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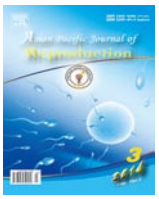

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Differences in the activity of prolactin cells in male and female fresh water teleost *Mastacembelus armatus* (Lacepede) during gonadal cycle

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ABSTRACT

Objective: To determine the differences in the activity of pituitary prolactin cells between male and female fresh water teleost *Mastacembelus armatus* (*M. armatus*) during their reproductive cycle. **Methods:** Fishes were sampled every month throughout the year. They were dissected, gonads weighed for the determination of GSI. Blood samples were collected for estimation of plasma calcium, 17 β-estradiol of females and testosterone of males and prolactin. Nuclear diameter of PRL cells, diameter of oocyte and testicular lobule was measured by image analyzer microscope. An experiment was also conducted in which female and male fishes were injected with 17 β-estradiol and 17 alpha-methyltestosterone respectively and the blood samples were analyzed for plasma calcium, prolactin and sex steroids levels. **Results:** Negligible change in plasma calcium and prolactin level and little variation in nuclear diameter of PRL cells were recorded throughout the year in relation to testicular cycle as well as after 17 alpha-methyltestosterone administration in male. Variations in level of plasma calcium and prolactin and a large range of difference in nuclear diameter of PRL cells were noted during ovarian cycle as well as after 17 β-estradiol administration in female. **Conclusion:** Difference in the activity of PRL cells was noted between male and female *M. armatus*. It act as hypercalcemic factor in females whose level increase along with maturation of ovary with the influence of increasing level of 17 β-estradiol from ovarian follicles to fulfill the increased demand of calcium for vitellogenesis and thus directly effecting reproduction. No such role of prolactin was observed for male fishes.

1. Introduction

More than 300 different biological activities have been identified for prolactin[1], a hormone secreted by rostral pars distalis of pituitary. Among these activities the important ones are maintenance of water and electrolyte balance, growth and development, behaviour, reproduction, and immunoregulation[1].

The role of prolactin (PRL) during reproduction has been described significantly in mammals[1-2] as well as in other vertebrates[1-4]. In both mammals and birds, PRL was found to involve in promoting survival and steroidogenesis of the corpus luteum[5].

Endocrine control of fish reproduction remains relatively poor understood compared to other vertebrate models. As far as PRL is concerned, comparatively less research has been done regarding its role during reproduction and inconsistent results were obtained. Few studies have been conducted in certain species of fishes like tilapia[6-7], seabream [8-9], Japanese flounder[10] and goldfish[11] which confirmed the role of prolactin in reproductive activities like spermatogenesis, vitellogenesis or ovulation evidenced by increase in its plasma level as well as increase in the amount of mRNA and mature protein in gonads. It was found that the level of plasma prolactin increases

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along with gonadotrophin during gonadal development in freshwater catfish *Clarius batrachus* (*C. batrachus*), and reaches a maximum during spawning[12]. However no such type of variation was noted in Japanese eels[13]. 17 β-estradiol treatments produced an increase in PRL mRNA depending on the maturity of the animal in seabream[8]. The low level of plasma prolactin in juvenile male blue gouramis in comparison to adults also confirmed its role in reproduction[14]. PRL directly stimulates testosterone production in courting male Mozambique tilapia[15]. It was found to stimulate 17 β-estradiol secretions in guppy [*Poecilia reticulate* (*P. reticulate*)] oocytes during their development[16] and has also been found to have a gonadotrophic and steroidogenic action in hypophysectomised *Fundulus heteroclitus* (*F. heteroclitus*) [17]. In *Heteropneustes fossilis* (*H. fossilis*) hypophysectomy causes rapid regression of seminal vesicles, PRL acts in a synergistic manner with HCG and androgens to stimulate seminal vesicle growth and secretion[18].

