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Estradiol Affects Ultimobranchial Gland of a Freshwater Catfish, *Heteropneustes fossilis* kept in Different Calcium Environments

Susmita Srivastav[1], Diwakar Mishra[2], Sunil K. Srivastav[3], Nobuo Suzuki[4] and Ajai K. Srivastav[3*]

[1]Department of Zoology, Shiv Harsh Kisan P. G. College, Basti-272001, [2]Department of Zoology, Government Girls' P. G. College, Ghazipur-233001, [3]Department of Zoology, D.D.U. Gorakhpur University, Gorakhpur-273009, India; [4]Noto Marine Laboratory, Institute of Nature and Environmental Technology, Kanazawa University, Ogi,Noto-cho, Ishikawa, Japan.

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Abstract

Some individuals of a freshwater fish species *Heteropneustes fossilis* were divided into groups A-D. Group A and B were kept in artificial freshwater. Group C and D were maintained in calcium-deficient freshwater. The groups A and C were administered with vehicle and the groups B and D were injected with estradiol. Plasma calcium levels and UBG were studied after 1, 3, 5, 10 and 15 days.

In group B fish, estradiol administration resulted in hypercalcemia from day 3 to 10; however, after day 15 calcium level of plasma slightly decreased. Plasma calcium level of group C exhibited slight decrease from that of the day 1 to 3. Thereafter, the level increased on day 10 and 15. Plasma calcium level of the estradiol treated group D exhibited progressive increase from day 3 to 15.

In group B, the nuclear volume of UBG exhibited progressive increase from day 5 to 10 and displayed weak staining response of cytoplasm. After day 15, few degenerating cells were noticed and nuclear volume of UBG cells exhibited slight decrease. In group C, staining response of the cytoplasm of UBG cells became slightly weak on day 10 and 15. In group D, there is a progressive increase in the nuclear volume from day 10 to 15, and the ultimobranchial cells displayed weak staining response. Moreover, after day 15, few degenerating ultimobranchial cells were observed in group D.

Keywords: Estradiol, Fish, Plasma calcium, Ultimobranchial gland

1. Introduction

The fish possess a unique and more complex system than that of the terrestrial vertebrates as they remain in intimate contact with surrounding water which provides an inexhaustible supply of calcium, thus building calcium gradients across the body surface. In land vertebrates, direct exchange of calcium between body and surrounding medium is not possible and they rely solely on dietary calcium uptake. In this condition, body calcium level is regulated by a balance between intestinal calcium absorption and renal calcium excretion.

The fish regulate their blood calcium level very efficiently, which involves different hormones such as prolactin from pituitary, vitamin D metabolites, calcitonin from ultimobranchial gland (UBG) and stanniocalcin from corpuscles of Stannius (CS) (Srivastav 1983, 1989; Srivastav and Srivastav 1988; Srivastav and Singh 1989, 1992; Srivastav *et al.* 1995, 1998; Prasad *et al.* 2015 and Kumar *et al.* 2017). Different hormones act through various target organs such as skin, fin, gut, gill, bone, and kidney for calcium regulation.

Vitellogenin is released into the circulation after its synthesis from the liver (Baily 1957; Chen 1983; Bjornsson and Haux 1985 and Kwon *et al.* 1993) and transported to the ovaries where it is conjugated as vitellin and stored as yolk (Persson *et al.* 1994, 1995 and Yeo and Mugiya 1997). Vitellogenin synthesis is induced by estradiol-17β (Naderi *et al.*, 2015). The increased plasma calcium level during vitellogenesis has been correlated (Bjornsson and Haux, 1985) with the enhanced synthesis of vitellogenin which binds calcium ions. During vitellogenesis, there is an increased calcium demand which has to be taken either from the environment, and intestinal uptake or from the internal calcium reservoirs.

