Original article

Responses of Corpuscles of Stannius to intra-peritoneal vitamin-D₃ administration in teleost *Labeo rohita* (Hamilton, 1822) reared in water with two different levels of calcium concentration

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ABSTRACT

This study assessed the responses of vitamin-D₃ intraperitoneally injected to Rohu, *Labeo rohita* @ of 0 IU/ kg bw (only solvent), 100 IU/kg bw and 500 IU/kg bw reared in 20 and 40 ppm of calcium (Ca) enriched water. The cellular changes in Corpuscles of Stannius (CS) gland, serum Ca, and inorganic phosphate (P_i) level were analysed up to the 60th day. Rohu administered with 100 IU/kg bw D₃ and exposed to 40 ppm Ca-rich water exhibited notable hyperplasia of CS compared with their control groups. Notable changes with high serum Ca level (13.87 ± 0.3 mg/dl) was detected on the 5th day in fish exposed to 40 ppm Ca-rich water, while related values attained (13.74 ± 0.1 mg/dl) only after 7 days in 20 ppm Ca-rich water of 500 IU/kg bw vitamin D₃ injection. Similarly, high serum P_i level (7.66 ± 0.2 mg/dl) in 40 ppm Ca injected with D₃ at 500 IU/kg bw. The results demonstrated that the Ca homeostasis of *Labeo rohita* is influenced by intra-peritoneal vitamin D₃. Progressive studies should be conducted by increasing the dose of vitamin D₃ to investigate optimum dose/supplement in feed for commercially important aquaculture teleost *Labeo rohita* for maximum and sustainable absorption of Ca from the variable water Calcium levels to maintain Ca^{2+} homeostasis.

1. Introduction

Freshwater aquaculture in India is dominated by Indian Major Carps (*Labeo rohita, Catla catla* and *Cirrhinus mrigala*), by contributing about 87% of the total freshwater fish production (ICLARM, 2001). Among the IMC, *Labeo rohita* is being widely cultured throughout India and accounts for a majority of the production, and have a great potential in terms of biodiversity as well as consumer preferences.

Corpuscle of Stannius (CS) is endocrine tissue secretes hypocalcemic factor(s), Stanniocalcin to prevent hypercalcemia by reducing branchial and whole body Ca uptake (Fontaine, 1964). Many workers (Fenwick, 1976; Fenwick and Forster, 1972; Wendelaar Bonga and Greven, 1978; Pang et al., 1975). Olivereau (1964) and Johnson (1972) and Gu et al (2015) reported that CS is more active in seawater (rich in calcium) than in freshwater (poor in calcium) and inferred that calcium content of the surrounding water has a direct impact on the CS activity and transcriptomic responses of CS. Suryawanshi and Mahajan (1976) and Ahmad and Swarup (1979) have also reported that the activity of CS increases in the fishes kept in calcium-rich freshwater. In past years, a steady flow of papers has advanced evidence that Ca^{2+} influx in branchial, intestinal (and likely in renal Ca^{2+} transporting cells) is controlled by stanniocalcin (STC) (Buttler, 1993; Flik et al., 1993; Wendelaar Bonga and Pang, 1986ab). STC is a hormone produced by the so-called CS endocrine gland. In gill and intestine STC inhibits Ca^{2+} entry (Flik et al, 1993; Verbost et al., 1993) and it thus, controls the permeability of Ca^{2+} of the apical membrane through a secondary messenger (cAMP) dependent pathway most likely a Camp operated calcium channel (Verbost et al., 1993). In the complex epithelium of the gills' chloride cells, specialized ion transporting cells mediated Ca^{2+} uptake (Flik et al., 1995; McCormic, 1993; Perry et al., 1992). Stanniocalcin is a glycoprotein hormone important in the maintenance of Ca and P_i homeostasis in fish. Two mammalian related stanniocalcin genes, STC1 and STC2, were found to be expressed in various tissues of fish as paracrine regulators (Luo et al., 2005; Joshi, 2020). Although Stannniocalcin-1

(STC-1) was originally described in fish, it is now known to be present throughout the animal kingdom in both vertebrates and invertebrates (Ishibashi and Imai, 2002; Yoshiko and Aubin, 2004; Tanega et al., 2004; Richards et al., 2012; Palma et al., 2019). The principal sources of STC-1 in bony fish are endocrine glands known as the Corpuscles of Stannius (CS) which are anatomically associated with the kidneys. STC-1 release is stimulated by a rise in serum levels of ionic Ca above the physiological set point through the activation of Ca-sensing receptors (Richards et al., 2012). The hormone then exerts regulatory effects on the epithelial transport of Ca and/or phosphate across the gills, gut, and kidneys to restore normocalcemia as the inhibitory action of STC reduces mucosa to serosa calcium transport, which means that the uptake in gills and intestine and re-uptake from ultra-filtered plasma in the nephron is controlled (Flik and Verbost, 1996). The transcriptomic responses of corpuscle of Stannius gland of Japanese eels (*Anguilla japonica*) to Changes in Water Salinity are recently reported by Gu et al (2015). The effects of *Euphorbia royleana* on the alteration of CS of *Heteropneustes fossilis* are demonstrated by Prasad et al. (2017).

