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The Role Of Corpuscles Of Stannius During Ovarian Cycle In Female *Mastacembelus Armatus*

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Abstract:-*Mastacembelus armatus* (Lacepede) is a seasonal breeder with a single breeding peak in a year which extends from late July to September. Plasma calcium and stanniocalcin levels were determined throughout the reproductive cycle. The level of both plasma calcium and stanniocalcin increases during advanced maturation phase with simultaneous increses in gonadosomatic index reaching the peak during spawning phase. It gradually decreases after spawning and significantly reduced during postspawning and resting phase. Histochemical analysis also indicates the seasonal variation in the activity of the gland, corpuscles of Stannius (CS) with respect to the reproductive cycle. CS exhibit signs of hyperactivity during prespawning and spawning phase however its reduced activity during post spawning phase and almost inactivity were noted during spent phase. 17-β estradiol administration for 10 days also induced hypercalcemia in female M. armatus fed with calcium deficient food. An increased level of stanniocalcin in 17-β estradiol treated fish reflects the hyperactivity of CS. Therefore it can be concluded that hyperactivity of CS is due to elevated calcium level through extra intestinal routes that accompanied ovarian maturation. Stanniocalcin from hyperactive CS reduces the increasing level of plasma calcium in the fish.

Keywords:*Mastacembelus armatus* (Lacepede), Reproductive cycle, Corpuscles of Stannius, Plasma calcium, Stanniocalcin, 17-β estradiol.

INTRODUCTION

The gonadal maturation in fishes is associated with increase in plasma calcium levels and phosphate levels (Guerreiro et al. 2002). Several authors have correlated the increased blood calcium content with ovarian maturation in case of many fishes including some Indian freshwater species also from time to time (Fleming et al. 1964; Oguri and Takada 1967; Woodhead 1968; Ahmad and Swarup 1990; Srivastava and Srivastava 1994, 1998). There exists a difference in the increase of plasma calcium level with respect to sex of the fish. In freshwater murrel Channa punctatus it was found that males exhibited no change in plasma calcium levels throughout the year in correlation with testicular maturation but females showed marked seasonal changes in plasma calcium level which were associated with ovarian maturation (Srivastava and Srivastava 1998).

It was proposed that increase in plasma calcium during ovarian maturation is due to increased secretion of estrogen. (Whitehead et al. 1980; Scott et al. 1983; Fosteir et al. 1983; Guerreiro et al. 2001; Gillespie and Peyster 2004). It was also reported that administration of estradiol induces hypercalcemia in fishes (Ho and Vanstone 1961; Fleming et al. 1964; Yaron et al. 1977; Pang and Balbontin 1978; Bjornsson and Haux 1985; Carragher and Sumpter 1991; Persson et al. 1997; Guerreiro et al. 2002; Gillespie and Peyster 2004)

Increase in blood calcium resulted from higher activity of gonadal hormones during sexual maturation may lead to impairment of various physiological activities. Therefore it becomes essential to maintain extracellular calcium concentration at a relatively constant level, and for this endocrine systems play an important role in calcium metabolism throughout the vertebrate classes. Calcium in different forms in extracellular fluid together constitute about 10 mg/dl (2.5mM), out of which half of this total is in ionic form.

It is evident that fishes regulate their serum calcium efficiently but the endocrine systems involved are different from those in tetrapods. Calcium homeostasis is mainly mediated through the secretions of ultimobranchial gland (Bonga and Pang 1991; Rubi et al., 2009), corpuscles of Stannius (Srivastava et al. 1996; Wei-guo and Kun-ying 1999; Clark et al. 2002; Suzuki 2005; Hang and Balment 2005; Shin and Shon 2008) and pituitary (Harris et al. 2004; Mukherjee et al. 2004; Takahashi et al., 2008)

