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Molecular characterization and expression profiling of growth hormone-releasing hormone (GHRH) in Indian catfish, *Clarias magur* (Hamilton, 1822)

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Abstract
Growth hormone-releasing hormone (GHRH) is an important neuroendocrine factor that stimulates the release of growth hormone (GH) from the anterior pituitary somatotrophs. Unlike in mammals, information on teleosts GHRH is insufficient. Here, GHRH full ORF (open reading frame) has been extracted from transcriptome data of Indian catfish, *Clarias magur*. It is a 423 bp transcript consisting of an ORF translatable to a 140 aa long polypeptide. This protein was shown to have apparent molecular weight (MW) of 16119.11 Da with pI of 6.61. The secondary structure of GHRH showed the presence of 17 αhelices, 27 coils and 10 β-sheets in the protein. The structural percentage of amino acid revealed that 37 amino acids (26.43%) have an alpha helix, 25 (17.86%) have extended strand, 10 (7.14%) have a beta turn, and 68 amino acids (48.57%) have random coil structures. Phylogenetically GHRH was closer to other catfishes like channel catfish and striped catfish. For homology modelling, pituitary adenylate cyclase activating polypeptide-38 (PDB id: 2d2p.1.A) was identified as suitable template. The alignment between our target GHRH and structural template was satisfied and predicted model showed high sequence similarity (58%). Relative mRNA expressions of GHRH in various tissues of different growth wise selected fish groups (i.e. high and low growth performing fishes) were estimated by using q-RT PCR analyses. It was abundantly expressed in the brain followed by male/female gonads of growth wise selected catfishes; demonstrating its possible functions may not be restricted to GH release only, but also in many other physiological and developmental regulations. Our results could be of great help to choose molecular marker like SNP or microsatellite engaged with this gene may contribute towards marker assisted selection for catfish significantly.

Keywords: GHRH, growth stages, *Clarias magur*, mRNA expression, 3D structure

Introduction
Growth hormone-release hormone (GHRH) is a hypothalamic hormone that was first isolated from pancreatic tumour tissue of acromegaly patients in 1982 [1]. In mammals, GHRH is a 44-aa polypeptide, and its C-terminus is amidated in several mammalian species, including human, pig, cattle and sheep [2]. The primary function of mammalian GHRH is to stimulate GH synthesis and release from anterior pituitary somatotrophs via binding to GHRH receptors [3]. In addition, GHRH has multiple extra-pituitary functions, as it activates cell proliferation, cell differentiation, jejunal motility control [4], regulation of sleeping and increase of leptin levels in modest obesity. However, due to the limitations of early experimental methods, GHRH in lower vertebrates has been misunderstood. Growth hormone-releasing hormone (GHRH) belongs to the glucagon/secretin superfamily, which also includes glucagon, pituitary adenylate-cyclase-activating polypeptide (PACAP), PACAP-related peptide (PRP), secretin and vasoactive intestinal peptide (VIP), peptide histidine methionine (PHM) and glucose-dependent insulinotropic peptide (GIP) [5]. The glucagon/secretin superfamily is believed to have originated from a common ancestral gene. The VIP, PACAP, and glucagon genes encode two or three bioactive peptides, whereas the GHRH, secretin, and GIP genes encode only a single bioactive peptide in mammals. Previously, PACAP and GHRH were hypothesized to be encoded on the same gene in non-mammalian vertebrates such as fish [6] and birds [7], whereas in mammals, PACAP and GHRH are encoded by separate genes on separate chromosomes [8] [9].

With rapid development of biotechnological techniques and large-scale genome sequencing of various model organisms, the true GHRH proteins in fish and chicken were found almost

simultaneously by two different research groups [10] [11]. These research on the evolution of GHRH in non-mammalian vertebrates showed that the GHRH-like peptides that encode PACAP in non-mammals are in fact the counterparts of mammalian PRPs, and the real GHRH peptides encoded in cDNA isolated from goldfish, zebrafish, and African clawed frog were identified [10]. However, very few reports are available for the molecular identification and differential gene expression of GHRH mRNA in growth wise selected fishes. To understand GHRH regulation and expression in teleosts, we first identified and characterized GHRH in Indian catfish, *Clarias magur*, a commercially important freshwater catfish that is widely cultured in India. The bioinformatics analysis of this protein was conducted to reveal their functional properties. Then we investigated the expression profiling of GHRH gene in tissues obtained from different growth performing fishes for exploring possibilities of their role in growth.

