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Optimal dietary protein requirement of juvenile GIFT Tilapia (*Oreochromis niloticus*) reared in inland ground saline water

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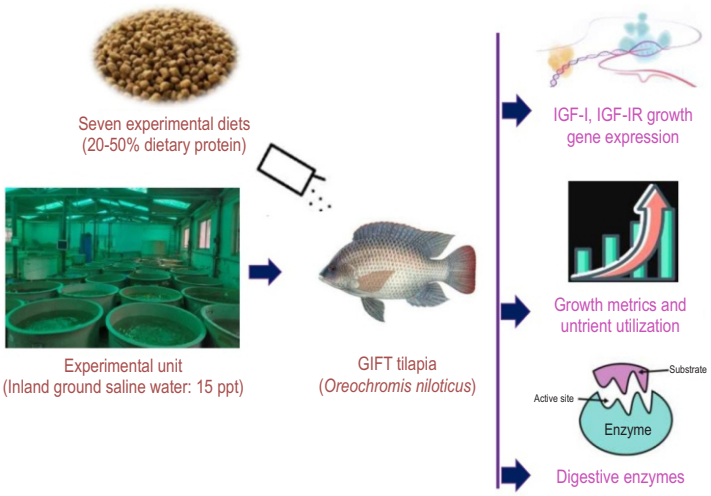
Abstract

Aim: The present study was conducted to optimize the dietary protein level in the diet of Genetically Improved Farmed Tilapia reared in inland ground saline water (IGSW) of 15 ppt ambient salinity.

Methodology: Seven iso-caloric (400 kcal digestible energy, DE/100g), iso-lipidic (6%) and hetero-nitrogenous (20-50% crude protein, CP) purified diets were prepared for conducting the feeding trial. Following completely randomized design, the random distribution of 315 acclimatized fish (average body weight 4.01±0.01g) was made in seven groups namely, CP_{20}, CP_{25}, CP_{30}, CP_{35}, CP_{40}, CP_{45} and CP_{50} in triplicate with the stocking density of 15 fish per replicate tank.



Results: Weight gain percentage, specific growth rate, and expression of insulin like growth factor-I and insulin like growth factor-I receptor with higher quadratic relation (R^2=0.94, 0.96, 0.90 and 0.93, respectively) to the dietary crude protein (CP) level significantly increased (p<0.05) up to 40% crude protein and beyond that the values significantly decreased. With higher quadratic (R^2=0.83) relation, muscle RNA/DNA ratio was significantly highest in 40% crude protein fed group. With higher quadratic (R^2=0.81 and 0.98, respectively) relations, significantly higher protease and lower amylase activities were found in 40, 45 and 50% crude protein fed groups.

Interpretation: Second order polynomial regression analysis based on WG%, SGR and IGF-I gene expression revealed that the optimum dietary protein for GIFT juveniles could be 41.84, 40.66 and 42.22%, respectively. This data will be helpful for development of economic and environment-friendly feed for GIFT tilapia culture in inland ground saline water of medium salinity.

Key words: Gene expression, GIFT Tilapia, Growth metrics, Inland Ground Saline Water

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Introduction

The expansion of freshwater aquaculture become more unpredictable due to climate change and increased salinity throughout the globe (Allan *et al.*, 2001). Moreover, urbanization and industrialization creates major conflict over horizontal expansion of aqua farming practices. Therefore, utilization of unused resources for aquaculture can play pivotal role to achieve the targeted global aquaculture production of 109 million tonnes by 2030 (FAO, 2018). In this context, global agriculturally unproductive inland saline lands (380 million hectare) can be converted to aquaculture resources for culturing euryhaline species for enhancing aquaculture production (Allan *et al.,* 2009). But, in compared to brackish and marine water, inland ground saline water (IGSW) is deficient of potassium ion with high calcium ions and variable concentrations of magnesium ions (Allan *et al.,* 2001), which affect the physiological homeostasis and growth performance of fish (Aklakur, 2017). However, several countries have successfully cultured different euryhaline species in IGSW with or without fortification of potassium ions (Jana *et al.,* 2006; Partridge and Lymbery, 2008; Jana *et al.,* 2021a). Thus, genetically improved farmed tilapia (GIFT) could be the most suitable species for culturing in IGSW because of its high growth rate, disease resistance and tolerance to wide range of salinity and ionic variation. Higher growth and feed utilization has been reported in GIFT strain of tilapia in comparison to red tilapia under similar salinities (Ng and Hanim, 2007).

