PRIMARY ARTICLE

Hypocalcemic Role Of Calcitonin In Female *Mastacembelus Armatus* (lacepede), During Reproductive Cycle

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ABSTRACT

On the basis of various histological changes the ovarian cycle of *Mastacembelus armatus* can be divided in to five different phases i.e. resting, maturation, prespawning, spawning and postspawning phases. Seasonal cyclic changes in the plasma calcium and calcitonin level were found associated with this ovarian cycle. An increase in their level occurs during maturation phase with a corresponding increase in the gonadosomatic index reaching the peak during prespawning phase and spawning phase. On the other hand a significant decrease was noted during postspawning and resting phase. Histological changes in the ultimobranchial gland also revealed the seasonal variation in its activity. It exhibits various signs of hyperactivity like Maximum increase in the population of secretory cells, decrease in size of lumen and dilation of blood vessels during prespawning and spawning phase. Disruptive follicular organization was noted during resting and post spawning phase. 17-β estradiol administration resulted in hypercalcemia and increase in plasma calcitonin level in *M. armatus* fed with calcium deficient food. Therefore it can be concluded that UBG is mainly concerned with reproductive physiology of the fish *M. Armatus* by lowering down elevated plasma calcium level accompanied through extra intestinal routes during ovarian maturation.

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KEYWORDS :
Ultimobranchial gland, Hypercalcemia, Ovarian cycle.

INTRODUCTION :

Calcitonin (CT) is one of the important calcium regulating hormones in mammals produced mainly by the parafollicular 'C' cells of the thyroid gland. In fishes calcitonin CT is secreted by a specialized gland, known as the ultimobranchial (UBG) gland and along with stanniocalcin secreted by Corpuscles of Stannius (Srivastava et al., 1996; Wei-guo and Kun-ying, 1999; Clark et al., 2002; Suzuki, 2005; Hang and Belment, 2005; Shin and Shon, 2008) and prolactin secreted by pituitary (Harris et al., 2004; Mukherjee et al., 2004a; Takahashi et al., 2008) regulate plasma calcium level very efficiently (Mukherjee et al., 2004 b)

In fishes total calcium in extracellular fluid is about 10 mg/dl (Abbink et al., 2006). Increase in its level may lead to myocardial dysfunction and lethargy as well as reduction in neuromuscular transmission (Bonga and Flik, 1993). Thus it should be maintained at relatively constant level to maintain normal physiology of the body.

The role of CT on calcium homeostasis in fish has long been controversial. The hypocalcemic effect of UBG extracts of various fishes in rats have been documented several times (Takei et al., 1991; Sasayama et al., 1992;1993). At the same time no such effect was observed on allogenic injection of UBG extract in various species of fishes (Bonga, 1981; Srivastava et al., 1989) .Hypocalcemic effect of salmon/porcine calcitonin in fishes has been also reported (Swarup et al., 1991; Srivastava et al., 1998). It

has been also proposed that hypocalcemic or hypercalcemic role of CT in fishes may depends upon the dose or species (Fouchereau-Peron et al., 1987). A decrease in the calcium uptake in fishes has been reported with the exposure of cadmium (Baldisserotto et al., 2004). On the other hand cadium treatment in fish *Heteropneustes fossilis* resulted in hypocacemia and ultimately degeneration of UBG (Rubi et al., 2009)

Now it is a well known fact that gonadal maturation in fishes is associated with increase in plasma calcium content (Ahmad and Swarup, 1990; Srivastava and Srivastava, 1994, 1998; Guerreiro et al., 2002). This increase in plasma calcium level is more pronounced in females as compared to males (Srivastava and Srivastava, 1998) because of increased secretion of estrogen (Galli-Gallardo et al., 1977; Whitehead et al., 1980; Scott et al., 1983; Fosteir et al., 1983; Guerreiro et al., 2001; Gillespie and Peyster, 2004). Previously it was also suggested that in fishes hypercalcemia can be induced by administration of estradiol (Carragher and Sumpter, 1991; Persson et al., 1997; Guerreiro et al., 2002; Gillespie and Peyster, 2004).