Materials and methods

Experimental animal and sample collection

The experimental animals, Indian catfish, *Clarias magur* were collected from the farms of ICAR-Central Institute of Fisheries Education (ICAR-CIFE), Balbhadrapuram, Andhra Pradesh, India where a genetic selection programme is running for improved body weight for *C. magur*. Under this programme base population was developed by diallel cross among three stocks of magur from Andhra Pradesh, Assam and West Bengal using single paired mating design. For expression profiling analysis tissues like brain, liver, gonad (testis and ovary) were collected aseptically from the selected parents (five high and five low growth performing fishes). All the sampled tissues were kept in RNAlater TM (Qiagen, Germantown, MD) and stored at -80 °C until further analysis.

RNA isolation and cDNA synthesis

Total RNA was extracted from the tissue using TrizolTM reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's guidelines. The extracted total RNA was treated with DNase I (Thermo Scientific, Waltham, MA) to remove the genomic DNA contamination. The A260/280 ratios of all the prepared RNAs were measured using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA). The RNA integrity was assessed by electrophoresis on 1% agarose gels. First-strand cDNA synthesis was performed in a volume of 20 µL with 1 µg of total RNA using Oligo dT primer and RevertAidTM reverse transcriptase First Strand cDNA Synthesis kit (Thermo Scientific, Waltham, MA) according to the manufacturer's protocol, and all cDNA samples were stored at −20 °C.

Extraction of growth related gene from transcriptome data

The division of Fish Genetics and Biotechnology, ICAR-CIFE has developed transcriptome database of *C. magur* using Illumina NGS platform. A total of 9 GB transcriptome data with 78.96 million high-quality reads was obtained. De Novo transcriptome assembly resulted in 52,237 contigs with an average length of 917 bp and N50 length of 1330 bp. Total 36642 contigs could be annotated to the known sequence databases with significant BLAST scores using Blast2GO software. From the annotated contigs, a full ORF of growth hormone-releasing hormone gene were selected for this study.

Amino acid sequence analysis and phylogenetic analysis

The open reading frame (ORF) of the full-length cDNA sequence obtained through transcriptome data was determined using NCBI ORF finder tool (https: //www.ncbi.nlm.nih. gov/orf finder/). The amino acid sequence of the coding region was deduced using Gene Runner and conserved domains were identified by NCBI Conserved Domain search tool (https://www.ncbi.nlm.nih.gov/Structure/ cdd/wrpsb.cgi). The deduced amino acid sequences of the GHRH from *C. magur* (Indian magur), *I. punctatus* (Channel catfish), *D. rerio* (Zebrafish), *P. hypophthalamus*, *Tachysurus fulvidraco* (Yellowhead catfish), *Lates calcarifer*, *Betta splendens*, *Silurus meridionalis* (Chinese large-mouth catfish), *Homo sapiens* (human), *Mus musculus* (Mouse) were subjected to multiple sequence alignment analysis using CLC Genomics Workbench 9 program software (QIAGEN, USA). The sequence alignment obtained was used to construct a Neighbour joining tree with 1000 bootstrap replicates using MEGA 7.0 software [12].