From the aquaculture perspective, provision of nutritionally balanced, environment-friendly and cost effective feed makes the aquaculture operation more profitable and sustainable (Kumar *et al.,* 2018). Therefore, adequate nutritional information, especially the knowledge on dietary nutrient requirement of a species in relation to age and environmental condition is the most crucial consideration (Jayant *et al.,* 2018). Fish feeds on energy satiation and unlike mammals, it can efficiently utilize dietary protein for energy production. However, provision of high dietary protein with less quantity of non-protein energy sources (lipid and carbohydrate) attributes higher amino acids catabolism with excretion of more ammonia to pollute the environment leading to growth retardation of fish (Talukdar *et al.,* 2020). Therefore, optimization of dietary protein with optimum protein to energy ratio (P: E) under existing environmental condition can make the aquaculture operation more profitable (NRC, 2011). Tilapia requires 20-56% dietary protein in the diet depending on the salinity of rearing water (Winfree and Stickney, 1981). Additionally, Nile tilapia (*Oreochromis niloticus*) requires 26-40% dietary protein in relation to higher to lower body weight (NRC, 2011).

There is no available report on the optimum dietary protein for GIFT tilapia in IGSW of medium salinity (15 ppt). The digestive enzyme activities of fish indicate their efficiency of dietary nutrient digestion and absorption for growth and the activities are influenced by availability of substrates in the gastrointestinal tract of fish (Kumar *et al.,* 2017). As in all vertebrates, the actions of growth hormone (GH) and resultant growth and nutrient utilization in fish are mediated by insulin like growth factor (IGF) axis (Cruz *et al.,* 2006) through the expression of especially hepatic insulin-like growth factor-I (IGF-I) gene, which is controlled by nutrient composition of diet and plays as a suitable biomarker of growth and nutritional status of fish (Kumar *et al.,* 2018). Keeping the above-mentioned views in mind, the current study aims to optimize the dietary protein for GIFT juveniles reared in IGSW of medium salinity.

Materials and Methods

Procurement and acclimatization of fishes: GIFT juveniles were procured from RGCA, India and carefully transported to ICAR-CIFE, Rohtak centre, India. The fishes were kept in freshwater containing cemented tanks (4m x 3m x 1m; 12,000 l capacity) for 15 days acclimatization with round the clock aeration facility. Fishes were fed with commercial diet (35% protein) on thereafter satiation basis thrice a day. IGSW was added to raise (1 ppt/ day) the salinity to 15 ppt and finally fishes were acclimatized for 15 days following the round the clock aeration and same feeding regime.

Experimental diet formulation and preparation: Commercial purified feed grade ingredients were used for the formulation (Table 1) and preparation of seven iso-lipidic (6% ether extract),iso-caloric (400 kcal digestible energy $100g^{-1}$) and hetero-nitrogenous (20-50% crude protein) purified experimental diets namely, CP_{20} (20% protein), CP_{25} (25% protein), CP_{30} (30% protein), CP_{35} (35% protein), CP_{40} (40% protein), CP_{45} (45% protein) and CP_{50} (50% protein). Briefly, all the ground ingredients, except heat labile ingredients and oils were mixed with water to form dough, and then it was steam cooked followed by cooling and homogenous mixing the remaining ingredients. Pellets (1mm diameter) were formed by pressing the dough in a pelletizer and dried in room temperature. Later it was oven dried (40°C) until achieved 10% moisture level was. Dry pellets were crushed, packed, labelled and stored at 4°C until used for feeding.