Administration of vitamin D_3 and its derivative had been demonstrated in many fishes to make it hypercalcemic and to know the changes in serum Ca and P_i level (Srivastav and Srivastav, 1988; Srivastav and Singh, 1992). Although vitamin D_3 is abundantly present in fish liver, its role in Ca homeostasis has been emphatically denied (Rao and Raghuramulu, 1995). It has been reported that vitamin D_3 and its metabolites induce hypercalcemia in fishes (Singh and Srivastav, 1996; Swarup and Srivastav, 1982; Srivastav et al., 1985, 1998; Hayes et al., 1986; Srivastav and Singh, 1992). However, there is not single literature on the effect of vitamin D_3 on the physiological response to Indian major Carps, the most important fishes for aquaculture in India and other neighbouring Asian countries, and changes in serum Ca and P_i level. This study clearly explains the histological change and efficiency of CS and its hormone Stanniocalcin in freshwater fish *Labeo rohita*. Thus the objective of the present study is to assess the response and behavior of Ca and P_i regulating endocrine gland, Corpuscles of Stannius (CS) in *Labeo rohita*, most cultured fish in aquaculture system when reared in two levels of Ca enriched water exposed to intra-peritoneal doses of vitamin D_3.

2. Materials and methods

2.1. Experimental fish

The experiment was conducted for 60 days at the wet laboratory of ICAR - Central Institute of Fisheries Education (Deemed University), Mumbai. Healthy *Labeo rohita* (weight: 30 ± 2 g;) fishes were brought from Mahad fish farm, Mumbai in oxygen-filled polythene bags to CIFE, Mumbai. The fish stock was acclimatized for one week in calcium-deficient water under an aerated condition with a basal diet.

2.2. Experimental setup

The experimental setup consists of 18 uniform size plastic rectangular tanks (80 cm × 57 cm × 42 cm, 150 L capacity) covered with perforated lids. Two hundred and sixteen fish were randomly and equally distributed and stocked into experimental tanks with a 2 × 3 factorial design in triplicates. The calcium level in water is enriched using Calcium chloride ($CaCl_2 \cdot 2H_2O$) and the experimental fish were stocked @12 fish/tank into 2 groups, namely group A – 20 ppm Ca, and group B – 40 ppm Ca enriched water. The level of Ca in the experimental tank is measured using EDTA titration method (APHA, 1998). Similarly, water quality parameters like

pH, Dissolved Oxygen, Ammonia, Nitrite, Nitrate, and Temperature were estimated periodically as per APHA (1998) and maintained throughout the experiment.

2.3. Intra-peritoneal injection

After stocking fish from both groups were injected intraperitoneally i.e., in the abdomen at a 45° angle between the pelvic fins and anal vent with vitamin D_3 at different doses of, (i) (0.0 IU/kg bw only solvent, Arachis oil) (ii) (100 IU/kg bw) and (iii) (500 IU/kg bw). By considering 0.2 ml Vitamin D_3 injection for 25 g size fish, the 3Lakh IU Arachitriol (oil-based) was diluted with Arachis oil. Out of 9 tanks from group A with 20 ppm Ca water, 3 tanks were given Vitamin D_3 Intraperitoneal injection @ the dose of 0 IU/kg bw (control, only 0.2 ml solvent, Arachis oil), 3 tanks were given Vitamin D_3 Intraperitoneal injection @ the dose of 100 IU/kg bw and 3 tanks were given Vitamin D_3 Intraperitoneal injection @ the dose of 500 IU/kg bw respectively. A, similar setup is arranged for group B with 40 ppm Ca-rich water. The controls were given only solvent (without vitamin D_3 to give similar conditions in control and experimental fishes of both the treatments A & B), thus, the administration of Vitamin D_3 was not done to the control group. The doses 100 IU/kg and 500 IU/kg is at par with many authors used in their studies (Swarup and Srivastav, 1982; Swarup and Norman, 1996; Singh and Srivastav, 1996; Srivastav et al., 1998).

2.4. Fish sampling and serum analysis

Sampling was done on Day 1, 2, 3, 5, 7, 9, 11, 13, 15, 30, and 60. In each sampling, one fish from each replicate was sacrificed during the study period and taken for ionic activity, and histological studies. Serum samples were taken by collecting blood in vials using a sterile syringe and they are kept aside without any disturbance for separation of serum from a blood clot. Then the vials were centrifuged for perfect separation of serum without any hemolysis to estimate Ca and inorganic phosphate (P_i) level using Trinder (1960) and Fiske and Subbarow, (1925) methods respectively.

2.5. Histological studies

The Corpuscle of Stannius along with the adjoining portion of the kidney was removed from the fish of each treatment group and they were fixed in aqueous Bouin's fluid. The fixed samples were processed by standard techniques following a series of alcohol treatment, cleared in xylene, and embedded in paraffin wax. Thin (5 μm) sections were prepared with a microtome and stained with Haematoxylin/Eosin (HE).

2.6. Statistical analysis

The results of each experiment are expressed as mean ± standard deviation and analysed by multivariate analysis of variance (ANOVA) using statistical package SPSS version 16 to test the significance of the difference between the control and experimental groups. A probability level of 0.05 was used to find out the significance in all cases.

3. Results

3.1. Change in serum Ca and inorganic phosphate (P_i)level and cellular changes of Corpuscle of Stannius in two different Ca enriched groups

3.1.1. Control group (A and B)

The serum Ca and P_i level was observed on days; 0, 1st, 2nd, 3rd, 5th, 7th, 9th, 11th, 15th, 30th, and 60th day. And there was no rise

in serum Ca and P_i levels from day 1 to day 60 (Table 1, Figs. 1–4). The corpuscles of Stannius also possess oval or rounded nuclei without any cellular changes (Figs. 5 and 6).

3.2. Group A (20 ppm Ca) – experimental group, AL and AH

3.2.1. Calcium analysis

In low dose (100 IU/kg bw) there is an increase in Ca level and reached the peak at 5th day up to 11.45 ± 0.4 mg/dl and again

started decreasing till 60th day (Table 1). At the same time for high dose (500 IU/kg bw) the uptake of Ca is higher than low dose and reached maximum on 7th day at a range of 13.74 ± 0 mg/dl. and again it reduced till 60th day (Fig. 1).