In silico analysis of GHRH

The online ProtParam tool at ExPASy [13] was used to observe the physiochemical parameters of the *C. magur* deduced GHRH amino acid sequence. The online tools like PSIPRED V.3 [14] and SOPMA [15] were used to predict the secondary structure while ProtScale program was used to analyze the protein hydropathy profile. SignalP 4.0 software was used to predict the signal peptide [16]. The 3D model of *C. magur* GHRH was generated using the amino acid sequence of closest homologs for which X-ray crystal structures were available. The homology model for the protein was generated using online tool, SWISS-MODEL. A suitable template was identified by PSI-BLAST against the Protein Data Bank (PDB) proteins available in the NCBI web server and appropriate template was selected on the basis of sequence similarity. The stereo chemical properties of *C. magur* GHRH 3D model were analysed through Ramachandran plots from RAMPAGE server [17]. Ramachandran plot tells the percentage of amino acid residues in the favourable and disallowed regions. The ERRAT program evaluates the statistics of non-bonded interactions between different atom types and plots the value of the error function versus position of a nine-residue sliding window, calculated by a comparison with statistics from highly refined structures. Verify3D examines the compatibility of an atomic model (3D) with its own amino acid sequence (1D). The interactions of *C. magur* GHRH protein with other glucagon/secretin family member proteins were determined using online software STRING. [18]

Real time mRNA expression of GHRH gene

The basal mRNA expression analysis of GHRH mRNA in tissues of *C. magur* was carried out in LightCycler450 Real-time PCR detection system (Roche, USA). The reactions were performed in 10 µL reaction mix volume containing 5 µL of 2× MaximaTM SYBR Green qPCR master mix (Thermo Scientific, USA), 0.5 µL of (0.3 pM) each gene-specific primer (Table 1) and 2 µL (20 ng) of cDNA and 2 µL of nuclease-free water. The default thermal profile was used for PCR amplification and it consisted of initial denaturation at 95 °C/10 min, followed by 40 cycles of denaturation at 95 °C/20 s, annealing at 59 °C/20 s and extension at 72 °C /30 s. All reactions were run in triplicate and repeated twice. Relative expression of genes was determined using comparative Ct method ($2^{-\Delta Ct}$) [19]. The putative

housekeeping gene (HKG), β-actin was statistically analysed by the comprehensive algorithm of Norm Finder, Best keeper and Genorm to assess their transcriptional expression stability. Here, β-actin was selected as HKG for gene normalization. The one-way analysis of variance (ANOVA) of statistical package SPSS 17.0 (USA) was used to test the statistical significance of differences in mRNA transcript levels. $P<0.05$ was considered as statistically significant. The results were expressed as mean ± SEM (bars).

Table 1: Primers used in the study

Primer name	Sequence (5' – 3')	Usage
ghrhF_qRT	GCACCAAAGCCAGAAGCCATG	Real time PCR
ghrhR_qRT	CCCTGCAATTCGGCATCAGTTC	Real time PCR
B_actinF	GCCGAGAGGGAAATTGTCCGTG	Internal Control
B_actinR	GCCAATGGTGATGACCTGTCCG	Internal Control

Results

From the assembled and annotated contigs of transcriptome data, the full length ORF (423 bp) of growth hormone-releasing hormone gene were obtained. The ORF of *C. magur* GHRH gene encoded a putative protein of 140 amino acids. The sequence obtained in the present study was submitted to NCBI Gen Bank. The *C. magur* GHRH protein was shown to have apparent molecular weight (MW) of 16119.11 Da with pI of 6.61. The secondary structure of *C. magur* GHRH predicted by PSIPRED server showed the presence of 17 αhelices, 27 coils and 10 β-sheets in the protein. The structural percentage of amino acids of GHRH predicted by SOPMA software revealed that 37 amino acids (26.43%) have an alpha helix, 25 (17.86%) have extended strand, 10 (7.14%) have a beta turn, and 68 amino acids (48.57%) have random coil structures. *C. magur* GHRH protein exhibited 16 serine (Ser), 6 threonine (Thr), and 3 tyrosine (Tyr) phosphorylation sites that were uniformly distributed throughout the polypeptide chain as predicted by NetPhos 3.1 Server. As the GHRH belongs to glucagon/secretin superfamily which is a large family of evolutionary related peptide hormones that regulate activity of G-protein coupled receptors from secretin receptor family. Sequencing result of GHRH was analyzed using BLAST tool at NCBI for similarity search. The deduced amino acid sequence of *C. magur* GHRH deduced amino acid revealed 47%, 74% and 98% identity with house mouse, zebrafish and channel catfish sequences, respectively. Multiple sequence alignment of *C. magur* deduced amino acid sequence of GHRH with sequences from other fish species showed well-conserved regions across the sequences (Fig.1).

