Original Article

Estradiol Affects Prolactin Producing Cells and Calcium levels in a Teleost, *Heteropneustesfossilis*, Kept in Different Calcium Concentrations

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ABSTRACT

Background: This study investigated the effects of estradiol on plasma calcium and prolactin cells of *Heteropneustes fossilis* kept in calcium-deficient and normal freshwater.

Methods: Fish were deprived of food and divided into groups A-D. Group A and B were kept in artificial freshwater with normal electrolytes. Group C and D were maintained in calcium-deficient freshwater. Vehicle was administered to groups A and C. Groups B and D were injected with estradiol. Plasma calcium levels and prolactin cells were studied after 1, 3, 5, 10 and 15 days.

Results: Normal-calcium freshwater: In group A calcium levels remained unaffected. In group B, estradiol provoked hypercalcemia from day 3 to 10 although calcium decreased after day 15. Prolactin cells in group B became degranulated after 10day. Nuclear volume increased from day 10 onwards.

Calcium-deficient freshwater: Calcium levels in group C decreased from day 1 to 3 thereafter increased from day 5 to 15. Plasma calcium of group D increased from day 3 to 15. In group C prolactin cells exhibited hyperactivity on day 3 and degranulation on day 5. Nuclear volume increased from day 5 onwards. On day 10 and 15 certain cells became degenerated. In group D degranulation of prolactin cells began on day 3 which proceeded to complete degranulation on day 10. Nuclear volume increased from day 5 onwards.

Conclusions: Estradiol enhanced prolactin production and increased blood calcium in food-deprived fishes kept in calcium-deficient medium suggesting that calcium needed for elevation of blood calcium was derived from internal sources.

Keywords: Calcium, Catfishes, Estradiol, Fishes, Prolactin.

INTRODUCTION

The absence of parathyroid glands in fishes stimulated the search for a hypercalcemia inducing endocrine factor in this group. Several studies [1-6] have indicated the pituitary gland as the main endocrine organ responsible for the release of a hypercalcemia factor, with prolactin as the active substance. Teleosts make up 96 percent of all fish species. The pituitary glands of teleosts have been implicated in calcium regulation since 1956 when Fontaine [7] found that removal of the pituitary of freshwater eels caused hypocalcemia. The role of prolactin have been proposed since 1973 when Pang et al. [8] found that hypophysectomized killifish *Fundulus*

heteroclitus adapted to calcium-deficient seawater showed hypocalcemia and tetanic seizures. These effects were corrected by administration of pituitary homogenates of mammalian prolactin [1]. Mammalian prolactin could induce hypercalcemia in a variety of teleost fishes [2, 9-12].

Liver synthesizes vitellogenin (an egg yolk precursor protein) and releases it into the circulation [13-16], which is then transported to the ovaries and is taken up by the developing oocytes and stored as yolk [17-19]. Estradiol-17β induces the synthesis of vitellogenin, which is a calcium-binding protein. During vitellogenesis, there is an increase in the protein bound plasma

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calcium fraction whereas plasma ionized calcium remains unaffected. Gillespie and Peyster have reported a significant linear correlation between plasma vitellogenin and plasma calcium for male and female fathead minnows (*Pimephales promelas*) [20]. Thus, during vitellogenesis there is an increase in calcium demand, which has to be obtained either from the environment, intestinal uptake and/or from the internal calcium reservoirs.

Controversy exists regarding the source of additional calcium needed after estradiol-induced vitellogenesis in fishes. Enhanced uptake of calcium from environment [17, 21] and mobilization from scales [17, 18, 22, 23], muscles [17] and bile [24] have been reported after estradiol treatment in different fishes. Calcium mobilization from scales is mediated by estrogen receptors (ER). Pinto et al. have localized Immuno-histochemically ERα, ERβa and ERβb proteins in scales of juvenile and adult sea bream (*Sparus auratus*) and Mozambique tilapia (*Oreochromis mossambicus*) [25] and suggested that the calcium mobilizing action of 17β-estradiol on fish scales is through its direct action on ERs present in osteoblasts. On the other hand, estradiol treatment has been reported not to affect calcium uptake from the environment [26] or muscles [23], bone resorption[17, 22,23], intestinal calcium uptake [26] or calcium excretion [17, 23].

With this background an attempt has been made in the present study to investigate the effects of estradiol administration in the catfish, *Heteropneustes fossilis* maintained either in artificial freshwater with normal electrolytes or calcium-deficient freshwater. Changes in plasma calcium levels, induced experimentally by estradiol, have been correlated with the activity of calcium regulating endocrine gland namely prolactin cells of pituitary gland.