3.2.2. Inorganic phosphate analysis

In low dose (100 IU/kg bw) there is an increase in P_i level and reaching the peak at 5th day up to 6.23 ± 0.5 mmol/l and again started decreasing till 60th day (Table 2). Similarly for high dose

Table 1
Serum Ca concentration (mg/dl) of *L. rohita* injected with graded level of Vitamin D_3 and reared in group A and B.

Sr. No.	Day	Control (A) 20 ppm Ca	Exp.(AL) 100 IU/kg b.w.	Exp.(AH) 500 IU/kg b.w.	Control(B) 40 ppm Ca	Exp.(BL) 100 IU/kg b.w.	Exp.(BH) 500 IU/kg b.w.
1	0	8.42 ± 0.4	8.44[a] ± 0.2	8.47[a] ± 0.6	8.38 ± 0.2	8.39 [a] ± 0.4	8.45[a] ± 0.4
2	1	8.54 ± 0.6	8.79[a] ± 0.5	8.92[a] ± 0.4	8.47 ± 0.3	8.88 [a] ± 0.2	8.99[a] ± 0.2
3	2	8.52 ± 0.3	10.55[b] ± 0.4	12.36[b] ± 0.2	8.49 ± 0.4	10.78[b] ± 0.3	11.76[b] ± 0.3
4	3	8.49 ± 0.3	10.59[b] ± 0.1	12.98[b] ± 0.6	8.46 ± 0.6	10.79[b] ± 0.4	13.55[c] ± 0.2
5	5	8.47 ± 0.1	11.45[c] ± 0.4	13.05[b] ± 0.5	8.40 ± 0.2	12.48[c] ± 0.5	13.87[c] ± 0.3
6	7	8.48 ± 0.2	10.26[b] ± 0.2	13.74[c] ± 0.1	8.37 ± 0.4	11.48[c] ± 0.7	13.80[c] ± 0.7
7	9	8.55 ± 0.7	9.78[a,b] ± 0.3	12.28[b] ± 0.2	8.44 ± 0.3	9.24[a,b] ± 0.2	12.72[b] ± 0.6
8	11	8.37 ± 0.5	8.75[a] ± 0.4	9.98[a] ± 0.3	8.42 ± 0.2	8.94[a] ± 0.5	10.02[b] ± 0.4
9	15	8.43 ± 0.4	8.41[a] ± 0.1	8.49[a] ± 0.4	8.38 ± 0.5	8.66[a] ± 0.2	9.06[a] ± 0.1
10	30	8.45 ± 0.7	8.36[a] ± 0.5	8.47[a] ± 0.6	8.33 ± 0.1	8.74[a] ± 0.3	8.54[a] ± 0.1
11	60	8.49 ± 0.2	8.23[a] ± 0.3	8.38[a] ± 0.4	8.40 ± 0.4	8.43[a] ± 0.2	8.48[a] ± 0.2

Different superscript in a column differ significantly at 95% confidence limit (P < 0.05) A = Control group (20 ppm Ca) with 0.0 IU Vitamin D_3/kg bw (only solvent); AL = Group with low dose IP, 100 IU Vitamin D_3/kg bw; AH = Group with high dose IP, 500 IU Vitamin D_3/kg bw; B = Control group (40 ppm Ca) with 0.0 IU Vitamin D_3/kg bw (only solvent); BL = Group with low dose IP, 100 IU Vitamin D_3/kg bw; BH = Group with high dose IP, 500 IU Vitamin D_3/kg bw.
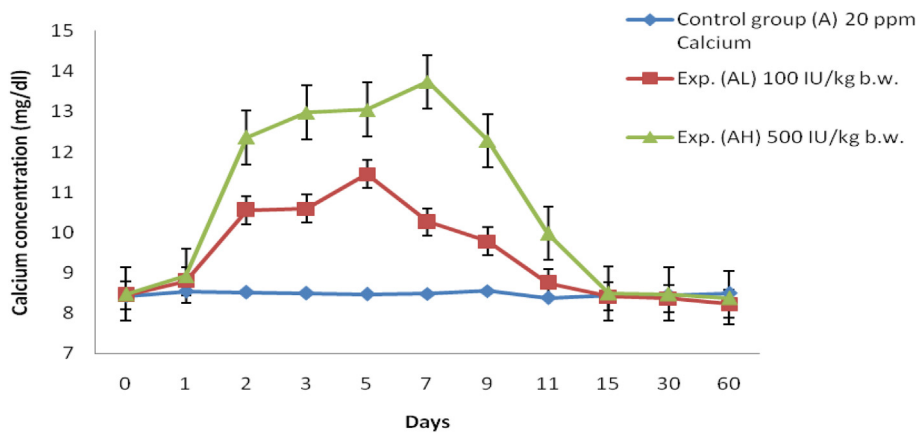

