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# Haemato-biochemical responses and induction of HSP70 to dietary phosphorus in *Catla catla* (Hamilton) fingerlings

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**Abstract** A feeding trial of 120 days was conducted to study the effect of graded levels of dietary phosphorus on haematology, serum protein concentrations and HSP70 expression in fingerlings of the Indian major carp, Catla (*Catla catla*). Eight isonitrogenous and isoenergetic purified diets were formulated to contain graded levels of dietary phosphorus (dP), i.e., $T_1$, 0.1%; $T_2$, 0.3%; $T_3$, 0.5%; $T_4$, 0.7%; $T_5$, 0.9%; $T_6$, 1.1%; $T_7$, 1.3%; or $T_8$, 1.5%. Four hundred and eighty fish (average weight 4.23 ± 0.016 g) were equally distributed into 24 tanks forming eight treatments with three replicates each. The fish were fed daily at the rate of 3.5% body weight in two instalments. At the end of feeding trial fish were sampled to study total RBC and WBC count, haemoglobin, serum lysozyme activity, serum total protein, albumin (A), globulin (G) concentration and HSP70 expression. Total RBC count, haemoglobin concentration and serum lysozyme activity did not vary significantly in response to different dietary phosphorus concentrations. Total WBC count was found to be significantly ($P < 0.05$) higher in $T_1$ relative to all other treatments. Serum albumin and A/G ratio was found to be significantly lower in fish of $T_1$ and $T_2$ in relation to $T_7$ group ($P < 0.05$). Serum globulin and total protein levels remained unaffected by variations in dietary phosphorus. HSP70 expression was observed in $T_1$ group (0.1% dP) in gills and brain tissue, but not in liver and muscle tissues. No HSP70 expression was observed in fish of $T_4$ (0.7% dP) and $T_8$ (1.5% dP) treatments. These *prima facie* results suggest that dietary phosphorus had only minor influence on the haemato-biochemical parameters studied; however dietary phosphorus deficiency caused organ specific induction of HSP70 in catla fingerlings.

**Keywords** Phosphorus · Catla · Haematology · Serum protein · Lysozyme · HSP70

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## Introduction

Phosphorus is one of the most important minerals required by fish in their diet. Besides the important structural and functional roles played by phosphorus, it has been shown to significantly affect growth (Baeverfjord et al. 1998; Roy and Lall 2003), feed utilization (Ye et al. 2006; Yang et al. 2006) and stress tolerance (Vielma et al. 2002); traits crucial for the success of any aquaculture venture. Hence, optimum phosphorus level in the diet is a critical

factor which should be carefully considered during feed formulation. However, considering the stringent legislations regulating the levels of phosphorus in effluent discharges of aquafarms, feed manufacturers are putting their best efforts into producing feed with just the optimum level of phosphorus. This in turn has led to serious clinical problems of cultured fish (Sugiura et al. 2004). The situation hence calls for a clearer understanding of the implications of dietary phosphorus restriction in fish feed.

Most of the studies until now have been conducted in relation to the impacts of dietary phosphorus on growth, mineralization and metabolic changes, but very few studies have focused on the effects of phosphorus deficiency on haematological and immunological responses in fish. Dietary phosphorus levels have been found to influence the antibody production and resistance of channel catfish to *Edwardsiella ictaluri* challenge (Eya and Lovell 1998). Studies of phosphorus deficiency in European white fish *Coregonus lavaretus* revealed that dietary phosphorus did not have any marked influence on the immune functions of fish (Jokinen et al. 2003). HSP70 expression under conditions of stress has been found to be affected by suboptimal diet compositions (Martin et al. 2003; Hemre et al. 2004), but there are so far no reports of HSP70 expression in animals subjected to a nutrient deficiency.

The present study aimed at understanding the influence of dietary phosphorus level on serum haemato-biochemical parameters and HSP70 expression in fingerlings of the Indian major carp, Catla (*Catla catla* Hamilton) with respect to specific organs.