MATERIALS AND METHODS

Live specimens of freshwater catfish *H.fossilis* (both sexes; body wt. 27-39 g) were collected locally from Gorakhpur in 2015 and acclimatized to laboratory conditions for two weeks in plastic pools. Water was half renewed daily and the fish were fed with dry shrimp powder on alternate days. The Ethical Committee of Department of Zoology, DDU Gorakhpur University, approved all the experimental protocols.

For the experiment the fish were transported to identical glass aquaria each containing 10 L of the medium. Twelve fish were kept in each aquarium. The medium was replaced on alternate days. After acclimation, they were divided into 4 groups (A-D), with 12 fish in each group, and treated as follows:

Group A: Fish from this group were given a single intra-peritoneal injection of vehicle (0.1 ml of groundnut oil/100 g body wt) and were kept in artificial freshwater.

Group B: Fish from this group were given a single intra-peritoneal injection of estradiol dissolved in groundnut oil (1 mg/100 g body wt) and were kept in artificial freshwater.

Group C: Fish from this group were given a single intra-peritoneal injection of vehicle (0.1 ml of groundnut oil/100 g body wt) and were kept in calcium-deficient freshwater.

Group D: Fish from this group were given a single intra-peritoneal injection of estradiol dissolved in groundnut oil (1 mg/100 g body wt) and were kept in calcium-deficient freshwater.

Samples were not fed from 24 h before and during the experiment.

Different artificial media i.e. freshwater and calcium-deficient freshwater were prepared as follows:

(a) **Artificial freshwater with normal electrolytes:** Distilled water containing (in mmol/liter): NaCl 2.10; Na_2SO_4 0.45; KCl 0.06; $CaCl_2$ 0.8; $MgCl_2$ 0.20. PH of the solution was adjusted to 7.6 with $NaHCO_3$.

(b) **Calcium-deficient freshwater:** same as artificial freshwater without $CaCl_2$.

Fishes from each group were anaesthetized with tricainemesylate (MS-222) and blood samples were taken after 1, 3, 5, 10 and 15 days following the treatment. Blood samples were collected in heparinized tubes by sectioning the caudal peduncle. Plasma was separated by centrifugation and analyzed for calcium level by Sigma kit. After collection of blood samples, the pituitary gland along with the brain was fixed in aqueous Bouin's solution and Bouin-Hollande fixative for histological studies. Tissues were routinely processed in graded series of alcohols, cleared in xylene and embedded in paraffin. Serial 6-μm sections were prepared. The pituitaries were

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stained with Herlant's tetrachrome and Heidenhain's AZAN trichrome stains.

Nuclear indices (maximal length and maximal width) were determined with ocular micrometer.In total, 50 nuclei were measured per specimen, thus 300 nuclei were measured from six specimens, and the nuclear volume was calculated as:

Volume = 4/3 π ab^2

Where 'a' is the semi-major axisand 'b' is the semi-minor axis.

Data were presented as mean ± S.E. of six specimens and Student's *t*-test was used to determine statistical significance. Each experimental group was compared to its related time control group (group B compared with group A and group D compared with group C).

RESULTS

(A) Artificial Freshwater Groups (Groups A and B):

The plasma calcium levels of vehicle-injected fish (group A) exhibited almost no change throughout the experiment (Fig.1). In group B no significant change was noticed in the plasma calcium level after day one following estradiol treatment. Estradiol treatment caused progressive increases in plasma calcium levels from day 3 to day 10, however, after day 15 the levels slightly decreased (Fig. 1).

The pituitary gland of *H. fossilis* is almost ovoid in shape and is attached to the brain by a distinct stalk (leptobasic). The gland has two major parts: the neuro-hypophysis and adenohypophysis. The glandular adenohypophysis is composed of rostral pars distalis, proximal pars distalis and pars intermedia. These three regions are arranged dorso-ventrally one after the other. Prolactin cells are predominant cell types of rostral pars distalis. These cells have indistinct boundaries with adjacent cells. The nuclei are distinct with dense chromatin granules and the cytoplasm is scanty and shows affinity for azocarmine and erythrosine (Fig. 2).