Fig. 1. Serum Ca concentration (mg/dl) of *Labeo rohita* injected with graded levels of vitamin D_3 and reared in group A (20 ppm Ca).
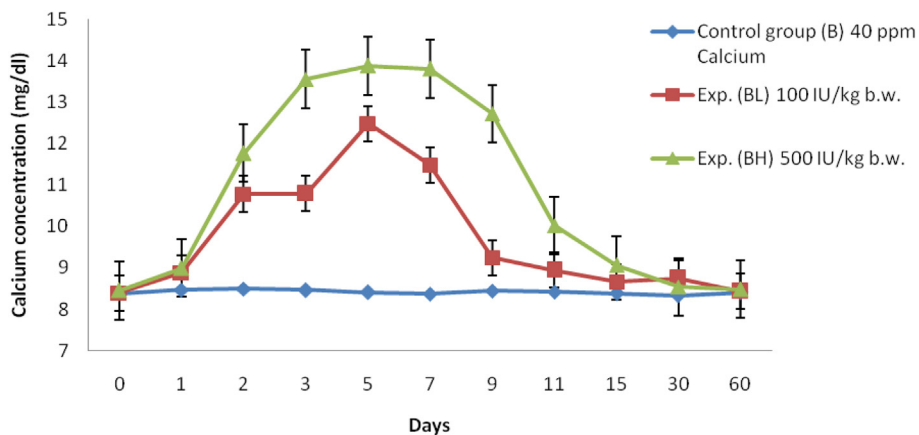

Fig. 2. Serum Ca concentration (mg/dl) of *Labeo rohita* injected with graded levels of vitamin D_3 and reared in group B (40 ppm Ca).
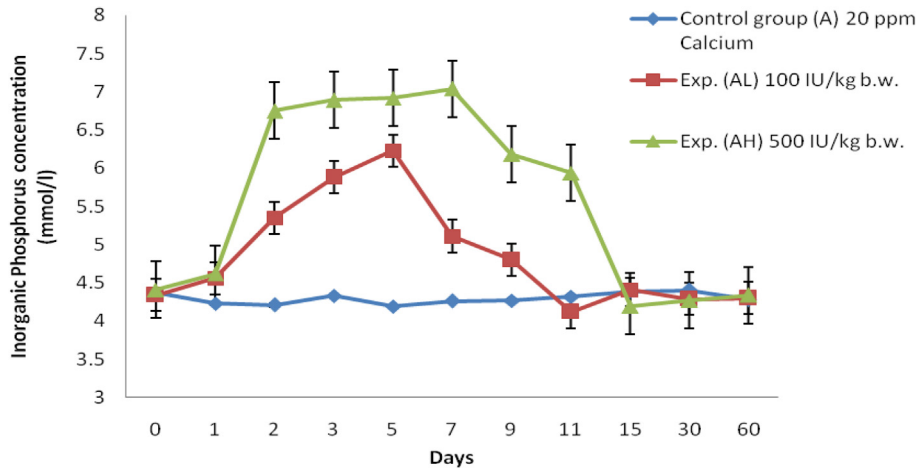
Fig. 3. Serum inorganic phosphorus concentration (mmol/l) of *Labeo rohita* injected with graded level of vitamin D_3 and reared in group A (20 ppm Ca).
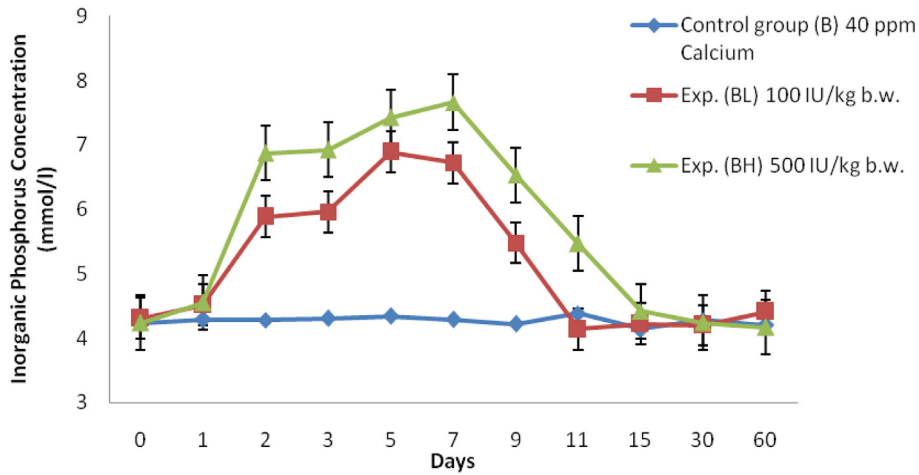

Fig. 4. Serum inorganic phosphorus concentration (mmol/l) of *Labeo rohita* injected with graded level of vitamin D_3 and reared in group B (40 ppm Ca).
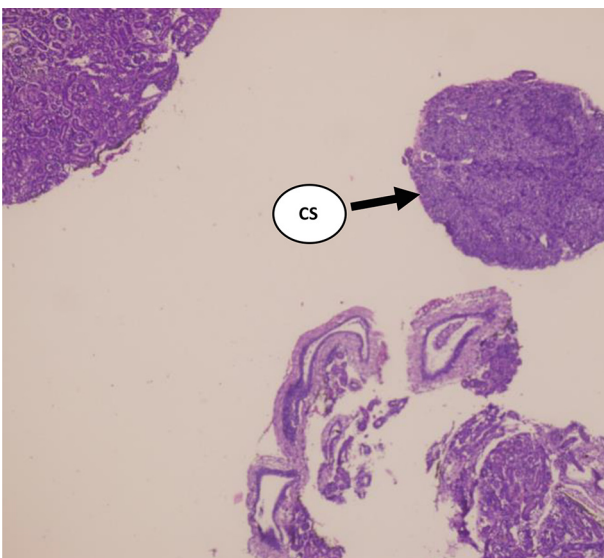