In fishes plasma calcium is regulated by the hypocalcemic hormone, STC-1 which are synthesized and secreted by the kidney associated gland the corpuscles of Stannius (CS).The corpuscles of Stannius (CS) are unique calcitropic endocrine gland, found exclusively in teleostean and holostean fishes. Fontaine for the first time studied the effect of stanniectomy in eels and found that it results in significant hypercalcemia (Fontaine 1964). Administration of extracts of CS in to stanniectomized eels restored the

serum electrolyte level to normal (So and Fenwick 1979). The hypercalcemia induced by stanniectomy was also corrected by autotransplantation of CS in eels (Jones et.al. 1996). Injection of CS homogenate or transplantation of CS or hypocalcin therapy was effective in restoring serum calcium level in stanniectomized killifish in seawater (Lafeber et al., 1988). It is now well established fact that corpuscles of Stannius secrete hormone stanniocalcin (STC-1) which is an inhibitor of gill Ca2+ uptake in most of the bony fishes and induce hypocalcemia in most of the bony fishes. A second STC, STC-2, has been identified recently in fish (Luo et al. 2005), but current evidence suggests that it does not play a significant role in calcium regulation (Wagner and Dimattia 2006).

Several earlier workers have reported that CS hyperactivity during gonadal maturation in female fishes is in response to an increase in serum calcium level which in turn is the effect of increased secretion of estradiol (Urasa and Bonga 1987). An increase in the amount of plasma vitellogenin was observed during ovarian maturation (Crim and Idler 1978) as well as by estradiol- 17β administration (Carragher and Sumpter 1991; Nath et al. 1992; Nagler et al. 1987; Benfey et al. 1989). Vitellogenesis is under the hormonal control of estradiol and is accompanied by a marked increase of plasma calcium and phosphate. Phosphate binds covalently to serine residues of the phosvitin moiety of vitellogenin to which Ca2+ is ionically bound. The vitellogenin molecules are rich source of calcium and phosphorus. As maturation proceeds gross changes in plasma calcium level occurs because one atom of calcium is associated with every protein phosphate group in vitellogenin complex (Wallace 1970). Thus during during the process of vitellogenesis, plasma levels of E2, and total calcium are positively correlated. Estradiol increase the level of plasma calcium by acting directly on gills or intestine as evidenced by the presence of ER mRNA in fish gills (Filby and Tyler 2005; Luo et al. 2005) as well as in intestine (Filby and Tyler 2005; Wang et al. 2005). It may act indirectly via some endocrine factor like PTHrP or a related factor responsive to E2 (Fuentes J, et al. 2007).

The fish M. armatus is one among the economically important fish in rural parts of India (Verma and Murmu 2010), therefore for evaluating commercial potentialities of its stock, life history, culture practice and management of its fisheries it becomes necessary to know every aspect of its reproductive physiology. As no previous work has been done in India on calcium regulation in M. armatus during reproductive cycle, the present study was undertaken to analyze the changes in its plasma calcium and stanniocalcin levels with relation to its reproductive cycle. The reproductive cycle as well as the seasonal activity of the gland CS was traced out with simultaneous determination of serum calcium level with the hypothesis that the hormone stanniocalcin plays major role in calcium homeostasis in this species as in others. The female fishes were also injected with estradiol- 17β during the inactive phase of their reproductive cycle to confirm its role in total plasma calcium level and stanniocalcin level elevation.

Materials and Methods: Adult specimens of female M. armatus served as the material for this research work. Specimens were collected from Dimna Lake, Jamshedpur (India). Dimna Lake (22° 51' 43.53"N and 86° 15' 24.68"E) is located 13km away from the limits of city Jamshedpur in the district of E. Singhbhum, Jharkhand (India). Ten adult fish were randomly sampled every month throughout the year using the beach seines, gill nets or stake tapes and transferred immediately to the laboratory where body weight of each specimen was measured.

For estimation of plasma calcium and stanniocalcin the collected specimens were anesthetized with phenoxyethanol, the tail was severed and the blood samples were collected from the caudal vessels using a heparinized syringe. After centrifugation (1 min, 10,000g) total plasma calcium concentrations were measured colorimetrically using a calcium kit (Sigma Diagnostics). A competitive ELISA technique (Mayer-Gostan et al. 1991) which is based on competition between free STC in standard or plasma samples and STC immobilized on microtiter plates for the STC antibodies was used for determination of plasma staniocalcin level.

For the determination of gonadosomatic index the fishes were dissected, gonads excised and weighed (gm). Bouin's solution (75ml. saturated picric acid, 25 ml. 40% formaldehyde and 5 ml. glacial acetic acid) was used to preserve the gonads. After 10 to 16 hours (depending upon the size of the sample) they were placed in to 70% alcohol. Before being embedded in paraffin, the tissues were dehydrated in increasing concentration of alcohol (90% and absolute alcohol) and sections of 5-7µm were prepared with a microtome. The sections were stained in haematoxylin and counterstained in eosin to observe the histological changes in the ovaries to confirm the maturity stage.