## Materials and methods

### Preparation of diet

Purified ingredients supplemented with potassium dihydrogen orthophosphate ($KH_2PO_4$; Qualigens Fine Chemicals, India) as the source of phosphorus were used to formulate eight diets (Table 1) containing graded levels of total phosphorus (0.1, 0.3, 0.5, 0.7, 0.9, 1.1, 1.3 or 1.5%). $KH_2PO_4$ replaced cellulose to achieve the desired levels of phosphorus in the diets. Diets were prepared by thoroughly blending all the ingredients except the vitamin and mineral mixtures. Oil was added to the dry mixture and dough was prepared with the required amount of water. The dough was cooked in a pressure cooker,

**Table 1** Composition of the experimental diets (% dry matter) fed to *Catla catla* fingerlings for 120 days

| Ingredients | Percentage of inclusion | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $T_1$ | $T_2$ | $T_3$ | $T_4$ | $T_5$ | $T_6$ | $T_7$ | $T_8$ |
| Egg albumin | 42.7 | 42.7 | 42.7 | 42.7 | 42.7 | 42.7 | 42.7 | 42.7 |
| White dextrin | 29.85 | 29.85 | 29.85 | 29.85 | 29.85 | 29.85 | 29.85 | 29.85 |
| Cellulose | 14.46 | 13.58 | 12.704 | 11.83 | 10.95 | 10.07 | 9.192 | 8.314 |
| Corn oil | 5.05 | 5.05 | 5.05 | 5.05 | 5.05 | 5.05 | 5.05 | 5.05 |
| Cod liver oil | 4.04 | 4.04 | 4.04 | 4.04 | 4.04 | 4.04 | 4.04 | 4.04 |
| Vitamin mixture[a] | 0.38 | 0.38 | 0.38 | 0.38 | 0.38 | 0.38 | 0.38 | 0.38 |
| Phosphorus-free mineral mix[b] | 1.4 | 1.4 | 1.4 | 1.4 | 1.4 | 1.4 | 1.4 | 1.4 |
| Carboxy methyl cellulose | 2.02 | 2.02 | 2.02 | 2.02 | 2.02 | 2.02 | 2.02 | 2.02 |
| Butyl hydroxyl toluene | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |
| Betaine hydrochloride | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 | 0.05 |
| Potassium di hydrogen phosphate | 0 | 0.878 | 1.756 | 2.634 | 3.512 | 4.39 | 5.268 | 6.146 |

[a] Vitamin mix: (IU or mg/kg diet): ascorbic acid (L-ascorbyl polyphosphate), 450 mg; DL-alpha tocopheryl acetate, 60 IU; Na menadione bisulphate, 5 mg; retinyl acetate, 15,000 IU; DL-cholecalciferol, 3,000 IU; thiamine, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; $B_{12}$ 0.05 mg; nicotinic acid, 175 mg; folic acid, 5 mg; inositol, 1,000 mg; biotin, 2.5 mg; calcium pantothenate, 50 mg; choline, 2,000 mg

[b] Mineral mix: calcium carbonate (40% calcium), 10 g; magnesium oxide (60% Mg), 1.24 g; ferric citrate, 0.2 g; potassium iodide (75% I), 0.4 mg; zinc sulphate (36% Zn), 0.4 g; copper sulphate (25% Cu), 0.3%; manganous sulphate, 0.3 g; dibasic calcium phosphate (20% Ca, 18% P), 5 g; cobalt sulphate, 2 mg; sodium selenite (30% Se), 3 mg; potassium chloride, 0.9 g; sodium chloride, 0.4 g

cooled and then $KH_2PO_4$, vitamin and mineral mixtures were added. Choline chloride was dissolved separately in water and mixed with the dough. Pellets were prepared using a hand pelletizer with 2 mm diameter size, air dried for 30 min followed by oven drying at 60°C for 12 h. The pellets were stored in airtight containers. According to the analysis conducted, all the diets contained 35% crude protein and 8.5% lipid contents.

## Experimental design

Fingerlings of *C. catla* (average weight 4.23 ± 0.016 g) were procured from a private fish farm at Palghar, Maharashtra, and acclimated under experimental conditions for 15 days. Four hundred and eighty fish were equally distributed into 24 tanks (80 × 57 × 42 cm) forming eight treatments with three replicates each. Feeding was carried out at 3.5% body weight, twice a day at 1000 and 1800 hours under normal light regime (light/dark 12/12 h) for 120 days. Uneaten feed and faeces were siphoned out daily along with a 50% water exchange. During the experimental period, the water temperature, pH, and dissolved oxygen were in the normal range of 23.3–28.4°C, 7.0–8.5, and 5.6–7.6 mg $l^{-1}$, respectively.