Experimental site and inland ground saline water collection and storage: The present experiment (60 days) was conducted in ICAR-CIFE, Rohtak centre, India. Underground IGSW (15 ppt) was pumped out, filtered (100µm filter bags) and filled into cemented tanks (3m × 2m × 1.5 m, 9000 l capacity). After seven days, IGSW was transferred to storage tanks (1.05 m^2 × 0.89 m, 935 l capacity) for using in experimental tanks as and when necessary.

Experimental facilities and feeding trial: After acclimatization, 315 fish (average body weight 4.01±0.01g) were arbitrarily dispersed in seven experimental groups with three replicates (15 fish/tank, 92 cm diameter and 45 cm height, 300 l capacity with 200 l water volume) as per completely randomized design (CRD). Respective diets were fed thrice a day (10.00, 14.00 and 18.00 hr) on satiation basis maintaining 12 hr photoperiod and aeration. Fortnightly, total biomass of the

fishes from each tank was measured to assess the growth rate. Every day before first feeding, faeces were siphoned out and replenished with equal volume of fresh IGSW. During trial period, 25-30% water from each tank was exchanged with fresh IGSW at an interval of three days.

Physico-chemical parameters of water: Physico-chemical parameters of water were monitored routinely throughout the experimental period (APHA, 2012). Except water temperature, all the parameters of IGSW were determined at three days interval. The temperature of IGSW was measured twice a day (8:00 and 20:00 hr).

Proximate composition: Prior to start of the experiment, fifteen fishes were taken out for initial proximate composition analysis and after completion of feeding trial, five fishes were collected from each experimental tank for analysis of final whole body composition (AOAC, 1995). For moisture analysis, the initial fish sample, final fish samples and diets were oven dried at 80°C until the constant weight was achieved. The micro-Kjeldahl method (Kelplus, PELICAN, India) was followed to estimate the crude protein (CP), lipid was estimated by solvent extraction using soxhlet apparatus (SOCS plus, PELICAN, India). Samples were incinerated at 550°C for 5 hr in muffle furnace for determining the total ash (TA) content of samples. The analysis of diets crude fibre (CF) content of fat free samples were performed through digestion using 1.25% sulphuric acid followed by 1.25% sodium hydroxide in crude fibre assembly (Tulin Equipment, India). Thereafter, digested sample were burmed in a muffle furnace at 550°C for 5 hr. Subtraction method was employed for calculating nitrogen free extract (NFE) of experimental diets and total carbohydrate (TC) of fish samples (AOAC, 1995). Finally, wet weight of body composition of fish was calculated. Gross energy (GE) of diet was determined by Bomb calorimeter (5E-AC/PL, CKI Co., Ltd., China) (AOAC, 1995). The digestible energy (DE) (Halver, 1976) value and protein to energy ratio (Jana *et al.,* 2021b) of the experimental diets were calculated by standard formulae.

Growth performance and survival rate: Following one day starvation, the fish were weighed on a electronic weighing balance at beginning and after completion of the trial to calculate the growth parameters such as weight gain percentage (WG%), specific growth rate (SGR), feed conversion ratio (FCR) and apparent net protein utilization (ANPU%) as per Brown (1957). After completion of the trial, live fish from each experimental tank was counted for calculation of survival of the experimental animal (Brown, 1957).

Assays of digestive enzymes: After completion of trial, three fishes were randomly collected from each experimental tank and anaesthetized with clove oil (Kumar *et al.,* 2017). The intestine was then dissected out in ice cold condition for preparing of the intestinal tissue homogenate to analyse the digestive enzyme activities.

Tissue homogenate preparation: Intestinal tissue homogenate was prepared in chilled sucrose solution (0.25M) using a Teflon coated mechanical tissue homogenizer (REMI Equipment, India) to make 5% tissue homogenate under iced condition. Then the homogenate was centrifuged (5000 rpm, 10 min, 4°C) in a cooling centrifuge (Thermo Fisher Scientific, Germany) and the collected supernatant was stored at −20°C until used for digestive enzyme assays.