To study seasonal histological changes occurring in CS same fixative (Bouin's fluid) was poured over the exposed kidney of sacrificed fishes. The posterior part of the kidney along with the gland is removed and kept for recommended period in the same fixative. After dehydration and paraffin embedding the tissues were sectioned at 5-7 µm and mounted on slides, where they were stained with different techniques: haematoxylin/eosin, aldehyde fuschin and periodic acid Schiff?s reagents.

To observe changes occurring in the nucleus, nuclear diameter of CS (µm) were measured with the help of image analyzer microscope (Metavis image analyzing system with Meltmage Lx Software). 25 nuclei were randomly selected from every fifth section of the gland, thus the total number of the nuclei measured were always more than 250 for each individual.

An experiment was conducted to analyze the effect of 17 β-estradiol administration. 20 live, adult and healthy female specimens of M. armatus were caught from Dimna Lake, Jamshedpur, during the month of December. They were acclimatized to laboratory conditions (temperature, 27°C-32°C; light:Dark photoperiod, 12.004h:12.00h). During the experiment the fishes were fed calcium-deficient diet to know the exact route of elevated calcium level if any. The residual calcium concentration of the food was less than 0.1 g · kg-1. After 15 days the fishes were divided between two groups with each group containing 10 fishes and kept in

separate aquaria. One group was kept in tap water and injected with 0.1 ml of vehicle (peanut oil). The other group was also kept in tap water but was administrated with 100 µg of 17 β-estradiol (sigma) in 0.1 ml of vehicle. The fishes were injected intraperitonially on alternate days and to avoid diurnal variation the injections were given at the same time of the day. The blood samples were collected for serum calcium and stanniocalcin estimation after 10 days. At the same time the gland corpuscles of Stannius, was also removed for histological analysis. The differences between control and experimental values were tested for statistical significance. Analysis of variance was used to determine statistical significance. Significance was accepted at $P<0.05$
Results: On the basis of changes in gonadosomatic index, ova diameter and developmental stages of oocytes the ovarian cycle of M. armatus can be divided in to following six stages. Based on histological changes a seasonal cycle of CS can also be observed which runs simultaneously along with this reproductive cycle.

1. Resting phase or Phase I (December, January and February): This phase can be considered as the stage of inactivity for ovaries. Ovaries are small, shrunken and without any visible ova. Internally many primary oocytes and oogonia within ovigerous folds can be seen . Soon the single nucleoli begin to multiply in number and the amount of cytoplasm increases. The average diameter of oocyte was found to be 0.2 + 0.01 mm in this stage.

During this phase CS enters in a diminished activity evidenced by single or two layers of epithelial lining and empty central lumen . A considerable reduction in the nuclear diameter of CS cells (4.261+ 0.0291 µm) and degeneration of glandular cells in the central part of the lobules was observed during resting phase.

2. Early maturation or Phase II (March and April): The nucleoli arranged along the inner surface of the nuclear membrane . The diameter of oocytes (0.6 + 0.01 mm) and amount of basophilic ooplasm increases. Characteristic yolk nucleus of Balbiani can be seen in juxtranuclear area of ooplasm .

The lobules of CS are lined by few layers of cells with nuclear diameter of 5.119 + 0.134 µm and a large lumen was clearly visible inside the lobules during early maturation phase .

3. Advance maturation phase or Phase III (May): Yolk vesicles appear towards the periphery of oocytes . Extravesicular yolk can be seen in perinuclear area of ooplasm. The vascular supply also increased. The average diameter of oocyte was noted as 1.2 + 0.09 mm in this stage.

The lumen inside lobules are occupied by proliferating cells. Nucleoli become prominent and the nuclei exhibit an increase in size. Due to increased granulation the staining capacity of cytoplasm towards aldehyde fuschin and periodic acid Schiff?s reagents was found to be increased. All these are indications of hyperactivity of CS.

4. Prespawning phase (June and early July): The nucleus becomes indistinct. The whole of the ooplasm is filled with protein yolk bodies. Cortical alveoli arrange themselves in 3 or 4 layers at the periphery of oocytes. The oocytes attains an approximate diameter of 3.5 + 0.23 mm.

