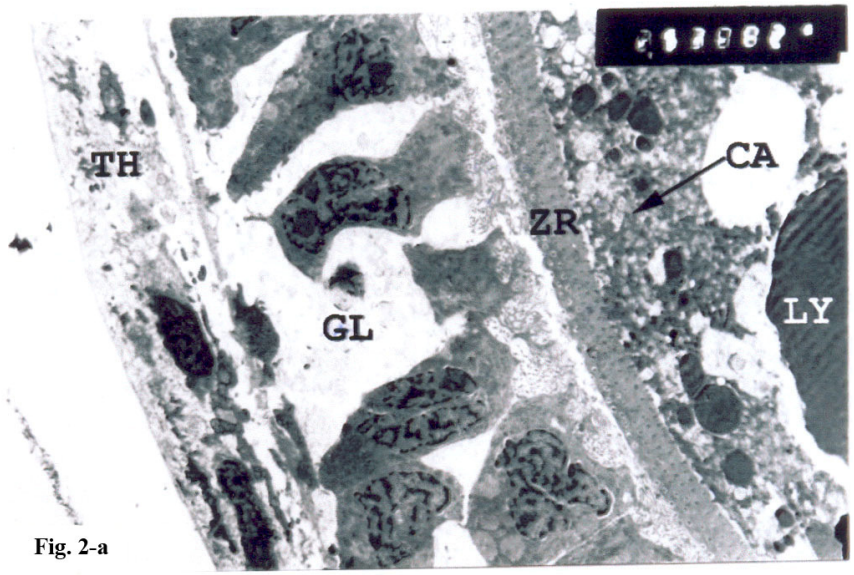

Fig. 2-a

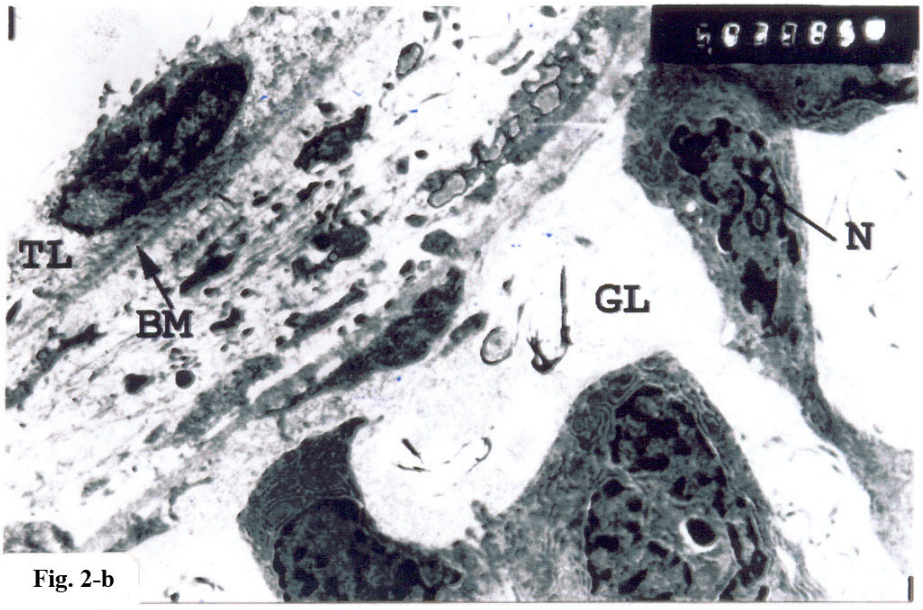

Fig. 2-b

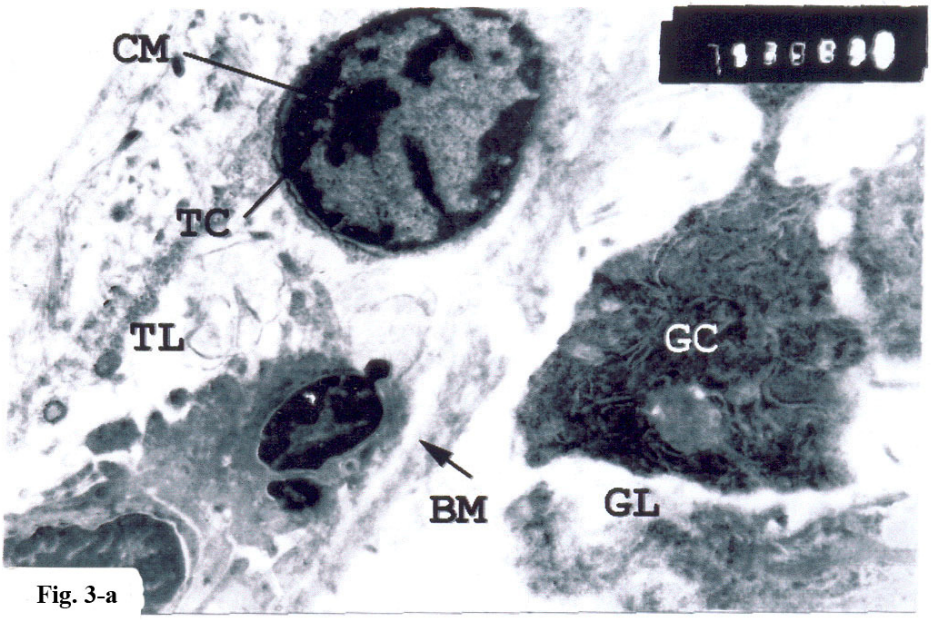

Fig. 3-a

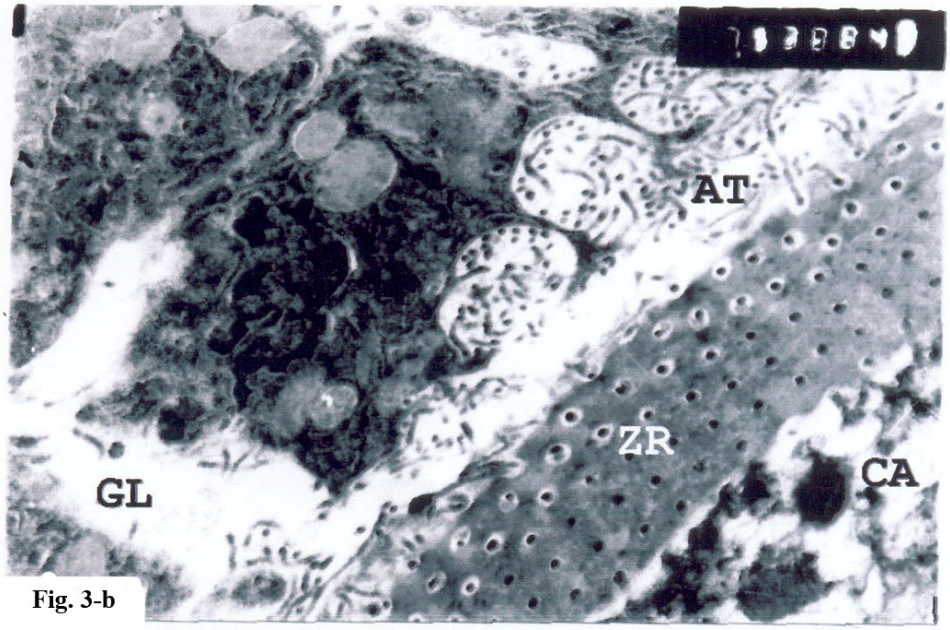

Fig. 3-b

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Explanation of figures:

Fig (1a): Electron micrograph of oocyte wall in the normal ovary of *C. rueppelli* at spawning season showing: Theca layer (TL). Nucleus (N), Basement membrane (BM), Mitochondria (M).Smooth endoplasmic reticulum (SER). Granulose layer (GL) Golgi bodies (GB). Stained with lead citrate (L.C.) and uranyl acetate (U.A.)(X7500).

Fig (1b): Magnification of normal wall showing: Granulose layer(GL). Zona radiate (ZR).Cortical alveoli (CA).Mitochondria(M) .Stained with (L.C. & U.A.) (X7500).

Fig (2a): Electron micrograph of early atretic oocyte wall of catfish freshwater *C. rueppelli* after three days of second injection (9000 I.U.) of hormone only showing, Enlargement of theca layer (TL).Granulose layer with deformed granulose cells (GL). Compact zona radiate layer (ZR). Cortical alveoli (C.A).Liquified Yolk Granules (LY)..Stained with L.C. & U.A. (X 17000).

Fig (2b): Magnification of early atretic wall after three days of second injection (9000 I.U.) of hormone only showing, Theca layer with small flattened irregular nucleus with dense chromatin material and deformed organelle (TL). Nucleus (N).Granulose layer (GL) with deformed granulose cells. Basement membrane (BM). Stained with L.C. & U.A. (X 25000).

Fig (3a): Electron micrograph of late atretic oocyte wall after 14^{th} days of last injection (1500 I.U. and 500µg) of LHRHa and gonadotropin hormone showing: Theca layer (TL) with circular nucleus. Theca cell (TC) rich in chromatin material (CM).Granulose layer (GL), Deformed granulose cells (GC), Rupture basement membrane (BM). Stained with L.C. & U.A. (X 25000).

Fig (3b): Magnification of late atretic oocyte wall after 14^{th} days of last injection (1500 I.U. and 500µg) of LHRHa and gonadotropin hormone showing, Granulose layer (GL) with 2 rows of deformed cells and atretic tissue (AT). Compact zona radiate (ZR) and cortical alveoli (CA).Stained with L.C. & U.A. (X 25000).