Fig. 5. Section of Corpuscles of Stannius (CS) in the fish, *Labeo rohita* in Control group A (H and E 4X) Day – 0 (Ca 20 ppm and 0.0 IU D_3).
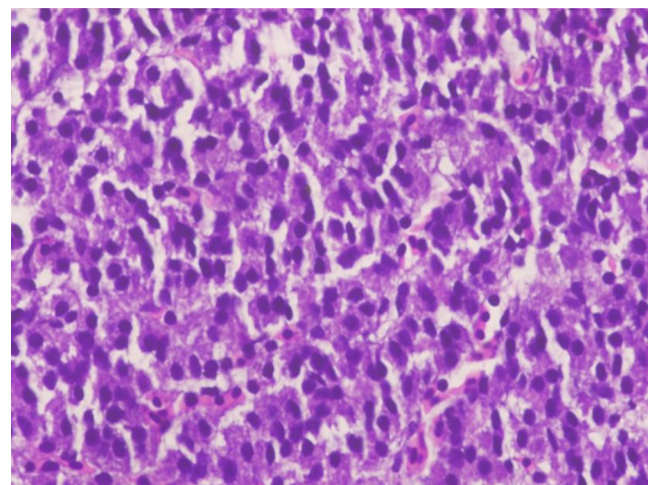

Fig. 6. Section of Corpuscles of Stannius in the fish, *Labeo rohita* in Control Group A (H and E 60X) Day – 0 (Ca 20 ppm and 0.0 IU D_3).

Table 2
Serum Inorganic phosphorus concentration (mmol/l) of *L.rohita* injected with graded level of Vitamin D$_3$ and reared in group A and B.

Sr. No.	Day	Control (A) 20 ppm Ca	Exp.(AL) 100 IU/kg b.w.	Exp.(AH) 500 IU/kg b.w.	Control (B) 40 ppm Ca	Exp.(BL) 100 IU/kg b.w.	Exp.(BH) 500 IU/kg b.w.
1	0	4.37 ± 0.2	4.34[a] ± 0.1	4.41[a] ± 0.2	4.24 ± 0.3	4.31[a] ± 0.2	4.24[a] ± 0.4
2	1	4.23 ± 0.1	4.56[a] ± 0.3	4.62[a] ± 0.2	4.29 ± 0.2	4.52[a] ± 0.2	4.55[a] ± 0.1
3	2	4.21 ± 0.2	5.35[b] ± 0.2	6.75[b] ± 0.1	4.28 ± 0.1	5.88[b] ± 0.3	6.87[b] ± 0.1
4	3	4.33 ± 0.4	5.88[b] ± 0.2	6.89[b] ± 0.3	4.31 ± 0.2	5.96[b] ± 0.1	6.92[b] ± 0.2
5	5	4.19 ± 0.2	6.23[c] ± 0.5	6.92[b] ± 0.4	4.34 ± 0.3	6.89[c] ± 0.2	7.42[b] ± 0.3
6	7	4.26 ± 0.1	5.11[b] ± 0.3	7.04[c] ± 0.2	4.29 ± 0.3	6.72[b] ± 0.5	7.66[c] ± 0.2
7	9	4.27 ± 0.2	4.80[a] ± 0.4	6.18[b] ± 0.1	4.22 ± 0.2	5.48[a] ± 0.1	6.53[b] ± 0.1
8	11	4.32 ± 0.3	4.12[a] ± 0.2	5.94[a] ± 0.1	4.39 ± 0.1	4.14[a] ± 0.3	5.47[a] ± 0.2
9	15	4.39 ± 0.1	4.41[a] ± 0.2	4.19[a] ± 0.2	4.41 ± 0.2	4.22[a] ± 0.3	4.42[a] ± 0.1
10	30	4.40 ± 0.2	4.29[a] ± 0.3	4.27[a] ± 0.4	4.28 ± 0.3	4.20[a] ± 0.2	4.24[a] ± 0.2
11	60	4.28 ± 0.4	4.30[a] ± 0.1	4.34[a] ± 0.2	4.20 ± 0.3	4.41[a] ± 0.3	4.17[a] ± 0.3

Different superscript in a column differ significantly at 95% confidence limit (P < 0.05) A = Control group (20 ppm Ca) with 0.0 IU Vitamin D$_3$/kg bw (only solvent); AL = Group with low dose IP, 100 IU Vitamin D$_3$/kg bw; AH = Group with high dose IP, 500 IU Vitamin D$_3$/kg bw; B = Control group (40 ppm Ca) with 0.0 IU Vitamin D$_3$/kg bw (only solvent); BL = Group with low dose IP, 100 IU Vitamin D$_3$/kg bw; BH = Group with high dose IP, 500 IU Vitamin D$_3$/kg bw.

(500 IU/kg bw) the uptake of P$_i$ was higher than the low dose and reached maximum on 7th day at a range of 7.04 ± 0.2 mmol/l and again it reduced till 60th day (Fig. 3).
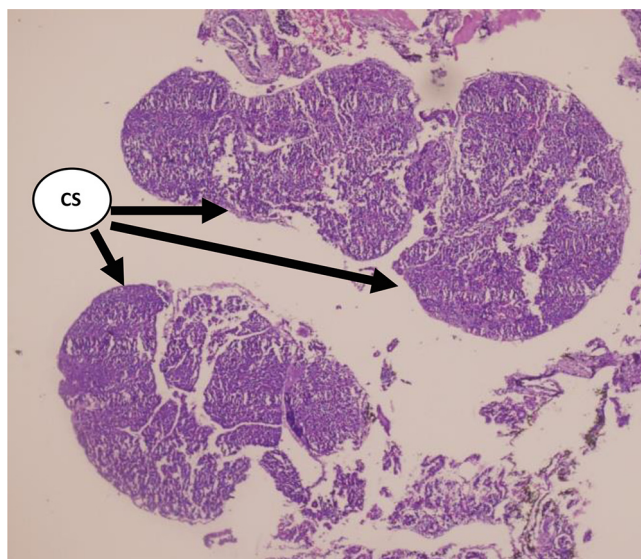

