The Bioscan 11(4): 2077-2082, 2016
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.com

QUANTITATIVE EXPRESSION OF MYOD REGULATORY FACTOR IN *PANGASIANODON HYPOPHTHALMUS* (SAUVAGE, 1878) FINGERLINGS FOR OPTIMIZATION OF DIETARY PROTEIN

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KEYWORDS
MyoD
Myoregulatory factor
Panagasianodon Hypophthalmus
Dietary protein

Received on :
03.06.2016

Accepted on :
24.10.2016

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ABSTRACT

Nutritional regulation of growth is mediated by physiological pathways, which in turn is regulated by gene and gene regulatory factors. In present study expression pattern of MyoD gene, a myogenic regulatory factor, was quantified by using qPCR by fold change ($2^{-\Delta CT}$) comparison and muscle growth with graded levels of dietary protein (20%, 25%, 30%, 35%, 40% and 45%) was studied in a feeding trial of 45 days in *Pangasianodon hypophthalmus* fingerlings. In the interesting trend gene expression studies indicated that on 15th day sampling expression increase with level protein from in 20 % protein (0.02 ± 0.01) to 45 % (0.08 ± 0.01) in gradual manner. While on 30th day sampling less than 30% protein inclusion level in the diet was insufficient while 35% and above protein were optimum for maintaining the trend. But later in 45th day sampling it showed decreased expression from earlier. The expression level at 35% protein support continuously elevated gene expression level and also supported the highest values of growth parameters such as SGR(1.87 ± 0.10) and percentage weight gain(132 ± 11.39). The quantitation of MyoD gene by real time shows full synergy with growth so it can be concluded that optimum requirement of protein for fingerling of *Pangasianodon hypophthalmus* to be 35 % maximum.

INTRODUCTION

Pangasius aquaculture first began in the 1940's in Vietnam and continues today globally and in India. Being much popular with the Vietnamese, it is exported to over 100 nations with principle markets in Europe, the United States and Russia. Demand for the fish is high and expected to go up. Pangasius exhibits a range of potential advantages in terms of reproductive capacity, resistance to low dissolved oxygen and production yields. Further development of production standards such as flesh quality and muscular growth with lesser fatty deposition will help define how the Pangasius aquaculture industry can improve further and secure a sustainable future.

Aquaculture is production driven and production is in function to muscular growth, so the growth of white muscle fibres become index of growth and is responsible for increase in the body weight. While deposition of excess fatty tissue impart poor quality index for the flesh in cultured Pangasius. Therefore, the targeted growth with white muscle fibres content increase comes as a way forward. The adult myoblast or myo-satellite cells are the myogenic precursor cells involved in the plastic mechanism of fish muscle growth (Johnston., 1999; Rowlerson and Veggetti, 2001). So the myogenic regulatory factors (MRFs) becomes key in muscle growth regulation, the superfamily of it include MyoD, Myf5, Myogenic and MRF4, these regulate muscle growth mechanisms (Watabe. 1999). MRFs are transcription factors and share a highly conserved central region (termed the basic helix–loop–helix (bHLH) domain) (Edmondson and Olson., 1993), which mediates via a sequence-specific DNA binding region called E-box, found in the promoter's regions of many skeletal muscle-specific genes (Blackwell and Weintraub, 1990; Lassar *et al*., 1989; Murre *et al*., 1989). MyoD and Myf5 are primary and Myogenin and MRF4 are secondary MRFs. The primary MRFs direct proliferating myogenic progenitor cells towards a myogenic lineage, whereas the secondary MRFs control the differentiation and fusion of myoblasts to form myofibers (Megeney and Rudnicki, 1995; Rudnicki and Jaenisch, 1995; Watabe, 1999).

High expression of MyoD and Myf5 indicate myoblast proliferation and hyperplasia during the initial growth phases, whereas Myogenin and MRF4 expression is more intense during the adult growth phase and it can be inferred to myoblast differentiation and hypertrophy (Johnston *et al*., 1995). Final body weight depends on both hypertrophy and hyperplasia in muscle growth. In large, fast growing ûsh, hyperplasia is particularly active during the larval and juvenile stages (Weatherley and Gill, 1984). During hypertrophic growth, as ûbers expand they absorb myoblast nuclei in order to maintain a relatively constant nuclear to cytoplasmatic ratio (Koumans *et al*., 1994).