Hyperactivity of UBG during gonadal maturation has been reported very early by Ahmad and Swarup (1988). This hyperactivity may be shown by hypertrophy and hyperplasia of cells (Oguri, 1973) as well as increased calcitonin level especially in females (Fouchereau-Peron et al., 1990). Administration of 17 β-estradiol induces UBG to release CT(Suzuki et al., 2004) whereas salmon calcitonin was found to stimulate the secretion of 17β-estradiol in vitellogenic ovarian follicles of carp *Cyprinus carpio*(Paul et al., 2008). Although these studies indicate a relationship between CT and fish reproduction there has been no clear evidence on the role of calcitonin in female *M. armatus* during its reproductive cycle. Therefore the present study was designed to confirm a relationship between calcitonin and estrogen-mediated reproductive cycle in females *M. armatus*.

Materials And Methods:

Adult specimens of female *M. armatus* were collected from Dimna Lake, Jamshedpur (India). Random sampling was done every month throughout the year and 12 specimens were collected monthly using the beach seines, gill nets or stake tapes. After collection the fishes were transferred immediately to the laboratory where body weight of each specimen was measured.

For estimation of plasma calcium and calcitonin the collected specimens were anesthetized with phenoxyethanol. The tail was severed and with the help of heparinized syringe blood samples were collected from the caudal vessels. After centrifugation (1 min, 10,000g) total plasma calcium concentrations were measured colorimetrically using a calcium kit (Sigma Diagnostics). Plasma calcitonin levels were measured using ELISA.

The fishes were dissected, gonads excised and after weighing were preserved in Bouin's solution (75ml. saturated picric acid, 25 ml. 40% formaldehyde and 5 ml. glacial acetic acid). After 10 to 16 hours they were placed in to 70% alcohol for dehydration. Before being embedded in paraffin, the tissues were completely dehydrated in increasing concentration of alcohol and sections of 5-7µm were prepared with a microtome. Haematoxylin and eosin was used for staining. The prepared slides were used to confirm the maturity stage of the ovary.

To study seasonal histological changes occurring in UBG the tissue between the heart and the oesophagus were dissected out and fixed in Bouin's solution. After dehydration and paraffin embedding the tissues were sectioned at 5-7 µm and mounted on slides, where they were stained with haematoxylin/eosin.

Nuclear diameter of UBG cells were measured with the help of image analyzer microscope (Metavis image analyzing system with Meltmage Lx Software). 30 nuclei were randomly selected from every fifth section of the gland. In this way a total of about 300 nuclei measured for each individual.

Simultaneously few experiments were conducted to analyze the effect of 17 β-estradiol administration on the activity of UBG. 20 live, adult and healthy female specimens of *M. armatus* were collected from Dimna Lake, Jamshedpur, during the month of December which the spent period for this fish. They were acclimatized to laboratory conditions

(temperature, 27°C-32°C; light:Dark photoperiod, 12.00h:12.0h). During the experiment the fishes were fed calcium-deficient diet with residual calcium concentration less than 0.1 g · kg-[1]. After acclimation they were divided into two groups each containing 10 fish and subjected to the following treatments:

Group A. Fish were kept in tap water and injected with 0.1 ml of vehicle (peanut oil).

Group B. Fish were maintained also 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 daily at the same time of the day to avoid diurnal variation.

The blood samples were collected from fish of both groups for serum calcium and calcitonin estimation after 15 days. At the same time the UBG 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:

Various parameters likegonadosomatic index, ova diameter, oocyte developmental stage and other histological details of the ovary revealed that the reproductive cycle of *M. armatus* consists of five different phases. UBG in *M. armatus* is a follicular (Fig. 1) structure in which various histological changes can be seen along with seasonal cycle of ovary.