The prolactin cells of estradiol treated fish (group B) exhibited no change up to day 5. These cells became degranulated after day 10 (Fig. 3). Moreover, the nuclear volume of prolactin cells progressively increased from day 10 to 15 (Fig. 4).
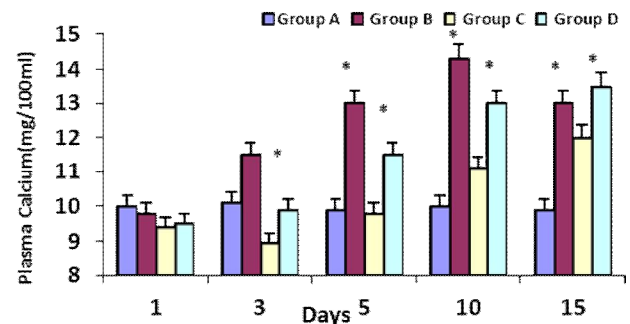

Figure 1. Changes in the plasma calcium levels of *Heteropneustes fossilis* treated with vehicle + artificial freshwater with calcium (Group A), estradiol + artificial freshwater with calcium (Group B), vehicle + calcium deficient freshwater (Group C), and estradiol + calcium deficient freshwater (Group D). Each value represents mean ± S.E. of six specimens. Asterisks indicate significant differences (P<0.05) (Group B compared with Group A; Group D compared with Group C).
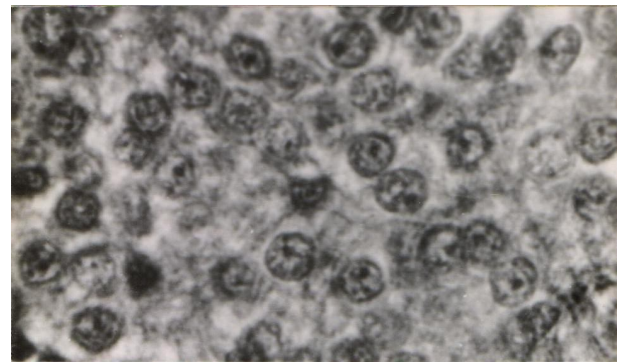

Figure 2. Prolactin cells at day one in vehicle-injected *Heteropneustes fossilis* kept in artificial freshwater with calcium exhibiting distinct nuclei with dense chromatin granules. Herlant tetrachrome ×800.
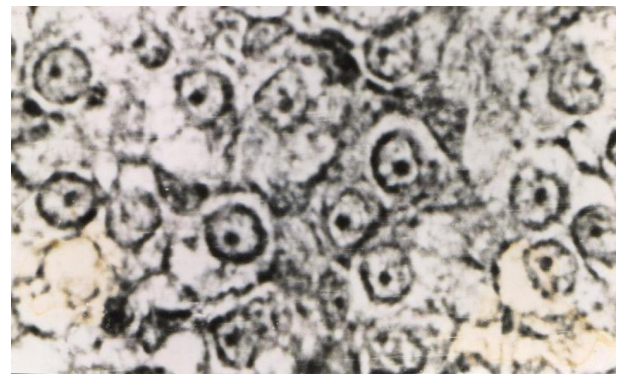

Figure 3. Degranulation in prolactin producing cells at day 10 in estradiol treated fish maintained in artificial freshwater with calcium, Herlant tetrachrome ×800.
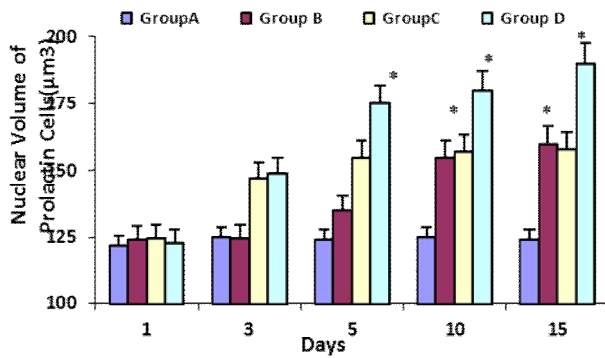
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Figure 4. Nuclear volume of prolacin producing cells of *Heteropneustes fossilis* treated with vehicle + artificial freshwater with calcium (Group A), estradiol + artificial freshwater with calcium (Group B), vehicle + calcium deficient freshwater (Group C), and estradiol + calcium deficient freshwater (Group D). Each value represents mean ± S.E. of six specimens. Asterisks indicate significant differences (P<0.05) with vehicle injected specimens.

(B) Calcium Deficient Freshwater Groups (Groups C and D):

The plasma calcium level of vehicle-injected fish (group C) exhibited a slight decrease on day 1 (as compared to the fish kept in artificial freshwater at day 1). This response continued until day three. Thereafter, the plasma calcium levels increased from day 5 resulting in hypercalcemia from day 10 (Fig. 1).