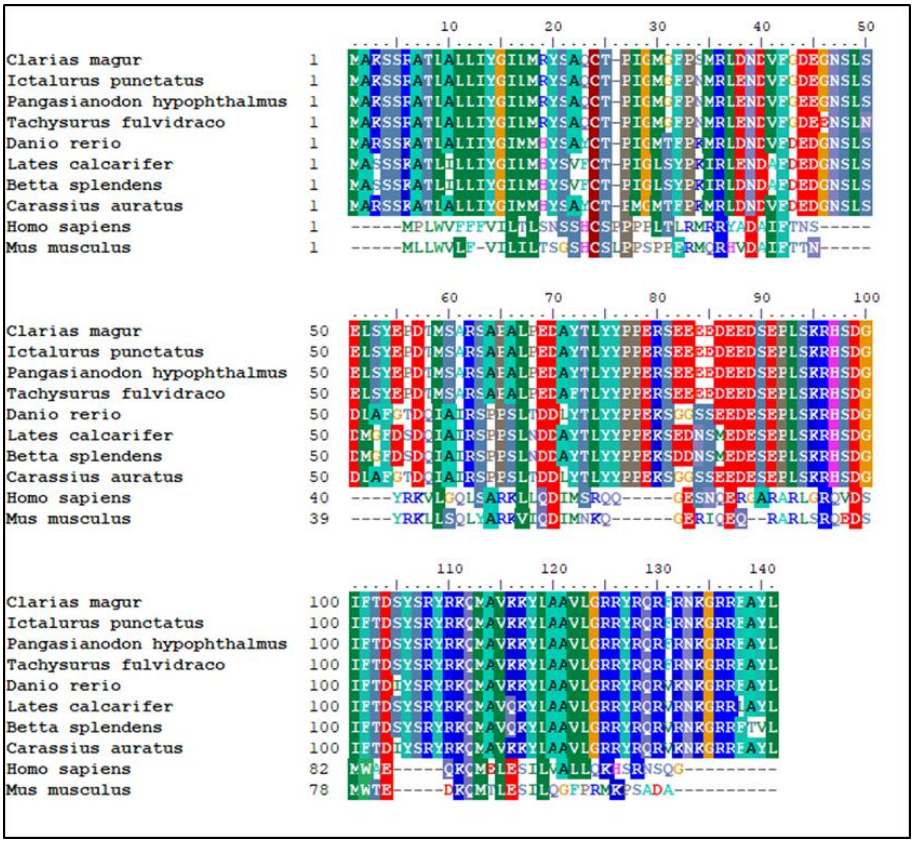

Fig 1: Conserved amino acid sequences of the Cyp26A1 protein across different vertebrates. Amino acid sequences encoding the GHRH protein were aligned using Clustal W algorithm of the CLC Genomics Workbench 9. The bars below indicate the conserved motifs in different vertebrates. The amino acid sequences of *I. punctatus, P. hypophthalamus, T. fulvidraco, D. rerio, L. calcarifer, B. splendens, C. auratus, H. sapiens* and *M. musculus* were retrieved from NCBI data base.

The catalytic domains of GHRH protein were found to be highly conserved across all the species used for multiple alignment analysis. The identity level of *C. magur* GHRH amino acid sequence with other species is presented. The Neighbour-joining tree for GHRH was prepared which differentiated the closely related species in separate clades.

The catfishes formed one cluster while cyprinids formed a separate group (Fig.2). Different catfish species grouped in to a single cluster while human formed sister group. GHRH of *C. magur* was closely related to Thai catfish and channel catfish followed by cyprinids. This finding is consistent with the classification and evolutionary status of these species.

