CALCEMIC RESPONSES OF INTRAPERITONIAL VITAMIN-D_3 ON CORPUSCLES OF STANNIUS GLAND AND GENE EXPRESSION OF STANNIOCALCIN IN *LABEO ROHITA* (HAMILTON, 1822) REARED IN CALCIUM ENRICHED WATER

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M. F. Sc. (FISH PHYSIOLOGY AND BIOCHEMISTRY)

by

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TOTALLY DEDICATING TO MY GUIDE

DR. P.P. SRIVASTAVA

THEN TO MY

AMMA, APPA, KUTTY, JILL

and

WHOLE HEARTEDLY
LEAVING EVERYTHING IN
THE FOOT OF GOD

DECLARATION

I hereby declare that the dissertation entitled **"CALCEMIC RESPONSES OF INTRAPERITONEAL VITAMIN-D_3 ON CORPUSCLES OF STANNIUS GLAND AND GENE EXPRESSION OF STANNIOCALCIN IN *LABEO ROHITA* (HAMILTON, 1822) REARED IN CALCIUM ENRICHED WATER"** is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

Date: 30.06.2016

Place: Mumbai

(U.SIVAGURUNATHAN)

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DATE: 30-06-2016 **(U.SIVAGURUNATHAN)**

PLACE: CIFE-Mumbai

सारांश

विटामिन D_3 के प्रभाव को, जलकृषि के लिए पाली जाने वाली उत्तम, मछ्ली रोहू (*Labeo rohita*) के रक्त में calcium (Ca), अकार्बनिक phosphorus (P_i), Corpuscles of Stannius (CS) ग्रंथि की रचना में परिवर्तन एवं इसके द्वारा उत्पन्न हॉरमोन Stanniocalcin के gene की अभिव्यक्ति को मापा गया और यह किया गया जब की इन मछलियों को कम (20 ppm) एवं अधिक (40 ppm) calcium के पानी में संवर्धित किया गया। मछलियों को दोनों तरह के पानी में रखने के बाद उन्हें 100 IU D_3/kg शरीर-भार एवं 500 IU D_3/kg शरीर-भार के हिसाब से विटामिन D_3 के injection को खाल के नीचे लगाया गया। इसके लिए कुल 6 तरह के प्रयोगिक ग्रुप बनाये गए। 1). कम कैल्सियम नियंत्रित ग्रुप (D_0C_L): 0.0 IU D_3/kg शरीर-भार; 2). कम कैल्सियम प्रायोगिक ग्रुप -1 (D_LC_L); 100 IU D_3/kg शरीर-भार; 3). कम कैल्सियम प्रायोगिक ग्रुप-२ (D_HC_L): 500 IU D_3/kg शरीर-भार; 4). अधिक कैल्सियम नियंत्रित ग्रुप (D_0C_H): 0.0 IU D_3/kg शरीर-भार; 5). अधिक कैल्सियम प्रायोगिक ग्रुप -1 (D_LC_H): 100 IU D_3/kg शरीर-भार एवं 6). अधिक कैल्सियम प्रायोगिक ग्रुप-2 (D_HC_H): 500 IU D_3/kg शरीर-भार। injection लगाने के बाद रक्त के serum को 0, 1वें, 2वें, 3वें, 5वें, 7वें, 9वें, 11वें, 15वें, 30वें एवं 60वें दिन निकाला गया एवं उसमे calcium (Ca) एवं अकार्बनिक phosphorus (P_i) की मात्रा मापी गई। और यह पाया गया कि कम कैल्सियम प्रायोगिक ग्रुप -1 (D_LC_L) में 5वें दिन अधिकतम कैल्सियम (11.45$\pm$0.4 mg/dL) जबकि कम कैल्सियम प्रायोगिक ग्रुप-२ (D_HC_L) में 7वें दिन अधिकतम कैल्सियम (13.74$\pm$0.1 mg/dL) पाया गया। उसी तरह अधिक कैल्सियम प्रायोगिक ग्रुप -1 (D_LC_H) एवं अधिक कैल्सियम प्रायोगिक ग्रुप-2 (D_HC_H) में रक्त के कैल्सियम कि मात्रा क्रमशः 12.48$\pm$0.5 mg/dL एवं 13.87$\pm$0.3 mg/dL 5वें, एवं 7वें दिन मापी गई जबकि दोनों नियंत्रित ग्रुप में (D_0C_L एवं D_0C_H) calcium कि मात्रा में कोई परिवर्तन देखने को नहीं मिला। calcium की तरह अकार्बनिक phosphorus (P_i) के सान्द्रण में यह पाया गया कि कम कैल्सियम प्रायोगिक ग्रुप -1 (D_LC_L) में 5वें दिन अधिकतम P_i (6.23$\pm$0.5 mg/dL) जबकि कम कैल्सियम प्रायोगिक ग्रुप-२ (D_HC_L) में 7वें दिन अधिकतम P_i (7.04$\pm$0.2 mg/dL) पाया गया। उसी तरह अधिक कैल्सियम प्रायोगिक ग्रुप -1 (D_LC_H) एवं अधिक कैल्सियम प्रायोगिक ग्रुप-2 (D_HC_H) में रक्त के P_i कि मात्रा क्रमशः 6.89$\pm$0.2 mg/dL एवं 7.66$\pm$0.2 mg/dL 5वें, एवं 7वें दिन मापी गई जबकि दोनों नियंत्रित ग्रुप में (D_0C_L एवं D_0C_H) P_i कि मात्रा में कोई परिवर्तन देखने को नहीं मिला। Corpuscles of Stannius (CS) ग्रंथि द्वारा उत्पन्न हॉरमोन Stanniocalcin के gene की अभिव्यक्ति को मापा गया और यह पाया गया कि gene प्रभावित होता है एवं इस gene को आंशिक रूप से sequence किया गया जिसकी क्रमगत संरचना 421bp पायी गई। Corpuscles of Stannius (CS) ग्रंथि की रचना में परिवर्तन को माइक्रोस्कोप से देखा गया और यह पाया गया कि उसकी कोशिकाओं में संख्या के साथ-साथ आकार भी बढ़ा पाया गया और यह परिवर्तन serum के calcium एवं P_i सान्द्रण के उतार चढ़ाव के अनुरूप पाया गया। इससे यह साबित होता है कि इस मीठे पानी कि मछ्ली में विटामिन D_3 का प्रभाव serum के calcium सान्द्रण नियंत्रण में पड़ता है चाहे मछ्ली कम calcium वाले पानी में या अधिक calcium वाले पानी में पाली जाए। और ऐसे पानी में विटामिन D_3 के इस प्रभाव को रोहू (*Labeo rohita*) के रक्त में, Stanniocalcin के gene की अभिव्यक्ति, Corpuscles of Stannius (CS) ग्रंथि की रचना में परिवर्तन को पहली बार अध्ययन किया गया है।

ABSTRACT

Effect of vitamin D_3 were investigated on the serum calcium and phosphate levels, cellular level changes in Corpuscles of Stannius and expression of stanniocalcin gene of commercially important freshwater fish *Labeo rohita* reared in calcium enriched water. In this, the fish were intra-peritonealy injected with vitamin D_3 at two different doses namely 100 IU/Kg BW, 500IU/Kg BW and then they are reared in two types of water with calcium at the range of 20ppm and 40 ppm. There were 6 experimental groups viz: 1). Low calcium control group(D_0C_L): 0.0 IU D_3/kg bw; 2). Low calcium group-1 (D_LC_L); 100 IU D_3/kg bw;3).Low calcium group-2(D_HC_L): 500 IU D_3/kg bw; 4). High calcium control group(D_0C_H): 0.0 IU D_3/kgbw; 5). High calcium group -1 (D_LC_H): 100 IU D_3/kg bw and 6). High calcium group -2 (D_HC_H): 500 IU D_3/kg bw.Serum samples were collected at day 1, 2, 3, 5, 7, 9, 11, 15, 30 and 60. Serum calcium and inorganic phosphorus levels were elevated and again declined back to its normal level. The serum calcium level analyzed to be 11.45$\pm$0.4 at 5th day in low dose vitamin $D_3$and 13.74$\pm$ 0.1at 7th day for high dose vitamin D_3 in 20ppm water. But fish in calcium enriched water (40 ppm) shows higher calcium uptake and analyzed to be 12.48$\pm$0.5 at 5^{th} day in low dose vitamin D_3 and 13.87$\pm$0.3 at 5th day in high dose vitamin D_3. The serum phosphorus level analyzed to be 6.23$\pm$0.5 at 5th day in low dose vitamin D_3 and 7.04$\pm$ 0.2 at 7th day for high dose vitamin D_3 in 20ppm water. But fish in calcium enriched water (40 ppm) shows higher calcium uptake and analyzed to be 6.89$\pm$0.2 at 5^{th} day in low dose vitamin D_3 and 7.66$\pm$0.2 at 7^{th} day in high dose vitamin D_3. Corpuscles of Stannius also collected along during serum collection and the endocrine gland is taken for histological studies. The histological slides of CS are showing more hyperplasia in fish reared in calcium enriched water and the increase and decrease in cell numbers and volume of gland is more proportional to the result of serum calcium and phosphorus level. Then the STC-1 gene was partially sequenced in *L.rohita* at a length of 421 bp and expression studies were analyzed. The present study concludes that the freshwater fish*Labeo rohita*also regulates the calcium level in their body, when they are injected with vitamin D_3 and it also shows the response of calcium and phosphorus ions in body due to vitamin D_3interaction and this is the first reporting on *L.rohita* reared in low and high calcium concentration of ambient water.

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1.INTRODUCTION

World fish production has developed steadily in the last five decades and has reached to 158 million tones with food fish supply increasing at an average yearly pace of 3.2 percent, outpacing world population increase at 1.6 percent. World per capita apparent fish consumption increased from an average of 9.9 kg in the 1960s to 19.2 kg in 2012. Aquaculture, accounts for nearly 50 percent of the world's food fish. World aquaculture production of food fish reached 66.6 million tons in 2012 contributing to US$ 144.4 billion which is increased by 6.2 percent from 62.7 million tons in 2011 (FAO,2014).

Among the Indian major carps, rohu is one of the most preferred species in the country and commands a higher price in the market. The species is also an excellent game fish owing to its easy acceptance of angler's bait. Andhra Pradesh, West Bengal, Assam and Odisha are the most important states for aquaculture production and rohu is the most preferred species in these states and also in other states of the country.

The "Rohu (*Labeo rohita*) Database" is designed by Central Institute of Freshwater Aquaculture (CIFA), Kausalyaganga, Bhubaneswar pertaining detailed information related to basic biology, genetics, nutritional requirements, physiological studies, flesh quality and its value addition, diseases, genomics and proteomics analysis, which will provide a baseline genomic data for other related fish species. We have made an effort to collect all related information and make a comprehensive database for the benefit of students, teachers, researchers and other stakeholders and would be updated on a regular basis.

Calcium and phosphate play important roles in a number of vital biological processes; the most important is in the maintenance of ionic levels (Aurbach, 1988). The activity of ions are seen in many target tissues, which mayinclude the gills, skin, kidney, intestine and bone to maintain plasma levels of these and other ions(Feinblatt, 1982).The regulations of these ions within the species are done by some glands and hormones. The focus of

thisstudy is to examine a unique endocrine tissue referred to as the corpuscles of Stannius [CS] andits homeostatic hormone , stanniocalcin [STC] bony fishes – Teleostein (Nelson, 1994) and some authors like Marra (2000) also studied about the corpuscles of Stannius and stanniocalcin in the more ancient bony fishes.Abbink and Flick.(2007) identified the parathyroid hormone-related protein in teleost fish.This STC-1 originally described in fish and now it known to present throughout the all vertebrate and invertebrates.(Roch and Sherwood, 2010).

Pang and Co-workers (1973) showed that fishes regulate their serum calcium efficiently but that endocrine systems involved may be different from those in tetrapods. A functional parathyroid gland has not yet been demonstrated in fishes. The majority of evidence indicates that calcitonin has little or no effect on fish calcium regulation. Instead, the corpuscles of Stannius and the pituitary gland are necessary for maintaining fish serum calcium levels. In the killifish, *Fundulus heteroclitus*, the removal of the corpuscles produces hypercalcemia in sea water but not in artificial sea water deficient in calcium. Transplants of the corpuscles or the administration of corpuscle homogenate corrects the increase in calcium. On the other hand, hypophysectomy elicits hypocalcemia under calcium deficient conditions but not in calcium rich sea water. Replacement therapy with pituitary homogenate or hypophysial transplant prevents the fall in calcium. It is postulated that the hypocalcemic corpuscles of Stannius (CS) and the hypercalcemic pituitary gland enable the euryhaline killifish to regulate its serum calcium levels in high calcium sea water and low calcium fresh water, respectively. Some authors have correlated the serum calcium and phosphorus level in various teleost by wild collection as well as by administrating hormones into fishes. (Hess*et al.,* 1928;Singh and Srivastav, 1990).

Norman and Henry (2015) studied about the calcium-Regulating Hormones: Vitamin D, Parathyroid Hormone, Calcitonin, and Fibroblast Growth Factorand corpuscles of Stannius (CS) secrete hypocalcemic factor(s). This is evident by a rise in serumcalcium level after removal of CS which is correctedby administration of CS extract. APTH-like substance

(parathyrin) has recently beenlocalized immunocytochemically in the eel CS.Although vitamin D_3 is abundantly present infish liver, its role in calcium homeostasis has been emphatically denied (Rao and Raghuramulu,1995). Recently, it has been reported that vitamin D_3 and its metabolitesinduce hypercalcemia in fishes. Moreover,Srivastav (1982) have also reported hyperactivity of CS after vitamin D_3treatment and the effects of 1,25-dihydroxyvitamin D_3 (1, 25 $(OH)_2D_3$) on the CS ofthe freshwater catfish, *Clarias batrachus*(Srivastavand Srivastav,1988).

Effect of vitamin D_3 administration on the serum calcium and inorganic phosphate levels of the freshwater catfish,*Heteropneustes fossilis*, maintained in artificial fresh water, calcium-rich fresh water, and calcium-deficient fresh water(Srivastav and Singh,1992) also recorded. The level of serum calcium and phosphorous changes during gonad development and reproductive cycle of fishes and it also figures out the role of Corpuscles of Stannius in this serum level differentiation. (Balbontin *et al*.,1978, Urasa and Bonga,1985). Few authors had injected vitamin D_3 and its derivative in fishes to make it hypercalcemic and to know the changes in serum calcium and phosphorus level. (Avioli *et al*., 1981, Fenwick *et al.,*1984;Lopez*et al*., 1977; Swarup and Srivastav, 1982; Takeuchi *et al.,* 1987).

Stanniocalcin is a glycoprotein hormone important in the maintenance of calcium and phosphate homeostasis in fish. Two related mammalian stanniocalcin genes, STC1 and STC2, were found to be expressed in various tissues as paracrine regulators(Luo*et al.,* 2004).Although stannniocalcin-1 (STC-1) was originally describedin fish, it is now known to be present throughout theanimal kingdom in both vertebrates and invertebrates. The principle sources of STC-1 in bony fish are endocrineglands known as the corpuscles of Stannius (CS) whichare anatomically associated with the kidneys. STC-1 releaseis stimulated by a rise in serum levels of ionic calciumabove the physiological set point through the activationof calcium-sensing receptors(Richards *et al.,*2012). The hormone then exertsregulatory effects on the epithelial transport of calciumand/or phosphate across the gills, gut, and kidneys in orderto restore normocalcemia.Stanniocalcin-1 (STC-1) is

an anti-hypercalcemic hormone that is produced by the corpuscles of Stannius (CS) in the teleost (Shin*et al.,* 2006). The hormoneis a homodimeric glycoprotein involved in the calcium and phosphate regulation in both teleost fish and mammals.Hence nowadays gene expression studies are playing major role in molecular research work, the study of Stanniocalcin gene also done in various species from fish to humans. Because these genes are prominent to fish species and later identified in human. They are named as STC and in fish two types of stanniocalcin are identified such as STC-1 and STC-2(Amemiya*et al.,* 2006; Chang *et al.,* 1995; Chang *et al.,* 1998a,b;McCudden*et al.,*2001;Ellis and Wanger, 1995).

There is no literature on the effect of vitamin D_3on physiological responseto rohu (*Labeorohita*), thus, on the basis of above literature it was a need to assess the response and behavior of calcium and phosphorus regulating endocrine gland, Corpuscles of Stannius (CS) in *Labeo rohita* when reared in Calcium enriched water and given intra-peritoneal doses of vitamin D_3.

Probably, this will be first reporting on *Labeo rohita,* and to take up such studies the following objectives were made to assess the combined response of vitamin $D_3$and calcium levels in ambient water on the serum calcium, inorganic phosphate (P_i), behavior of the CS and gene expression of its hormone Stanniocalcin.

Objectives:

To assess the Responses of Intra-peritonealVitamin D_3 on:

- Serum Calcium, Phosphorous levels and Corpuscles of Stannius in *Labeo rohita* reared in calcium enriched waters.
- Gene expression of Stanniocalcin in *Labeo rohita* reared in calcium enriched waters.
- Histology of Corpuscles of Stannius of*Labeo rohita* reared in calcium enriched waters.

2. REVIEW OF LITERATURE

2.1. *Labeo rohita*

Hamilton (1822) in his Book “An Account of the Fishes Found In the River Ganges and Its Branches”, he briefly described about various fish species found in river Ganges which includes *Labeo rohita*. Body bilaterally symmetrical, moderately elongate, its dorsal profile more arched than the ventral profile; body with cycloid scales, head without scale; snout fairly depressed, projecting beyond mouth, without lateral lobe; eyes dorsolateral in position, not visible from outside of head; mouth small and inferior; lips thick and fringed with a distinct inner fold to each lip, lobate or entire; a pair of small maxillary barbels concealed in lateral groove; no teeth on jaws; pharyngeal teeth in three rows; upper jaw not extending to front edge of eye; simple (unbranched) dorsal fin rays three or four, branched dorsal fin rays 12 to 14; dorsal fin inserted midway between snout tip and base of caudal fin; pectoral and pelvic fins laterally inserted; pectoral fin devoid of an osseous spine; caudal fin deeply forked; lower lip usually joined to isthmus by a narrow or broad bridge; pre-dorsal scale 12-16; lateral line distinct, complete and running along median line of the caudal peduncle; lateral line scales 40 to 44; lateral transverse scale-rows six or six and a half between lateral line and pelvic fin base; snout not truncate, without any lateral lobe; colour bluish on back, silvery on flanks and belly.