1. Resting phase or Phase I (December, January and February): This is the spent phase for the ovaries in which they appear smaller in size and without any visible ova. Finger like internal projections containing many primary oocytes and oogonia are visible inside the ovary (Fig.2). The average diameter of oocyte in this phase was found to be 0.3 mm.

Well organized follicles are not seen inside UBG (Fig. 3). Cells with reduced nuclear diameter were seen during this stage (Fig. 9).

2. Maturation or Phase II (March to May): A single nucleoli began to multiply in number and arranged along the inner surface of the nuclear membrane (Fig.4). The diameter of oocytes (0.9mm), amount of cytoplasm and vascular supply considerably increased during this stage. Appearance of yolk nucleus of Balbiani in juxtranuclear area of ooplasm (Fig.5) and yolk vesicles towards the periphery of oocytes is one of the characteristic features of this stage. Slight increase in the activity of the UBG can be seen during maturation phase. Follicles reappear with pseudo stratified epithelial lining and follicular cells with increased nuclear diameter (Fig. 9). Blood vessels become more prominent while nuclear pycnosis and vacuolization can be seen among follicular cells.

3. Prespawning phase (June and early July): Entire cytoplasm is filled with yolk bodies making nucleus indistinct (Fig.6). Cortical alveoli in 4 or 5 layers can be seen at the periphery of oocytes which attains an approximate diameter of 4.0 mm during prespawning phase.

UBG becomes hyperactive evidenced by increase in the number of secretory cells as well as nuclear size of these cells (Fig 9). Sometimes epithelial cells undergoing hypertrophy and hyperplasia were also seen.

4. Spawning phase (Late July, August and September): The ovary starts to release fully ripened eggs (Fig 7) having diameter around 6.0 mm during late July. Maximum value of gonadosomatic index was noted during early days of August whereas a gradual fall in its value was recorded with the advancement of spawning phase. Nucleus lost its identity as the entire cytoplasm is filled with large yolk globules formed from small yolk bodies of prespawning phase.

Maximum increase in the population of secretory cells, decrease in size of lumen, dilation of blood vessels and total enlargement in the size of UBG (Fig 8) indicates the peak maximum activity of this gland.

5. Postspawning phase (October and November): After ovulation the ovaries collapsed during the month of October and November. Unovulated eggs in the form of corpora atretica can be seen in postspawning phase. Cells of discharged follicles get resorbed in the ovarian tissue.

Minimized activity of UBG during postspawning phase was exhibited by cytolysis and nuclear pycnosis as well as appearance of appearance of cellular debris in the luminal space of follicles.

A considerable increase in the level of serum calcium (Fig. 9) and calcitonin (Fig. 10) were recorded with the advancement of maturation phase reaching the peak during prespawning

and spawning phase. Its gradual decreases with spawning and significant reduction was noted in postspawning and resting phase.

A significant difference ($P < 0.05$, one-way ANOVA) in plasma calcium and calcitonin levels were observed between 17-β estradiol injected female fishes and the fish which were kept in tap water and injected with 0.1 ml of vehicle (Table 1). Administration of 17-β estradiol for 15 days induced hypercalcemia in female fish.

Follicular arrangement of UBG with a single layer of epithelial cells with moderate staining property was observed in vehicles injected controls. The UBG in 10 days estradiol injected female *M. armatus* exhibit various signs of hyperactivity like considerable increase in the nuclear size (Table 2) and population of secretory cells, their dense staining towards the basement membrane.