Fig. 7. Section of Corpuscles of Stannius in the fish, *Labeo rohita* in Experimental group AL (H and E 4X) Day-5 (Ca 20 ppm and 100 IU D$_3$).
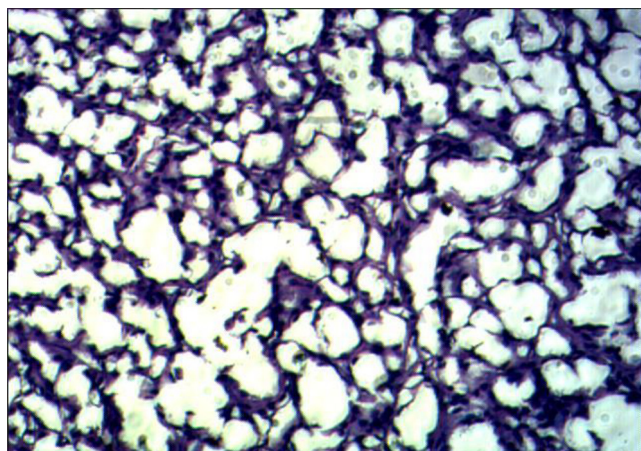

Fig. 8. Section of Corpuscles of Stannius in the fish, *Labeo rohita* in Experimental group AL (H and E 60X) Day-5 (Ca 20 ppm and 100 IU D$_3$).

3.2.3. Histological analysis

Cellular structures are shown in Figs. 7 and 8 on day- 5 showing the nuclear volume of cells records an increase and they become partially de-granulated as is evident by their weak staining response. There was an increased dilatation of sinusoids (Fig. 8) and these changes get exaggerated. The results of cellular activities are in correspondence with the serum levels of Ca and inorganic phosphate and demonstrating that there was the hypocalcemic response of CS gland.

3.3. Group B (40 ppm Ca) – experimental group, BL and BH

3.3.1. Calcium analysis

In low dose (100 IU/kg bw) there was an increase in Ca level and reaching peak at 5th day up to 12.48 ± 0.5 mg/dl and started decreasing till 60th day (Table 1, Fig. 2). At the same time for high dose (500 IU/kg bw) the uptake of Ca was higher than the low dose and reached maximum on 5th day at a range of 13.87 ± 0.3 mg/dl and it reduced till 60th day (Fig. 2).

3.3.2. Inorganic phosphate analysis

In low dose (100 IU/kg bw) there was an increase in P$_i$ level and reaching peak at 5th day up to 6.89 ± 0.2 mmol/l and started decreasing till 60th day (Table 2, Fig. 4). At the same time for high
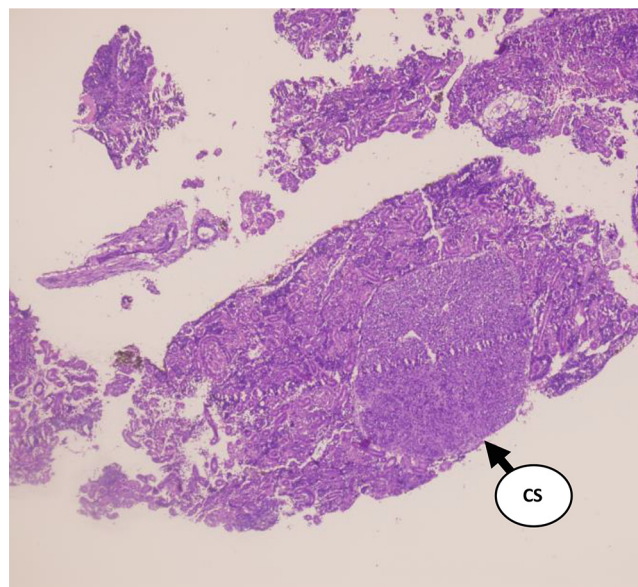

Fig. 9. Section of Corpuscles of Stannius in the fish, *Labeo rohita* in Experimental group BH (H and E 4X) Day-7 (Ca 40 ppm and 500 IU D$_3$).
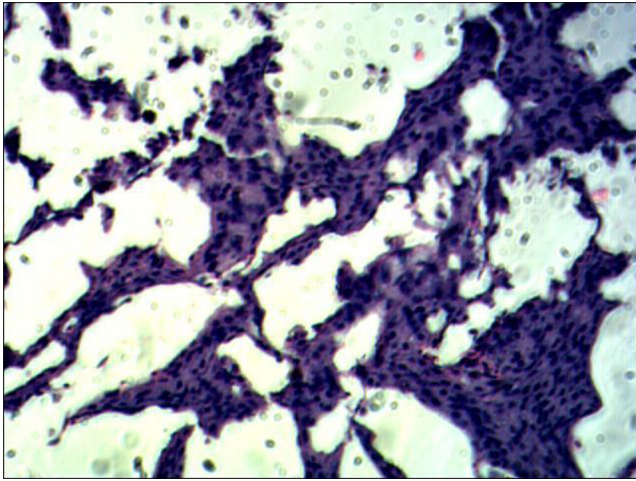
Fig. 10. Section of Corpuscles of Stannius in the fish, *Labeo rohita* in Experimental group BH (H and E 60X) Day – 7 (Ca 40 ppm and 500 IU D_3).

dose (500 IU/kg bw) the uptake of P_i was higher than the low dose and reached maximum on 7th day at a range of 7.66 ± 0.2 mmol/l and it reduced to normal condition as in case of control till 60th day (Fig. 4).