The plasma calcium levels of estradiol treated fish (group D) exhibited no change at day one but there was progressive increase in plasma calcium levels from day 3 to 15 (Fig. 1).

In vehicle-injected fish (group C), prolactin cells exhibited hyperactivity on day 3 which was evident by degranulation (Fig. 5), hyperchromaticity of the nuclei and increased nuclear volume (Fig.4). After day 5, the cells became completely degranulated (Fig. 6). The nuclear volume further increased from day 5 to 15 (Fig.5). On day 10 and 15, certain cells were degenerating (Fig. 7).

In estradiol-injected fish (group D) there was slight degranulation in the prolactin cells (Fig. 8) on day 3. However, the nuclear volume showed no change as compared to the vehicle-injected fish (group C). The majority of these cells became completely degranulated from day 10 (Fig. 9). The nuclear volume progressively increased from day 5 onwards (Fig. 4).
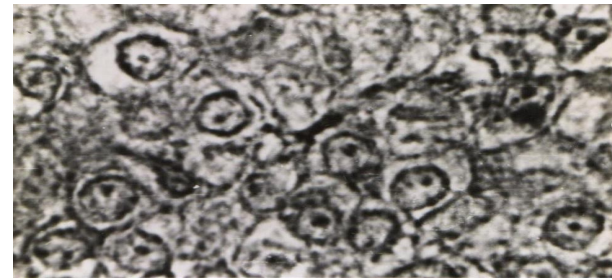

Figure 5. Prolactin producing cells at day 3 in vehicle-injected fish kept in calcium-deficient freshwater showing degranulation. Herlanttetrachrome ×800.
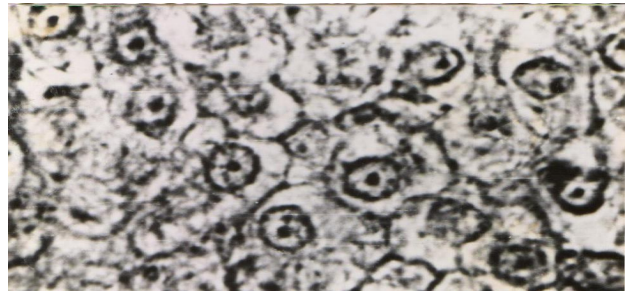

Figure 6. Complete degranulation of prolactin producing cells at day 5 in vehicle-injected fish kept in calcium-deficient freshwater. Herlant tetrachrome ×800.
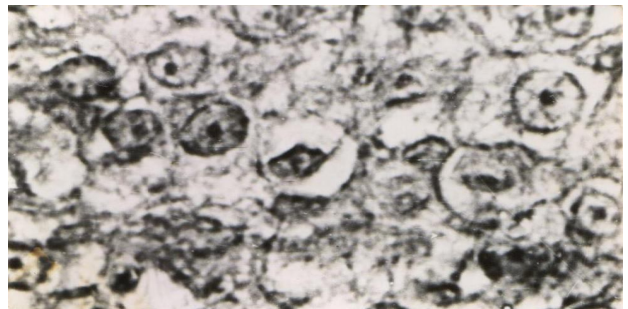

Figure 7. Degeneration of prolactin producing cells at day 15 in vehicle-injected fish maintained in calcium-deficient freshwater. Herlant tetrachrome ×800.
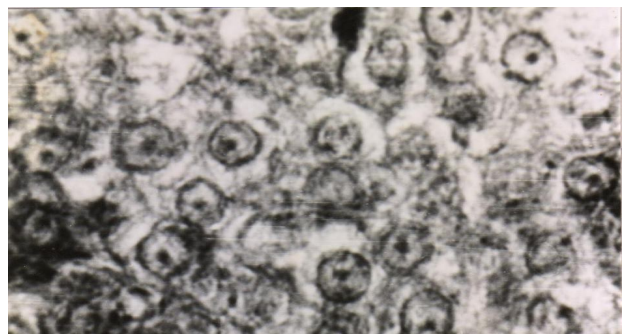

Figure 8. Slight degranulation of prolactin producing cells at day 3 in estradiol treated fish kept in calcium–deficient freshwater. Herlant tetrachrome ×800.
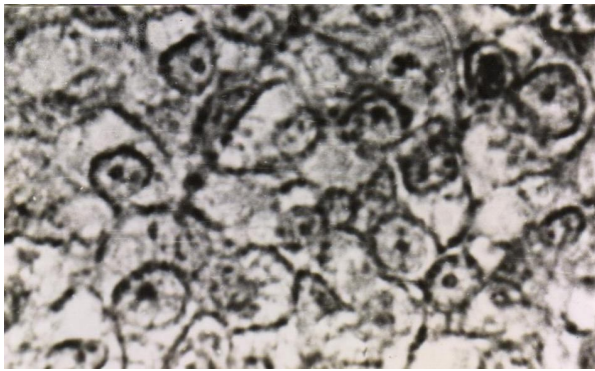
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Figure 9. Prolactin producing cells at day 15 in estradiol treated *Heteropneustes fossilis* kept in calcium–deficient freshwater exhibiting complete degranulation. Herlant tetrachrome ×800.