The source of additional calcium needed after estradiol-induced vitellogenesis in fishes is in controversy. Increased calcium uptake from the environment (Fleming *et al.* 1964 and Persson *et al.* 1994), mobilization from scales (Mugiya and Watabe 1977; Carragher and Sumpter 1991 and Persson *et al.* 1994, 1995), muscles (Persson *et al.* 1994) and from bile (Mugiya and Hazama 1994) have been reported after estradiol treatment in fishes. On the contrary, Estradiol treatment to fish has also been reported not to affect the calcium uptake from environment (Mugiya and Ichii 1980), tissues like muscle (Carragher and Sumpter 1991), bone (Mugiya and Watabe 1977; Carragher and Sumpter 1991 and Persson *et al.* 1994), and intestine (Mugiya and Ichii 1980) as well as calcium excretion through kidneys (Carragher and Sumpter 1991 and Persson *et al.* 1994).

* Corresponding author e-mail: ajaiksrivastav@hotmail.com.

In this study, an attempt has been made to investigate the effects of estradiol administration in a freshwater catfish, *Heteropneustes fossilis* maintained either in artificial freshwater or in calcium-deficient freshwater. The experimentally induced changes by this hormone in the plasma calcium level have been correlated with the activity of the ultimobranchial gland.

2. Materials and methods

Live specimens of the freshwater catfish *Heteropneustes fossilis* (both sexes; body wt. 27-39 g) were collected from Gorakhpur, India (26.7606° N, 83.3732° E) and acclimated to laboratory conditions for two weeks in plastic pools (48 inch x 40 inch x 22 inch; capacity 125 gallon). The tank-water was half renewed daily and the fish were fed on dry shrimp powder on alternate days. For experimentation, 12 fish were kept in glass aquaria containing 10 litres of the medium. The water was replaced on alternate days. The Ethical Committee of the Department of Zoology, DDU Gorakhpur University, approved all the experimental protocols.

Experimentation: After acclimation the fish were divided into four groups (A-D) and were given the following treatments:

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

Group B: The fish from this group were given a single intra-peritoneal injection of 0.1 ml of estradiol preparation (1 mg/100 g body wt) and kept in artificial freshwater.

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

Group D: The fish from this group were given a single intra-peritoneal injection of 0.1 ml of estradiol preparation (1 mg/100 g body wt) and kept in calcium-deficient freshwater.

The preparation of estradiol used for the groups B and D were dissolved in groundnut oil. The doses of estradiol were selected on the basis of experiments done earlier on the same fish species (Singh *et al.*, 2009). Plasma concentrations of E_2 ranged from 0.12±0.04 ng ml−1 to 0.96±0.13 ng ml−1 in males and 1.04±0.19 ng ml−1 to 10.50±0.97 ngml−1 in females from resting phase to spawning phase (Tewary *et al.*, 2001). The fish were not fed for a period from 24 h before to the end of the experimentation.

For experimentation freshwater and calcium-deficient freshwater were prepared with the following compositions:

a) **Artificial freshwater:** 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 the artificial freshwater without $CaCl_2$.

Twelve fishes from each group were anaesthetized with MS 222 and blood samples from caudal peduncle were collected in heparinized tubes on days 1, 3, 5, 10 and 15 following the treatment. The plasma were separated by centrifugation and used to analyse its calcium content with Sigma kits. After collection of the blood samples, the fish were autopsied and the body parts containing the UBG gland were removed and fixed in aqueous Bouin's fluid. The tissues were routinely processed in graded series of alcohol, cleared in xylene and embedded in paraffin. Serial sections were cut at 6 µm and stained with hematoxylin-eosin (HE).

Nuclear indices (length and width) were determined (300 nuclei were measured from six specimens) with the aid of ocular micrometer, and then nuclear volume was calculated as:

$$\text{Volume} = 4/3\ \pi\ ab^2$$

Where 'a' is the major semiaxis and 'b' is the minor semiaxis.

Data were presented as the mean ± S.E. of six specimens and Student's t-test was used to determine statistical significance, and P value was set at <0.05. Each experimental group was compared to its specific time control group.

3. Results

3.1. Biochemical and histological parameters of Group A and B fish:

Plasma calcium level of the vehicle-injected fish (group A) exhibited almost no change throughout the experiment (Figure 1).