## Sampling

At the end of the feeding trial, six fish per treatment were sampled for the haematological and serum biochemical parameters and lysozyme activity. Each fish was anaesthetized with clove oil (50 µl $l^{-1}$) before withdrawing blood from caudal vein, using a medical syringe previously rinsed with an anticoagulant, for analysis of haematological parameters. For serum biochemical parameters, blood was withdrawn in the same way but without using anticoagulant. Then the blood was allowed to coagulate till serum became separated from blood cells.

## Haematological parameters

Total erythrocyte and leukocyte were counted in a haemocytometer using erythrocyte and leukocyte diluting fluids (Qualigens), respectively. Twenty microlitres of blood were mixed with 3,980 µl of diluting fluid in a glass test tube and the mixture was shaken well to suspend the cells uniformly in the solution. Then the cells were counted using a haemocytometer. The number of erythrocytes and leucocytes per ml of the blood sample was calculated as:

$$\text{Number of cells ml}^{-1} = \frac{\text{Number of cells counted} \times \text{Dilution}}{\text{Area counted} \times \text{depth of fluid}}$$

The haemoglobin content of blood was analysed by estimating cyanmethemoglobin using Drabkins Fluid (Qualigens). Five millilitres of Drabkins working solution was added to 20 µl of blood in a clean and dry test tube. The absorbance was measured using a spectrophotometer (MERCK, Nicolet, evolution 100) at a wavelength of 540 nm. The final concentration was calculated by comparing with the standard cyanmethemoglobin (Qualigens, India).

## Serum total protein, albumin and globulin

Serum protein was estimated by Biuret and BCG dye binding method (Reinhold 1953) using a kit (Total protein and albumin kit; Qualigens Diagnostics, Glaxo Smithkline). Albumin was estimated by Bromocresol green binding method (Doumas et al. 1971). The absorbance was measured against a blank in a spectrophotometer at 630 nm. Globulin level was calculated by subtracting the albumin values from the total serum protein.

## Serum lysozyme activity

Serum lysozyme activity was measured using ion exchange chromatography kit (Bangalore Genei, India). Serum samples were diluted with phosphate buffer (pH 7.4) to final concentration of 0.33 mg $ml^{-1}$ protein. In a suitable cuvette, 3 ml of *Micrococcus luteus* suspension in phosphate buffer ($A_{450}$ = 0.5–0.7) was taken, to which 50 µl of diluted serum sample was added. The content of the cuvette was mixed well for 15 s and a reading was taken in a spectrophotometer at 450 nm exactly 60 s after the addition of serum sample. This absorbance was compared with standard lysozyme of known activity following the

same procedure as above. The activity was expressed as U $min^{-1}$ $mg^{-1}$ protein of serum.

### Expression of HSP70

Heat shock protein 70 (HSP70) expression was studied at three levels of dietary phosphorus, i.e., at lowest dietary phosphorus ($T_1$—0.1% dP), intermediate dietary phosphorus ($T_4$—0.7% dP), and highest dietary phosphorus ($T_8$—1.5% dP). HSP70 expression was analysed by SDS-PAGE and Western blotting method (Towbin et al. 1979). Liver, gill, brain and muscle tissues were homogenised in tris buffer (pH 7.5) under chilled conditions with protease inhibitor (0.1 mM phenyl methane sulfonyl fluoride, PMSF). Homogenate was centrifuged (3,000*g* at 4°C for 10 min) and the supernatant was collected and frozen (−20°C) for HSP70 analysis. Thawed sample supernatant was analysed for total protein content (Lowry et al. 1951). Sample buffer was immediately added to each sample and heated to 95°C for 2 min. Subsamples of protein (50 μg) were separated by SDS-PAGE with 12% separating and 5% stacking polyacrylamide gels (Blatter et al. 1972) using an electrode buffer (Laemmli 1970). Heat shocked, Hela cell lysate (Cat. No. LYC 101F; Stressgen, Canada) (20 μg) was loaded to one lane to serve as an internal standard for blotting efficiency. Proteins were separated at 1.5 mA per well for approximately 3 h and then electroblotted on to a total PVDF (polyvinylidene fluoride) transfer membrane (E578-10 × 10 cm SQ, USA) at 200 mA for 3 h. After blotting, gels were stained with Coomassie blue to ensure complete transfer. Membranes were blocked with 3% bovine serum albumin (BSA) and Tris Buffer Saline (TBS pH 7.4). Tween 20 (0.05%) in TBS was used as washing solution. Primary monoclonal antibodies HSP70 developed against carp HSP70 (1:2,000 dilution, Bioreagents-SPA-810; Stressgen) were used as probes. Horseradish peroxidase-conjugated goat antimouse IgG (1:2,000 dilution, Bioreagents-SAB-100; Stressgen) was used to detect HSP70 probes. Bound antibodies were visualized by Gel Documentation system (Syngene, UK).