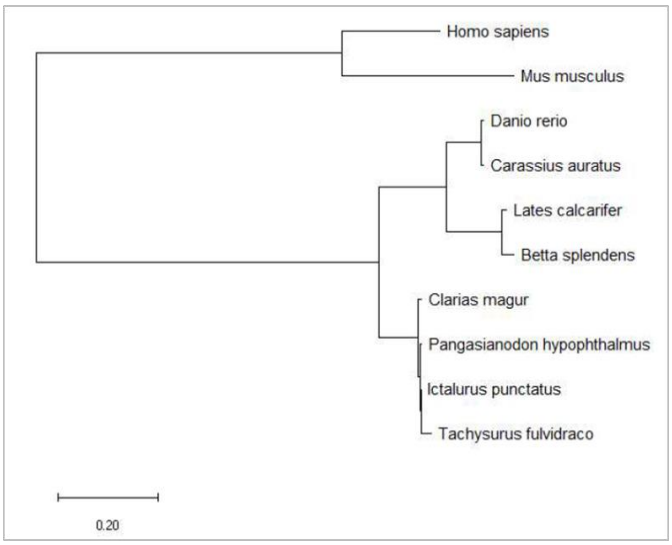

Fig 2: Phylogenetic analysis of C. magur GHRH

The hydropathy profile of the GHRH protein was determined by ProtScale program which demonstrated that the arginine (Arg) at residue position 68 of the polypeptide chain exhibited the highest hydrophily (hydrophobic parameter: -3.311) while valine (V) at residue position 17, showed highest hydrophobicity (hydrophobic parameter: 2.7). In general, hydrophilic area was greater than hydrophobic area (Fig.3). Therefore, the GHRH peptide chains of *C. magur* found to be hydrophilic in nature. The computational structural biology approach is being applied for the prediction of protein structure. A suitable structural template for GHRH, Pituitary adenylate cyclase activating polypeptide-38 (PDB id: 2d2p.1.A), was identified by a BLAST search as implemented in the SWISS-MODEL Protein Modelling Server. The alignment used in the model shows a high sequence similarity (58%) between the target and structural template molecules with all the gaps introduced outside the secondary structures. Model quality was assessed with QMEAN Z score (-0.36) and Ramachandran plot analysis. Ramachandran analysis of *C. magur* GHRH 3D model revealed that all 38 (100%) modelled residues are in the most favoured region and no residues are in outlier region (Fig. 4). A good quality model would be expected to have over 90% of amino acid residues in the most favoured region, and during homology modelling 98% of the amino acid residues must be present in the allowed region. These results confirmed that the quality of modelled protein prediction was a good. The data figure indicated that the 3D model generated was highly stable

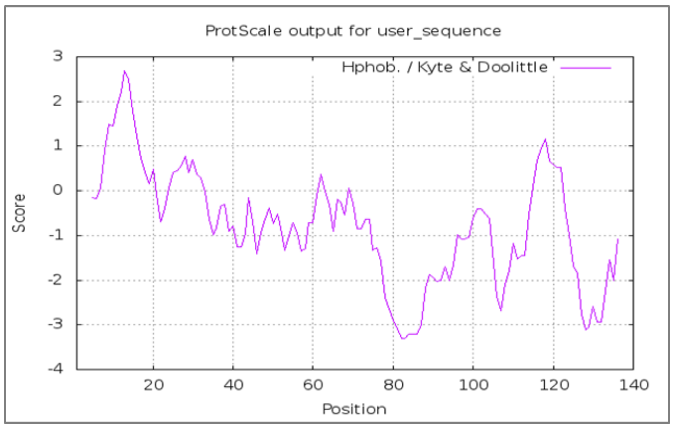

Fig 3: The hydropathy profile of *C. magur* GHRH protein.