Calcium is an important factor in fishes for various physiological activities especially during reproduction. Role of prolactin in calcium homeostasis in fishes has been proposed from time to time. It was suggested that prolactin may be the pituitary hypercalcemic principal in fish[19]. An inverse relationship between calcium levels in the external environment and prolactin cell (PRL cell) activity has been reported in freshwater stickleback[20] and tilapia[21]. It is reported that the steroid hormones, estrogen and testosterone, are able to enhance the response of PRL cells to GnRH, increasing percentage release over 3-fold. Thus steroids may regulate the sensitivity of the PRL cells to GnRH stimulation during the reproductive cycle[22].

Although all such studies indicated the involvement of prolactin in calcium homeostasis during reproduction in fishes, still further investigations are needed in order to collect more information on the source of hypercalcemic hormone from the pituitary and hypercalcemic regulation as they are the most diverse group of vertebrates and showed inconsistent results towards hypercalcemic role of prolactin on many occasions.

The fish *Mastacembelus armatus* is one among the economically important species in rural parts of India [23]. Therefore for evaluating commercial potentialities it is necessary to know every aspect of its reproductive physiology. The species prefers to avoid light as far as possible and likes to hide away by bury themselves in sand during the daytime. Due to its habit the possible role of melatonin on prolactin secretion and therefore on reproduction can be best studied in case of fishes by using this species as animal model. As far as my knowledge is concerned no previous work has been done regarding the difference in the activity of prolactin cells in male and female fishes during reproduction in this particular species. Therefore the present study was undertaken to find out the exact role of this hormone in *M. armatus* by analyzing the monthly variations in plasma calcium, 17 β-estradiol, testosterone and prolactin levels. The reproductive cycle as well as the seasonal activity of the prolactin cells was traced out with simultaneous determination of 17 β-estradiol, testosterone and serum calcium level with the hypothesis that the hormone prolactin might play an important role in calcium homeostasis during reproduction in this species as explored in some other teleosts.

2. Materials and methods

2.1. Animals and sampling procedure

Ten adult specimens of both male and female *M. armatus* 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. 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 for estimation of plasma calcium, 17 β-estradiol, testosterone and prolactin level. The fishes were dissected, gonads excised and weighed (g) for the determination of gonadosomatic index.

2.2. Plasma calcium estimation

After centrifugation (1 min, 10 000 g) total plasma calcium concentrations were measured colorimetrically using a calcium kit (Sigma Diagnostics).

2.3. Enzyme linked immunosorbent assay

A competitive ELISA technique[24] which is based on competition between free PRL in standard or plasma samples and PRL immobilized on microtiter plates for the PRL antibodies was used for determination of plasma prolactin level.

2.4. Radioimmunoassay

Plasma concentration of testosterone and 17 β-estradiol were determined by RIA method[25, 26], following Guerriero *et al.* [27]. The sensitivity of testosterone was 7 pg (intraassay, 7%; interassay, 13%), and that of 17 β-estradiol was 5 pg (intra-assay, 9%; interassay, 13%). The antibody used for testosterone determinations cross-reacted with dihydrotestosterone, and therefore the data are reported as androgens[28].

2.5. Histological parameters

Gonads were preserved in Bouin's solution (75 mL saturated picric acid, 25 mL 40% formaldehyde and 5 mL glacial acetic acid). 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 5-7 μm were prepared with a microtome. Maturity stages of gonads were determined by studying histological changes after staining sections in haematoxylin and counterstaining in eosin.

Bouin's fluid (fixative) was poured over the exposed brain. After considerable period of time the brain along with intact pituitary was taken out and fixed in the same fixative. After dehydration and paraffin embedding the tissues were sectioned at 5-7 μm. The sections were stained in haematoxylin and counterstained in eosin. In addition several sections were stained at need by Mallory's triple.

Nuclear diameter of PRL cells (μm) were measured by image analyzer microscope (Metavis image analyzing system with Meltmage Lx Software). 50 nuclei were randomly selected from every fifth section of the gland. Total number of the nuclei measured was always more than 300 for each individual.