Quantification of tissue protein: Lowry's method (Lowry *et al.,* 1951) was used for quantification of protein in intestinal tissue homogenate and the value was used for calculating the digestive enzyme activities.

Activities of digestive enzymes: The activity of protease (mm of tyrosine released min^{-1} mg^{-1} protein) was estimated as per Drapeau (1974). The activity of amylase (mm maltose released min^{-1} mg^{-1} protein) was analysed according to Rick and Stegbauer (1974). Titrimetric method of Cherry and Crandall (1932) was followed for analysing the activity of lipase (unit hr^{-1} mg^{-1} protein).

Quantification of RNA, DNA and RNA-DNA ratio: After random collection from every experimental tank, three fishes were anaesthetized and the muscle was dissected in ice cold condition for quantification of muscle nucleic acids (DNA, RNA and RNA: DNA ratio) according to Schneider (1957).

Expression of IGF-I and IGF-IR genes

Isolation of total RNA and synthesizing single-strand cDNA: After completion of trial, random collection of three fishes from each tank followed by anaesthesia and the liver was dissected out in ice cold condition. The liver tissues were immediately stored in 1 ml cryo-tubes containing RNAlater™ solution (Qiagen, Netherlands). For gene expression studies, RNA was isolated using TRIzol reagent (Invitrogen, USA) following protocol of Sambrook and Russell (2001) with some modifications. Total RNA isolated was DNase treated and equal amount was converted into first strand complementary DNA (cDNA) using oligo (dT) primer and RevertAid Reverse Transcriptase (Thermo Scientific, USA) following manufacturers protocol. Finally, cDNA was either used immediately for quantitative real time-polymerase chain reaction (qRT-PCR) or stored at -80°C until use.

Designing of primer: Gene Runner software (Version 6.5.48 × 64 Beta) was used for primer sequencing to study the hepatic β-actin (reference gene, KJ126772.1, FP 5'-AATCCTGCGGAATCCACGAAAC-3', RP 5'-CTCCTTCTGCATCCTGTCAGCG-3', 140 bp), insulin like growth factor-I, IGF-I (targeted gene, EU272149.1, FP 5'-GGACGAGTGCTGCTTCCAAAGC-3', RP 5'-TGCTCTTGGCATGTCTGTGTGC-3', 121 bp) and insulin like growth factor-I receptor, IGF-IR (targeted gene, KC506777.1, FP 5'-GCGACCCAAAGAGCAACAGTGG-3', RP 5'-TGCCAGATCTCGGTGGACAAAC-3', 130 bp) gene expression.

Hepatic mRNA expression by RT-PCR: Relative mRNA expression of IGF-I and IGF-IR genes were performed taking β-

actin as reference gene using SYBR green qPCR Master Miix (Takara Biosciences, USA) in a RT-PCR (Agilent Technologies, USA). The RT-PCR conditions were as follows: denaturation at 95°C for 15 sec, annealing at 59.3°C for 15 s and extension at 59.3°C for 60 sec, for 40 cycles. Calculation of relative mRNA expression of IGF-I and IGF-IR genes with consideration of β-actin was performed according to Livak and Schmittgen (2001). CP_{20} (20% protein fed group) was used as control for gene expression studies.

Statistical analyses: Data were subjected to One-way ANOVA using SPSS software. Orthogonal-polynomial contrasts were performed for testing overall, linear and quadratic trends of parameters in relation to protein levels of diets. Significant differences among the means ($p< 0.05$) were ensured through DMRT with Post-hoc analysis. Second-order polynomial regression model (Zeitoun *et al.*, 1976) was fitted with WG%, SGR and IGF-I expression to optimize the dietary protein requirement of GIFT reared in IGSW of 15 ppt salinity.