DISCUSSION

Estradiol treatment provoked elevation in the plasma calcium level of the fish kept in artificial freshwater. This derives support from previously reported studies describing increases in plasma calcium after estradiol treatment [13,15, 17, 18, 21, 24, 27-30]. Elevation of plasma calcium in *H. fossilis* may be attributed to increased mobilization from internal stores. In E_2 (estradiol) treated rainbow trout [17, 23], goldfish and killifish [22] scale calcium mobilization has been noticed. Persson et al. [17] have also noticed calcium mobilization from muscles of E_2 treated rainbow trout; however, calcium content of muscles, vertebrae, rib bones, jaw or otolith organs have been reported to remain unaffected [22, 23].

Increased plasma calcium content in estradiol treated *H. fossilis* could not be attributed to increased intestinal calcium uptake, as the fish were not fed in the present study. Estradiol administration of the fish kept in calcium-deficient freshwater resulted in hypercalcemia. Since the animals were not fed during the present study, the observed hypercalcemia in estradiol treated *H. fossilis* could not be linked to calcium absorption at intestinal mucosa or gills. Enhanced calcium uptake from the environment after estradiol injection has been reported in other fish species [17, 21], although Mugiya and Ichii [26] have noticed no effect of estradiol on *in situ* branchial calcium uptake.

Fish kept in calcium deficient freshwater that were only treated with vehicle, became hypocalcemic. Wendelaar Bonga et al.[31] have also noticed hypocalcemia in tilapia after 5 days of its transference to low ambient calcium medium. This can be attributed to increased branchial efflux of these ions. Low calcium concentration in the ambient water of tilapia would allow intracellular Ca^{++} to diffuse out of the animal [32]. Branchial efflux of Ca^{++} through paracellular routes could increase as a result of lower ambient Ca^{++}. Hypocalcemia noticed in the fish maintained in calcium deficient freshwater was similar to other studies [31, 33], where found increased integumental water permeability at low ambient Ca^{++}. This increased water uptake may enhance urine production, which leads to Ca^{++} loss from the body [34].

In the present study, treatment with estradiol stimulated the prolactin cells of *H. fossilis*, which was evident by degranulation and increased nuclear volume of these cells. This derives support from the studies of Williams and Wigham [3] who have reported that in estradiol treated rainbow trout prolactin cells demonstrated *in vitro* increase in both synthesis and release of the hormone.Estradiol treatment caused activation of prolactin cells in *Gillichthys mirabilis*[35]. In *Oreochromi smossambicus*, *in vitro* incubation with estradiol also increased prolactin cell activity [4]. It seems that the observed hypercalcemia in estradiol treated *H. fossilis* is due to the release of prolactin hormone. Prolactin has already been reported as a hypercalcemic factor in teleosts[1, 2, 5, 6, 36,37]. In our study, prolactin cells of vehicle-injected *H. fossilis* kept in calcium deficient freshwater became hyperactive on day 3. The observed hypocalcemia in these specimens seems to be a plausible reason for this increased activity. The observed increase in the plasma calcium level on day 10 and day 15 could be attributed to enhanced release of prolactin from these cells. In low calcium freshwater adapted tilapia, prolactin secretion has been reported to be enhanced [37]. Moreover, an elevated plasma calcium level in tilapia has been observed after acclimation to low calcium freshwater [38]. This restoration of plasma calcium is most probably mediated by an enhanced production of prolactin hormone.

CONCLUSION

Estradiol treatment in fish provoked elevated blood calcium levels and enhanced prolactin producing cells activity. Prolactin has been reported as a hypercalcemic inducing agent

in fishes. Thus, it seems that the effects of estradiol might be mediated by the release of prolactin, which resulted in the elevated blood calcium levels. In the present study, estradiol increased blood calcium levels in food deprived fishes that were maintained in calcium-deficient medium, thus suggesting that calcium was derived from internal sources.

ACKNOWLEDGMENTS

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