CS during the prespawning phase of gonadal cycle is characterized by hyperactivity of CS evidenced by hyperplasia of epithelial cells. Lumen of the lobules is completely filled by proliferating cells which is an indication of hyperplasia of epithelial cells. Size of the nuclei were considerable increased (6.557 + 0.291µm) and staining capacity of the cytoplasm towards aldehyde fuschin and periodic acid Schiff?s reagents as a result of increased granulation further increases. Blood vessels become prominent. Disappearance of cytoplasmic granules from epithelial cells of lobules located towards periphery was observed which is an indication of hormone release.

5. Spawning phase (Late July, August and September): The gonadosomatic index as well as ova diameter attains their peak value (5.0 + 0.45 mm) during late July, August and September (fig 1 and 2). Small protein yolk bodies in ooplasm which appeared during advance maturation phase coalesce to form large globules. A gradual fall in gonadosomatic index is recorded with the advancement of spawning phase. Broken ovigerous lamillae as well as indications of released ovum are clearly visible .

Maximum activity of CS was observed during this phase. CS lobules become completely filled with proliferated cells and no lumens were visible inside lobules. Few cells located in the central lobules of CS undergo vacuolization which may occur due to exhaustion after hyperactivity.

6. Postspawning phase (October and November): The ovaries collapsed during the month of October and November. Unovulated eggs undergoing resorption and corpora atretica in vascular stroma can be frequently seen in postspawning phase. Cells of discharged follicles get resorbed in the ovarian tissue and ultimately disappear.

CS of M. armatus during the prespawning phase of gonadal cycle is characterized by continuous reduction in its activity with the advancement of postspawning phase, reduction in the nuclear diameter of lobular cells, degeneration of lobular cells and appearance of cellular debris in the luminal space.

A remarkable increase in the serum calcium (Fig. 1) and stanniocalcin levels (Fig. 2) were observed during advanced maturation phase with a corresponding increase in the gonadosomatic index reaching the peak during prespawning phase and spawning phase. It gradually decreases with spawning and significantly reduced during postspawning and resting phase.

Administration of 17-β estradiol for 10 days induced hypercalcemia in female fish. A significant difference ($P< 0.05$, one-way ANOVA) in plasma calcium and stanniocalcin levels were observed between 17-β estradiol injected female fishes and controls (Table 1).

The epithelial linings of CS strands are

characterized by single or two layers of cells and a central lumen in vehicles injected controls. The cells exhibit mild granulation towards the periphery of the corpuscular strand. Whereas the CS in 17-β estradiol injected female M. armatus exhibit hyperactivity characterized by hypertrophy and hyperplasia of the epithelial cells, intense granulation towards the periphery of the corpuscular strands and a considerable increase in the nuclear diameter (Table 1).

DISCUSSION:

In fishes there exists a close relationship between gonadal maturation, plasma calcium level and corpuscles of Stannius (CS) activity. A remarkable morphological changes in CS of Chum Salmon, Onchorhynchus keta was observed by Hiroi (1970) during gonadal maturation. Such morphological changes in CS are closely related to the ovarian cycle (Bonga 1975). The Present study on plasma calcium and stanniocalcin levels of female Mastacembelus armatus (Lacepede) also revealed that the activity of CS varies along with reproductive cycle. The gonadosomatic index, ova diameter frequencies and histological details of the ovary of M. armatus indicate its marked seasonal cycle with a single breeding peak in a year which extends from late July to September. Comparatively large sized cells of CS with large nucleus and increased cytoplasmic granulation can be considered as an indication of high activity of the gland with enhanced production of its hormone stanniocalcin. CS becomes hyperactive during advance maturation and prespawning phase evidenced by increase in nuclear diameter, elongation and compactness of corpuscular cells and increased granulation of cells. However reduced activity of CS during post spawning phase and almost their inactivity was noted during spent phase. An increase in the level of plasma stanniocalcin was also observed during advance maturation and prespawning phase.