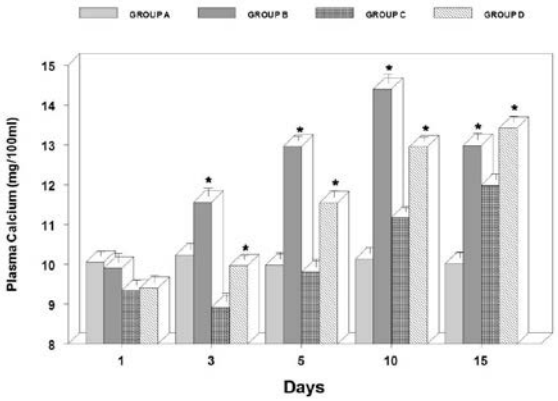

Figure 1. Changes in the plasma calcium levels of *Heteropneustes fossilis* kept under four different experimental conditions. Group A vehicle-injected fish kept in artificial freshwater, Group B estradiol-injected fish kept in artificial freshwater, Group C vehicle-injected fish kept in calcium-deficient freshwater or Group D estradiol-injected fish kept in calcium deficient freshwater. Each value represents mean ± S.E. of six specimens. Asterisk indicates significant differences (P<0.05) with vehicle-injected specimens

No significant change has been noticed in the plasma calcium level in the estradiol treated group B fish up to day 1. However, a progressive increase of calcium level was observed from day 3 to day 10, but after day 15 the level slightly decreased (Figure 1).

The ultimobranchial gland of the vehicle-injected (control; group A) *Heteropneustes fossilis* exists in the interseptum between the pericardial and abdominal cavities. It is not visible with the naked eyes and can only be detected in the serial sections of the interseptum with the aid of an optical microscope. A thick connective tissue sheath envelops the ultimobranchial gland. The ultimobranchial gland consists of follicles and cell cords (Figure 2). All the ultimobranchial cells are alike, having indistinct cell boundaries and eosinophilic cytoplasm. The

gland of the vehicle-injected fish exhibited no change in histological structure throughout the experiment.

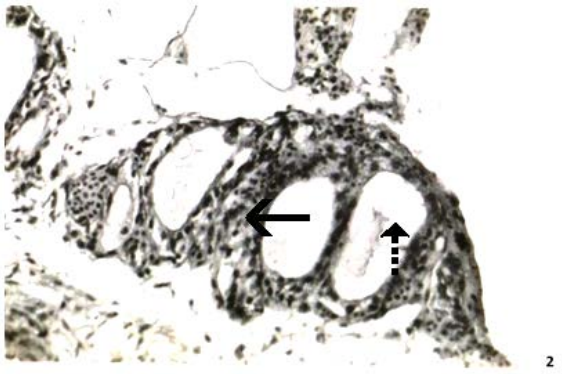

Figure 2. Histomorphograph of ultimobranchial gland of 5 day vehicle-injected *Heteropneustes fossilis* kept in artificial freshwater showing follicles (broken arrow) and cords (arrow). HE x 200.

In estradiol-treated group B fish, there was no change in the nuclear volume of ultimobranchial cells up to day 3. From day 5 to 10 the nuclear volume exhibited a progressive increase (Figure 3). Moreover, the ultimobranchial cells display poor staining response after days 5 and day 10 (Figure 4). After day 15, few degenerating cells were noticed (Figure 5) and the nuclear volume decreased slightly (Figure 3).

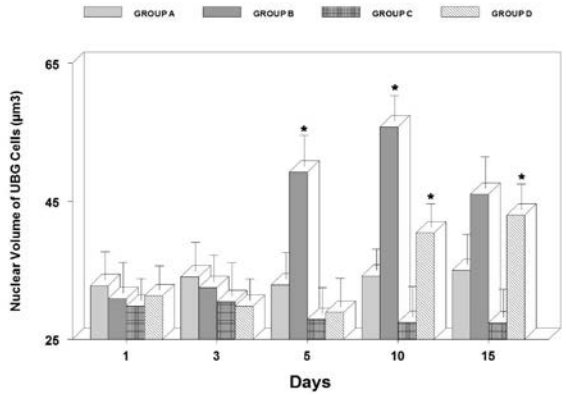

Figure 3. Nuclear volume of ultimobranchial gland of *Heteropneustes fossilis* kept under four different experimental conditions. Group A vehicle-injected fish kept in artificial freshwater, Group B estradiol-injected fish kept in artificial freshwater, Group C vehicle-injected fish kept in calcium-deficient freshwater or Group D estradiol-injected fish kept in calcium deficient freshwater. Each value represents mean ± S.E. of six specimens. Asterisk indicates significant differences ($P<0.05$) with vehicle-injected specimens.