2.2. Nutritional Requirements of *Labeo rohita*

According to FAO, the optimum protein requirement for larvae and fry varies from 35–45 percent (Sen *et al*., 1978) and the performance in terms of growth rate, protein utilization and conversion efficiency have been reported to be the best when rohu has been fed with diet having 40 percent protein (Mohanty *et al*.,1990a & b). The optimum protein requirement for fingerlings has also been reported at 40 percent (Swamy and Mohanty, 1990; Satapathy *et al*., 2003). The optimum lipid requirement for rohu fingerlings was found to be in the range of 12-15 percent (Gangadhara *et al*., 1997; Satapathy *et al*., 2003). However, lipid requirement has been shown to be

temperature dependent. Mishra and Samantaray (2004) reported that at 21^{o}C, the lipid requirement for rohu fingerling was 8 percent, whereas at 31^{o}C the requirement was 13 percent. Mohanty (2006) considered a range of 7–9 percent dietary lipid to be optimum for Indian major carps including rohu. Under laboratory conditions Anwar and Jafri (2001) reported that rohu fingerlings require 5 percent lipid in a 40 percent crude protein diet with 18.3 KJ/g gross energy at a carbohydrate to lipid ratio of 8:3.

2.3. Calcium and its significance

Calcium (Ca) is an alkaline earth metal with atomic number 20 and fifth most abundant element in Earth's crust and also in seawater after Sodium, Chloride, Magnesium and Sulfate. The presence of more number of calcium ion in water lead to increase in hardness of the water. In seawater, the level of calcium is up to 400ppm while in freshwater it seems to be contain 1-2 ppm. Calcium is a dietary requirement of many aquatic animals for skeletal growth and building up of eye lenses. Other important roles of calcium are cell membrane development, cell division, blood clotting, muscle contraction, nerve impulse transfer, pH stabilization etc. calcium and its components are more or less water soluble. For example, Calcium hypochlorite has high solubility of up to 218 g/l and Calcium hydroxide at 1.3 g/l. Gonzalez *etal*., (1998) found that rise in calcium concentration from 10 µmol L^{-1} to 100 µmol L^{-1} at low pH had no effect in loss of ions through gills in few fishes from Amazon river. They also suggested that, this effect is due to high brachial affinity for calcium due to the saturation of all sites at 10µmol L^{-1}. Hence there is no space for other calcium molecule to exchange. Eddy (1975) experienced that, the addition of calcium to the medium greatly reduces the sodium and chloride efflux rates in gold fish (*Carassius auratus).*

2.4. Vitamin D_3 and its role

Fermanta Biotech Limited briefly explained that, in 1920's Edward Mellanby and Elmer McCollum discovered an anti-rachtic factor in cod liver oil while researching a cure for the disease rickets. McCollum identified the factor to be distinct from Vitamin A in 1922 and called it Vitamin D as it was the fourth Vitamin to be named. The epidermis and dermis layers of the skin

contain 7-dehydrocholesterol also known as provitamin D_3 which on exposure to Ultra Violet radiation between 290 – 315 nm (UVB range) is synthesized to Vitamin D_3.Simaon Foden in the site named “Pets On Mom Me”, clearly described that fish don’t get vitamin D from the sun. Bottom-dwelling and deep sea fish have no need for synthesizing sunlight into vitamin D, because they are never exposed to the sun. While other fish benefit hugely from UV light and use plasma to process it, this is not where they get their vitamin D. Fish get vitamin D from food, such as plankton, and store it in their liver. Fish actually are a great source of dietary vitamin D for other animals. Kauffman briefly described about the Vitamin D and its benefit. In this, Kauffman coated a sentence which taken from the NIH News stated on Aug 12, 2008 – “Vitamin D is an essential component in bone health that helps ensure that the body absorbs calcium, which is critical for building strong, healthy bones. People get this nutrient from three sources: sunlight, dietary supplements, and foods.” Ling-hong *et al*. (2015) quantified the level of vitamin D_3 requirement for Wuchang bream and also they reported that there is increase in plasma calcium level with increasing dietary vitamin D_3 level from 0 to 1 IU.

2.5. Arachis Oil

Peanut oil, also known as groundnut oil or Arachis oil, is a mild-tasting vegetable oil derived from peanuts. According to the USDA data upon which the following table is based, 100 g of peanut oil contains 17.7 g of saturated fat, 48.3 g of monounsaturated fat, and 33.4 g of polyunsaturated fat. Then they surveyed and finalized that the supplementation of vitamins A and D in water-soluble form seems to increase the risk of allergic disease up to the age of 4 years compared with supplementation with the same vitamins given in peanut oil.

2.6. Corpuscle of Stannius

The corpuscles of Stannius (CS) are endocrine glands to teleostean and holostean fishes. The CS arise from the pronephric, mesonephric or opisthonephric ducts (Garrett, 1942; Ford, 1959; Belsare, 1973; Krishnarnurthy, 1967). In the more primitive holosteins, such as the bowfin

and garpike, the CS is small, more in numbers and widely distributed in the kidney (Garrett, 1942; Bauchot, 1953; De Smet 1962; Youson and Butler, 1976). In eel, the CS be larger and occurring as a single pair attached in the ventral surface of the posterior mesonephric kidney (Bauchot, 1953). The (CS) are glands which are usually embedded in the kidneys of holosteans fishes (Bhattacharya *et al*., 1982; De Smat, 1962; Marra *et al*., 1992, 1994; Youson and Butler, 1976) and teleostean fishes(Hirano, 1989). Corpuscles of stannius of *Mystus vittatus* in relation to calcium and sodium rich environments was documented (Ahmed and Swarup, 1979).

Marra and Co-workers (1995) examined the corpuscles of Stannius of arawana (*Osteoglossum bicirrhosum*), an ancient teleost and described that they are similar to those in more recent teleosts with respect to cell structure and their anatomical distribution. Ahmad *et al*. (2002) studied the ultra structure of corpuscles of Stannius of *Heteropneustes fossilis* and found they are type 2 cells, were other teleost possessing type 1 cells. Mishra *et al*. (2010) studied the changes in nuclear volume of Corpuscles of Stannius(CS) in *Heteropneustes fossilis* by treating them with cypermethrin (pyrethroid). They found there are two types of CS namely, AF-positive and AF-negative according to their staining. They also reported that there is increase in nuclear volume of CS cells and then lowers due to prolonged treatment with cypermethrin and also change in plasma calcium level. Srivastav *et al*. (1996) found that aqueous extracts of goldfish corpuscles of Stannius induced rapid but transient hypocalcemia and hyperphosphatemia in the sting ray (*Dasyatis akajei)*. Butler and Zhang (2001) reported that varying doses of angiotensin I and II induced Corpuscles of Stannius to secrete rennin or isorenin to regulate the cardio vascular function in freshwater eel (*Anguilla rostrata)*. Bedjargi and Kulkarni (2014) studied the comparative histology of corpuscles of Stannius in freshwater and sea water fishes and they identified three types of cells found in these fishes in which type-I is predominant and found to be active. They also indicated that corpuscles of Stannius in sea-water fishes are more active than fresh water fishes.

2.7. Stanniocalcin

Luo and Co-workers (2005) identified a stanniocalcin paralog in *F.rubripes* and named it as STC 2, which shows paracrine activity in fish. They indicated the identity between STC 1 and STC 2 for a given species was less than 30%. Pierson *et al*. (2004) proposed that STC secretion stimulated by decrease of plasma NaCl concentration, which also imply a broader function of STC other than its classical hypocalcemic action. Wagner *et al*. (1997) studied the secretion of stanniocalcin in salmon reared in freshwater and seawater. They concluded that the STC cells were equally Ca2+ - sensitive in the two groups. Schein *et al*. (2011) shown that *Tetraodon nigroviridis* and other teleost possess STC isoforms, designated as stc1-a, stc1-b, stc2-a, stc2-b. They also studied the structure, phylogenetic analysis, tissue distribution and expression of these genes. They also provided the information that STC1-a in the CS involved in extracellular calcium regulation. Amemiya *et al*. (2001) reported that Stanniocalcin (STC) cells were localized in both corpuscles of Stannius (CS) and in specific cells of the distal renal tubules of silver arawana (*Osteoglossum bicirrhosum),* by immunostaining with salmon STC antiserum and this is the first report of monomeric form of STC in any vertebrate and first evidence in renal tubules. McCudden *et al*. (2001) studied the new form of Stanniocalcin -2 (STC-2) which is released from ovary of trout during early stage of oocytes and said that they are glycosylated in nature. Madsen *et al.* (1998) identified that stanniocalcin in mammals act as regulatory protein in regulating the mammalian intestinal calcium and phosphate transport.

2.8. Role of Stanniocalcin hormone

Yeung and Co-workers (2012) studied that of mammalian STCs (STC1and STC2) hormones are made in virtually all tissues and they act primarily asparacrine/autocrine factors to regulate various biological functions. Clark (1983) explained the calcium regulation in lower vertebrates appears to be a continuum. The predominant hypercalcemic hormone in reptiles, birds and mammals is parathyroid hormone, while the major hypercalcemic control in fishes is a pituitary factor, probably prolactin. Wagner and Dimattia (2006)

identified sensitive and reproducible, dose-related effects of salmon STC on gill calcium transport.

Hang and Balment (2005) studied STC mRNA expression levels in seawater-adapted fish CS were about 3-fold higher than in freshwater-adapted fish Corpuscles of Stannius. Pierson *et al*.(2004) evaluates the effect of the pivotal stress signal cortisol, the eventual output of the stress axis on STC secretion in freshwater rainbow trout (*Oncorhynchus mykiss*) and proposed that STC secretion might be stimulated also by a decrease of sodium chloride concentrations, implying a broader function than the classical hypocalcemic action of STC. Schein *et al.* (2012) described that Stanniocalcin (STC), first isolated from the corpuscles of Stannius (CS) of teleost fishes and a systemic regulator of mineral metabolism, is present in all vertebrates as two isoforms, STC1 and STC2, encoded by separate genes and both STC1 and STC2 genes are represented by paralogues in teleosts genomes and the analysis performed suggests that only STC1-a in the CS is involved in extracellular calcium regulation. The widespread distribution of STCs in fish tissues supports pleiotropicroles. Shin *et al*. (2006) done RT-PCR analysis revealed that the turbot STC-1 gene is expressed in the CS, pituitary, brain, kidney, liver, heart, muscle, and gonad. Amemiya *et al*. (2002) reported monomeric form of STC in any vertebrate and the first evidence of STC in renal tubules of adult fish. Bedjargi and Kulkarni (2014) compared the histology of corpuscles of Stannius shows that the cells are active in both freshwater and seawater fishes. In marine fishes which are associated with coral reefs the concentration of calcium increases in the reefs, the CS cells are more active than the fresh water fishes of fresh water, thus indicating calcium is a factor for the activity of corpuscles of Stannius in fishes. The histological observation shows that three types of cells found and one type (type-I) of cell is predominant and found to be active. Butkus *et al*. (1987) had done purification and cloning of a corpuscles of Stannius protein from *Anguilla australis*. McCudden *et al*.(2004) had studied the co-localization of stanniocalcin- 1 ligand and receptor in human breast carcinomas. Verma and Alim (2014) studied the differential activity of Stanniocalcin in male and female lacepede (*Mastacembelus armatus*) during gonadal maturation. They found in

females corpuscles of Stannius act as hypocalcemic factor and release the Stanniocalcin to bring the level of calcium to normal which get increased during preparatory and pre spawning phases of gonadal cycle. In male, plasma STC concentration varied widely during gonadal cycle but showed no consistent relationship to plasma calcium level. Wagner *et al*. (1997) developed a new in vivo bioassay for STC using rainbow trout, which involves the suppressing plasma STC levels by injecting phosphate into fish. Thus it lowers the plasma calcium level. Chou *et al*. (2015) studied the role of stanniocalcin-1 in controlling the ion regulation functions of ion-transport epithelium which is other than calcium homeostasis. In this, they identified stc-1 negatively regulate the number of ionocytes to reduce the ionocyte function which helps in ion transport.

2.9. Action of vitamin D_3 on Corpuscle of Stannius

Srivastav and Srivastav (1988) explained the hyper activity of Corpusle of Stannius, by increase in nuclear size and decrease in staining property of the corpuscular cell which is treated at different doses of 1, 25 Dihydroxy vitamin D_3.

2.10. Calcium regulation

Radman and co-workers(2002) found that Ca^{2+} -stimulate STC secretion in fish is mediated by a calcium ion-sensing receptor similar to that in mammals. They also revealed that this transcript also present in gill, kidney, pancreas, brain, muscle and spleen. Chang and Shoback (2004) had given brief description about the extracellular Ca^{2+}-sensing receptors in ancient and modern fishes, in which they play major role in Ca^{2+} homeostasis. Loretz. (2008) found out that involvement of Calcium sensing receptor in fish Ca2+ homeostasis at the level of environmental sensing, of integrative endocrine signaling through both hypercalcemic and hypocalcemic hormones and other local regulation of Ca^{2+} -transporting tissues. Srivastav *et al*. (1997) found out that deltamethrin disturb the calcium and phosphate homeostasis which are important ions for synthesis of vitellogenin and thus affecting the reproductive state of the fish. Eugien *et al*. (2014) estimated the level of calcium and phosphorous in bones of three low value fishes like *Sardinella fimbriata,*

Sardinella albella and *Sardinellagibbosa* from southeast coast of India and they found sardine bones stands as a major repository of calcium and that can be used as a raw material for the production of calcium tablets. Pratap and Bonga (2007) studied the plasma calcium, composition of calcium and phosphate in scales, operculum and vertebrae, corpuscles of Stannius and bony tissues of *Oreochromis mossambicus* which is acclimated to low and high calcium water and exposed to ambient and dietary cadmium. They found that cadmium does not stimulate corpuscles of Stannius and high level of calcium water had a protective effect against ambient and dietary cadmium. Canario and Flik (2007) reported a discussion on the endocrinology of calcium homeostasis in various freshwater and marine water fishes.

2.11. Calcium Transport in Fish Gills and Intestine

The extant bony fishes represent the largest class of vertebrates with an estimated 20000 species, and fish are considered to be an evolutionary success. They have developed sophisticated osmo and ionoregulatory mechanisms, allowing them to inhabit and to thrive in virtually every aquatic niche, be it an ice-cold sea or a tropical soda lake. Recently, it has become clear that fish have also developed mechanisms for calcium transport to realize a calcium homeostasis that appears to be comparable with that of higher vertebrates. The calcium transport mechanisms underlying the homeostasis have made fish essentially independent of their aquatic environment, which may be strongly hypocalcic, with micro-molar levels of calcium in soft water, or hypercalcic, with some $10mmoll^{-1}$ calcium in sea water. An essential difference between terrestrial vertebrates and aquatic fish is that the latter have a specialized organ for calcium uptake, the gills. This situation provides a continuous constraint on calcium regulatory mechanisms.

Unlike terrestrial vertebrates, fish show significant variations in plasma calcium levels, not only between species but also within species, e.g. as occurs when a fish is confronted with water containing different levels of calcium (Urasa and Bonga 1987). Fish are able to survive the extreme hypercalcaemia (up to $10mmoll^{-1}$ total calcium, $4.5mmol^{-1}$ Ca^{2+}; Hanssen *et al.,* 1989) that develops when their source of hypocalcaemic hormone, the corpuscles of Stannius, has been removed. Such observations indicate that

fish can tolerate fluctuations in plasma calcium concentration to an extent not encountered in terrestrial vertebrates. Fish cells must have very strict control over Ca^{2+} entry. Bones and scales, crucial elements in vertebrate life, derive their sturdiness from deposited calcium phosphate minerals. Most fish grow continuously and, to do this, accumulate calcium in their body throughout their life. This is realized through a continuous uptake of Ca^{2+} via their gills (Herrmann and Flik, 1989). The gills are the most important site of contact between fish and water, covering a surface area 10–60 times larger than the area of skin covering the body surface. The large surface area of the gills reflects their role in the gas- and ion-exchange processes that are determined by the environment and the lifestyle of the fish. Such a large area of integument in contact with the water requires strict control over calcium movements across this epithelium. Fish have two sites for the uptake of calcium: their gills (containing an abundance of ion-transporting cells or 'chloride cells', also known as ionocytes) and their intestine. The branchial epithelium may be considered to be a specialized skin area.Calcium regulation in common carp is reported by Srivastava *et al.* (2000b). The estimated contribution of the intestine to total calcium uptake comes to 30%.

In the euryhaline teleosts *Oreochromis mossambicus* (McCormick *et al.,*1992) and *Oncorhynchus mykiss* (Marshall *et al.,* 1992), parts of the skin covering the inner operculum have also been shown to transport Ca^{2+}. Using a chamber, it has been demonstrated that this transport is probably mediated by the ionocytes found in this part of the skin under these conditions. The skin has been shown to contain numerous ionocytes at typical densities of around $200mm^{-2}$. Extrapolation of the positive correlation between skin ionocyte numbers and inward calcium transport rates suggests that skin devoid of ionocytes is essentially impermeable to Ca^{2+}. The contribution of extra-branchial calcium transport via the skin is considered to be small, and this is reflected in the absolute numbers of ionocytes in gills and skin. Care should be taken with the extrapolation of data on calcium transport via extra-branchial sites, as have shown that calcium transport via the skin is not affected by La3+, a powerful blocker of branchial calcium transport. It has also been shown that trout stanniocalcin had no effect on calcium transport in

Fundulus heteroclitus opercular membranes, although this hormone has been shown to inhibit the movement of calcium over the apical membrane of branchial ionocytes of a variety of species, including trout, eel and tilapia. It may be that a homologous hormone is required to show an effect in *Fundulus heteroclitus*.