Results and Discussion

Water temperature, salinity and pH in the experimental groups ranged between 30.30±1.45 to 32.12±2.21°C, 15.05±0.29 to 15.60±0.38 g l^{-1} and 7.80±0.24 to 8.45±0.37, whereas dissolved oxygen, total hardness, and total alkalinity ranged between 5.51±0.78 to 6.72±0.83, 2890.02±11.45 to 2980.54±15.76, 244.46±9.25 to 298.66±16.27 mg l^{-1}. The calcium, magnesium, and potassium ranged between 320.21±12.52 to 355.89±9.08, 480.56±7.78 to 524.86±10.43, 8.89±2.12 to 11.90±1.88 mg l^{-1} Ammonium, nitrite and nitrate of water were found in the range of 0.02±0.01 to 0.10±0.04, 0.001±0.001 to 0.007±0.002 and 0.02±0.01 to 0.08±0.03 mg l^{-1} respectively. Free carbon dioxide remained non-detectable during the experimental period. All the physico-chemical parameters of water were within the acceptable limits for successful culture of GIFT tilapia in IGSW (Singha *et al.,* 2020).

Dietary crude protein (CP) levels were 20.13, 25.06, 30.20, 35.21, 40.16, 45.30 and 50.28% in the experimental diets of CP_{20}, CP_{25}, CP_{30}, CP_{35}, CP_{40}, CP_{45} and CP_{50}, respectively and extract, gross energy, digestible energy and protein to energy ratio values ranged between 6.04-6.13%, 470.73-513.78 kcal $100g^{-1}$, 398.93-400.09 kcal $100g^{-1}$ and 50.33-125.83 mg protein $kcal^{-1}$ digestible energy, respectively (Table 1). The result indicated that the experimental diets were hetero nitrogenous, iso-lipidic and iso-caloric as the basis of protein requirement study (Talukdar *et al.,* 2020). Whole body composition indicates the nutritional quality, nutrient utilization efficiency and health status of fish (Jana *et al.,* 2021). Initial moisture, protein, lipid, ash and carbohydrate contents of fish were 76.41, 13.99, 3.98, 3.75 and 1.87%, respectively. Though no significant variation ($p>0.05$) was found for final moisture (linear R^2=0.09 and quadratic R^2 = 0.32) and crude protein (linear R^2=0.35 and quadratic R^2=0.62), lipid with overall, linear and quadratic (linear R^2=0.27 and quadratic R^2=0.47) trend, total ash with overall and quadratic (R^2=0.56) trend and total carbohydrate with overall and linear (R^2=0.21) trend were significantly ($p<0.05$) affected in fish in relation to dietary protein levels (Table 2). In this study, the contrast analysis revealed that the whole body lipid content of CP_{40} was statistically similar ($p>0.05$) to $CP3_5$ and CP_{45}, but significantly lower ($p<0.05$) than other groups. This is well supported with the observations of Winfree and Stickney (1981) and Mohammadi *et al.* (2014) who demonstrated analogous trend of body lipid content of tilapia in relation to dietary CP level. Total ash content of CP_{30}, CP_{35} and CP_{40} groups was statistically similar ($p>0.05$), but higher ($p<0.05$) than the other groups. This is in agreement with the findings of Nwanna *et al.* (2014) in African catfish fed with varying dietary protein levels. In contrast, Jauncey (1982) found similar ($p>0.05$) whole body ash content in relation to dietary protein level, but exact reason of these variable observations under similar feeding regime remains unclear.

Growth, the muscle hyperplasia of animals including fish is controlled by both nutritional and environmental factors. In spite of providing energy to the animals, dietary crude protein play a pivotal role in growth of fish, thus optimum dietary protein with corresponding P:E ratio can supply sufficient amino acids for body protein synthesis to maximize growth (Kumar *et al.,* 2017). Overall, linear and quadratic trend of growth performances including WG%, SGR, FCR and ANPU of GIFT juveniles were significantly affected ($p<0.05$) in relation to graded level of CP in the diet (Table 3).There was significant increment ($p<0.05$) of WG% (linear R^2=0.67 and quadratic R^2=0.94) and SGR (linear R^2=0.68 and quadratic R^2=0.96) in relation to enhancing dietary protein up to 40% level (CP_{40} group) and then gradually reduced ($p<0.05$) with further increase in protein level. This finding clearly indicates that feeding excess CP beyond the optimum level for GIFT reared in IGSW of 15 ppt salinity was not beneficial in terms of growth as excess amino acids instead of synthesizing body protein were oxidized for energy production concomitant with excretion of more ammonia that further enhanced the energy demand of fish (Winfree and Stickney, 1981). Similarly, feeding low dietary protein probably could not provide sufficient amino acids for tissue building leading to poor growth. Siddiqui *et al.* (1988) reported 40% dietary protein requirement of tilapia in brackish water condition supported our result.