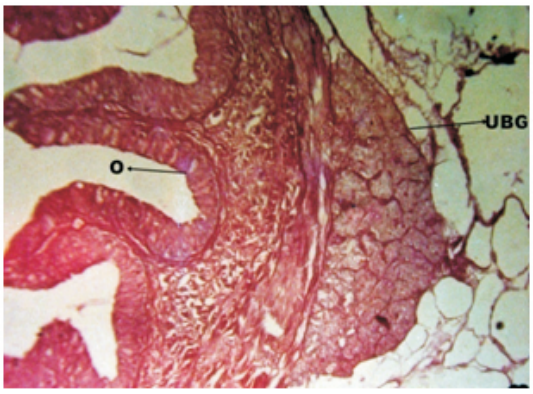

Fig -1:Ultimobranchial gland (UBG) of female *M. armatus* lying dorsal to oesophagus (O)

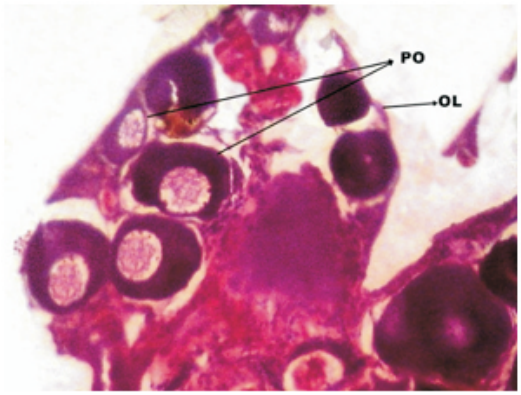

Fig -2 :Ovigerous lamellae (OL) inside ovary containing many primary oocytes (PO)

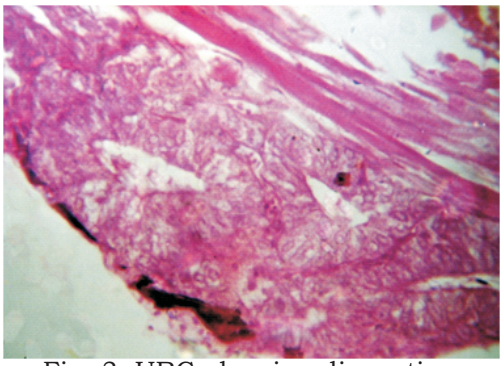

Fig -3 :UBG showing disrupting follicular organization during resting phase of ovary.

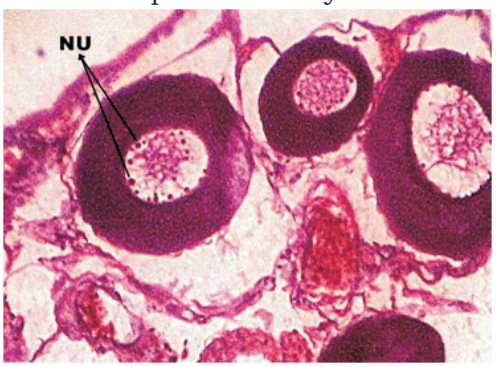

Fig -4 : Nucleoli (NU) arranged along the inner surface of the nuclear membrane in a developing oocyte

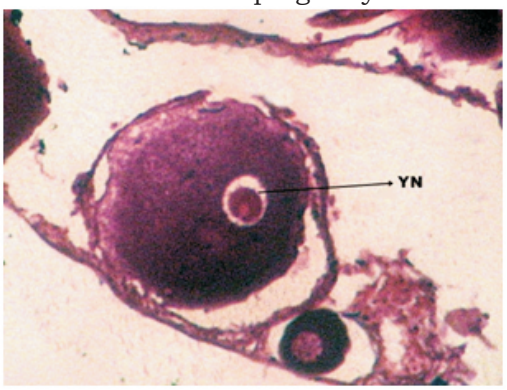

Fig- 5:Appearance of yolk nucleus of Balbiani in juxtranuclear area of ooplasm during maturation phase