### Statistical analysis

The effect of dietary phosphorus levels on the parameters was determined by one-way ANOVA. All statistical analysis was done using SPSS (version 14). Values are expressed as mean ± SEM.

## Results

### Total leucocyte, total erythrocyte and haemoglobin content

Total leukocyte, total erythrocyte and haemoglobin content of *C. catla* juveniles at the end of the feeding trial are shown in Table 2. No significant variation was observed among the treatments with respect to total erythrocyte and haemoglobin content. Significantly higher ($P < 0.05$) total leukocyte count was observed in fish fed the diet $T_1$ in comparison to all other treatments. However, there was no variation in total leukocyte count among the phosphorus supplemented groups ($T_2$ to $T_8$).

**Table 2** Total erythrocyte count ($\times 10^6$ cells $mm^{-3}$), total leukocyte count ($\times 10^6$ cells $mm^{-3}$), haemoglobin content (g $dl^{-1}$) of *C. catla* fingerlings fed the experimental diets

Each column bearing different superscript letters indicates statistical significance ($P < 0.05$)

Values are mean ± SEM ($n = 6$)

| Treatment | Total erythrocyte count | Total leukocyte count | Haemoglobin content |
|---|---|---|---|
| $T_1$ | 1.01 ± 0.05 | $0.67^b$ ± 0.07 | 6.75 ± 1.15 |
| $T_2$ | 1.07 ± 0.07 | $0.45^a$ ± 0.23 | 6.35 ± 0.25 |
| $T_3$ | 1.07 ± 0.13 | $0.42^a$ ± 0.46 | 6.07 ± 0.75 |
| $T_4$ | 1.23 ± 0.10 | $0.40^a$ ± 0.13 | 6.10 ± 0.47 |
| $T_5$ | 1.05 ± 0.09 | $0.40^a$ ± 0.22 | 5.53 ± 0.19 |
| $T_6$ | 1.13 ± 0.11 | $0.41^a$ ± 0.11 | 5.70 ± 0.30 |
| $T_7$ | 0.97 ± 0.03 | $0.40^a$ ± 0.22 | 4.95 ± 0.25 |
| $T_8$ | 0.99 ± 0.20 | $0.42^a$ ± 0.18 | 5.40 ± 0.81 |

**Table 3** Serum total protein (g $dl^{-1}$), albumin (g $dl^{-1}$), globulin (g $dl^{-1}$), albumin to globulin ratio and lysozyme activity (unit $min^{-1}$ $mg^{-1}$ serum protein) of *C. catla* fingerlings fed the experimental diets

| Treatment | Total protein | Albumin | Globulin | Albumin/globulin ratio | Lysozyme activity |
|---|---|---|---|---|---|
| $T_1$ | 3.16 ± 0.05 | $0.96^{a}$ ± 0.02 | 2.18 ± 0.01 | $0.43^{a}$ ± 0.01 | 236.22 ± 5.36 |
| $T_2$ | 3.16 ± 0.06 | $0.96^{a}$ ± 0.02 | 2.21 ± 0.08 | $0.44^{a}$ ± 0.02 | 239.35 ± 25.32 |
| $T_3$ | 3.17 ± 0.03 | $1.03^{ab}$ ± 0.05 | 2.10 ± 0.06 | $0.49^{ab}$ ± 0.04 | 266.67 ± 11.56 |
| $T_4$ | 3.14 ± 0.03 | $1.00^{ab}$ ± 0.03 | 2.14 ± 0.01 | $0.46^{ab}$ ± 0.02 | 257.93 ± 12.05 |
| $T_5$ | 3.17 ± 0.05 | $1.07^{ab}$ ± 0.06 | 2.26 ± 0.19 | $0.47^{ab}$ ± 0.03 | 243.94 ± 6.69 |
| $T_6$ | 3.12 ± 0.04 | $1.03^{ab}$ ± 0.06 | 2.13 ± 0.06 | $0.47^{ab}$ ± 0.06 | 236.07 ± 15.14 |
| $T_7$ | 3.17 ± 0.03 | $1.09^{b}$ ± 0.02 | 2.09 ± 0.03 | $0.53^{b}$ ± 0.01 | 235.00 ± 5.40 |
| $T_8$ | 3.03 ± 0.08 | $1.05^{ab}$ ± 0.03 | 2.05 ± 0.07 | $0.46^{a}$ ± 0.02 | 236.07 ± 11.97 |