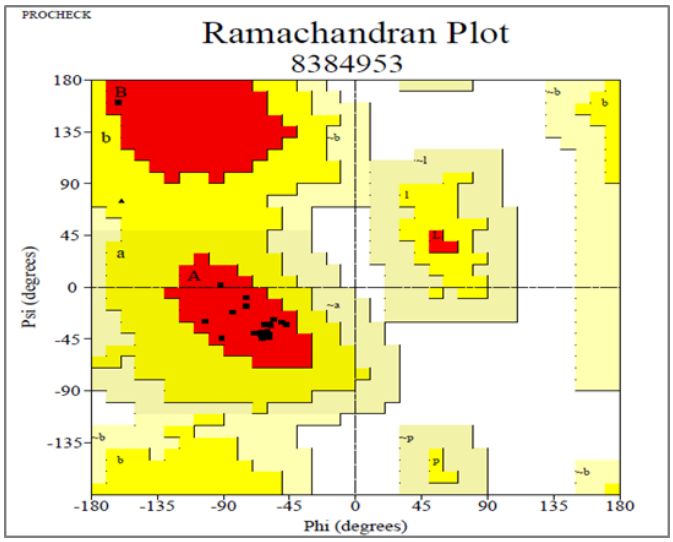

Fig 4: Ramachandran plot (RAMPAGE) of *C. magur* GHRH.

The subcellular localization of the protein was analysed through CELLO2GO tool and the results revealed that the predicted localization of GHRH protein is maximum in nucleus (43%) followed by extracellular region (18.2%) (Fig.5). The gene ontology terms assigned to the protein indicated its involvement in ovarian follicle development, behavioural fear response, histamine secretion, regulation of acute inflammatory response to antigenic and non-antigenic stimulus, elevation of cytosolic calcium ion concentration, neuropeptide signalling pathway, positive regulation of cell proliferation and G-protein coupled receptor protein signalling pathway. The protein-protein interaction revealed that GHRH protein has a strong association with adenylate cyclase activating polypeptide 1 receptor; G-protein coupled receptor 2 family member, vasoactive intestinal peptide receptor 2 followed by vasoactive intestinal peptide receptor 1b with confidence score of 0.994, 0.992 and 0.986, respectively.

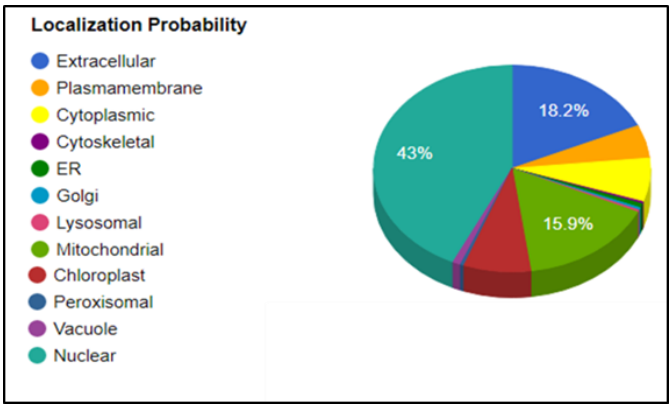

Fig 5: Percentage-wise subcellular localization of *C. magur* GHRH protein in different sections of cell.

The mRNA expression pattern of GHRH gene in high and low growth performing catfishes was determined through qRT-PCR in the different tissues (brain, liver and gonads). The GHRH gene in high growth performing fish was expressed in brain and gonads of all selected stages with significantly higher mRNA expression. However, not much significant differences in mRNA expression were observed in liver and gonad tissue from high performing fishes (Fig.7). In low growth performing fishes, the GHRH gene was expressed in all three tissues but significantly higher in brain compared to the liver and gonads.