Ca^{2+} can be taken up directly from the water via the gills, but also through the intestine by drinking water. Food may be another source of Ca^{2+} through intestinal absorption. The role of the intestine in the calcium metabolism of fish is not very clear and has only been sparsely studied. The primary function of the intestine must be the uptake of nutrients, which may depend on the uptake of ions (e.g. Na^{+}-coupled sugar uptake). The intestine may become active in the uptake of calcium from food (a freshwater fish confronted with osmotic water influx should not drink) in times of extra need for calcium, such as during gonadal maturation or when ambient calcium levels are very low (Berg, 1968; Ichii and Mugiya, 1983). This has been shown for the Atlantic cod, *Gadus morhua*, in which intestinal calcium absorption increases drastically in pre-spawning fish (Sundell and Bjornsson, 1988), which require vast amounts of calcium for the development of the gonads. Very little is known about the relative contribution of the gills and the intestine to calcium uptake in seawater fish, with the exception of data from the stenohaline Atlantic cod. They estimated that intestinal absorption of calcium in seawater fish made up around 30% of the total calcium intake.

For two species (eel and tilapia) studied in lab condition, it has been shown that net branchial Ca^{2+} influx in sea water was not lower, but comparable or slightly higher, than in fresh water. Seawater fish do drink and sea water is high in calcium (approximately $10mmoll^{-1}$). The water is drunk to compensate for osmotic water efflux but at the same time presents a significant calcium load. Considering the branchial influx that suffices for growth and homeostasis, one would predict that intestinal uptake is not required. All studies on intestinal calcium transport in fish indicate that in the proximal parts of the intestinal tract active transport underlies net uptake of calcium, in freshwater as well as in seawater species. Measurements have

been made of branchial and intestinal calcium uptake for the stenohaline Atlantic cod, *Gadus morhua*. The estimated contribution of the intestine to total calcium uptake comes to 30%.

In a recent study on tilapia (Schoenmakers *et al.,* 1993), evidence was provided that this species reduces calcium absorption to a minimum when in sea water, indicating that species differences in intestinal calcium handling may exist. Any surplus calcium taken up by fish is excreted, largely via extra-branchial routes, i.e. the renal system or the intestinal tract, Hickman and Trump, 1969). It has been shown that freshwater eels produce hypotonic urine containing 1.5–2mmol l^{-1} calcium at a considerable rate (40mmolday^{-1} kg^{-1} fish), so the renal route is a significant pathway for calcium secretion, while the kidneys of seawater fish are renowned for their secretion of divalent ions (Ca^{2+}, Mg^{2+}, SO_4) and are considered to be essential to keep calcium flows balanced. The contribution of the complete intestinal tract to calcium excretion has been analyzed only in cod (Sundell and Bjornsson, 1988). These studies indicate that the intestinal excretion (1.22 mmol.kg^{-1}h^{-1}) represents 20% Calcium transport in fish gills and intestine of the total calcium excretion (renal and extrarenal, 4.2 and 2.0 mmol.kg^{-1}h^{-1} , respectively) and 50% of the extrarenal excretion. The very few physiological studies that have been carried out further indicate that the urinary bladder may modify the calcium content of the urine significantly, and this means that the bladder epithelium should also be regarded as a calcium transport site. The endocrine control of plasma calcium levels in fish is very different from that in higher vertebrates. Prolactin and somatolactin from the pituitary gland and the steroid cortisol produced by the interrenal cells are calciotropic hormones with hypercalcaemic effects in fish. Stanniocalcin, a unique fish hormone produced by the corpuscles of Stannius, is the predominant hypocalcaemic hormone in fish (Bonga and Pang, 1986).

Calcitonin, a hormone with hypocalcaemic effects in higher vertebrates, first isolated from salmon ultimobranchial bodies, has no clear calcitropic effects in fish and its role in fish physiology remains enigmatic. The involvement of hormones in calcium regulation in fish has been reviewed

extensively. Flick and Verbost (1995) described the cellular mechanisms in calcium transport and homeostasis in fish. Flick *et al.* (1985) related the whole-body calcium flux rates in cichlid teleost fish *Oreochromis mossambicus*adapted to freshwater.Effect of a carpet industry effluent on gill tissue and Ca+2-ATPase activity in common carp *Cyprinus carpio* has been reported (Srivastava *et al*., 2000a).

2.12. Regulation of calcium absorption by Vitamin D_3

Zhu and Co-workers (2015) conducted an experiment to evaluate the combined effects of phytase, citric acid and vitamin D3 on the digestibility coefficients, growth performance, body composition, and vertebral and serum mineral contents in juvenile yellow catfish (*Pelteobagrus fulvidraco*). Sundh *et al*. (2007) measure the in vitro production of [3H]-1,25$(OH)_2D_3$ and [3H]-24,25$(OH)_2D_3$ in fresh water(FW) and after 1, 2, 3, and 7 days after transfer to seawater (SW). Ling-Hong *et al*.(2015) determined the growth performance, body and muscle composition, plasma and body physiological and biochemical index, including calcium, phosphorus, cholesterol, triglycerides, glucose and insulin by dietary feeding of Vitamin D_3. Radman *et al*. (2002) suggest that Ca^{2+}-stimulated STC secretion in fishes is mediated by a calcium ion-sensing receptor similar to that in mammals. Coloso *et al*. (2001) studied the dietary combination of low P and high vitamin D_3 decreased soluble and fecal P levels in the effluent indicating a strategy whereby effluent P concentrations can be reduced by regulation of Phosphorus metabolism. Adiele *et al*. (2012) studied the features of cadmium and calcium uptake and toxicity in rainbow trout (*Oncorhynchus mykiss*) mitochondria. Dose-dependent influence of vitamin D_3 on the growth and survival of *Cyprinus carpio* spawn (Srivastava *et al*, 1994).

2.13. Gene expression of STC

Hand and Balment (2005) studied the expression of euryhaline flounder (*Platichthys flesus*) STC in various tissues and organs. They also concluded that the STC mRNA expression levels in seawater-adapted fish CS were about 3-fold higher than in freshwater-adapted fish CS. At the same time, Shin *et al*. (2006) have cloned and characterized a full-length cDNA of

STC-1 from the turbot (*Scophthalmus maximus*) CS and examined its expression pattern in various tissues like CS, brain, kidney, liver, heart, muscle and gonad. After three years, Shin and Sohn (2008) had cloned and characterized a full-length STC2 cDNA from Japanese flounder (*Paralichyhus olivaceus*) ovary and analyzed expression pattern of STC2. They found that STC2 encoded 286 amino acids and highly identical to STC2 gene of puffer fish, zebra fish and humans (57.7-89.0%) and also this gene also expressed almost in wide variety of tissues. Greenwood *et al*. (2009) also examined the response of corpuscles of Stannius, calcium-sensing receptor and stanniocalcin to calcimimetics and physiological challenges to seawater and freshwater adapted flounder. They found there is no significant difference in CS CaSR mRNA expression or plasma STC-1 levels. Luo *et al.* (2005) identified that STC 2 is a functional homodimeric glycoprotein and it's expressed in the thecal layers of ovary, which play paracrine role during follicular development.

3. MATERIALS AND METHODS

3.1. Site of Experiment

The experiment was conducted for a period of 60 days at the wet laboratory situated in old campus of ICAR - Central Institute of Fisheries Education (CIFE) Panch Marg, off Yari Road, Versova, Andheri (west), Mumbai. The experimental setup was maintained with triplicates. Intraperitonial injection, sampling, histology, biochemical and molecular work were carried out in the Nutrition and Biochemistry Laboratory of Fish Nutrition, Biochemistry and Physiology Division.

3.2. Experimental Animal and Acclimatization

Labeo rohita of weight 30±2g were brought from Mahad. A prophylactic treatment with 3% NaCl solution was given to the collected fish for 10 minutes with sufficient oxygen supply by artificial aeration. The fish were acclimatized to the experimental condition for 1 week by feeding them with basal diet, a mixture of basal diet and purified diet and purified diet during first, second and third group of days of conditioning period respectively.

3.3. Rearing

The fishes of uniform size were kept in each tub and they were covered with a lid to prevent the fish from jumping out. No attempts were made to stimulate or control the environmental condition except calcium enrichment. The experimental conditions were kept the same all throughout the experimental period.

3.4. Experimental Design and Experimental Set-Up

The experiment was conducted in 18 plastic tubs of equal size (Cap. 100L). The whole set up was arranged in the wet laboratory of Old campus of CIFE situated in Versova, Andheri (West), Mumbai. Two types of water with normal and calcium enriched at the range of 20 and 40 ppm respectively. Intraperitonial Vitamin D_3 dosage at the level of 100IU /kg bw and 500IU/kg bw respectively (Table 1). Basal feeds were used to feed the fishes.

Table 1: Experimental design

Vitamin D_3 → / Replicate ↓	Control (C_L)	Vitamin D_3 (100 IU/kg bw)	Vitamin D_3 (500 IU/kg bw)	Control (C_H)	Vitamin D_3 (100 IU/kg bw)	Vitamin D_3 (500 IU/kg bw)
R1	$R1D_0C_L$	$R1D_L C_L$	$R1D_HC_L$	$R1D_0C_H$	$R1D_LC_H$	$R1D_HC_H$
R2	$R2D_0C_L$	$R2D_LC_L$	$R2D_HC_L$	$R2D_0C_H$	$R2D_LC_H$	$R2D_HC_H$
R3	$R3D_0C_L$	$R3D_LC_L$	$R3D_HC_L$	$R3D_0C_H$	$R3D_LC_H$	$R3D_HC_H$

D = Vitamin D3**; D0** = No vitamin D3**;** $\mathbf{D_L}$ = Low level D3 (100 IU/kg bw); $\mathbf{D_H}$ = High level D3 (500IU/kg bw)**;** $\mathbf{C_L}$ = Low/Normal Calcium in water (20 ppm); $\mathbf{C_H}$ =High Fortified Calcium in water (40 ppm)

3.5. Vitamin D_3 and Dosage level

Fishes were injected with two dosage level of vitamin D_3

I. Low dosage – 100IU/kg B.W.

II. High dosage – 500IU/kg B.W.

By considering 0.2ml Vitamin D_3 injection for 25g size fish, the 3Lakh IU Arachitriol (oil-based) is diluted with Arachis oil.

3.5.1. Product Details

VITAMIN D_3 INJECTION

Manufactured by - Abbott (India) Limited, Mumbai.
Product name - Arachitol – 3L (In oily base)
Volume - 1ml Ampoule
Lic. No. - G/28A/2191-A
Batch No. - JACA5006
Mfg. Date - AUGUST, 2015
Exp. Date - APRIL, 2018

Arachis Oil

Manufactured by – Raj Oil Mills Limited, since 1943. (Free from Argemone Oil)

Volume - 1 liter

Lot No - 13

Mfd Date - APRIL – 2016

Fssai Lic No - 11511018000218

Nutritional Factors:

Energy – 900 kcal ; SFA - 20g ; PUFA - 30g ; MUFA - 50g ; Vit E - 15mg

3.5.2. Preparation of Stock solution

I. Taken 0.2ml Vitamin D3 (3L IU) in the sterile syringe and diluted with 19.8ml Arachis oil and made up to 20ml.This 20ml Vitamin D_3 contains 60000 IU.

II. Then take 5ml from this above 20ml Vitamin D_3 and again dilute with 25ml Arachis oil and make up to 20ml stock solution.

3.5.2.1. Preparation of 100IU/kg b.w working solution from stock solution

1ml of stock solution is taken and diluted with 39ml Arachis oil and made up to 40ml working solution. From this 40ml Vitamin D3 working solution, taken 0.2ml for 25g size fish which containing 2.5 IU Vitamin D3 (i.e. 100 IU/kg). Then this 0.2ml is intra-peritonically injected into 25g size fish using sterile syringe.

3.5.2.2. Preparation of 500IU/kg b.w working solution from stock solution

8ml of stock solution is taken and diluted with 56ml Arachis oil and made up to 64ml working solution. From this 64ml Vitamin D3 working solution, taken 0.2ml for 25g size fish which containing 12.5IU Vitamin D3 (i.e. 500IU/kg). Then this 0.2ml is intra-peritonically injected into 25g size fish using sterile syringe.

3.6. Calcium enrichment procedure

Level of calcium in the normal water is measured using EDTA titration method and found that the value of calcium is 50mg/l. So calcium enriched water is prepared by just doubling the normal water calcium to 100mg/l by adding Calcium chloride.

3.6.1. Calcium in normal water is determined by the following procedure

3.6.1.1. Chemicals required

1. Ammonium purpurate
2. 1N Sodium hydroxide
3. Sodium chloride
4. 0.02M EDTA

3.6.1.2. Procedure

20ml water sample is taken from the treatment tanks and 2ml 1N Sodium hydroxide solution is added. Then a pinch of Ammonium purpurate is added and water colour changes to pink colour. This solution is titrated against 0.02M EDTA until the water colour changes from pink to violet as an end point. Then the titre value is noted and applied in the formulae to calculate the amount of Calcium in treatment tanks.

3.6.1.3. Estimation of Calcium

- Calcium hardness = Titre value x Normality x 50 x 1000 / volume of sample mg/l as $CaCO_3$ equivalent.
- Calcium present in the sample (ppm) = Calcium hardness in mg/l as $CaCO_3$ x molecular weight of calcium / molecular weight of $CaCO_3$

3.6.1.4. Enrichment Calculation

The capacity of treatment tank is 75 litre. Hence the calculation is

- 75 litre x 20mg/l = 1500mg Ca/tub (i.e. 1.5g calcium)
- Molecular weight of calcium is 40.07g
- Molecular weight of calcium chloride is 111g

Thus for 1.5g calcium the amount of calcium chloride needed is:

$CaCl_2$= 111x 1.5 / 40.07.

$CaCl_2$ = 4.16 g/ tub.

Thus, by adding 4.16g of calcium chloride to the normal water of 20 ppm will increase the calcium level to 40mg/l or ppm.

3.7. Sampling strategy

Sampling was done in Day 1, Day 2, Day 3, Day 5, Day 7, Day 9, Day 11, Day 13, Day 15, Day 30 and Day 60. Fishes were caught using a fine scoop net and excess water was removed by blotting with tissue paper. The fishes were weighed in a pre-weighed beaker of water by using an electronic balance with 0.001 g accuracy. In each sampling one fish from each replicate were sacrificed during the period of experiment and taken for ionic activity, gene expression and histological studies.

3.8. Physico-chemical parameters of water

Water quality parameters viz. Temperature, pH, dissolved oxygen, free carbon dioxide, total hardness, ammonia, nitrite and nitrate were recorded during the experimental period.

3.8.1. Temperature

Water temperature of all the experimental tubs were recorded using dissolved oxygen meter (MERCK, Germany) in which the temperature was also shown.

3.8.2. pH

The pH was measured by a digital pH meter (LABINDIA) for all the experimental tubs.

3.8.3. Dissolved oxygen

The dissolved oxygen was measured by membrane electrode method using dissolved oxygen meter (MERCK, Germany) for all the experimental tubs.

3.8.4. Free Carbon dioxide

The dissolved free Carbon dioxide was measured by titrimetric method (APHA, 1998) and calculated using the following formula.

$$CO_2 \text{ (mg/ml)} = \frac{A \times N \times 44 \times 1000}{\text{Volume of sample (ml)}}.$$

Where, A = Volume of titrant (NaOH) and

N = Normality of titrant (N/44)

3.8.5. Carbonate hardness

Carbonate hardness was estimated by carbonate hardness test kit (Carbonate hardness test, MERCK, Germany).

3.8.6. Ammonia

Un-ionized ammonia concentration was estimated spectrophotometrically at 635nm wavelength by phenate method (APHA, 1998) and compared with the standard graph. The concentration was expressed as mgL^{-1}.

3.8.7. Nitrite-N

Nitrite concentration was estimated spectrophotometrically at 543 nm wavelength (APHA, 1998) and compared with the standard graph. The concentration was expressed as mgL^{-1}.

3.8.8. Nitrate-N

Nitrate concentration was estimated spectrophotometrically at 543 nm wavelength (APHA, 1998) and compared with the standard graph. The concentration was expressed as mgL^{-1}.

3.9. Calcium and Phosphorous analysis from serum:

Approximately 30g of fish was weighed from each of the treatment were subjected to serum calcium and phosphorous analysis at each sampling. Blood from the fishes was taken using sterile syringe (1ml) without any anticoagulant. Then the blood was dropped in sterile tubes (2ml) and left it undisturbed for separation of serum and clot. Then the tubes were centrifuged at 6000 rpm for 10-15mints. The separated serum were filled in new tubes and taken for analysis.

3.9.1. Calcium analysis

Serum calcium level is analyzed by Arsenazo III method, in this arezeno III have high affinity for calcium ions and shows no interference from other cations normally present in serum or plasma. This can be measured at 650nm. The whole serum calcium analysis is done by Erba kit. In this, serum sample of 10ul is mixed with reagent mixture (Arsenazo III, phosphate buffer) of 1000ul and they are cross analyzed with blank and standard at 650nm.

3.9.1.1. Calculation

Calcium (mg/dl) = $\Delta A_{sam} / \Delta A_{std} \times C_{std}$

- ΔA_{sam} – Absorbance of the sample
- ΔA_{std} – Absorbance of the standard
- C_{std} – Standard (Calibrator) concentration

3.9.2. Phosphorus analysis

Serum phosphorus level is analyzed by ammonium molybdate method, in this ammonium molybdate react with phosphorus and form reduced phosphomolybdate which can be measured at 340nm. The whole serum phosphorus analysis is done by Erba kit. In this, serum sample of 10ul

is mixed with reagent mixture (ammonium molybdate and Sulphuric acid) of 1000ul and they are cross analyzed with blank and standard at 340nm.