Muscle morphology and MyoD, genes expression in muscle of *Pangasianodon hypophthalmus* were analysed to understand the cellular and molecular mechanisms involved in regulating fish muscle growth. The growing cultural practices of *P. hypophthalmus* in south East Asian region faces major challenges like reduced growth and poor flesh quality.

The present study is first reported for practical approach for insight of the protein in diet on MyoD gene expression and white muscle fibre regulation in growth and flesh quality through newly emerging field of nutrigenomics which has potential to explore the interaction of nutrient with physio-metabolic gene. The study was undertaken to understand the effect of dietary level of proteins on expression of MyoD gene, one of the myogenic transcriptional factor for muscle growth and differentiation. The aim of our study was to determine the optimum level of protein by molecular methods and to understand the molecular control of growth in P. hypophthalmus at the different protein level in diet.

MATERIALS AND METHODS

Experimental animals and experimental design

The experimental animals used were fingerlings of striped catfish (*Pangasianodon hypophthalmus*, Sauvage, 1878) were acclimatized under aerated conditions for a period of 15 days. During acclimation, fish were fed with a basal diet containing 30% crude protein. The animals with an average size of 3.35 ± 0.3g were randomly distributed across 6 treatments with 3 replicates for each, following a completely randomized design (CRD).

Feeding trials and sampling

45 days of feeding trial was conducted in 18 plastic rectangular tubs (57 X 36 X 47 cm, 75 L capacity) covered with perforated lids in the wet laboratory of Central Institute of Fisheries Education, Mumbai. Six heteronitrogenous, isolipidic and isoenergetic diets were prepared using semi-purified ingredient with graded levels of protein and fed to the animals during feeding trial. The experimental diets were T1 (20 % CP), T2 (25 % CP), T3 (30 % CP), T4 (35 % CP), T5 (40 % CP) and T6 (45 % CP) respectively. Sampling was carried out in every 15 days during feeding trial and the tissues for gene expression studies were kept in RNA Later ® (Qiagen, USA) for further use.

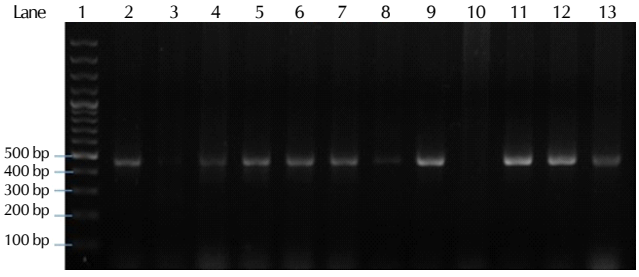

Figure 1: Colony PCR products in 2% agarose gel. Lane 1 -100bp DNA ladder with bold band at 500bp, lane 2 to 13 bacterial colony, 12 white colonies were picked from the transformation plates and colony PCR was performed using the MyoD-F and MyoD-R primers. All the 12 colonies screened were positive, however, colonies at band 3 and 10 are very diminished whereas, rest others are showing a band of around 450bp in the 2% agarose

RNA isolation and cDNA synthesis

Total RNA was extracted from white skeletal muscle tissue taken from the caudal axial region of randomly selected fish using *TRIzol* reagent (Invitrogen life technologies, USA) base on guanidine thiocyanate method (Chomczynski and Sacchi, 1987), and stored at -20ºC after dissolving in nuclease free water. The quality of RNA was conformed using Nano drop spectrophotometer by obtaining by 260/280 nm OD ratio from 1.8 to 2.0.The integrity of 18S and 28S RNA bands was checked by running on a 2% agarose gel. The extracted total RNA was treated with DNase I (Invitrogen life technologies, USA) to remove the genomic DNA contamination. The mRNA was converted into cDNA by using the first strand cDNA synthesis Kit (Fermentas, USA) as per manufacturer's instruction.

Design of PCR primers and polymerase chain reaction (PCR)

Primer were designed by gene runner version 3.05 software, against the conserved sequences

Identified by multiple sequence alignment of MyoD sequences from *Ictalurus punctatus (Acc. No.*-AY562555).For Real time PCR primer designed from the sequence of MyoD gene of *Pangasianodon hypophthalmus* (Acc. No.-KM051988).Primers were synthesized by Bioserve biotechnology (India) Pvt. Ltd. and confirmed by running a PCR against the synthesized primer to confirm the specificity. The primer sequences are summarized has been given in Table 1.