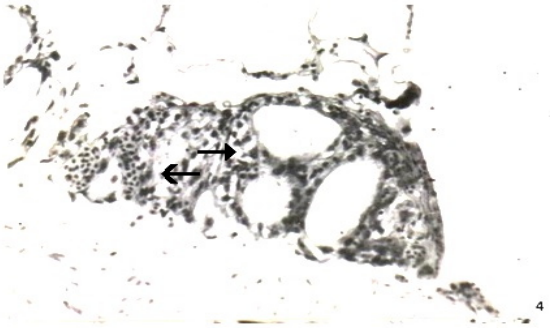

Figure 4. Histomorphograph of ultimobranchial gland of 10 day estradiol treated fish kept in artificial freshwater exhibiting weak staining (arrows) response. HE x 200.

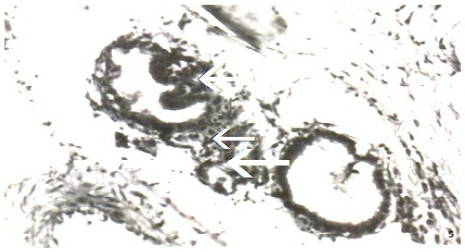

Figure 5. Histomorphograph exhibiting degenerating cells (arrows) in the ultimobranchial gland of 15 days estradiol treated *Heteropneustes fossilis* maintained in artificial freshwater. HE x 200.

3.2. Biochemical and histological parameters of Group C and D fish:

The plasma calcium level of the vehicle-injected group C fish exhibited a slight decrease on days 1 and 3 as compared to fish kept in artificial freshwater. Thereafter, the plasma calcium level had increased on days 5, 10 and 15 resulting in hypercalcemia (Figure 1).

The plasma calcium levels of the estradiol treated group D fish exhibited no change at day 1. Estradiol treatment caused a progressive increase in the plasma calcium level from day 3 to 15 (Figure 1).

There was no change in the ultimobranchial gland of the vehicle-injected group C fish up to day 5. On days 10 and 15, the ultimobranchial cells exhibited slightly less staining intensity (Figure 6). These cells did not exhibit significant change in their nuclear volume throughout the experiment (Figure 3).

The nuclear volume of ultimobranchial cells in estradiol-treated group D fish did not show significant change up to day 5. From day 10 to 15, a progressive increase in the nuclear volume (Figure 3) of the ultimobranchial cells was observed with less staining intensity (Figure 7). Moreover, after day 15, few degenerating cells were noticed in the gland (Figure 8).

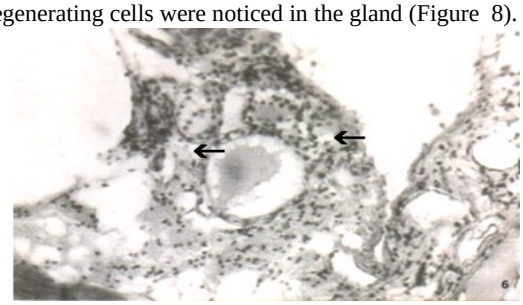

Figure 6. Histomorphograph of ultimobranchial gland of 10 day vehicle-injected fish kept in calcium-deficient freshwater showing decrease staining response of the cytoplasm (arrows). HE x 200.

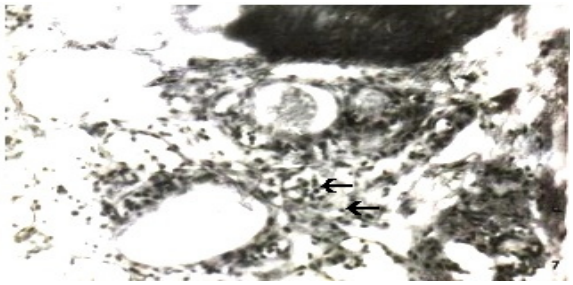

Figure 7. Histomorphograph of ultimobranchial gland of 10 day estradiol treated *Heteropneustes fossilis* kept in calcium-deficient freshwater depicting weak staining (arrows) response of the cytoplasm. HE x 200.