Each column bearing different superscript letters indicates statistical significance ($P < 0.05$)

Values are mean ± SEM ($n = 6$)

### Total serum protein, albumin, globulin, albumin/globulin ratio and lysozyme activity

Total serum protein, albumin (A), globulin (G), A/G ratio and lysozyme activity of *C. catla* juveniles at the end of the feeding trial are shown in Table 3. Total protein and serum globulin values did not vary significantly ($P > 0.05$) among the treatments. Serum albumin levels in $T_1$ and $T_2$ were found to be significantly ($P < 0.05$) lower in comparison to $T_7$. Serum A/G ratio was also found to be significantly ($P < 0.05$) lower in $T_1$ and $T_2$ in relation to $T_7$. No significant difference in lysozyme activity was observed among the treatments.

### HSP70 expression

The results of the study on HSP70 expression in fish fed the diet $T_1$ has been presented in Fig. 1. HSP70 expression bands were observed in gills and brain tissues of the group, but no band was found in liver and muscle. There was no HSP expression in liver, muscle, gill and brain tissues of $T_4$ and $T_8$ treatments.

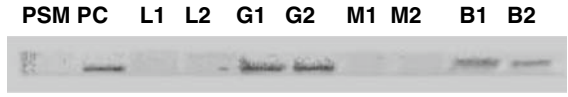


**Fig. 1** Western blot for heat shock protein 70 in different tissues of *Catla catla* fingerlings fed the experimental diet $T_1$. Lane 1, prestained marker (*PSM*); Lane 2, positive control (*PC*); Lane 3 and 4, liver (*L1* and *L2*); Lane 5 and 6, gill (*G1* and *G2*); Lane 7 and 8, muscle (*M1* and *M2*); Lane 9 and 10, brain (*B1* and *B2*).

## Discussion

In the present study, no significant variation was observed in total erythrocyte count, haemoglobin concentration or lysozyme activity with respect to dietary phosphorus supplementation in *C. catla* fingerlings. A significant effect was observed on total WBC count and serum albumin concentration with respect to dietary variations in phosphorus. A similar increase in WBC count has been observed during deficiency of other minerals such as zinc (El Hendy et al. 2001) and magnesium in higher animals (Sanchez-Morito et al. 2000; Malpuech-Brugere et al. 2000). Zinc deficiency seems to activate the immune mechanism in the animal resulting in elevated WBC levels (El Hendy et al. 2001). It has also been well established that magnesium deficiency leads to inflammatory responses in the animal which explains the elevated WBC levels under magnesium deficient conditions (Mazur et al. 2006). Previous reports of phosphorus deficiency in higher animals and humans indicate disturbances in leucocyte function, such as reduced chemotaxis, intracellular bactericidal activity and decreased phagocytosis (Craddock et al. 1974; Kreiberg 1977; Kiersztejn et al. 1992), clearly indicating changes in WBCs under conditions of dietary phosphorus deprivation.

Significantly lower levels of serum albumin levels have been observed in fish fed low levels of dietary phosphorus ($T_1$ and $T_2$). Similar results of reduced serum albumin have been reported during both zinc

(Bates and McClain 1981) and magnesium (Malpuech-Brugere et al. 2000; Nassir et al. 2002) deficiency in higher animals. Hypoalbuminaemia has been chiefly attributed to a variety of reasons, such as inflammation, trauma, extraneous loss of albumin, intravascular volume excess, impaired liver or kidney function (Fuhrman et al. 2004). Hypoalbuminaemia during magnesium deficiency has been attributed to the inflammatory responses triggered under magnesium deficient conditions. Intracellular calcium ions play an important role in triggering inflammatory response during magnesium deficiency (Mazur et al. 2006).