2.6. Experiment

To analyze the effect of 17 β-estradiol administration on PRL cell activity 36 live, (18 male and 18 female) adult and healthy specimens of M. armatus were collected from Dimna Lake, Jamshedpur, during the month of December which is the resting phase for this species in terms of reproduction. They were acclimatized to laboratory conditions (temperature, 27 ℃-32 ℃; light: Dark photoperiod, 12.00 h:12.00h). After 15 days the fishes were divided between three groups, one group consisting of 6 female, other group with 6 male and the last group with 6 male and 6 female and kept in separate aquaria of 100 L capacity. The group containing both male and females was injected with 0.1 mL of vehicle (peanut oil), the group containing only females was administrated with 100 μg of 17 β-estradiol (sigma) in 0.1 mL of vehicle the group consisting of only males was with 100 μg of 17alpha-Methyltestosterone (sigma) in 0.1 mL of vehicle. The fishes were injected intraperitonially on alternate days and injections were given at the same time of the day to avoid diurnal variation. The blood samples were collected for plasma calcium, 17 β-estradiol, testosterone and prolactin level estimation after 15 days. At the same time the pituitary gland was also removed for histological analysis.

2.7. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA). Significance was accepted at $P<0.05$.

3. Results

3.1. Gonadal cycle

The gonadal cycle of *M. armatus* is divided into five stages which are represented in table 1 and 2 along with observed characteristics.

3.2. Variations in plasma calcium, steroid, prolactin and nuclear diameter of PRL cells

In males: Negligible change in plasma calcium and prolactin level was recorded throughout the year in relation to testicular cycle (Figure 1). Variations in plasma testosterone level were observed which showed similar

Table 1
Different phases of ovarian cycle of *M. armatus* with observed parameters.

Phases	Months	Morphological and histological features	Oocyte diameter (μm)	Mean GSI (%)
Phase 1/Resting phase	December-February	Ovaries are small, shrunken, without any visible ova, primary oocytes and oogonia within ovigerous folds can be seen inside of ovary	79.31±3.77	1.92±0.14
Phase II/Preparatory phase	March- May	Slight increase in weight of ovary, Nucleoli arranged along the inner surface of the nuclear membrane, Yolk nucleus of Balbiani appears in juxtranuclear area of ooplasm, extra vesicular yolk can also be observed in perinuclear area of ooplasm.	123.42±9.88	2.86±0.84 $P<0.01$
Phase III/ Pre spawning phase	June-early July	The nucleus becomes indistinct, whole of the ooplasm is filled with protein yolk bodies	532.30±37.95	10.42±0.79 $P<0.01$
Phase IV/Spawning phase	Late July- September	Small protein yolk bodies in ooplasm which appeared during advance maturation phase coalesce to form large globules	600.43±68.14	16.49±1.70 $P<0.01$
Phase V/ Post spawning phase	October-November	Ovaries collapsed, unovulated eggs undergoing resorption and corpora atretica in vascular stroma can be frequently seen, cells of discharged follicles get resorbed in the ovarian tissue and ultimately disappear	104.20±3.24	3.08±0.34 $P<0.01$

Values represent mean ± SE of observation based on 20 fishes.

Table 2
Different phases of testicular cycle of M. armatus with observed parameters.

Phases	Months	Observed characteristic features	Testicular lobule diameter(μm)	GSI
Phase 1/Resting phase	December–February	Testes are very small and thread like, vascular supply is reduced.	48.20+1.91	1.13±0.12
Phase II/Preparatory phase	March– May	Considerable increase in the volume of and vascular supply, interlobular demarcation is clearly visible	91.20+4.09	1.42±0.22
Phase III/Pre spawning phase	June–early July	Testes enlarge in volume, blood vascular further increased	183.50+8.23	1.71±0.28
Phase IV/ Spawning phase	Late July– September	Testes appears slightly reddish due to maximum increase in blood supply, considerable increase in volume of testis was noted.	232.50±17.21	2.12±0.48
Phase V/Post spawning phase	October–November	Decreases in blood supply as well as volume of testis were observed.	81.44±5.08	0.62±0.04

Values represent mean ± SE of observation based on 20 fishes.

pattern in changes as that of GSI. Testosterone ranged from 0.6–1.5 μg/L (Figure 1).