3.9.2.1. Calculation

Phosphorus (mmol/l) = $\Delta A_{sam} / \Delta A_{std} \times C_{std}$

- ΔA_{sam} – Absorbance of the sample
- ΔA_{std} – Absorbance of the standard
- C_{std} – Standard (Calibrator) concentration

3.10. Histology of Corpuscles of Stannius

Approximately 30g of fish was weighed from each of the treatment and they were dissected using sterile scissors and forceps. Then the pair of Corpuscle of Stannius was dissected out from the fish which is attached below the posterior end of kidney with loose connective tissues. They are seems to be whitish yellow in color and in pairs. They are removed and kept in a 2ml sterile tube containing NBF solution. Then the samples were taken for histological studies.

3.10.1. Histology Standard Protocol

The Corpuscles of Stannius stored in NBF solution are removed and dried .Then the sample is processed in following standard histology protocol to make paraffin section (Lurie, Comprehensive Cancer Center Northwestern University) Xylene 2 min. Xylene 2 min. Xylene 2 min..100% OH 2 min.100% OH 2 min. 95% OH 1 min. 80% OH 1 min. Water 2:00 min. Hematoxylin 4:00 min. Water Wash 3:00 min Differentiate 1 min. : Water 3:00 min. Bluing 1:00 min. Water 3:00 min. Eosin :30 sec. 95% OH :15 sec. 100% OH :30 sec. 100% OH 1:00 min. 100% OH 2:00 min .Xylene 2:00 min. Xylene 2:00 min. Then the sample taken for sectioning using microtome and they are sliced in the range of 4-7mm and they are sliced over the slides permanently. While making histology slides, care should be taken in deparaffinized and hydration procedures for better staining and analyzing.

Stained Corpuscles of Stannius slides are viewed under two different Phase Contrast Microscope at different magnifications (4X, 60X).

3.11. Partial Sequencing of STC-1 gene from *Labeo rohita*

3.11.1. Chemical reagents and kits

Chemicals used in the present study were generally of Molecular Biology + grade. Agarose, ethidium bromide, TAE buffer, LB agar and LB broth were purchased from HiMedia, India. Ampicillin was purchased from Sigma Aldrich. Ethanol was purchased from Merck. 2X PCR master mix, nuclease free water, 6X gel loading dye, Xgal IPTG were purchased from Fermentas, USA. The kits used in the study were First Stranded cDNA synthesis kit (Fermentas, USA), MinElute Gel Extraction kit(Qiagen, USA), and InTAclone PCR cloning kit(Qiagen, USA).

3.11.2. Bacterial Strain and vector

DH5α strain of *E.coli* available at the molecular lab, FNBP Division, CIFE was used as host strain. Plasmid pTZ57R/T provided along with InsTAclone PCR Cloning Kit was used as a vector.

3.11.3. Enzymes

*Taq*DNA polymerase and restriction endonucleases such as *Bam*HI, *Eco*RI and *Hind*III were purchased from Fermentas, USA. T4 DNA Ligase was provided with the Instaclone PCR Cloning kit (Fermentas, USA). Reverse transcriptase and RibolockRNase inhibitor were provided with the first strand cDNA synthesis Kit (Fermentas, USA).

3.11.4. Nucleic acids and Nucleotides

Gene ruler 100bp plus DNA ladder and 1 kb DNA ladder were purchased from Fermentas, USA. Custom synthesized primers were obtained from Bioserve Biotechnlogies Pvt Ltd., India.

3.11.5. Tissues used for the Study

The expression analysis was done in Corpuscle of Stannius embedded in the Kidney of the fish.

3.11.6. Tissue collection and preservation

The sampling was done on Day 1, Day 2, Day 3, Day 5, Day 7, Day9, Day 11, Day 13, Day 15, Day 30 and Day 60. The organs were collected carefully using sterile scissors and forceps. Each specimen was preserved in RNA later for further use.

3.11.7. Total RNA Extraction

Total RNA was extracted using *TRIzol reagent* following the manufacturer's instructions. Tissue samples preserved in RNA *Later* were blotted and kept in 10-20 volume of *TRIzol* reagent. It was then homogenized and kept for incubation at room temperature for 5 minutes. About 200ul of chloroform for 1ml*TRIzol* was added. After giving 15 seconds of rigorous shaking, the sample was again incubated at room temperature for 5-15minutes. It was then centrifuged at 12000g for 10-15minutes at 4°C and the aqueous phase was transferred to a new microfuge tube. To this 500ul of isopropanol for 1ml of *TRIzol*was added, vortexed for 5-10 second and incubated at room temperature for 5-10 minutes. After centrifuge at 12000g for 8 minutes at 4°C the supernatant was discarded, 1ml of 70% ethanol per 1ml of *TRIzol* was added and again centrifuged at 7500g for 5minutes. The ethanol was then removed and the pellet was air dried for 30 minutes. Then the pellet, after it was completely dried, was dissolved in 30-50µl of nuclease free water. The quality of RNA was confirmed using Nanodrop spectrophotometer. The integrity of the isolated was checked by running it on a 2% agarose gel.

3.11.8. Reverse Transcription

The mRNA transcript of the gene was converted into its complementary DNA strand using the first strand cDNA synthesis Kit (Fermentas, USA) as per the protocol provided by the manufacturer.

3.11.8.1. Removing genomic DNA contamination

The extracted RNA was treated with DNAse I (RNase free) to remove the genomic DNA contamination.

3.11.8.2. cDNA Synthesis

The template RNA can be converted into cDNA by the following method. A volume of 12.5µl mixture was made by adding 2µg template RNA, oligo (dT) 0.5µg and remaining with DEPC water. It was then gently mixed and briefly centrifuged. Then it was incubated at 65°C for 5 minutes and chilled on ice. Then the following components were added in the mentioned amounts, 5X reaction mixture 4µl, RibolockRNase inhibitor 0.5µl, 10mM dNTP mix 2µl, and Revert Aid H minus M-MuLV reverse transcriptase 1µl, so that the total volume becomes 20µl. It was then mixed gently and centrifuged briefly. It was then incubated at 42°C for 60 minutes and the reaction was terminated by heating at 70°C for 10minutes.

3.11.9. Polymerase Chain Reaction(PCR)

3.11.9.1. Primers

The PCR reaction was performed with the primers for STC-1 and Beta actin. The primers were designed by using Gene Runner v. 3.01 software by selecting the conserved regions of the STC-1 of closely related fish. The primers used were given in the table 2.

Table 2: List of Primers used for sequencing STC-1 genes in *Labeo rohita*

Sl. No	Primer Name	Primer Sequence From 5' to 3'
1.	STC1 (F)	GATGTCGCCCGCTGCCTGAA
2.	STC1 (R)	CGGCTCGCACCACCTCAACAATG
3.	β -actin (F)	GCCGAGAGGGAAATTGTCCGTGAC
4.	β -actin (R)	TTGCCAATGGTGATGACCTGTCCG

3.11.9.2. PCR amplification

The reaction mix was prepared by adding 12.5µl of MasterMix, 1µl of each forward and reverse primer each, 1µl of the cDNA and then finally water was added to make the volume up to 25µl. The temperature schedule was as follows in the table 3.

Table 3: Temperature schedule of PCR programs for STC- 1

Steps	**STC-1**
Initial Denaturation	94°C for 5minutes
Denaturation	94°C for 30 seconds
Annealing	57.5°C for 40 seconds
Extension	72°C for 30 seconds
Step 2 to 4 repeated 35 times	
Final Extension	72°C for 7 minutes
Hold	At 4°C

3.12. Agarose gel electrophoresis of PCR product

1% agarose gel was used for analyzing the amplified products of PCR. The electrophoresis was carried out at 70V for 50minutes. The gel was then visualized under the gel documentation systems.

3.13. Gel Elution

The bands obtained in the gel were cut and DNA eluted using quick gel extraction kit (Qiagen) following manufacturer's instructions and DNA was collected in Eppendorf tubes.

3.14. Cloning of the amplified STC-1 genes

Cloning was done using the InsTA Clone cloning kit(Fermentas) following the manufacturer's instructions.

3.15. Plasmid

The plasmid used in the present study is pTZ57R/T (MBI Fermentas, USA), having ampicillin resistance marker used for T/A cloning of the PCR amplified products.

3.16. Ligation

The total volume of 30µl reaction mixture was prepared, by adding 3µl-pTZ5R/T vector, 6µl-5Xbuffer, 5µl-insert, 16ul-distilled water and 1µl-t4 DNA ligase. The mixture was then kept for incubation at 4°C for overnight and then stored at -20°C.

3.17. Transformation

The ligated product was transformed into DH5α strain of *E.coli.* Overnight culture of DH5α strain was used for transformation. Briefly 150µl of the culture was mixed with 1.5ml of C and kept for incubation at 37°C for 30 minutes. The mixture was then centrifuged at 12000 rpm for 3 minutes. The supernatant was discarded and the pellet was suspended in 300µl T solution. After keeping it on ice for 5minutes it was again centrifuged as above, the supernatant was discarded and the pellet was suspended in 120µl of T solution followed by incubated on ice for 5 minutes. After adding 2.5µl of the ligation mix, the solution was incubated on ice for 5 minutes. This was then spread on Xgal-IPTG-Ampicillin LB Agar plates and kept for incubation overnight at 37°C. The white colonies were further taken for screening.

3.18. Analysis of Recombinant Clones

3.18.1. Screening of the recombinants by plating

Blue-white colony selection was performed to select for positive clones. The white colonies obtained were streaked on LB agar plates (master plate) containing 50mg/ml Ampicillin and incubated at 37°C for 16-18 hrs.

3.18.2. Screening of the clones by PCR for the insert

Colony PCR technique was used for screening the clones for insert. A few cells from each colony were picked up and suspended in normal PCR

reaction mixture except the template. The PCR conditions for STC-1 amplification were employed and the PCR products were analysed by running on 1% agarose gel. The colonies that showed the desired bands were selected and taken forward for future screening by plasmid isolation and RE digestion.

3.18.3. Recombinant DNA plasmid isolation

The plasmid DNA was isolated from the colonies that gave positive result in the colony PCR using Miniprep kit (Fermentas, USA), following manufacturer's instructions and plasmid was collected in a fresh tube. The integrity of the isolated plasmid was checked in 1% agarose gel.

3.18.4. Restriction Enzymes Digestion

RE digestion was done for the recombinant plasmid to check whether the insert was there or not. For this 3µl of plasmid was mixed with 0.5µl of *Eco*R1 and *Bam*HI each, 2µl of buffer and 14µl of nuclease free water. This reaction mixture was then kept at 37°C for 15min and ran on 1% agarose gel for about an hour. The plasmids that released the desired inserts were confirmed as positive clones.

3.18.5. Sequencing

The cloned products were sequenced using universal primers M13 forward and reverse by Bioinnovation Technologies.

3.18.5.1. Sequence Analysis

The sequence was analyzed using BLAST (Basic Logical Alignment Search Tool) software in the NCBI (National Center for Biotechnology Information) GenBank nucleotide database for finding homology with other sequences. The top 10 sequences were taken for multiple sequence alignment by CLUSTALW software and phylogenetic tree was constructed. The Generunner software was used to translate the sequence into protein sequence to analyze the amino acid sequence for the presence of functional domains that are specific to STC-1.

3.19. Statistical analysis

The data were statistically analyzed by statistical package SPSS version 16. Comparison among all the treatment was done by multivariate ANOVA. Comparisons were made at the 5% probability levels.

4. RESULTS

4.1. Physicochemical Parameters of Water

The physicochemical parameters of water such as temperature (^{0} C), pH, dissolved oxygen (mgml^{-1}), ammonia (mgml^{-1}), Nitrite-N (mgl^{-1}), Nitrate-N (mgl^{-1}) were recorded.

4.1.1. Temperature

The water temperature of the different experimental groups ranged from 28-33^{0}C during the experiment period of 60 days.

4.1.2. pH

There was not much variation in pH values during the experimental period. The pH values were recorded within the range of 7.5 to 8.5.

4.1.3. Dissolved oxygen

The dissolved oxygen concentration of all the experimental tubs was recorded within the range of 5.6 -7.7 mgL^{-1} during the experimental period of 60 days.

4.1.4. Free carbon dioxide

The free carbon dioxide in water was found to be negligible during the experimental period.

4.1.5. Carbonate hardness

The carbonate hardness was found to be 228-245 mgL^{-1}during the experimental period of 60 days.

4.1.6. Total Ammonia

The total ammonia content of all the experimental tubs was recorded before water exchange. It was found to within the range from 0.0 to 0.1mg $L^{-1.}$

4.1.7. Nitrite-N

The nitrite –N content was found to be in the range 0.001-0.005 mg $L^{-1.}$

4.1.8. Nitrate-N

The nitrate –N content was found to be ranging from 0.1-5.0 mg L^{-1}throughout the experimental period.

4.1.9. Calcium level in experimental water

Calcium level in normal water is found to be 20ppm and calcium enriched water was made as 40ppm using $CaCl_2$.

4.2. Analysis of Serum Calcium and Phosphorous levels in *Labeo rohita* reared in calcium enriched waters

Serum calcium and phosphorus level analysed in Labeo rohita for 60 days which is reared in normal and calcium enriched water. The change in concentration of serum calcium and phosphorus level is given in table 4 &5.

Normal Control group (D_0C_L& D_0C_H):

The serum calcium level was observed on days ; 0, 1^{th}, 2^{nd}, 3^{th}, 5^{th}, 7^{th}, 9^{th}, 11^{th}, 15^{th}, 30^{th} and 60^{th} day. And there was no rise in calcium level from day 1 to day 60 (Table-4).

Normal Experimental group (D_LC_L& D_HC_L):

4.2.1. Serum calcium concentration of *L.rohita* reared in normal water (20 ppm)

The serum calcium level was observed on days ; 0, 1th, 2nd, 3th, 5th, 7th, 9th, 11th, 15th, 30th and 60th day (Table - 4). Serum calcium level of *Labeo rohita*were studied at different day intervals after intra peritoneal administration of vitamin $D_3$and reared in both normal and calcium enriched water. The change in concentration of serum calcium level of *L.rohita*reared in normal water (20ppm) at different day is shown in figure 1. After injection of vitamin D_3 at two different doses, fish were showing a change in concentration of serum calcium level which is according to the dosage of vitamin D_3. In low dose(100 IU/Kg bw) there is a increase in calcium level and reaching peak at 5th day up to 11.45$\pm$0.4 and again started decreasing till 60th day. At the same time for high dose (500 IU/Kg bw) the uptake of calcium is higher than low dose and reached maximum on 7th day at a range of 13.74$\pm$ 0.1and again it reduced till 60th day.

Calcium Enriched Experimental group (D_LC_H & D_HC_H)

4.2.2. Serum calcium concentration of *L. rohita* reared in calcium enriched water (40 ppm)

The serum calcium level was observed on days ; 0, 1th, 2nd, 3th, 5th, 7th, 9th, 11th, 15th, 30th and 60th day (Table - 4). Serum calcium level of *Labeo rohita*were studied at different day intervals after intra peritoneal administration of vitamin $D_3$and reared in both normal and calcium enriched water. The change in concentration of serum calcium level of *L.rohita* reared in calcium enriched water (40ppm) at different day is shown in figure 2. After injection of vitamin D_3at two different doses, fish were showing a change in concentration of serum calcium level which is according to the dosage of vitamin D_3. In low dose(100 IU/Kg bw) there is a increase in calcium level and reaching peak at 5thday up to 12.48$\pm$0.5 and again started decreasing till 60thday. At the same time for high dose (500 IU/Kg bw) the uptake of calcium is higher than low dose and reached maximum on 5th day at a range of

13.87±0.3 and again it reduced till 60th day. But there is no significant difference seen in fish reared in control (i.e. without any dosage).

Normal Control group (D_0C_L& D_0C_H):

The serum inorganic phosphate level was observed on days; 0, 1th, 2nd, 3th, 5th, 7th, 9th, 11th, 15th, 30th and 60th day. And there was no rise in $P_i$level from day 1 to day 60 (Table -5).

Normal Experimental group (D_LC_L& D_HC_L)

4.2.3. Serum inorganic phosphorus concentration of *L.rohita* reared in normal water (20 ppm)

The serum inorganic phosphate level was observed on days; 0, 1th, 2nd, 3th, 5th, 7th, 9th, 11th, 15th, 30th and 60th day (Table - 5). Serum inorganic phosphorus level of *Labeo rohita* were studied at different day intervals after intra peritoneal administration of vitamin D3 and reared in both normal and calcium enriched water. The change in serum phosphorus level of *L.rohita* reared in normal water (20ppm) at different day is shown in figure 3. After injection of vitamin D3 at two different doses, fish were showing a change in concentration of serum phosphorus level which is according to the dosage of vitamin D_3. In low dose(100 IU/Kg bw) there is a increase in phosphorus level and reaching peak at 5th day up to 6.23±0.5 and again started decreasing till 60th day. At the same time for high dose (500 IU/Kg bw) the uptake of phosphorus is higher than low dose and reached maximum on 7th day at a range of 7.04± 0.2 and again it reduced till 60th day. But there is no significant difference seen in fish reared in control (i.e. without any dosage).