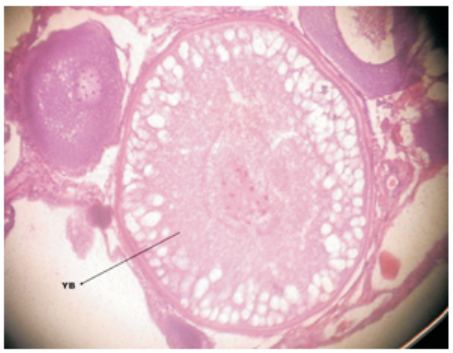

Fig -6 :Cytoplasm of egg filled with yolk bodies (YB) during prespawning phase

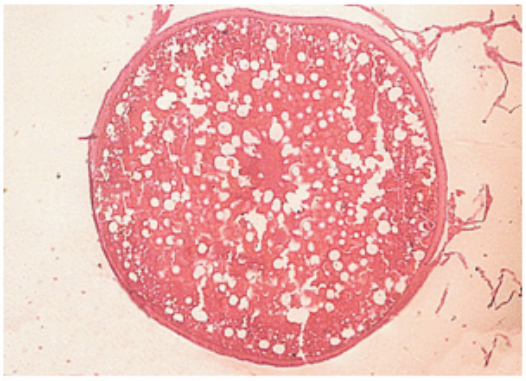

Fig -7 :Fully ripened egg observed during spawning phase.

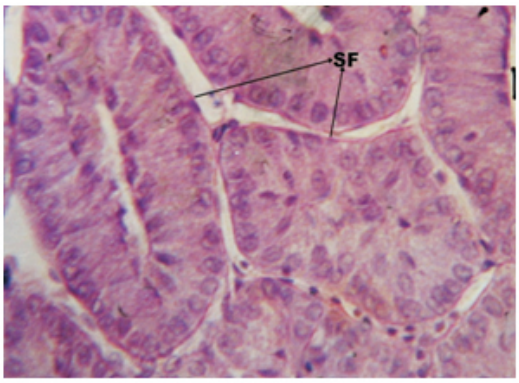

Fig - 8 :Hyperactive UBG during spawning phase having reduced lumen inside follicles, increased population of secretory cells and dilated blood vessels.

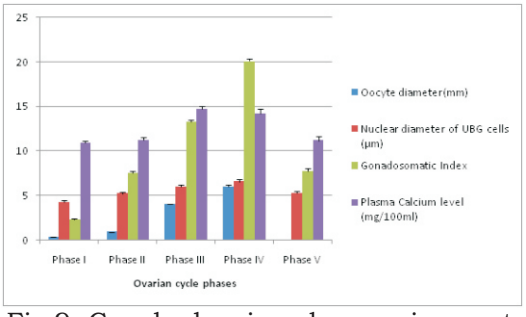

Fig 9: Graph showing changes in oocyte diameter, nuclear diameter of UBG cells and plasma calcium level during different phases of ovarian cycle of *M. armatus* with a corresponding change in gonadosomatic index

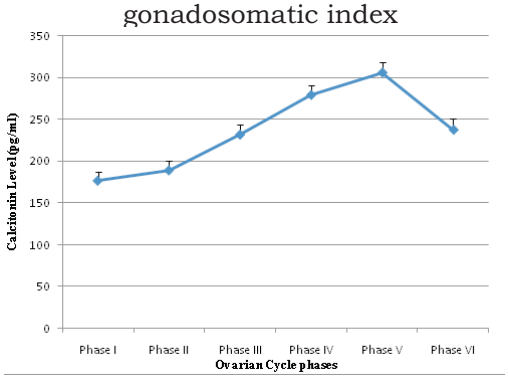

Fig 10: Graph showing changes in plasma calcitonin level during different phases of ovarian cycle. Each value represents means + SE of six specimens

Table No. 1: Changes in serum calcium (in mg/100ml) and calcitonin levels of female M. armatus following 17 β-estradiol administrations.

	Plasma Calcium Level (mg/100ml)	Plasma calcitonin Level (ng/ml)
In Controls	10.981± 0.165	200
In 17-β estradiol injected female fishes.	19.405+ 0.251	305

Table No. 2: Changes in nuclear diameter of UBG cells (in µm) of female M. armatus following 17 β- estradiol administrations.

	Nuclear diameter of UBG cells (in µm)
In Controls	4.425± 0.138
In 17-B estradiol injected female fishes	5.739+ 0.149

Each value represents mean ± SD of six specimens.