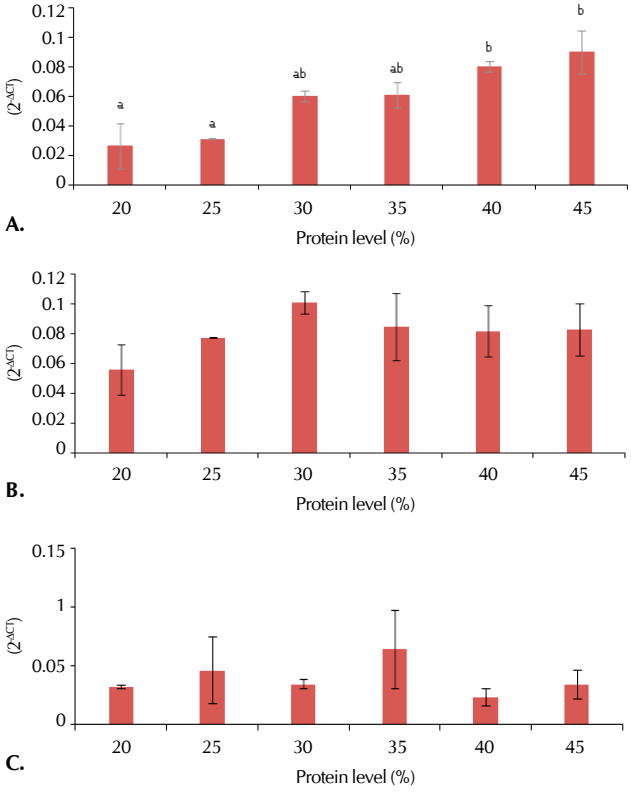

Figure 2: Change ($2^{-\Delta CT}$) in MyoD gene expression of different treatment groups fed with different protein level at different time interval A - 15thday, B - 30th day and C - 45th day

The PCR amplification reaction mixture were prepared by adding 15μl of PCR master mix (Thermo Scientific), 1μl of each forward and reverse primer, 1μl of the cDNA and rest nuclease free water to make the volume to 25μl. The PCR amplification of MyoD gene were carried for 5 minutes at 94ºC for initial denaturation, followed by 35 cycles consisting of 30 sec at 94ºC for denaturation, 1 min at 58-52 ºC for annealing (touch down PCR) and extension at 72ºC for 1 min.A final step of 7 min at 72 ºC was carried out for further elongation. Amplification product was separated in 1% agarose gel at 70V for 50 min in 1X TAE (Tris acetate EDTA) buffer.

Cloning of the amplified MyoD partial cDNA

100 μl of PCR products were separated in 1% agarose gel at 70V for 50 min in 1X TAE buffer. The bands obtained in the agarose gel were cut and separated DNA was eluted using QIAquick gel extraction kit (Qiagen, USA) by following the manufacturer's instructions. Cloning was done using the InsTAclone cloning kit (Fermentas, USA) following the manufacturer's instructions. Briefly, all amplified MyoD cDNA fragments were inserted into the vector provided (PTZ57R) by ligation and transformed into DH5α *E.coli* strain and spread on LB agar plates contains 1µl/ml of 100mg/ml Ampicillin. The positive (white colonies) recombinant clones were selected from blue-white colonies. The recombinant clones obtained were spread on pre-warm LB agar plates (master plate) containing Ampicillin and incubated at 37ºC for overnight. A colony PCR was performed with the positive colonies for confirming the clones by lengthening initial denaturation and amplified products were separated in 1% agarose using electrophoretic apparatus fig. 1.

Recombinant DNA plasmid isolation and sequencing

The confirmed colonies were picked and suspended in LB broth containing ampicillin and allowed to grow overnight. The plasmid DNA was isolated from the colonies using Miniprep kit (Qiagen, USA) following manufacturer's instructions. The integrity of the isolated plasmid DNA was checked in 1% agarose gel. A RE digestion was performed to confirm the insert in the plasmid using restriction nucleases viz., EcoRI and HindIII. Isolated plasmids were sequenced using M13 (forward and reverse) universal primers by Bioserve Biotechnology (India) Pvt. The nucleotide sequence was analysed using BLAST (Basic Logical Alignment Search Tool) software in the NCBI (National Centre for Biotechnology Information) GenBank nucleotide database for finding similarity with other sequences.