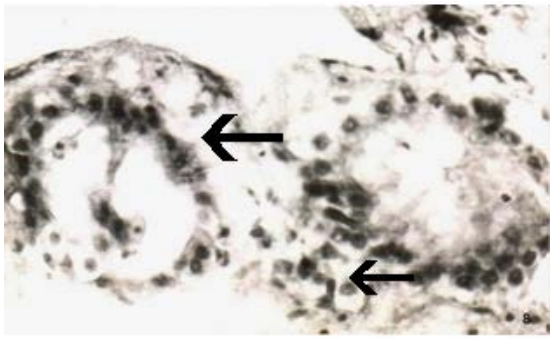

Figure 8. Histomorphograph exhibiting degenerating cells (arrows) in the ultimobranchial gland of 15 day estradiol treated fish maintained in calcium-deficient freshwater. HE x 200.

4. Discussion

In the present study, estradiol treatment caused elevation in the plasma calcium level of the fish kept in artificial freshwater. This derives support from reports of earlier workers, who have also reported an increase in plasma levels of this electrolyte after estradiol treatment (Baily 1957; Fleming *et al.* 1964; Yamada *et al.* 1982; Bjornsson and Haux 1985; Bjornsson *et al.* 1989; Norberg *et al.* 1989; Madsen and Korsgaard 1991; Mugiya and Hazama 1994; Persson *et al.* 1994, 1995 and Srivastav *et al.* 2016, 2017). The increased blood calcium might be due to increased mobilization from internal stores. Calcium mobilization from scales has been noticed in E_2 (estradiol) treated rainbow trout (Carragher and Sumpter 1991 and Persson *et al.* 1994) and goldfish and killifish (Mugiya and Watabe 1977). Muscle calcium mobilization has been reported by Persson *et al.* (1994) in E_2 treated rainbow trout. Mugiya and Watabe (1977) and Carragher and Sumpter (1991) have reported that there was no change in calcium content of muscle, vertebrae, rib bones, jaws or otolith of rainbow trout (*Oncorhynchus mykiss*) after estradiol treatment.

As the fish were not fed in the present study, the increased plasma calcium content in estradiol treated *H. fossilis* could not be attributed to the increased intestinal calcium uptake.

In calcium-deficient freshwater estradiol administration resulted into hypercalcemia. There exists no report of such a study from teleosts. Since calcium is not available in the surrounding media and also the animals were not fed in the present study, the hypercalcemia observed in estradiol treated *H. fossilis* could not be linked to the calcium absorption at intestinal mucosa and/or branchial calcium uptake. Enhanced calcium uptake from the environment after estradiol injection has been reported from other fish species (Fleming *et al.* 1964 and Persson *et al.* 1994). In contrast, Mugiya and Ichii (1980) have noticed no effect of estradiol on the *in situ* branchial calcium uptake.

The ultimobranchial gland of estradiol treated fish kept either in artificial freshwater or calcium deficient freshwater exhibited an increased activity which is evident by increased nuclear volume and degranulation of ultimobranchial cells. Several workers have noticed an increase in the activity of ultimobranchial gland during gonadal maturation (Lopez *et al.* 1968; Yamane and Yamada 1977; Srivastav 1983; Ahmad and Swarup 1988 and Singh 1990). Moreover, it has been reported that female chum salmon and Japanese eel have higher circulating calcitonin levels during spawning period (Deftos *et al.* 1974; Watts *et al.* 1975 and Yamauchi *et al.* 1978). The observed hyperactivity of ultimobranchial gland may be attributed to the estradiol induced maturation as suggested by these workers. This response of the ultimobranchial gland may also be linked to the protection of skeletal calcium as suggested by Chan *et al.* (1968) and Yamauchi *et al.* (1978).

In vehicle-injected *H. fossilis* kept in calcium-deficient freshwater, the ultimobranchial gland became active on day 10 and 15, which is evident from weak staining response of the ultimobranchial cells. This may be due to the observed hypercalcemia at these intervals.

5. Conclusion

It can be concluded that (i) estradiol caused hypercalcemia in fish kept either in artificial freshwater or in calcium-deficient freshwater, (ii) The ultimobranchial gland of estradiol treated fish kept either in artificial freshwater or calcium deficient freshwater exhibited an increased activity.

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