3.3.3. Histological analysis

The cellular structures are shown in Figs. 9 and 10 on day-7 showing the nuclear volume of cells records an increase and they become partially de-granulated as is evident by their weak staining response. Also, there is an increased dilatation of sinusoids (Fig. 10) and these changes get further exaggerated and complete exhaustion of the gland was recorded.

4. Discussion

4.1. Serum Ca concentration

Serum Ca concentration level in *Labeo rohita* is calculated and it is found to be higher on the 5th day of fish, which is reared in group A with a high dose (500 IU/kg bw) of vitamin D_3. At the same time, it is found to be higher on the 7th day of fish which is reared in group B with high dose (500 IU/kg bw) of vitamin D_3. Srivastav et al (1997a), who also observed vitamin D metabolites affect the serum Ca level in freshwater catfish *Heteropneustes fossilis*, in which there is an increase in serum Ca level at the day of 3 and 5, which were injected with intraperitoneal Vitamin D_3 and Srivastav et al. (1997b) also done a similar experiment in freshwater mud eel *Amphipnous cuchia* and reported that there is an increase in serum Ca level at day 10 when it is reared in Ca-rich environment and injected with 100 ng of vitamin D_3 for 100 g bw/-day. Bansal et al. (1979) showed increased serum Ca levels in *Labeo rohita* due to chronic chlordane exposure. This result shows similarity to the experiment done by Srivastava et al. (2012) in which the fish *Notopterus notopterus* treated with three level of vitamin D_3 dosage like 100, 500, 1000 IU/kg bw. In this, there is a peak levels of serum Ca is found on day 5 on the three levels. But the amount or level of Ca intake varies with the dosage. The dosage with 1000 IU/kg bw shown higher absorption of Ca from the environment which is followed by 500 and 100 IU. A number of authors studied hypercalcemic effects of vitamin D_3 metabolites (Singh and Srivastav, 1996; Swarup and Srivastav, 1982; Srivastav et al., 1985, 1998; Hayes et al., 1986; Srivastav and Singh, 1992), and showed that hypercalcemia depends on exposure time as well as on the type and concentration of the vitamin D_3 metabolite used (Swarup et al., 1984; Srivastav et al., 1993). Responses to vitamin

D treatments vary not only within but also among species. For example, injecting $1,25(OH)_2D_3$ in emerald rock cod (*Pagothenia bernacchii*) reduced free plasma Ca but left total plasma Ca levels unchanged, suggesting an increased fractional binding of Ca to plasma proteins (Fenwick et al., 1984).

In contrast, Sundell and Norman (1993) repeatedly injected $1,25(OH)_2D_3$ in the Atlantic cod and observed an increase of free Ca while total Ca levels remained unchanged. In male Mozambique tilapia (*O. mossambicus*) IP injections of $1,25(OH)_2D_3$ increased total plasma Ca without altering the free Ca levels (Srivastav et al., 1998). Vitamin D_3 injected male catfish (*Clarias batrachus*) acclimated to low Ca water increased their serum total Ca levels as compared with control fish. However, this increase doubled in vitamin D_3 injected catfish from water supplemented with extra Ca when compared to controls from the same water, although the duration of this increase was shorter than the one in fish from low Ca water (Swarup and Srivastav, 1982). A similar observation was performed in freshwater mud eel, *Amphipnous cuchia* (Srivastav, 1983). Magnitude and duration of the increase of plasma Ca in response to vitamin D_3 are dependent on the Ca concentration in the water (Srivastav and Srivastava, 1997). If Ca is not sufficiently available from the water or the diet, then fish can supplement plasma Ca from internal sources. Intra-peritoneal injections of unfed common carp with physiological doses of either vitamin D_3 or $1,25(OH)_2D_3$ resulted in hypercalcemia and hyperphosphatemia (Swarup et al., 1991), which suggests that the minerals must have been derived from internal sources. Similarly, daily injections with vitamin D_3 or 1, $25(OH)_2D_3$ in fed American eel (*A. rostrata*), increased plasma Ca and phosphorus, while this effect was absent in unfed eels (Fenwick et al., 1984). The plasma Ca levels in the fish are actually not controlled and/or regulated by the endocrine system only; other hormones like stanniocalcin (Pierson et al., 2004), parathyroid hormone and related protein (Guerreiro et al., 2007; Abbink and Flik, 2007), and the prolactin (Flik et al., 1984, 1989; Seale et al., 2006) are involved in the control mechanism as well.

4.2. Serum inorganic phosphate (P_i) concentration

In this experiment serum phosphorus concentration level in *Labeo rohita* is calculated and it is found to be higher on the 7th day of fish which is reared in group A with a high dose (500 IU/kg bw) of vitamin D_3. At the same time, it is found to be higher on the 7th day of fish which is reared in group B with a high dose (500 IU/kg bw) of vitamin D_3. Srivastav et al (1997a) who also observed vitamin D metabolites affect the serum phosphorus level in freshwater catfish (*Heteropneustes fossilis*), in which there is an increase in serum phosphorus level from day 3 to 10, and showing a hyperphophatemic reaction which was injected with intraperitoneal Vitamin D_3. Srivastav et al. (1997b) done a similar experiment in freshwater mud eel *Amphipnous cuchia* and reported that there is an increase in serum phosphorus level at 5th, when it is reared in Ca-rich environment and injected with 100 ng of vitamin D_3 for 100 g bw/day.