Discussion:

Most of the Indian teleosts are seasonal breeders and breed during monsoon (rainy season). Various reproductive parameters like gonadosomatic index, ova diameter as well as histological analysis of the ovary revealed that *M. armatus* is also a seasonal breeder whose spawning period extends from late July to September. This specific time of spawning in fishes depends upon some factors which contribute towards maximum survival of the eggs and fry (Sumpter, 1990). Sufficient water supply, enhanced water quality and optimum water temperature during monsoon period ultimately results in increased food availability and becomes deciding factors for *M. armatus* to breed during this period. The gonadosomatic index which is the percentage ratio of body weight to the gonad weight becomes minimum during resting phase and thereby increases gradually with the advancement of maturation and become maximum during prespawning and spawning phase. The maximum obtained value of GSI during month of August indicated the completion of maturity as well as maximum percentage of yolk laden ripe

eggs in the ovary. The minimum value during the months of December, January and February clearly indicated spent condition of the fish.

In the present study on *M. armatus* a close relationship was found to persist between reproduction, plasma calcium level and the activity of UBG. Similar observations were made by other workers on different fishes (Ahmad and Swarup, 1988; Suzuki et al., 2004).

Simultaneous increase in the serum calcium and calcitonin levels was noted with the advancement of reproductive cycle reaching the peak during prespawning phase and spawning phase. After which it gradually reduced during postspawning and becomes minimum at resting phase. Variation in this serum calcium level in relation to different phases of reproductive cycle is related with the activity of UBG. The gland exhibit signs of hyperactivity like hypertrophy and hyperplasia of the cells, increase in nuclear diameter, elongation and compactness of follicular cells etc. during prespawning and spawning phase whereas its reduced activity during post spawning phase and almost inactivity were indicated by decrease in the nuclear diameter of the cells, appearance of a lumen inside the follicles of the gland. The calcitonin level in the blood was also found to increase with the corresponding increase in the serum calcium level. It has been suggested that increased plasma level of calcitonin and calcium as well as increased activity of UBG during the spawning period is an indicative of direct role of calcitonin in calcium regulation (Paul et al., 2008).

Therefore it can be concluded that gonadal maturation in female *M. armatus* is associated with increase in the serum calcium and calcitonin level.

UBG in females becomes more active during gonadal maturation in comparison to males probably because in females the serum calcium is concerned with the process of vitellogenesis (De mones et al., 1988). Calcium is necessary for the formation of vitellogenin molecules (Yeo et al., 1997) which is a female-specific serum protein synthesized by the liver as the egg-yolk precursor (Yu et al., 1981). Vitellogenesis is evoked by direct estrogenic action upon the liver (Yu et al., 1981). An increase in the amount of plasma vitellogenin occurs as a result of estradiol- 17β administration (Carragher and Sumpter, 1991; Nath et al., 1992). The process of vitellogenesis is under the hormonal control of estradiol and is accompanied by a marked increase of plasma calcium and phosphate. Increase in plasma calcium level occurs with advancement of ovarian maturation because one atom of calcium is associated with every protein phosphate group in vitellogenin complex (Wallace, 1970). Thus during the process of vitellogenesis, plasma levels of E2, and total calcium are positively correlated. Thus it can be concluded that in *M. armatus* administration of estradiol induced hypercalcemia which stimulates UBG to secrete its antihypercalcemic hormone, calcitonin. Therefore it is suggested that in this fish, calcitonin is a hormone related to reproductive physiology as found in other teleosts (Bjornsson et al. 1989)

Calcium uptake in fishes occurs through gills as well as intestine, although the gills are supposed to play major role (Flik et al., 1996). The increased level of estradiol accompanied during ovarian maturation may 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 is also reported that it may act indirectly via some endocrine factor like PTHrP responsive to E2 (Fuentes J, et al. 2007). 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. In the present study the role of UBG in calcium homeostasis was 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 during reproduction which is still lacking.

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