RT-qPCR analysis of MyoD gene

The RT-qPCR analysis of *Pangasianodon hypophthalmus* MyoD gene was done for treatment groups fed with graded levels of dietary protein based diet at 15, 30 and 45 days interval.qRT-PCR was performed with Roche LC480 Light Cycler using Maxima SYBER green /ROX qPCR master mix (2x) (Fermentas, USA). α-actin was used as a reference gene. Specificity of there action was verified using melting curve. The primers for real-time expression study used were listed in table 1.0 beta-actin were used to compare the expression of MyoD gene.

Growth parameters

Percentage weight gain

The percentage weight gain was calculated using the following formula

$$\text{Weight gain (\%)} = \frac{\text{Final weight - Initial Weight}}{\text{Initial weight}} \times 100$$

Specific growth rate (SGR)

The Specific Growth rate was calculated by the following formula

$$\text{SRG} = \frac{\text{Log}_e \text{ Final weight} - \text{Log}_e \text{ Initial weight}}{\text{Number of days}} \times 100$$

Statistical analysis

Statistical significance was analysed using one-way analysis of variance (ANOVA) using SPSS 16.0 for Windows. Duncan's multiple range test was used for posthoc comparison of mean ($P < 0.05$) between different protein. All data presented in the text, figures and tables are means $\pm$ standard error and statistical significance for all statistical tests were set at ($P < 0.05$).

RESULTS

Nucleotide sequence of MyoD gene

The concentration of isolated total RNA was 2500 to 3500ng/ μL and A260/A280 ratio was 1.93 which showed the separation of 28S and 18S rRNA bands. cDNA was synthesised and amplified the MyoD gene by specific primer of an amplicon size of around 450bp and cloned are conformed by colony PCR.

Nucleotide sequence MyoD gene

The blast search in NCBI GenBank database with *Pangasianodon hypophthalmus* MyoD partial mRNA sequence resulted in 19 hits. Among the hits, those showing query coverage of 99% are *Ictalurus punctatus* (acc. No.:AY534328.1), *Ameiurus catus* (acc. No.:AY562556.1) and *Pelteobagrus fulvidraco* (acc. No.:HM363525.1). The partial sequence for MyoD mRNA of *Pangasianodon hypophthalmus* (acc. No.: KM051988) with 454bp was then used for real time primer designing.

Expression of MyoD gene in *Pangasianodon hypophthalmus* at different protein level

The effect of different protein levels on the expression of MyoD gene in *P. hypophthalmus* is shown as fold change ($2^{-\Delta CT}$) and expression level was recorded at 15th day, 30th day and 45th day (Fig. 2). MyoD gene expression is positively correlated with dietary protein level at initial stages of experiment, on 15th days of sampling, the expression level of MyoD was found to be positively correlated with protein level. A significant higher($P < 0.05$) expression was found in 40% and 45% protein fed group and the lowest expression in 20% protein fed group (fig.2A) There was no significant difference observed in MyoD gene expression between treatment groups on the 30th and 45th day. While, on 30thday, the expression level was highest in 30% protein fed group and lowest in 20% protein fed group (fig.2A) at 15 days sampling. Contrary to the first trend of 15th day on 45th (3rd sampling) day, highest and lowest expression levels were found in 35% and 40% protein fed groups, respectively (fig.2C)

Table 1:

Gene name		Primer sequence
MyoD-F	Forward	TGCTTTAACACCAGCGACATGCA
MyoD-R	Reverse	ATCCATCATGCCATCTGAGCAGTT
MyoD-qRT-F	Forward	CCTGTGGGCGTGTAAAGCATG
MyoD-qRT-R	Reverse	GCGTTTCTCAGGATCTCCACC
beta actin-F	Forward	GCCGAGAGGGAAATTGTCCGTGAC
beta actin-R	Reverse	TTGCCAATGGTGATGACCTGTCCG

Table 2: Partial nucleotide Sequence (5′→3′) for MyoD cDNA in the Pangasianodon hypophthalmus obtained from amplified PCR products Forward primers and reverse complement of reverse primers are indicated in bold letters