Little variation in nuclear diameter of PRL cells (ranged from 3.22± 0.25 to 3.11± 0.16) was recorded for males (Figure 2).

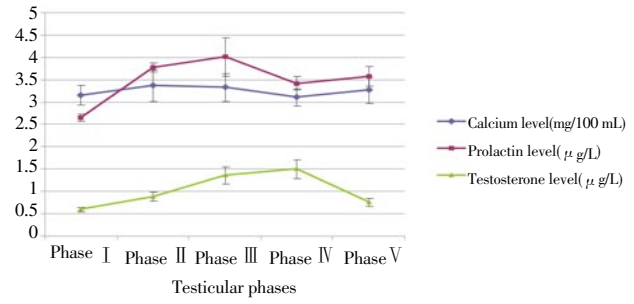

Figure 1. Plasma calcium, prolactin and testosterone level of male M. armatus during different phases of testicular cycle.

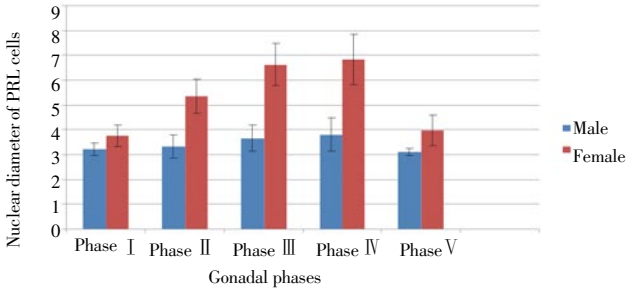

Figure 2. Changes in nuclear diameter of PRL cells during different phases of gonadal cycle in female *M. armatus*.

In females: Considerable variations in the level of plasma calcium were observed in female fishes (Figure 3). Increase in the serum calcium level was observed during advanced maturation phase reaching the peak during spawning phase. Afterwards gradually decreases with spawning and reduced to minimum at resting phase. 17 β–estradiol ranged from 2–4 μg/L (Figure 3) during ovarian cycle and changes in its concentration showed a similar pattern as changes of GSI. A large variation in the GSI was observed in case of females during annual gonadal cycle (Table 1). A large range of difference (from 3.76 to 6.83) was noted in nuclear diameter of PRL cells for females (Figure 2).

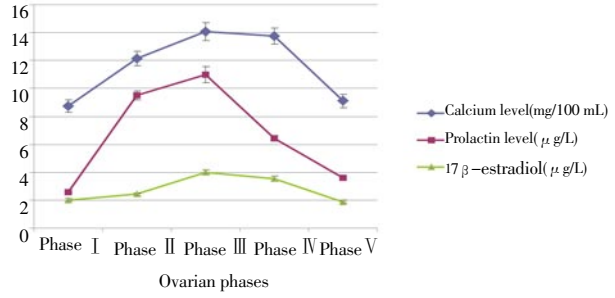

Figure 3. Plasma calcium, prolactin and 17 β–estradiol level of female M. armatus during different phases of ovarian cycle.

3.3. Effects of synthetic steroid administration

Negligible increase in the plasma calcium levels nuclear diameter of PRL cells and prolactin levels were observed in male fishes (Fig 4) after 17alpha–Methyltestosterone administration. On the other hand a sudden rise in plasma calcium level, nuclear diameter of prolactin cells and plasma prolactin levels were observed in female fishes (Figure 4).

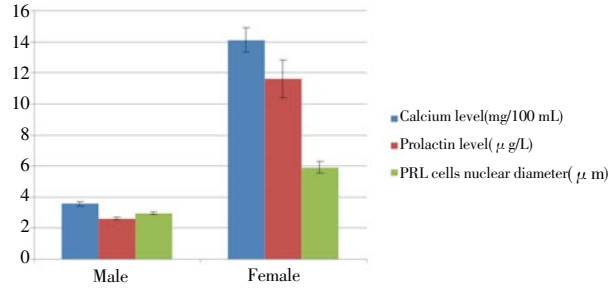

Figure 4. Effect of synthetic steroid administration in male and female fishes.