Calcium Enriched Experimental group (D_LC_H & D_HC_H):

4.2.4. Serum inorganic phosphorus concentration of *L.rohita* reared in calcium enriched water (40 ppm)

The serum inorganic phosphate level was observed on days; 0, 1th, 2nd, 3th, 5th, 7th, 9th, 11th, 15th, 30th and 60th day (Table - 5). Serum inorganic phosphorus level of *Labeo rohita* were studied at different day intervals after

intra peritoneal administration of vitamin D_3 and reared in both normal and calcium enriched water. The change in concentration serum phosphorus level of *L.rohita* reared in normal water (20ppm) at different day is shown in figure 4. After injection of vitamin D_3 at two different doses, fish were showing a change in concentration of serum phosphorus level which is according to the dosage of vitamin D_3. In low dose(100 IU/Kg bw) there is a increase in phosphorus level and reaching peak at 5th day up to 6.89$\pm$0.2 and again started decreasing till 60th day. At the same time for high dose (500 IU/Kg bw) the uptake of phosphorus is higher than low dose and reached maximum on 7th day at a range of 7.66$\pm$0.2 and again it reduced till 60th day. But there is no significant difference seen in fish reared in control (i.e. without any dosage).

4.2.5. Serum calcium level in *Labeo rohita* reared in normal and calcium enriched water for 60 days after administration of vitamin D_3

Table 4:Serum Calcium concentration (mg/dl) of *L. rohita* injected with graded level of Vitamin D_3 and reared in calcium enriched water

Sr. No.	Day	Control (N) 20 ppm Calcium	Exp. (NL) 100 IU/kg b.w.	Exp. (NH) 500 IU/kg b.w.	Control (C) 40 ppm Calcium	Exp. (CL) 100 IU/kg b.w.	Exp. (CH) 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 ($P<0.05$) N = Normal Water (20 ppm Calcium; NL = Normal Water (Fishes given i.p. 100 IU Vitamin D_3/kg b.w.); NH = Normal Water (Fishes given i.p. 100 IU Vitamin D_3/kg b.w.); C = Control with calcium enriched water (40 ppm)CL = Calcium enriched Water (Fishes given i.p. 100 IU Vitamin D_3/kg b.w.); NH =Calcium enriched Water (Fishes given i.p. 500 IU Vitamin D_3/kg b.w.

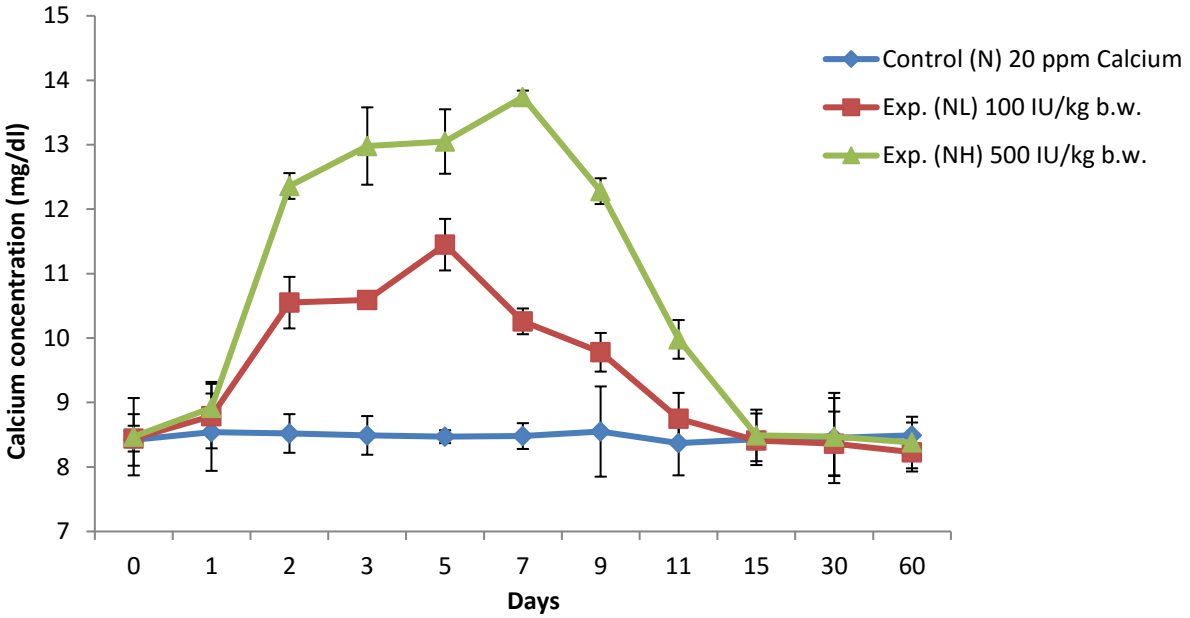

Fig 1: Serum calcium concentration (mg/dl) of *Labeo rohita* injected with graded level of vitamin D_3 and reared in normal water

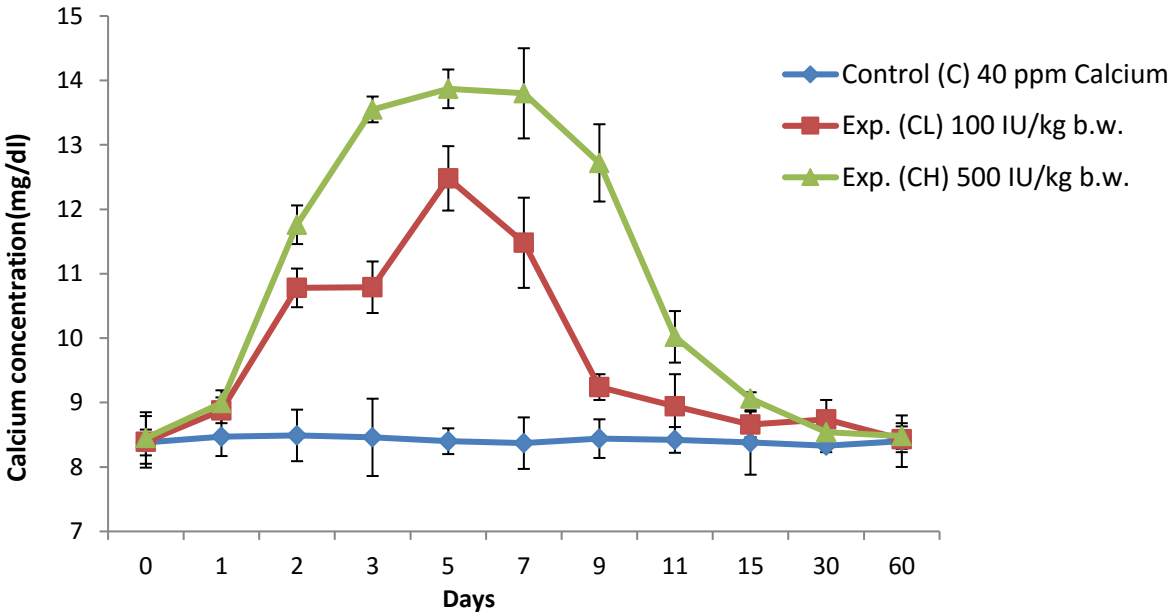

Fig 2: Serum calcium concentration (mg/dl) of *Labeo rohita* injected with graded level of vitamin D3 and reared in calcium enriched water

4.2.6. Serum inorganic phosphorus level in *Labeo rohita* reared in calcium enriched water for 60 days after administration of vitamin D_3

Table 5: Serum Inorganic phosphorus concentration (mmol/l) of *L.rohita* injected with graded level of Vitamin D_3 and reared in calcium enriched water

Sr. No.	Day	Control (N) 20 ppm Calcium	Exp. (NL) 100 IU/kg b.w.	Exp. (NH) 500 IU/kg b.w.	Control (C) 40 ppm Calcium	Exp. (CL) 100 IU/kg b.w.	Exp. (CH) 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 ($P<0.05$).N = Normal Water (20 ppm Calcium; NL = Normal Water (Fishes given i.p. 100 IU Vitamin D_3/kg b.w.); NH = Normal Water (Fishes given i.p. 100 IU Vitamin D_3/kg b.w.); C = Control with calcium enriched water (40 ppm)CL = Calcium enriched Water (Fishes given i.p. 100 IU Vitamin D_3/kg b.w.); NH =Calcium enriched Water (Fishes given i.p. 500 IU Vitamin D_3/kg b.w.)

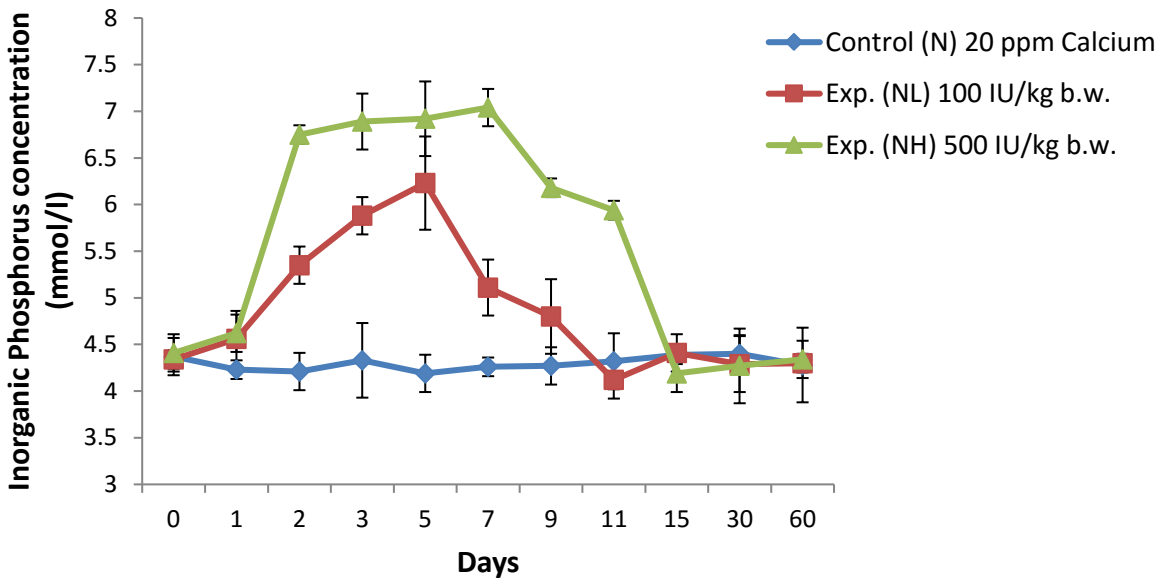

Fig 3: Serum inorganic phosphorus concentration (mmol/l) of *Labeo rohita* injected with graded level of vitamin D_3 and reared in normal water

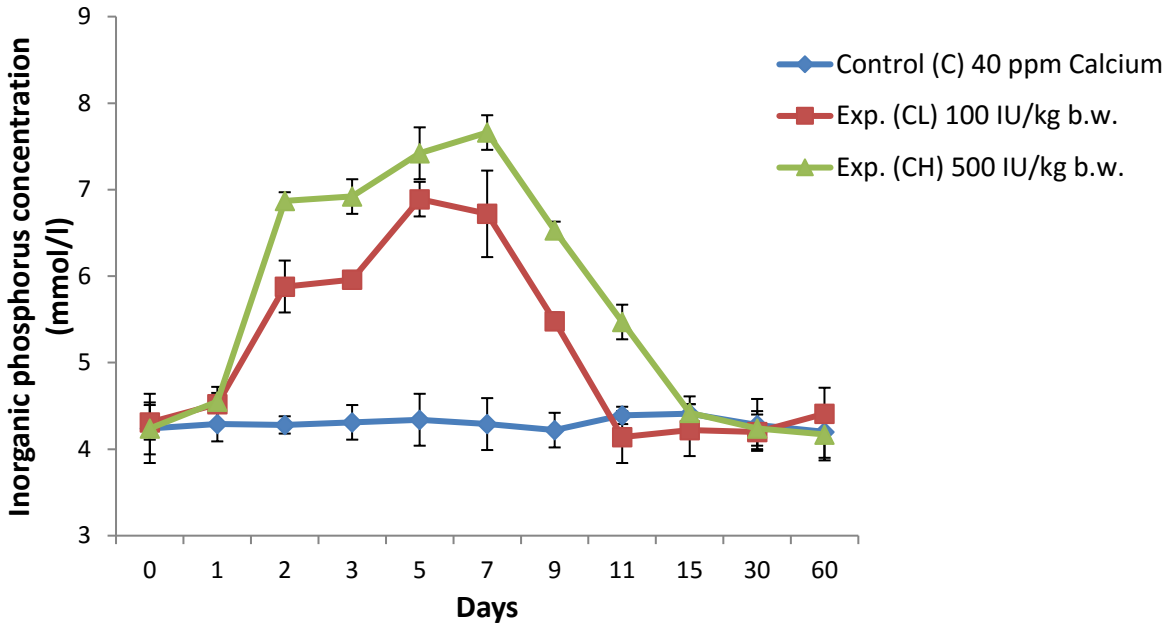

Fig 4: Serum inorganic phosphorus concentration (mmol/l) of *Labeo rohita* injected with graded level of vitamin D_3 and reared in calcium enriched water

4.3. Partial Sequencing of STC-1 gene from *Labeo rohita*

4.3.1 Total RNA Extraction

Total RNA isolated from the kidney and corpuscles of Stannius was isolated and tested for it quantity, purity and integrity. The concentration of the isolated RNA was 1500 to 5000ng/µl. The A_{260}/A_{280}ratio of the RNA sample was 1.90, indicating that the purity of the RNA was good. The integrity of the RNA was verified by running it in a 2% Agarose gel, which showed the separation of 28S and 18S rRNA bands (Fig 5).

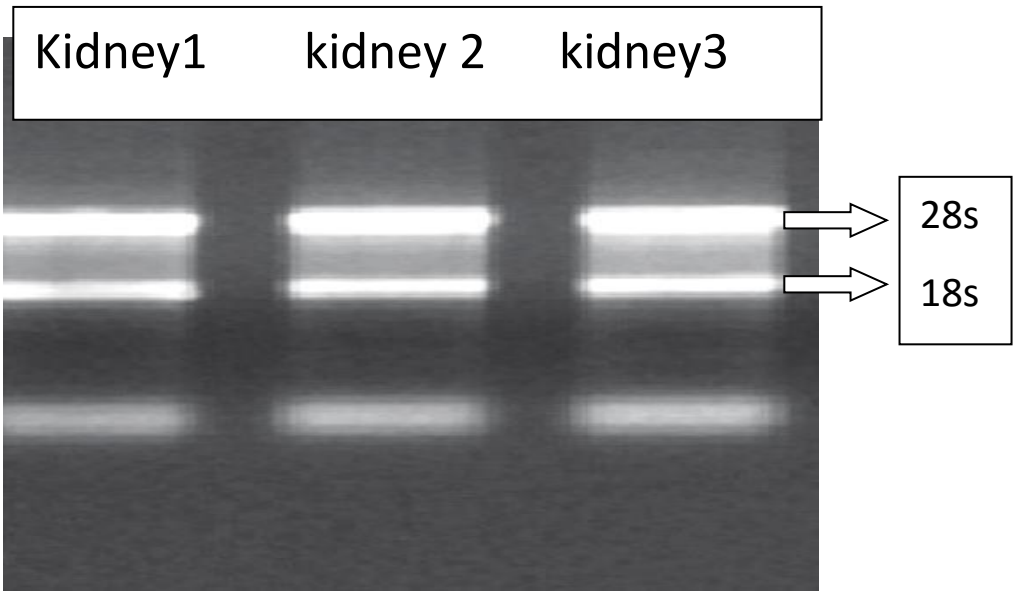

Fig 5: Gel Electrophoresis of total mRNA from kidney tissues of *Labeo rohita*

4.3.1. Reverse Transcription of cDNA

RT-PCR of kidney tissue was done with STC-1 forward and reverse primers in gradient temperature and shown a distinct band at 55^0C, 57^0C, 58^0C, 59^0C and 60^0C in around 400 bp in the 1% agarose gel (Fig 6).

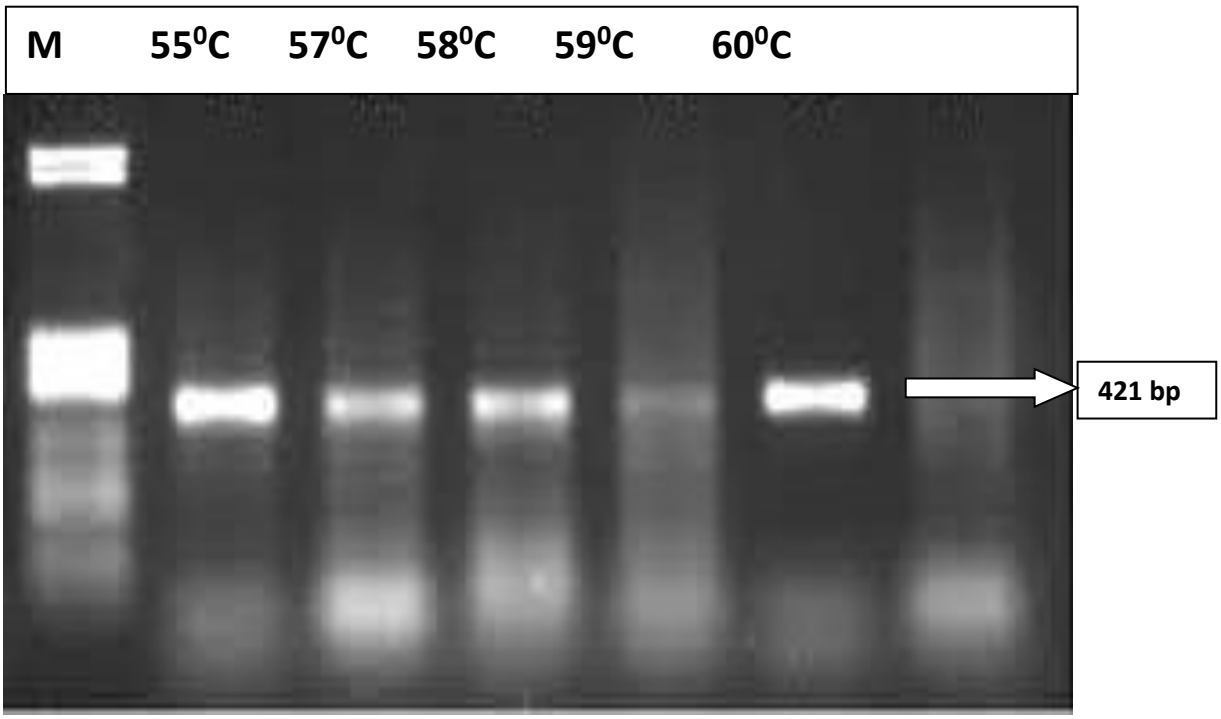

Fig 6: PCR analysis for *Labeo rohita* STC1 mRNA expression analysed in 1% Agarose gel

4.3.2. Cloning and screening of the recombinants

The DH5α strain of *E.coli* transformed with pTZ57R/T vector with insert blue and white colonies in the X-gal/IPTG/ Ampicillin positive LB Agar transformation plates. White colonies were carefully selected for colony PCR.