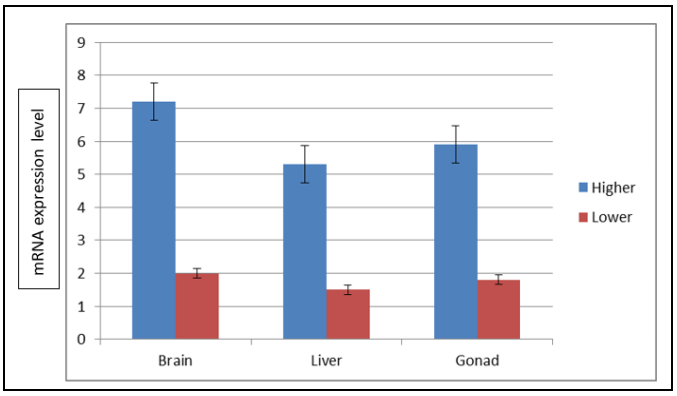

Fig 7: qRT-PCR analysis of GHRH mRNA expression in brain, liver and gonad of higher and lower growth performing family individual of *C. magur*.

Discussion

Previous reports suggested that several non-mammalian GHRH like peptides are encoded with PACAP and processed from the same transcript and prepropolypeptide. However, the previously named GHRH-like peptides of non-mammalian vertebrates were unable to demonstrate GH-releasing activities. Here, we identified and characterized a GHRH gene from the teleost catfish *C. magur* and investigated the role of GHRH in growth wise selected fishes. The true non-mammalian GHRHs were discovered from chicken, African clawed frog, zebrafish, takifugu, puffer fish, rainbow trout, and flounder by in silico analysis [10]. In the present study, we reported the full-length cDNA of growth hormone-releasing hormone from the Indian catfish *Clarias magur*. The ORF of *C. magur* GHRH gene encoded a putative protein of 140 amino acids. The predicted ORF and deduced amino acid sequences were similar to other fish species like channel catfish, *Ictalurus punctatus* (XP_017326441.1and D. rerio (NP_001259010.1). The full-length cDNA sequence of GHRH has been reported in olive flounder, *Paralichthys olivaceus* [20] and lungfish, *P. dolloi* and *Xenopus laevis* [21]. The catfish GHRH precursor cDNA identified in this study encodes 140 amino acids containing a 20-amino acid signal peptide. The amino acid sequence of the bioactive core region of catfish is identical with channel catfish and striped catfish, *Pangasius hypophthalmus* GHRHs and also shows high homology (approximately 74.6%) with zebrafish GHRH at the amino acid level. The phylogenic analysis of *C. magur* GHRH with other species revealed high similarity with I. punctatus, D. rerio and other catfishes. The prediction of ligand binding sites in a protein helps to understand its putative function and molecular interaction with other proteins or molecules [22]. Many ligand binding sites have been predicted in *C. magur* GHRH amino acid sequence out of which few have been revealed through homology modeling in human [23, 24]. The hydropathicity analysis using the Kyte-Doolittle algorithm predicted that *C. magur* GHRH protein is more hydrophobic at N-terminus region due to the presence of a highly hydrophobic region of nonpolar amino acids. GHRH is well documented to play a critical role in stimulating pituitary GH synthesis and release both in vitro and in vivo in mammals [25]. GHRH binding to the GHRH receptor results in increased GH production, mainly via the cAMP-dependent pathway [26]. Based on this mechanism of GH induction described, we investigated whether this GHRH has any impact regard expression differences between growth based selected fishes. Apart from GHRH role in growth regulation, GHRH may play important biological roles in germ cell maturation and hormone production in the ovary [27]. A study demonstrated that a mammalian GHRH stimulates release of GH, promotes somatic and gonadal growth and may affect reproductive performance in tilapia of suboptimal temperatures [28].

In summary, the present study reports full-length coding sequence of GHRH gene from *C. magur*. The sequence was analysed using various bioinformatics tools to predict its structural and functional features. The tissue distribution of GHRH mRNA was determined using RT-PCR. Catfish GHRH mRNA was detected in the brain as well as in the testes and the ovary. The differential mRNA expression analysis of the gene in various tissues from growth wise selected fishes revealed low expression of GHRH in the liver and gonads of low performing fishes. All-time the brain has showed comparatively higher expression of the GHRH gene. This significant observation if studied in detail might give a clue regarding the slow growth rate in these catfishes in captivity. The mRNA expression and further molecular marker like SNP or microsatellite engaged with this gene may contribute towards marker assisted selection for catfish significantly.

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