GCTTTAACACCAGCGACATGCATTTTCTTCGAAGACCTGGACCCCAGGTTAGAGCACGGGAGCTTGCTCAAGTCGGACGAGCACAACC
ACCTGGAGGACGAACACATCCGGGCTCCGAGCGGACACCACCAAGCGGGCAGGTGTCTCCTGTGGGCGTGTAAAGCATGCAAGAGG
AAAACCACCAACGCAGACCGGCGCAAAGCCGCAACCATGAGAGAAAGGAGACGTCTGAGCAAGGTCAACGATGC TTTCGAAACCCTG
AAGAGGTGCACGTCTACTAACCCTAACCAGAGGCTGCCCAAGGTGGAGATCCTGAGAAACGCCATCAGCTACATCGAGTCTCTCCAAGC
TTTACTCAGAAGTCAAGAGGAGAACTACTACCCGGTCCTGGAGCAATACAGCGGCGATTCAGACGCCTCCAGTCCCAGGTCCAACTGC
TCAGATGGCATGATGGAT

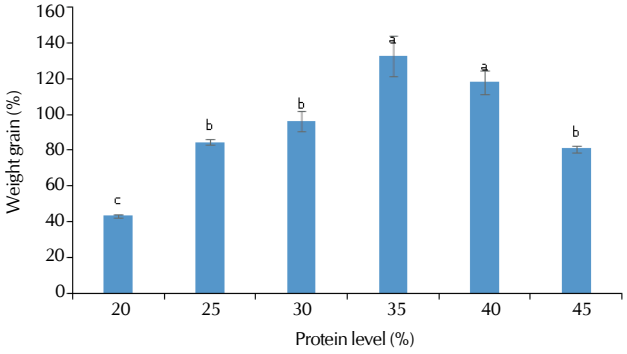

Figure 3: Percentage weight gains with different protein level on the 45th day of the experiment. An increased weight gain was showed with increasing dietary protein level up to an optimum (35% dietary protein level) and decreased gradually

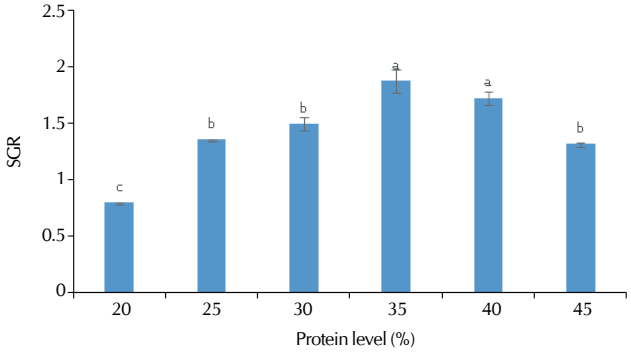

Figure 4: Specific Growth Rate (SGR) with different protein level on the 45th day of the experiment. Protein level expressed in percentage in the X-axis. A higher SGR was showed in 35% and 40% protein fed groups

Growth parameters

Percentage weight gain (WG %) and specific growth rate (SGR)

Percentage weight gains with different protein level on the 45th day of the experiment fig.3 showed that the MyoD gene expression was increasing with increasing dietary protein level at the initial stage and shown a maximum significant expression at 30% and higher dietary protein level. But the expression was not stable at 30% dietary protein level. The protein level at which the groups showed sustained expression were 35% dietary protein fed groups as shown in fig.3. Similarly Experimental group fed with 35% dietary protein level has showed better growth performance.The highest growth was observed in the case of 35% protein fed diet and the lowest was recorded in 20% protein fed diet. The SGR of the different experimental groups is shown in fig.4 Significant higher SGR (was found in 35% and 40% dietary protein fed groups

DISCUSSION

Growth and MyoD gene expression was studied with different dietary protein level for forty five days in fingerlings of *Pangasianodon hypophthalmus*.This study was planned for the characterization and MyoD mRNA expression in skeletal muscle of *Pangasianodon hypophthalmus*. We evaluated the dietary protein influence on *MyoD* gene expression of an important transcription factor that regulate myosatellite. MyoD gene is primary myoregulatory factor and may indicate the potentiating role of dietary protein on activation of myoblast cell in *Pangasianodon hypophthalmus*. In our result we could see the enhanced the expression of MyoD gene, with response to dietary protein level, it strengthens the understanding on the nutrient (protein) regulated myogenesis. In several studies made earlier it has been tried to draw sketch between muscle proliferation and MyoD gene. In flounder (*Paralichthys olivaceus*) MyoD expression was detected in precursor muscle cells during the initial phases of embryogenesis (Zhang *et al.*, 2006). Johansen and Overturf (2005) showed continuous differential MRF (MyoD, Myf5, Myogenin and MRF4) expression in rainbow trout (*Oncorhyncus mykiss*) skeletal muscle during different growth phases.