4.3.3. Colony PCR of the Recombinant Bacteria

The white colonies were selected from the transformation plates by using a sterile pipette tip and were dissolved in buffer. Then PCR was performed using the same primer and PCR conditions, but taking the bacteria dissolved in the buffer as template. Twelve colonies were screened of which all were positive showing a band of 421bp in the 1% Agarose gel.

4.3.4. Confirmation of the Recombinant Clones

The isolated plasmid on digestion with restriction nucleases viz. *Eco*RI and *Hind*III released the partial STC-1 gene insert of the size 421 bp. The restriction endonucleases *Eco*RI and *Hind*III were selected on the restriction enzyme analysis based on the restriction map of the multiple cloning site of vector pTZ57R/T.

4.3.5. Sequence analysis partial STC-1 gene

The recombinant plasmids containing the inserts were submitted to BioInnovations for sequencing. There were 3 different clones (i.e., triplicates) for each insert which were sent for sequencing and the sequences were sent in the chromatogram. The sequence for STC-1 received was 421bp long. The BLAST search in NCBI GenBank database of *Labeo rohita* partial STC-1 gene resulted in 93 to 97 hits, respectively are shown in table 5. *Labeo rohita* partial STC-1 gene showed maximum homology with *Sinocyclocheilus rhinocerous* stanniocalcin-like mRNA (100%) followed by *Sinocyclocheilus anshuiensis* stanniocalcin-like transcript variant X2, mRNA (Table 6).

Table 6:Sequence homology analysis of *Labeo rohita* partial STC-1 gene using BLAST

Accession Number	Description	Max Score	Query Cover
XM_016525895	Sinocyclocheilus rhinocerous stanniocalcin-like mRNA	678	100%
XM_016447215	*Sinocyclocheilus anshuiensis* stanniocalcin-like transcript variant X2, mRNA	667	100%
XM_016277286	*Sinocyclocheilus grahami* stanniocalcin-like mRNA	662	100%

4.3.6. 421bp long CDs sequence of Stanniocalcin-I of *Labeo rohita*

5'_GATGTCGCCCGCTGCCTGAACGGAGCTCTCCAGGTGGGCTGCGCAACTTTC
GCCTGTCTGGAGAATTCGACCTGCGACACTGACGGGATGCATGAGATCTGCAA
CGCTTTCCTCCACACTGCTGCAGTTTTTAATACAGAGGGTAAGACGTTTGTGAAA
GAGAGCATCAAGTGCATCGCCAACGGTATCACCTCTAAGGTCTTCCAGACCATC
AAACGCTGTTCCACCTTCCAGAAGATGATTGCTGAAGTGCAGGAGGAATGCTAC
AAGAAGCTTGACATCTGCGAAGTGGCCAGATCCAACCCTGAGGCCATTGGAGA
CGTGGTCCAGGTCCCCAGCCACTTCCCAAACAGGTACTACAGCACACTTCTGCA
GAGCTTGATGGAGTGCGACGACGACATTGTTGAGGTGGTGCGAGCCG**_3'**

4.4. Histology of Corpuscles of Stannius *Labeo rohita* reared in calcium enriched waters

The gross structure corpuscles of Stannius(CS)is shown in Fig. 7&8.

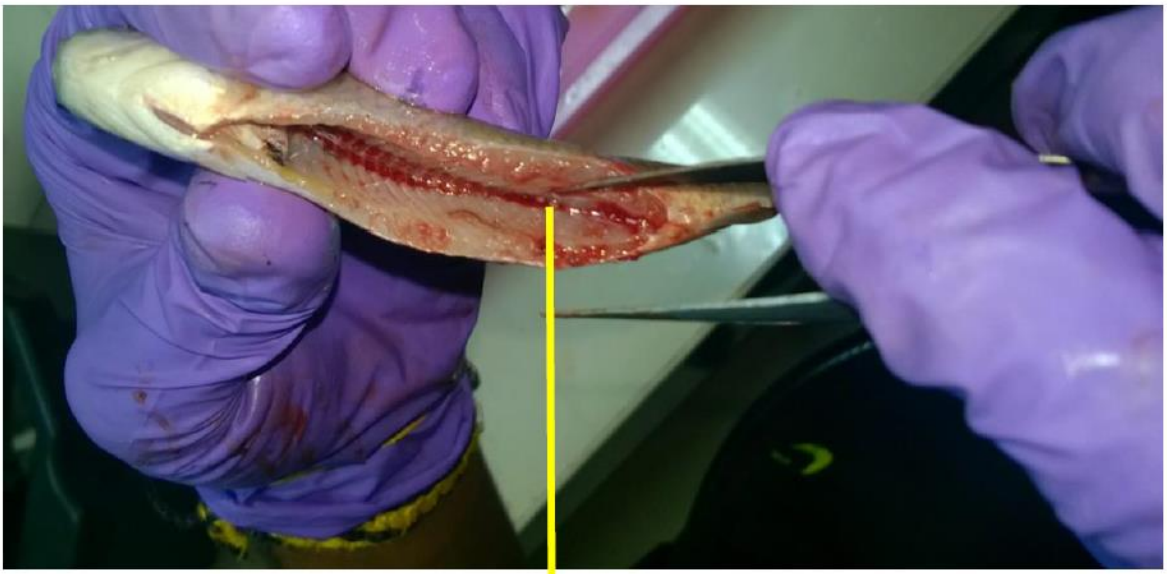

Fig 7: The location of CS in *Labeo rohita* embedded in the kidney

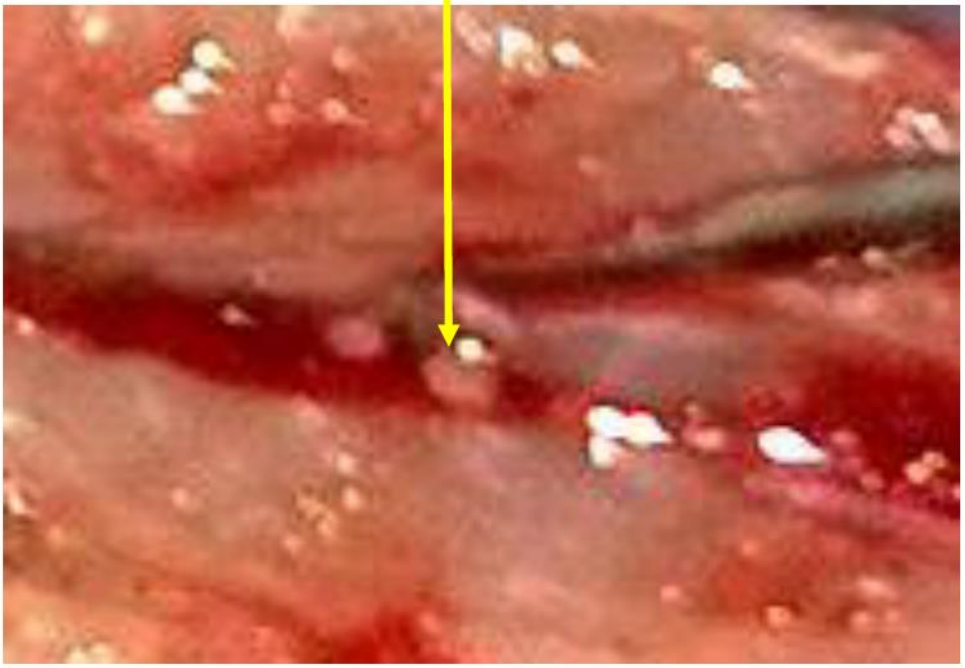

Fig. 8 In *Labeo rohita:* Pair of CS of 0.5-1µm in diameter; Whitish yellow and round in shape; Located at ventral portion of the posterior kidney; Loosely attached with kidney and vertebral column.

4.4.1. Normal Control group (D_0C_L): 0.0 IU D_3/kg b.w. and low calcium level

The CS in vehicle (Arachis Oil) injected fish reared in 0.0 IU D_3/kg b.w. and low calcium (20 ppm) level waterexhibits several complete or incomplete lobules which contain epithelial cells. These cells possess oval or rounded nuclei. Accordingly, the serum calcium and inorganic phosphate levels of vehicle-injected fish exhibits no change throughout the experiment. The cellular structure are shown in Fig. 9 & 10 on day – 0. The similar cellular structure was recorded in CS on day 30 (Fig. 15 & 16) when CS get rejuvenated after the effect of vit. D_3even at higher dose (500 IU/kg b.w.

4.4.2. Normal Experimental group (D_LC_L): 100 IU D_3/kg b.w. and low calcium level

The CS response in fish injected with 100 IU D_3/kg b.w. fish reared low calcium (20 ppm) level water exhibits several complete lobules which contain epithelial cells. There was peak calcium level and inorganic phosphate levels on day – 5. The cellular structures are shown in Fig. 11 & 12 on day – 5 showing the nuclear volumeof cells records an increase andthey become partially de-granulated as is evident bytheir weak staining response. Also, there is an increased dilatation of sinusoids (Fig. 12) and these changes get exaggerated. The results of cellular activities are in correspondence with the serum levels of calcium and inorganic phosphate and demonstrating that there was hypocalcemic response of CS gland.

4.4.3. Normal Experimental group (D_HC_L): 500 IU D_3/kg b.w. and low calcium level

The CS response in fish injected with 500 IU D_3/kg b.w. fish reared low calcium (20 ppm) level water exhibits several complete lobules which contain epithelial cells. There was peak calcium level and inorganic phosphate levels on day – 7. The cellular structures are shown in Fig. 13 & 14 on day – 7 showing the nuclear volumeof cells records an increase andthey become partially de-granulated as is evident bytheir weak staining response. Also, there is an increased dilatation of sinusoids (Fig. 14) and these changes get

further exaggerated and complete exhaustion of the gland was recorded. The results of cellular activities are in correspondence with the serum levels of calcium and inorganic phosphate and demonstrating that there was hypocalcemic response of CS gland. However, the gland action was more severe to make normocalcemia.

4.4.4. Calcium Enriched Control group (D_0C_H):

The CS in vehicle (Arachis Oil) injected fish reared in 0.0 IU D_3/kg b.w. and low calcium (40 ppm) level waterexhibits several complete or incomplete lobules which contain epithelial cells. The serum calcium and inorganic phosphate levels of vehicle-injected fish exhibits no change throughout the experiment. The cellular structure are shown in Fig. 17 & 18 on day – 0. The similar cellular structure was recorded in CS on day 30 (Fig. 23 & 24) when CS gets rejuvenated after the effect of vit. D_3even at higher dose (500 IU/kg b.w).

4.4.5. Calcium Enriched Experimental group (D_LC_H):

The CS response in fish injected with 100 IU D_3/kg b.w. fish reared low calcium (40 ppm) level waterthere was peak calcium level and inorganic phosphate levels on day-5. The cellular structures are shown in Fig. 19 & 20 on day-5 showing the nuclear volumeof cells records an increase andthey become partially de-granulated as is evident bytheir weak staining response. Also, there is an increased dilatation of sinusoids (Fig. 20) and these changes get exaggerated. The results of cellular activities are in correspondence with the serum levels of calcium and inorganic phosphate and demonstrating that there was hypocalcemic response of CS gland, but was slower in rate than in 500 IU/kg b.w.

4.4.6. Calcium Enriched Experimental group (D_HC_H):

The CS response in fish injected with 500 IU D_3/kg b.w. fish reared low calcium (40 ppm) level water exhibits several complete lobules which contain epithelial cells. There was peak calcium level and inorganic phosphate levels on day–7. The cellular structures are shown in Fig. 21 & 22 on day–7 showing

the nuclear volumeof cells records an increase andthey become partially de-granulated as is evident bytheir weak staining response. Also, there is an increased dilatation of sinusoids (Fig. 22) and these changes get further exaggerated and complete exhaustion of the gland was recorded. However, the gland action was more severe to exhibit normocalcemic response.

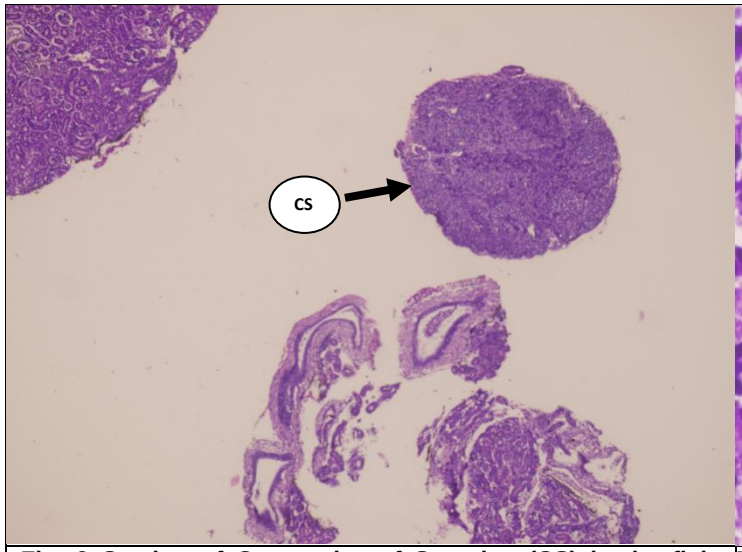

Fig. 9 Section of Corpuscles of Stannius (CS) in the fish, *Labeo rohita* inNormal Control group (D_0C_L)(H & E 4X) Day – 0 (Ca 20 ppm & 0.0 ID D_3)

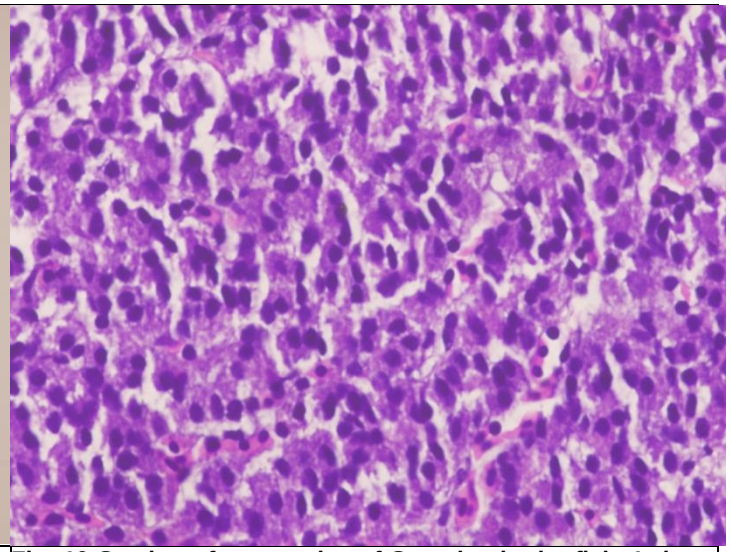

Fig. 10 Section of corpuscles of Stannius in the fish, *Labeo rohita* inNormal Control group (D_0C_L) (H & E 60X) Day – 0 (Ca 20 ppm & 0.0 ID D_3)

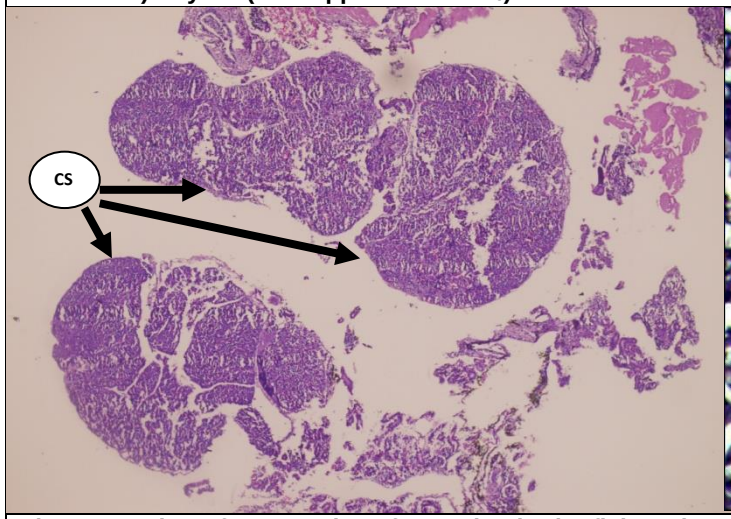

Fig. 11 Section of Corpuscles of Stannius in the fish, *Labeo rohita* in Normal Experimental group (D_LC_L)(H & E 4X) Day-5 (Ca 20 ppm & 100 IU D_3)

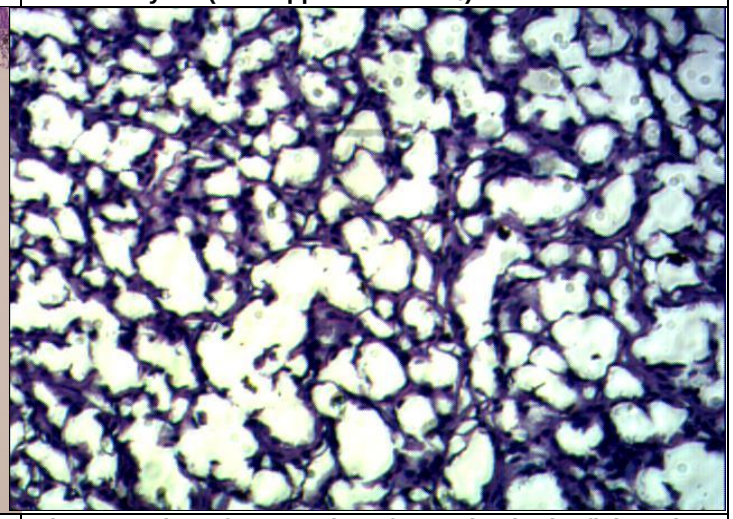

Fig.12 Section of corpuscles of Stannius in the fish, *Labeo rohita* in Normal Experimental group (D_LC_L)(H & E 60X) Day-5 (Ca 20 ppm & 100 IU D_3)