In contrast to Ca, fish must obtain phosphate via the diet as water phosphate levels are normally very low, and direct uptake of phosphate from the water is likely insignificant in fishes. Little information on the involvement of vitamin D_3 in phosphate metabolism in fish exists. Responses to vitamin D_3 metabolites on plasma phosphate vary between species. Daily intraperitoneal injection with vitamin D_3 or 1, $25(OH)_2D_3$ increase plasma phosphate in catfish (*C. batrachus*) (Swarup et al., 1984), American eel (Fenwick et al., 1984) and *C. carpio* (Swarup et al., 1991), but not in Mozambique tilapia (Rao and Raghuramulu, 1995). In unfed American eel (Fenwick et al., 1984) and freshwater mud eel, *A. cuchia* (Srivastav, 1983) plasma phosphate increased after intra-

peritoneal vitamin D$_3$ injection. Apparently, in addition to phosphate reabsorption in the kidney (Fenwick and Vermette, 1989), it can be mobilized by vitamin D$_3$ metabolites from a non-dietary source, presumably bone or soft tissues (Lopez et al., 1977).

4.3. Histology of Corpuscles of Stannius (CS)

In the present study, *Labeo rohita* injected with vitamin D$_3$ and kept in two different calcium environment exhibits degranulation of the CS cells by increase in the volume of the cells and sinusoidal dilatation. Earlier workers have considered hypertrophy of CS cells as an indication of the activity of CS in response to hypercalcemia (Olivereau and Olivereau, 1978; Srivastav et al., 1985; Srivastav and Srivastav, 1988). The volume and density of CS cells increase as a result of external Ca concentration (Urasa and Wendelaar Bonga, 1987). Suryawanshi and Mahajan (1976) and Ahmad and Swarup (1979) have also reported that the activity of CS increases in the fishes kept in calcium-rich freshwater. In *Labeo rohita* hypercalcemia results in degranulation of AF-positive cells. Aida et al. (1980) have suggested that the secretory activity of cells of CS may be directly affected by plasma ion levels, especially Ca ions. The present study supports this suggestion and also it agrees with the observations of Wendelaar Bonga et al (1980). According to Wendelaar Bonga et al (1980) the type-1 cells of CS are more active in the fish adapted to diluted or full-strength seawater than in freshwater specimens. For this response they have stated that the high activity of this cell in seawater is apparently due to the high Ca concentration of seawater. The degranulation of the cells of CS of *Labeo rohita* can be attributed to the increased release of hypocalcemic factor (Stanniocalcin) from CS to encounter the elevated level of Ca caused by vitamin D$_3$ treatment. The sinusoidal dilatations in response to hypercalcemia in *Labeo rohita* is similar to the observations on *Clarias batrachus* (Srivastav et al., 1985; Srivastav and Srivastav, 1988). The degeneration among few corpuscular cells observed in *Labeo rohita* in response to prolonged hypercalcemia is in agreement with the results obtained by Hiroi (1970) on *Oncorhynchus* sp, Srivastav et al (1985) and Srivastav and Srivastav (1988) on *Clarias batrachus* and histological and ultrastructure studies of CS in African catfish, *Clarias gariepinus* (Karkit et al., 2019). Advanced evidence that Ca^{2+} influx in branchial, intestinal (and likely in renal Ca^{2+} transporting cells) is controlled by stanniocalcin (STC) (Buttler, 1993; Flik et al., 1993; Wendelaar Bonga and Pang, 1986ab).

The degeneration is due to the exhaustion of corpuscular cells. In the CS of *Labeo rohita* kept in low Ca freshwater there is an increased storage of granules. This can be attributed to the observed decrease in the serum Ca and inorganic phosphate levels. Hypoactive cells have been noticed in the fish expose to low-Ca seawater (Wendelaar Bonga et al., 1980). Storage of secretory granules within calcitonin cells (which secrete a hypocalcemic factor in mammals) in response to hypocalcemia has also been reported earlier by Gittes et al., (1968) and Srivastav and Swarup (1982). In mammals, it has been suggested by Hirsch and Munson (1969) that the heavy accumulation of secretory granules in calcitonin cells during hypocalcemia results due to little or no calcitonin secretion and continuance of its biosynthesis. In the present study, the same principle seems to be involved. In a freshwater medium, there is no change in the cells of CS after vehicle-injected fish. This may be due to the non-involvement of this cell type in Ca homeostasis as according to Wendelaar Bonga et al (1976, 1980). In Ca-rich medium the cells of CS of fish and Ca/P$_i$ levels after vehicle or vitamin D$_3$-treatment exhibit a decrease in the volume. This conforms with the reports of Wendelaar Bonga et al (1976, 1980) and Meats et al (1978) who have reported indications for a reduction of secretory activity of CS from fish transferred from freshwater to seawater. Buttler, (1993), Flik et al. (1993), and Wendelaar

Bonga and Pang (1986a,b) had reported Ca^{2+} influx in branchial, intestinal, renal Ca^{2+} transporting cells is controlled by stanniocalcin in marine fishes.

5. Conclusion

The hypercalcemic and hyperphophatemic responses were more elevated in a higher doses of vitamin D$_3$ when fishes are reared in both the concentrations of calcium levels. Further, it is observed that the normocalcemic and normophosphatemic responses were faster in a lower dose of D$_3$ in both rearing conditions. It is of interest to note in the present study that CS cells exhibit an increased nuclear volume in Ca-rich when compared to those observed in low Ca freshwater. This increased activity of CS cells in Ca-rich freshwater may be attributed to the possible increase in the serum Ca and inorganic phosphate levels. Thus, this study helps in understanding the Ca and Pi regulation in commercially important freshwater Indian major carp fish, *Labeo rohita* reared in different calcemic environments of water available for aquaculture. A future study is needed to establish the mechanism of action and regulation of calcium homeostasis in important culturable food fish species reared in different water/soil conditions with special reference to saline and/or sodic soil water areas that are rising day by day in many countries including India.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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