In an interesting study on pacu Fernanda Losi Alvesde Almeida, 2008 found that during early development and the juvenile stage, muscle growth occurs by intense recruitment of new muscle fibers from the proliferation of undifferentiated myogenic progenitor cells that express primary MRF, MyoD,

and Myf5 (Rescan *et al*., 1994, Watabe, 2001 and Megeney and Rudnicki, 1995).

However in their study in adult *P. mesopotamicus,* they found muscle growth was mainly by hypertrophy. In this stage, myoblast proliferation and hyperplasia are not significant, with MyoD expression being smaller than in juvenile fish (Johansen and Overturf, 2005). This can explain the low MyoD expression in adult pacu compared to their juvenile counterparts. Similarly after 45 days there is decrease in overall MyoD expression recorded. In the present study an increased gene expression was found in the initial stages of feeding trial in response to increase in the dietary protein as in 15th day sampling. While the 30th day sampling record increase in expression up to 30% protein and then decrease with higher protein level. But overall MyoD gene expression showed no significant variation on 30th and 45th day of sampling indicating 30 % diet as optimum and 35% protein. This can be due to saturation of body amino acid pool in response to dietary protein supply (Wu G, 2009)

Similarly Johansen and Overturf (2005) also reported an increase in expression of MyoD and Myf5 during the initial growth phase as initial stage provide more nuclei for muscle hypertrophy and hyperplasia(Koumans and Akster, 1995). De Almeida *et al*. (2010), observed the muscle growth could be associated with hyperplastic and hypertrophic growth and MyoD gene expression could also be related to hyperplastic and hypertrophy mechanism in paccu. Our result also indicates potentiating role of dietary protein on activation of MyoD in *p. hypophthalmus* in early stage indicating associated with hyperplastic and hypertrophic growth.

As Chapalamadugu and co-workers(2009), discussed that dietary carbohydrate influenced MyoD gene expression, in present experiment dietary protein level also influenced MyoD gene expression. MyoD gene expression increases with increasing protein level, this may be due to surplus in amino acid composition. This finding is supported by findings of Alami-Durante *et al*. (2010) where they described that high levels of aminoacid in rainbow trout increased the expression of muscle growth regulating genes. Alami-Durante et al. (2010) also reported that use of feeds with different plant protein mixtures and the changes in IAA/DAA (indispensable/ dispensable amino acids) ratio can significantly modify the expression of MyoD and fast-MHC in the rainbow trout white muscle.

In a similar study in trout Alami-Durante H (2010) demonstrated that changes occurred in skeletal white muscle cellularity and expression of MyoD and fast-MHC. While the overall growth and protein accretion were not modified, when a diet rich in soybean meal and glutamic acid was ingested compared to diets containing fish meal. But their study clearly demonstrated that the white and red muscles of rainbow trout were differently affected by nutritional changes. In another study de Almeida FL (2008) found differential MyoD gene expression in pacu white muscle, it was related to differences in growth patterns during the phases analysed, with hyperplasia predominant in juveniles and hypertrophy in adult fish.

Impact of dietary protein on growth is very established fact but the cost effective utilization of protein necessitate standardization of best growth with optimum level of protein. Because later is one of the primary nutrients of feed as it significantly influences cost, growth, survival, and yield of fish as well as the economics of an aquaculture industry. The increase in dietary protein has often been associated with higher growth rate in many species. However, there is a certain level beyond further growth is not supported, and may even decrease the growth*(*Yang *et al*., *2002)*.

In consensus it, in present work on *P. hypophthalmus* increase in growth with increasing protein level up to optimum level of 35 % and then decrease in growth beyond this optimum level of dietary protein has been observed. The similar finding has been reported by several workers (Maldonado-García *et al*. 2012; Bhalchandra and Prakash 2012 ;Jauncey 1982; Mohanta *et al*., 2008, Kannan *et al*., 2015). In an interesting study by Galloway *et al*. (1999) in Cod Larvae, increased the growth and muscle growth due hypertrophy and hyperplasia has been reported.

ACKNOWLEDGEMENT

The authors are grateful to the Director, Central Institute of Fisheries Education, Mumbai, for providing facilities for carrying out the work. The first author is grateful to Central Institute of Fisheries Education for awarding the institutional fellowship.

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