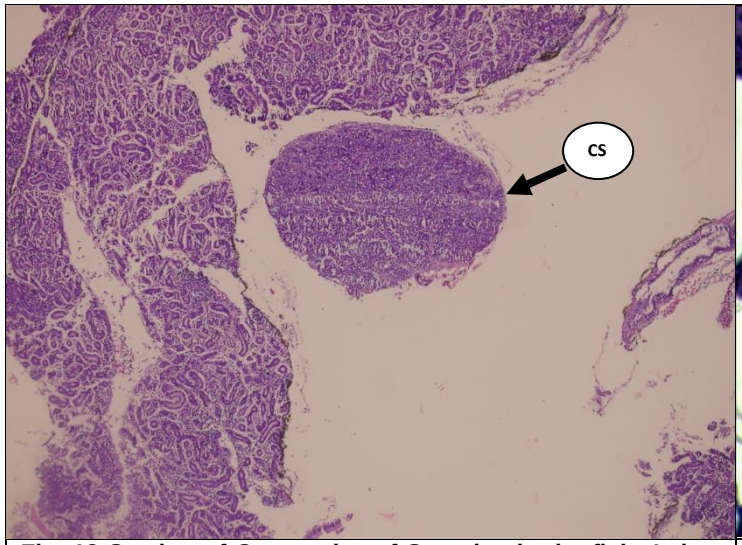

Fig. 13 Section of Corpuscles of Stannius in the fish, *Labeo rohita* in Normal Experimental group (D_HC_L) (H & E 4X) Day-7 (Ca 20 ppm & 500 IU D_3)

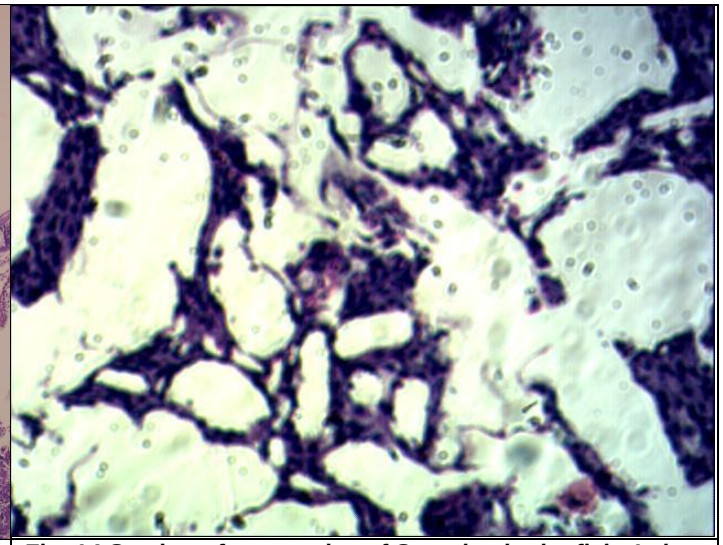

Fig. 14 Section of corpuscles of Stannius in the fish, *Labeo rohita* in Normal Experimental group (D_HC_L) (H & E 60X) Day – 7 (Ca 20 ppm & 500 IU D_3)

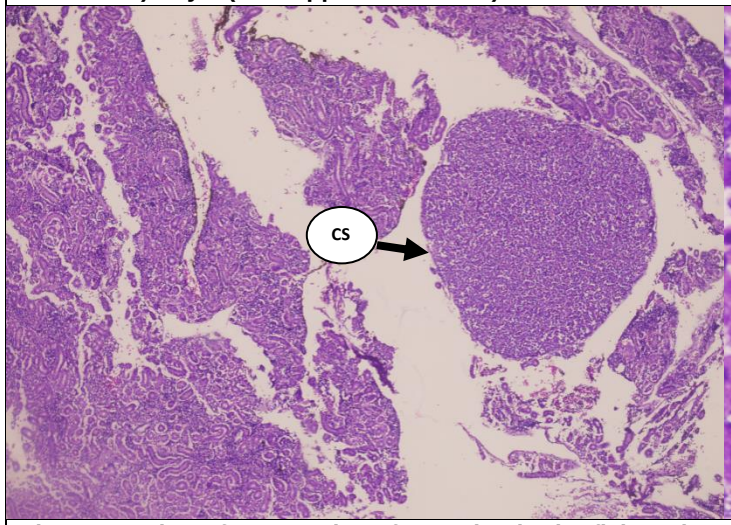

Fig. 15 Section of Corpuscles of Stannius in the fish, *Labeo rohita* in Normal Experimental group (D_HC_L) (H & E 4X) Day-30 (Ca 20 ppm & 500 IU D_3)

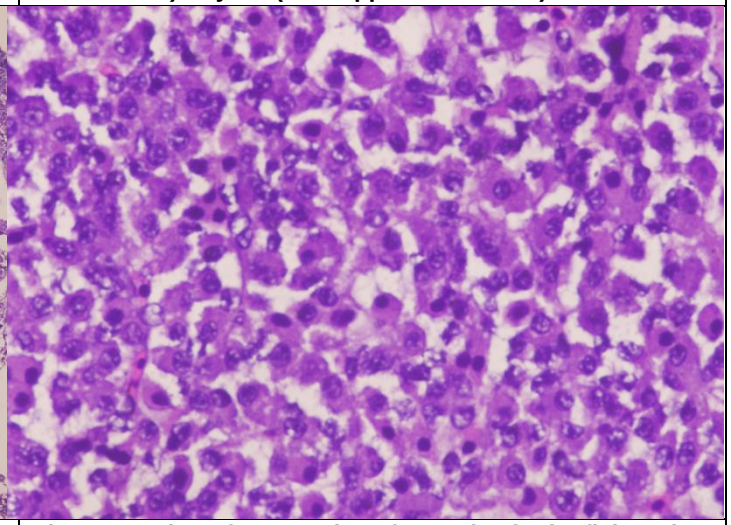

Fig. 16 Section of corpuscles of Stannius in the fish, *Labeo rohita* in Normal Experimental group (D_HC_L) (H & E 60X) Day – 30 (Ca 20 ppm & 500 IU D_3)

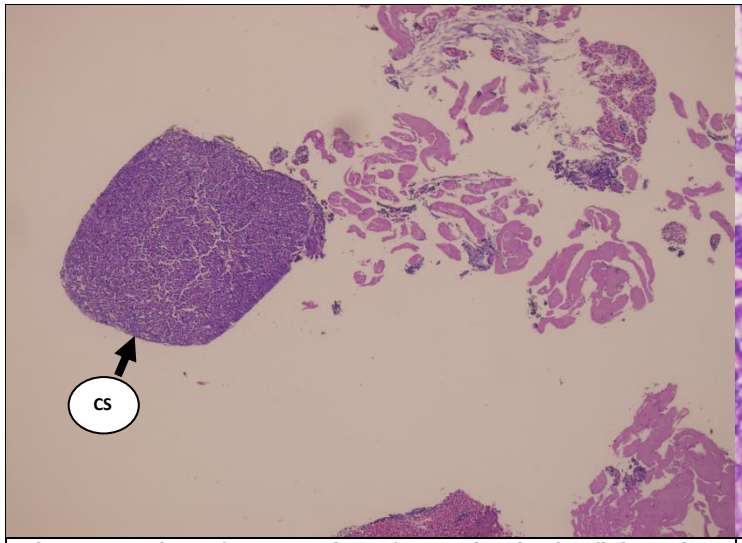

Fig. 17 Section of Corpuscles of Stannius in the fish, *Labeo rohita* in Calcium enriched Control group (D_0C_H)(H & E 4X) Day – 0 (Ca 40 ppm & 0.0 ID D_3)

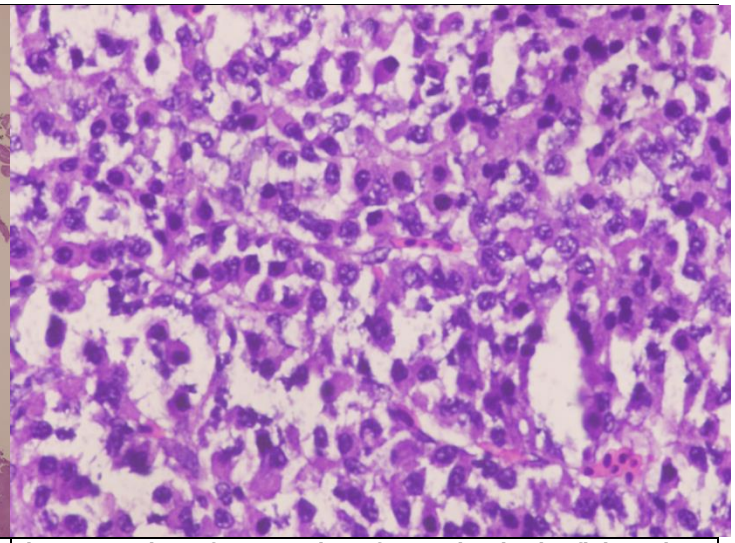

Fig. 18 Section of corpuscles of Stannius in the fish, *Labeo rohita* in Calcium enriched Control group (D_0C_H) (H & E 60X) Day – 0 (Ca 40 ppm & 0.0 ID D_3)

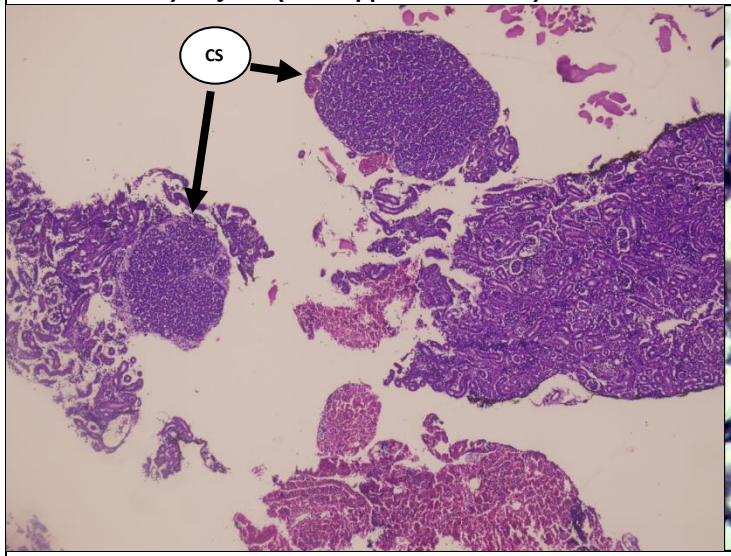

Fig. 19 Section of Corpuscles of Stannius in the fish, *Labeo rohita* in Calciumenriched Experimental group (D_LC_H)(H & E 4X) Day-5 (Ca 40 ppm & 100 IU D_3)

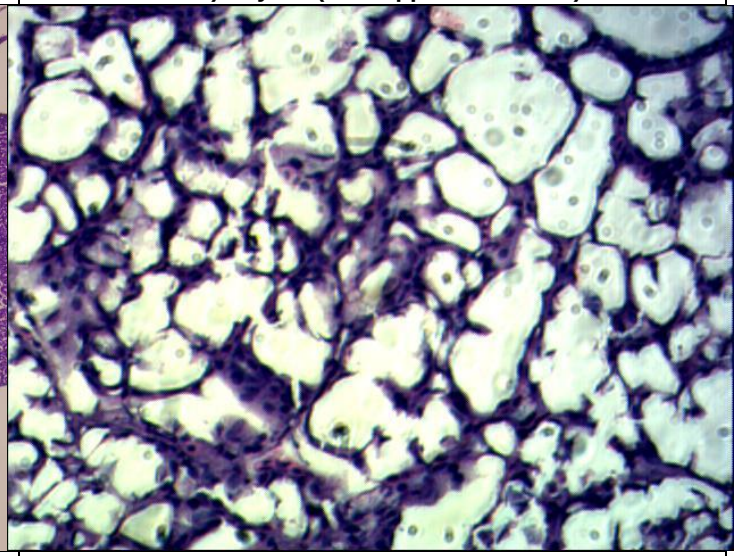

Fig.20 Section of corpuscles of Stannius in the fish, *Labeo rohita* in Calciumenriched Experimental group (D_LC_H)(H & E 60X) Day-5 (Ca 40 ppm & 100 IU D_3)

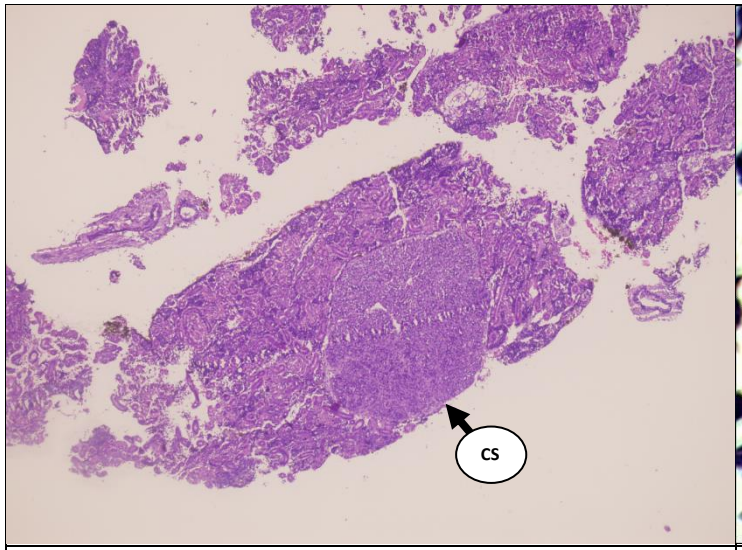

Fig. 21 Section of Corpuscles of Stannius in the fish, *Labeo rohita* in Calcium enriched Experimental group (D_HC_H) (H & E 4X) Day-7 (Ca 40 ppm & 500 IU D_3)

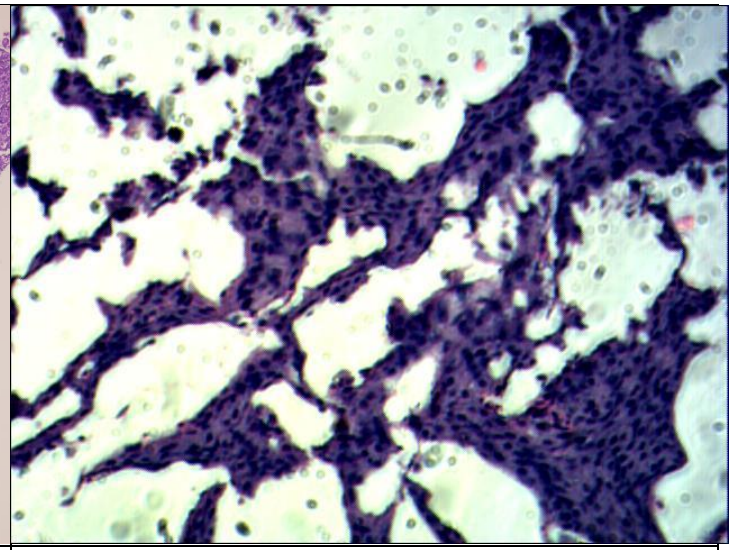

Fig.22 Section of corpuscles of Stannius in the fish, *Labeo rohita* in Calcium enriched Experimental group (D_HC_H) (H & E 60X) Day – 7 (Ca 40 ppm & 500 IU D_3)

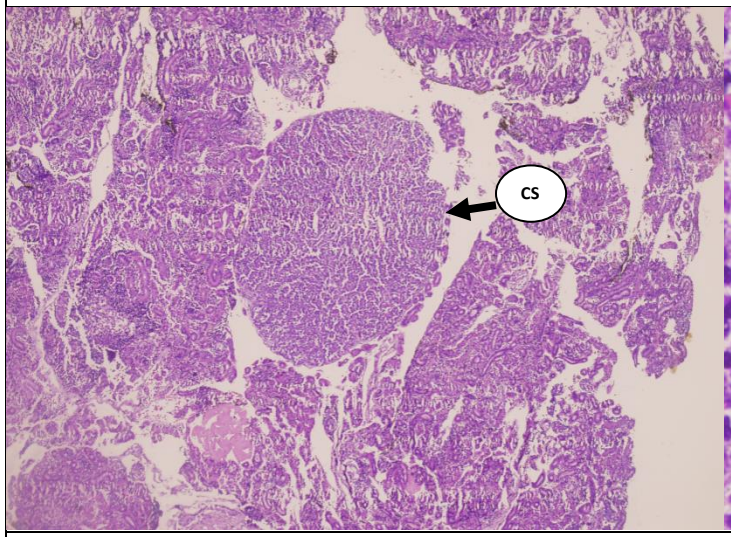

Fig. 23 Section of Corpuscles of Stannius in the fish, *Labeo rohita* in Calcium enriched Experimental group (D_HC_H) (H & E 4X) Day-30 (Ca 40 ppm & 500 IU D_3)

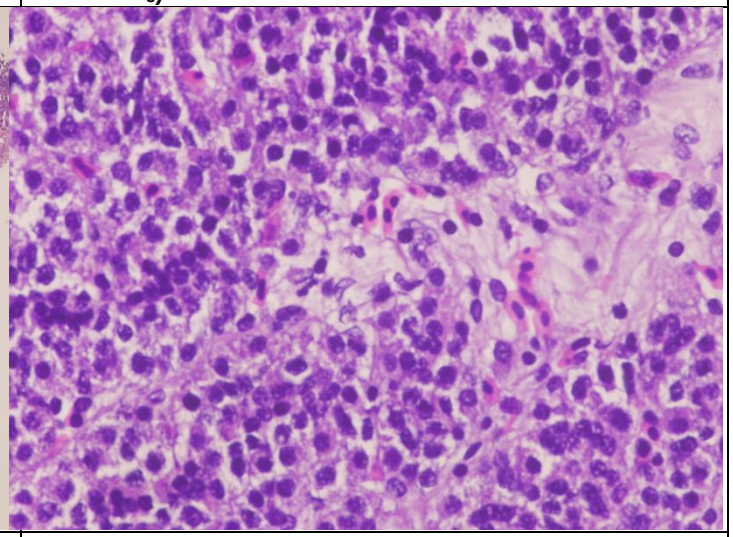

Fig.24 Section of corpuscles of Stannius in the fish, *Labeo rohita* in Calcium enrichedExperimental group (D_HC_H) (H & E 60X) Day – 30 (Ca 40 ppm & 500 IU D_3)

5. DISCUSSION

5.1. Physicochemical parameters of water

All the physiochemical parameters of water such as dissolve oxygen, free carbon dioxide, ammonia, total hardness, pH, nitrite-N, Nitrate-N were observed to be within the optimum range of requirements for fish. Brahmane *et al*. (2014) recorded the optimum temperature level for growth of rohu fry as 30^0c. pH of water in all the experimental groups were in the range of 7.5-8.5, which is within the acceptable range (6.5-9.0) as suggested by Swingle (1967). The dissolved oxygen level in water varies with a large number of factors such as water temperature, metabolic rate, biomass density etc. The dissolved oxygen level in different experimental tubs was recorded to be within the range of 5.6 -7.7 mgL-1, which is within the optimum range of 6-8 mg L-1for cyprinids as suggested by Huet (1975). From the above result, it is assumed that dissolved oxygen was optimum throughout the experimental period, which is due to aeration provided.