At the same time a remarkable increase in the serum calcium level was also observed during the advanced maturation phase reaching the peak during prespawning phase and spawning phase. It gradually decreases with spawning and significantly reduced during postspawning and resting phase. It has been suggested earlier that female fish develop hypercalcemic during sexual maturation due to increased estrogen secretion by ovary (Bjornsson, 1985). Ovariectomy leads to fall in plasma calcium level as well as reduction in the size of CS (Swarup 1985). Inactivation of CS after ovariectomy can be restored by administration of estradiol (Pandey, 1988). Increase in the activity of CS in the present study on female M. armatus during advance phases of reproductive cycle as well as on estradiol administration clearly indicates that there exists a close relationship between its activity and plasma calcium level. It has been reported that plasma calcium increases during advance phases of reproductive cycle as well as on estradiol administration in fishes due to the appearance of calcium containing yolk protein, vitellogenin which is synthesized in liver, secreted to the blood and transported to ovaries (Bjornsson and Haux, 1985). Estradiol induced hypercal cemia stimulates CS to secrete its antihyper calcemic hormone, stanniocalcin.

The gill and intestine are the sites for calcium uptake in fish, although the gills are supposed to play major role (Flik et al. 1996). Thus estradiol may increase the level of plasma calcium by acting directly on gills (Filby and Tyler 2005; Luo et al. 2005) or intestine (Filby and Tyler 2005; Wang et al. 2005). However, in the present study the fish were fed a calcium deficient diet which suggests that in M. armatus extra intestinal routes were responsible for the hypercalcemia.

It can be concluded that in M. armatus the process of spawning is positively correlated with the activity of the gland CS . It becomes clear from the experiments that CS exhibit hyperactivity in response to elevated calcium level that accompanied ovarian maturation. Stanniocalcin which is an antihypercalcemic hormone secreted from hyperactive CS reduces the increasing level of serum calcium in this fish. However the role of CS is evaluated in this study on M. armatus and its role in calcium homeostasis was clearly established, but it is also recommended that the role of PTHrP in calcium regulation should also be analyzed in this species for complete understanding of calcium homeostasis which is still lacking.

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DECLARATION:

We declare that the rules and regulation framed by the government of India were strictly followed during the research work.

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Table No. 1: Changes in serum calcium (in mg/100ml), stanniocalcin levels and of nuclear diameter of CS cells (in µm) female M. armatus following 17 β- estradiol administrations.

	Plasma Calcium Level (mg/100ml)	Plasma Stanniocalcin Level (ng/ml)	Nuclear diameter of CS cells (in µm)
Controls	11.235± 0.175	190	4.550± 0.154
17β- estradiol injected	18.220± 0.233	295	5.651± 0.118

Each value represents mean + SD of 6 specimens

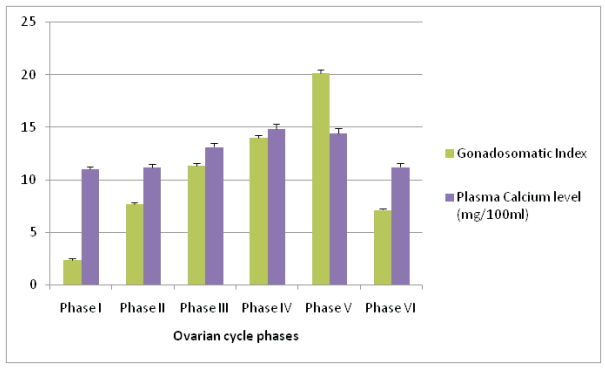

Fig 1: Graph showing changes in plasma calcium level during different phases of ovarian cycle of M. armatus with a corresponding change in gonadosomatic index.

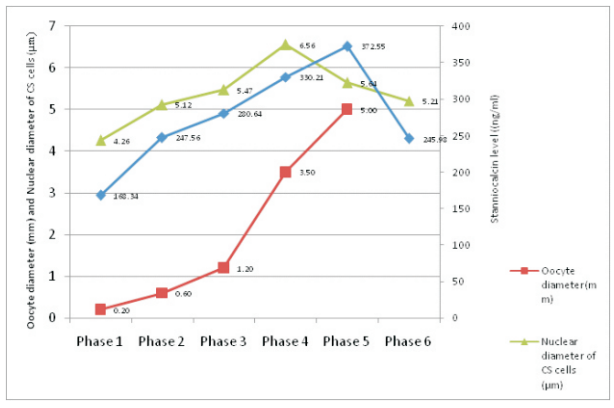

Fig 2: Graph showing changes in oocyte diameter (mm), nuclear diameter of CS cells (µm) and plasma stanniocalcin level (ng/ml) during different phases of ovarian cycle. Each value represents means + SE of six specimens.