4. Discussion

Variations in the level of 17- β-estradiol in females and testosterone in males of *M. armatus* correspond well with gonadal activity indicated by gonadosomatic index value. Variations in plasma testosterone level showed similar pattern in changes as that of GSI. High levels of testosterone signified that fishes undergo spermatogenesis and spermiogenesis[29]. In case of *M. armatus* high values were obtained from August to September which indicated that fishes produce milts during these months. The low levels indicated spermatogonia proliferation phase which occurs during resting phase in male fishes. December to February is the months having low levels of testosterone indicating spermatogonia proliferation phase for *M. armatus*.

High gonadosomatic indices recorded during late July to September implied that females would likely to spawn during these months. Increase in gonadosomatic index is associated with changes in 17- β-estradiol levels in circulation[30]. Vitellogenesis is initiated by rise in 17- β-estradiol levels[30]. High values of 17- β-estradiol during prespawning and spawning phase indicated that *M. armatus* undergo vitellogenesis and attains maturity. High gonadosomatic indices in the months with high levels of 17- β-estradiol indicated that vitellogenesis is going inside the ovaries and conformed the role of 17-β-estradiol in vitellogenesis.

Inconsistent results were obtained from time to time regarding the correlation between plasma calcium level and testicular maturation. Woodhead[31] reported that a positive correlation exists between blood calcium level and testicular maturation in arctic cod and sea cod. However in the present study on *M. armatus* no such correlation was observed which is in agreement with reports of other workers[32-33].

However a seasonal variation in plasma calcium and 17-β estradiol level as well as correlation between them was found which is in agreement with earlier observations made on other fishes[34-35].

Gonadotrophic hormone from pituitary stimulates ovarian follicle to secrete estrogen. Plasma concentration of 17-β estradiol was found to be maximum during pre spawning phase in *M. armatus*. Several authors have correlated the enhanced secretion of 17-β estradiol during the sexual maturation of females with serum calcium level [36]. Increased plasma level of 17-β estradiol initiated transcription and translation of VTG in liver[37] and increase protein-bound fraction of plasma calcium levels[38]. Increases in plasma calcium and VTG concentration due to influence of estrogen have been reported previously in salmonid fish[39].

Marked seasonal variations in plasma prolactin level associated with ovarian maturation and serum calcium level was also observed in female *M. armatus*. No such significant changes in plasma prolactin level associated with testicular cycle were observed in male fishes. In females the prolactin cell activity increased to maximum during pre spawning period manifested by increase in plasma concentration of prolactin as well as nuclear diameter of prolactin cells. This increased activity of prolactin cells was probably due to increased 17-β estradiol secretion during ovarian maturation which is in agreement with the results obtained by earlier workers[40-41]. Diminished activity of prolactin cells were noted after spawning when serum calcium level abruptly falls. During diminished activity of the gland the nuclear diameter reduced considerably.

Administration of 17-β estradiol for 10 days induced hypercalcemia in female *M. armatus*. A considerable difference in plasma calcium levels was observed between estradiol injected female fishes and controls. Hyperactivity of the prolactin cells indicated by increase in nuclear diameter of PRL cells were noted in female *M. armatus* after estradiol administration which is supported by the findings of earlier workers[42] (Singh & Singh 1981) Thus it can be concluded that the observed increased level of prolactin during gonadal maturation in female *M. armatus* was probably due to the effect of increased estradiol level.

Thus the role of prolactin in female *M. armatus* has been established and can be considered as hypercalcemic factor whose level increase during maturation phase of ovary with the influence of estradiol from ovarian follicles and thus directly effecting reproduction. No such role of prolactin was observed in male fishes as the plasma level of calcium as well as prolactin found to remain almost constant throughout the testicular cycle. *M. armatus* is a nocturnal fish and due to this habit a melatonin might have some influence on the secretion of prolactin. Further work has been proposed in this fish to find out the role of melatonin in prolactin synthesis and secretion because the direct role of prolactin in reproduction has been established in this study and melatonin may influence the reproductive capacity of this species by acting indirectly.

Conflict of interest statement

We declare that we have no conflict of interest.

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