The dissolved oxygen requirement of fish varies with a large number of factors such as water temperature, metabolic rate, biomass density, aeration etc. In the present study conducted the aeration was provided round the clock and the DO varied between 5.2-6.8 which was well within the acceptable range. Carbon Dioxide was found to be negligible and hence no adverse effect was found on the survival and performance of the fish. This may be due to low biomass and frequent water exchange during the experimental period. The carbonate hardness was found to be 228-245 $mg.L^{-1}$during the experimental period of 60 days**.** The suggested value of ammonia nitrogen in water ranges from 0.0 to 0.1mg. L^{-1}(Jhingran, 1991) and this support the range (0.14-0.27 $mg.L^{-1}$) in the present study. Nitrite concentration was recorded in the range of 0.001-0.005 mg. L^{-1} which is well within the permissible range for pond aquaculture (Boyd and Tucker, 1998). Nitrate-N level in a productive pond can be within 0.1-5.0 mg. L^{-1} (Boyd and Tucker, 1998). In the present study, nitrate-N was below the toxic level and hence did not affect the fish adversely.

5.2. Serum calcium concentration

Serum calcium concentration level in *Labeo rohita* is calculated and it is found to be higher in 5th day of fish which is reared in normal water (20ppm) with high dose (500 IU/Kg bw) of vitamin D_3. At the same time, it is found to be higher in 7th day of fish which is reared in calcium enriched water (40ppm) with high dose (500 IU/Kg bw) of vitamin D_3. Srivastav *et al*. (1997) who also observed vitamin D metabolites affect the serum calcium level in freshwater catfish (*Heteropneustes fossilis*), in which there is increase in serum calcium level at the day of 3 and 5, which were injected with intraperitonial Vitamin D_3. Again Srivastav *et al*. (1997) done similar experiment in freshwater mud eel *Amphipnous cuchia* and reported that there is increase in serum calcium level at day 10,when it is reared in calcium rich environment and injected with 100 ng of vitamin D_3 for 100g bw-l day-l bw and also result by Bansal *et al*.(1979) showing increased serum calcium levels have been reported in *Labeo rohita*.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 level of serum calcium is found on day 5 on the three levels. But the amount or level of calcium intake varies with the dosage. The dosage with 1000IU/Kg bw shows higher absorption of calcium from environment which is followed by 500 and 100IU. The result by Srivastava also agreed with this study and shows the similar response in *Labeo rohita*. A number of authors report hypercalcaemic effects of vitamin D_3 metabolites, and show that hypercalcaemia 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 calcium but left total plasma calcium levels unchanged, suggesting an increased fractional binding of calcium to plasma proteins (Fenwick *et al.,* 1984).In contrast, Sundell *et al.,* (1993) repeatedly injected $1,25(OH)_2D_3$ in the Atlantic cod and observed an increase of free calcium while total calcium levels remained unchanged. In male Mozambique tilapia (*O.*

mossambicus) ip injections of 1,25$(OH)_2D_3$ increased total plasma calcium without altering the free calcium levels (Srivastav *et al*. 1998). Apparently, the effect of vitamin D_3 on the ratio of free and bound plasma calcium differs between studies.In addition, responses may also vary with ambient water quality. Vitamin D_3 injected male catfish (*Clarias batrachus*) acclimated to low calcium water increased their serum total calcium levels as compared with control fish. However, this increase doubled in vitamin D_3 injected catfish from water supplemented with extra calcium when compared to controls from the same water, although the duration of this increase was shorter than the one in fish from low calcium water (Swarup & Srivastav, 1982). A similar observation was performed in freshwater mud eel (*Amphipnous cuchia*) by Srivastav (1983). Magnitude and duration of the increase of plasma calcium in response to vitamin D_3 are clearly dependent on the calcium concentration in the water (Srivastav *et al*. 1997). If calcium is not sufficiently available from the water or from the diet, then fish can apparently supplement plasma calcium from internal sources. IP injections of unfed common carp with physiological doses of either vitamin D_3 or 1,25$(OH)_2D_3$ resulted in hypercalcaemia and hyperphosphataemia (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 calcium and phosphorus, while this effect was absent in unfed eels (Fenwick *et al*. 1984). Plasma calcium levels in fish are certainly not controlled by the vitamin D endocrine system only; other hormones such as stanniocalcin (Pierson *et al*. 2004), parathyroid hormone-related protein (Abbink & Flik 2007; Guerreiro *et al*. 2007), and prolactin (Flik *et al*. 1984, 1989; Seale *et al*. 2006) are involved as well.

5.3. Serum phosphorus concentration

Serum phosphorus concentration level in *Labeo rohita* is calculated and it is found to be higher in 7th day of fish which is reared in normal water (20ppm) with high dose (500IU/Kg bw) of vitamin D_3. At the same time, it is found to be higher in 7th day of fish which is reared in calcium enriched water (40ppm) with high dose (500IU/Kg bw) of vitamin D_3. Srivastav *et al.* (1997) who also observed vitamin D metabolites affect the serum phosphorus level in freshwater catfish (*Heteropneustes fossilis),* in which there is increase in serum phosphorus level from day 3 to 10, and showing a hyper-phophetemic reaction which were injected with intraperitonial Vitamin D_3. Again Srivastav *et al*. (1997) done similar experiment in freshwater mud eel *Amphipnous cuchia* and reported that there is increase in serum phosphorus level at day 5, when it is reared in calcium rich environment and injected with 100 ng of vitamin D_3 for 100g bw^{-l} day^{-l}.In contrast to calcium, 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 ip 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 & Raghuramulu 1999). In unfed American eel (Fenwick *et al*. 1984) and freshwater mud eel *A. cuchia* (Srivastav 1983) plasma phosphate increased after IP vitamin D_3 injection. Apparently, in addition to phosphate reabsorption in the kidney (Fenwick & 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). Thus increase in serum Ca or Pi is not proportional to rise in dose from 100 IU/Kg b.w to 500 IU/Kg b.w, showing its potential capacity to rise even with 5 times rise in dose of vit.D_3. Therefore there must be an optimum level which is in between these two doses.

5.4. Cloning and sequencing of cDNA encoding partial STC-1 gene in *Labeo rohita*

There was scared information available on the stanniocalcin genes in countable number of fishes. To date, STC -1 genes in countable number of fishes have been sequenced and that too not even partially sequenced in Indian major carps. Some of the fishes were taken for stanniocalcin gene sequence is *Danio rerio* stanniocalcin 1, like (STC1), mRNA with gene bank accession number NM_200539, Human stanniocalcin mRNA, complete cds with gene bank accession number U46768, stanniocalcin [Oncorhynchus kisutch=coho salmon, corpuscles of Stannius, mRNA] with gene bank accession number S59519.

5.4.1. Amplification of *Labeo rohita* STC-1 gene

In the present study the cDNA sequence encoding the STC-1 gene in *Labeo rohita* wereamplified from the kidney tissue. This tissue is selected for the PCR assay on the basis of earlier reports suggested by Shin and Sohn, (2009). A set of primers were designed using the Gene Runner Software in the conserved gene sequences of the other fish. One best primer was selected for amplification, the amplicon size obtained, 421bp for STC-1 gene, were similar to the expected amplicon size.

5.4.2. Cloning and Sequencing of STC-1 Gene of *Labeo rohita*

The PCR products obtained using the primers were cloned into pTZ57R/T vector. Positive colonies, which showed white colour on X-Gal IPTG Ampicillin Plates, were selected. The plasmid was isolated from the colonies and the plasmid was sent for sequencing. The nucleotide BLAST analysis of the STC-1 gene of *L. rohita* showed high homology with the STC-1 gene respectively of the other organisms.

5.4.3. Nucleotide sequence analysis and expression of partial STC-1 Gene of *Labeo rohita*

Analysis and comparison of the STC-1 sequence obtained in the present study with gene sequence from other organisms confirmed that the genes amplified were STC-1 of *Labeo rohita*. The BLAST search in NCBI GenBank Database with the *Labeo rohita*STC-1 showed high homology with genus Sinocyclocheilus like *Sinocyclocheilus rhinocerous* (100%), *Sinocyclocheilus anshuiensis* (100%), *Sinocyclocheilus graham* (100%). This is the first time to partially sequence STC-1 Gene in Indian Major Carps and that too in *Labeo rohita.*

5.5. Histology of Corpuscles of Stannius (CS)

In the present study on the fish, *Labeo rohita* injected with vitamin D_3 and kept either in calciumrich freshwater (40 ppm), or calcium-deficient (20 ppm) freshwater exhibits degranulation of the cells, increased volume of these 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 increases as a result of external calcium concentration (Urasa and Wendelaar Bonga, 1987).

In *Labeo rohita*hypercalcemia results into degranulation of AF-positive cells. Similar response of corpuscular cells has been reported in the past by

Aida and co-workers (1980) have suggested that the secretory activity of cells of CS may be directly affected by plasma ion levels, specially Ca'. The present study supports this suggestion. It is also in agreement with the observations of Bonga *et al.* (1980). According to them type-1 cells are more active in the fish adapted to diluted or fullstrength seawater than in freshwater specimens. For this response Bonga *et al.* (1980) have stated that the high activity of these cells in seawater is apparently due to the high

calcium 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 calcium 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) on and Srivastav and Srivastav (1988)*Clarias batrachus.*The degenerationis due to exhaustion of corpuscular cells. In the CS of *Labeo rohita*kept in low calcium freshwater there is an increased storage of granules. This can be attributed to the observed decrease in the serum calcium and inorganic phosphate levels. Hypoactive cells have been noticed in the fish exposed to low-calcium seawater (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 freshwater medium, there is no change in the cells of CS after vehicle injected fish. This may be due to non-involvement of this cell type in calcium homeostasis as according to Bonga *et al.*(1976, 1980).

In calcium-rich medium the cells of CS of fish and calcium/$P_i$levels after vehicle or vitamin D_3-treatment exhibit a decrease in the volume. This is in conformity with the reports of 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. These studies including the present study are contrary to the report of Aida *et al.* (1980) who have noticed degranulated cells in CS of *Oncorhynchus kisutch* maintained in organ culture in media containing high calcium.

It is of interest to note in the present study that cells exhibit a decreased nuclear volume in calcium-deficient freshwater, similar to those observed in calcium-rich freshwater. This reduced activity of cells in calcium-deficient freshwater may be attributed to the possible increase in the serum calcium and inorganic phosphate levels as it has been reported that removal of calcium from water bathing the gills of gold fish resulted in a two-fold increase in monovalent ioninflux (Cuthbert and Maetz, 1972).

6. SUMMARY

Physiology is defined as the study about life process, which plays a major role in knowing about the day to day life process to have a sustainable life. In this study, one of the commercially important fish *Labeo rohita,* which is cultured all over the world. It is a major species to be cultured in India which had been chosen to study its calcium regulation by culturing it in calcium enriched environment. In this, the calcium regulation and its homeostatic function, action of Corpuscles of Stannius (CS) and expression of Stanniocalcin gene are studied. In calcium homeostatic study, the major endocrine gland involved is Corpuscles of Stannius, Parathyroid gland. In this study, the action Corpuscles of Stannius by release of stanniocalcin hormone and change in internal structure of cellular arrangement are identified.

A total of six treatments with two types of calcium water namely normal (20ppm) and calcium enriched (40ppm) and two dose of vitamin D_3 at the level of 100 IU/Kg bw and 500 IU/Kg bw were injected intra-peritonially to *L. rohita* and reared in both the waters. Then the serum calcium and inorganic phosphorus levels were measured in different day intervals from Day-1 to Day-60 and studied about the Calcemic responses of rohu in both water due to vitamin D_3 administration. Fishes reared in normal and calcium enriched water showing an increased calcium level in serum from Day-2 and reached a peak on 5^{th} day at low dose and 7^{th} day at high dose for vitamin D_3. But the level of calcium uptake is higher in fish reared in calcium enriched water and the declining of calcium level is also seems to be delayed in calcium enriched tank fish. At the same time, the inorganic phosphorus level in serum of fish reared in normal and calcium enriched tank shown high peak on 5^{th} day for low vitamin dose and 7^{th} day for high dose, which is more or less similar to the Calcemic response in serum. In case of higher water calcium level the calcium lowering effect of CS was delayed as shown in the Fig 2 & 4.

The rise and fall in levels of serum calcium and inorganic phosphate in fish body is due to calcium homeostasis which is regulated by an endocrine gland and its hormone. The endocrine gland normally involved is Corpuscles of Stannius, which helps in releasing stanniocalcin hormone, which helps in bringing back the fish to homeostatic stage, when it is influenced to hypercalcemic condition. In this, the administration of vitamin D_3 regulates the fish to uptake calcium from environment into the body. Due to this, there is hypercalcemic condition is seen in fish body. So Corpuscle of Stannius releasing Stanniocalcin to reduce the level by gill influx, bone and intestinal reabsorption. So this endocrinal gland is dissected at different day interval and taken for histological studies to know about the internal cellular changes in CS. The result shows, there is hyperplasia (increase in cell number) in CS, so there is release of more stanniocalcin into body fluid, which act as calcium homeostatic hormone and regulated the hypercalcemic condition of fish and return back it to normal stage. This had been clearly seen by sectioning the organs and viewed in different magnification to about its taxonomical and anatonomical changes.

Not only histological studies but also molecular work like partial sequence of stanniocalcin gene had been done first time in *Labeo rohita* by following a one week procedure from RNA isolation, cDNA synthesis, PCR, Ligation, Transformation, Colony formation, Plasmid isolation and sequencing. Then real time primers are designed and they are checked for the real time folds to know about its expression at different day intervals.

By knowing all the above information, it is identified in *Labeo rohita* that the pair of Corpuscles of Stannius which is embedded in ventral part of posterior kidney play major role in calcium homeostasis by releasing Stanniocalcin hormone into body fluid. By taking up with this piece of work we deduced that we have achieved as contribution to science as: 1).Understanding the physiological doses of vitamin D_3 and its interaction in serum Calcium/Pi and glandular changes in CS of IMC, *Labeo rohita* reared in calcium enriched water. 2). Understanding the impact of intraperitonial Vitamin D_3 on synthesis of

Stanniocalcin/ Gene Expression in IMC, *Labeo rohita* reared in calcium enriched water. We also recorded that the physiological responses are in corroboration with the cellular changes in endocrine gland, Corpuscles of Stannius and 3). The higher water calcium level increases the response time of Stanniocalcin hormone to perform normocalcemia.

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ACRONYMS

^0C	Degree Celsius
ANOVA	Analysis of Variance
APHA	American Public Health Association
APTH	Alternative Parathyroid Hormone
B.W	Body weight
BLAST	Basic Logical Alignment Search Tool
bp	Base pair
Ca	Calcium
$CaCl_2$	Calcium chloride
$CaCO_3$	Calcium carbonate
Cap.	Capacity
CaSR	Calcium sensing receptor
CD's	Coding Sequence
cDNA	Complementary Deoxyribonucleic acid
CIFA	Central Institute of freshwater Aquaculture
CIFE	Central Institute of Fisheries education
CS	Corpuscles of Stannius
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediamine tetra acetic acid
F	Forward
FAO	Food and Agriculture Organization
Fig	Figure
FW	Freshwater
g	Gram

H & E	Hematoxylin and Eosin
ICAR	Indian council of Agricultural Research
IU	International Unit
kcal	Kilo calorie
Kg	Kilogram
L	Liter
LB	Luria-Bertani
Ltd	Limited
M	Marker
mg	Milligram
ml	Milliliter
mmol	Milli mole
mRNA	Messenger Ribonucleic acid
MUFA	Mono Unsaturated fatty acid
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
Pi	Inorganic Phosphate
ppm	Parts per million
PUFA	Poly Unsaturated fatty acid
pvt	Private
R	Reverse
RE	Restriction enzyme
RT-PCR	Reverse Transcriptase-Polymerase Chain reaction
SFA	Saturated fatty acid

SPSS	Statistical Package for the Social Sciences
STC	Stanniocalcin
SW	Seawater
TAE	Tris base acetic acid and EDTA
US	United States
V	Volts
Vit	Vitamin
µl	Micro liter