We observed no significant change in the levels of total serum protein, globulin levels or lysozyme activity when fish were fed graded levels of phosphorus. A similar result was found in the study of Jokinen et al. (2003) who found no variation in lysozyme activity in white fish fed low phosphorus diets. The authors concluded that low phosphorus diet did not compromise the immune response of the animal and that its deficiency only had minor effects on immune parameters. However, Eya and Lovell (1998) demonstrated a higher antibody response and survival of channel catfish fed higher levels of phosphorus, against *Edwardsiella ictaluri* challenge.

Heat shock proteins (HSP70) are a family of highly conserved cellular proteins present in all organisms including fish (Iwama et al. 1998; Basu et al. 2002). HSP70 helps in the folding of nascent polypeptide chains, acts as a molecular chaperone and mediates repair of denatured proteins (Kiang and Tsokos 1998). In the present study, among the four tissues studied, liver, muscle, brain and gills, HSP70 induction was observed in gill and brain tissues in fish fed the lowest phosphorus supplemented diet, though not in liver and muscle tissues. Tissue-specific expression of HSP70 has been reported in gill and heart tissues of *Fundulus heteroclitus* (a teleost), but not in liver, muscle or brain (Koban et al. 1991). Sanders et al. (1994) have suggested that the differences in the accumulation of stress proteins are useful in identifying tissues, which are particularly vulnerable to damage by a specific stressor. No expression was observed in fish fed intermediate and highest phosphorus diets in any tissues. Reduced expression of HSP70 has been reported in chickens fed deficient levels of dietary phosphorus when subject to heat stress in comparison to those fed adequate levels of phosphorus (Edens et al. 1992; Mahmoud et al. 2004). There has not been any previous report regarding HSP70 induction under conditions of phosphorus deficiency in fish. However, magnesium deficiency was reported to cause an upregulation of genes associated with stress response (HSP70 and HSP84) in thymocytes of rats (Petrault et al. 2002).

ATP plays a significant role in the functioning of a cell. HSP70 expression has been shown to correlate with ATP depletion in several mammalian cells and this energetic stress is thought to trigger HSP synthesis (Beckmann et al. 1992; Iwaki et al. 1993; Nguyen and Bensaude 1994; Wang et al. 1996). A reduction in ATP is thought to cause the heat shock factor to bind with the heat shock element in the promoter region of the heat shock gene, thereby inducing a heat shock response (Beckmann et al. 1992; Morimoto 1993; Mestril and Dillman 1995). ATP concentration has been found to be affected by phosphorus deprivation in fish (Sugiura et al. 2000). Hence, it may be possible that long-term phosphorus deficiency may have affected the cellular ATP concentrations triggering a HSP70 response, or perhaps reduced magnesium levels associated with phosphorus deficiency (Skonberg et al. 1997; Baeverfjord et al. 1998) may have triggered the response.

No parallel reports are available on haematological changes and HSP70 expression due to phosphorus deficiency in fish to substantiate the findings and explain the precise cause of these changes associated with phosphorus deficiency. Low concentrations of minerals such as magnesium, calcium and zinc have been reported in the bones and whole body of fish fed low levels of dietary phosphorus (Skonberg et al. 1997; Baeverfjord et al. 1998; Roy and Lall 2003; Helland et al. 2005; Yang et al. 2006). It is possible that, like magnesium, phosphorus deficiency results in a proinflammatory state. It is also possible that interaction of phosphorus with magnesium or other essential minerals has led to such a response. This is the first report on HSP70 expression under the condition of any nutritional deficiency, particularly in fish, but further studies are needed to substantiate this hypothesis. However, fish fed diets containing low phosphorus levels had only minor influences on haemato-immunological status, but led to organ-specific expression of HSP70 to maintain cellular homeostasis. Therefore, it is concluded that the induction of HSP70 can be used as an indicator of stress caused by dietary phosphorus deficiency in *C. catla* fingerlings.

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