FCR indicates feed utilization in relation to growth of animals including fish which depends on the feed quality, condition of fish and environmental factors. FCR showed high quadratic (R^2=0.96) trend with significantly ($p<0.05$) inverse trend of growth parameters (WG% and SGR) in relation to dietary CP level. Our findings can be corroborated with the observation of Mohammadi *et al.* (2014) who found the lowest FCR value (1.35) in all-male Nile tilapia reared in IGSW of 8 ppt salinity due to feeding of optimum dietary protein. ANPU value indicates protein utilization efficiency of animals including fish. A significantly ($p<0.05$) decreasing trend of ANPU with high linear and quadratic (R^2=0.95 and R^2=0.97) was observed in relation to graded level of crude protein in the diet with highest value was found in CP_{20} group indicating maximum utilization of dietary CP derived amino

Table 1: Formulation and proximate composition (%) of experimental diets fed to GIFT juveniles cultured in 15 ppt inland saline water for 60 days

Ingredients (%)	Diets (Experimental groups)[1]						
	CP_{20}	CP_{25}	CP_{30}	CP_{35}	CP_{40}	CP_{45}	CP_{50}
Casein[2]	19.60	24.40	29.20	34.00	39.20	44.00	48.80
Gelatin[2]	4.90	6.10	7.30	8.50	9.80	11.00	12.20
Dextrin[2]	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Starch[2]	49.95	43.95	37.95	31.95	25.45	19.45	13.45
Cellulose[2]	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Fish oil[3]	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Sunflower oil[4]	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Vit -min mix[5]	1.50	1.50	1.50	1.50	1.50	1.50	1.50
CMC[6]	2.00	2.00	2.00	2.00	2.00	2.00	2.00
BHT[7]	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Choline chloride[2]	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Betaine[2]	0.75	0.75	0.75	0.75	0.75	0.75	0.75
Stay C[8]	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Total	100	100	100	100	100	100	100
Proximate Composition (on dry weight basis)							
Moisture (%)	8.54	8.50	8.54	8.61	8.52	8.27	8.40
Crude protein (%)	20.13	25.06	30.20	35.21	40.16	45.30	50.28
Ether extract (%)	6.08	6.13	6.07	6.09	6.04	6.09	6.08
Crude fibre (%)	5.13	5.16	5.19	5.31	5.29	5.18	5.07
Total ash (%)	2.47	2.48	2.43	2.57	2.48	2.66	2.63
NFE[9] (%)	66.19	61.17	56.11	50.82	46.03	40.77	35.94
GE[10] (kcal/100g)	436.27	447.77	454.47	468.87	479.17	482.93	491.23
DE[11] (kcal/100g)	400.00	400.09	399.87	398.93	399.12	399.09	399.60
P:E[12] (mg protein/kcal DE)	50.33	62.64	75.52	88.26	100.62	113.51	125.83

[1]CP_{20} (20% dietary crude protein), CP_{25} (25% dietary crude protein), CP_{30} (30% dietary crude protein), CP_{35} (35% dietary crude protein), CP_{40} (40% dietary crude protein), CP_{45} (45% dietary crude protein), CP_{50} (500% dietary crude protein); [2]Ingredients procured from Himedia Pvt. Ltd., India; [3]Procured from Seacod Oil by Sanofi India Ltd., India; [4]Purchased from local retail shop, India; [5]Vitamin mineral premix was procured from DSM Animal Nutrition and Health, India. [6]Carboxymethyl cellulose [7]Butylated hydroxytoluene, purchased Himedia Pvt. Ltd., India; [8]ROVIMIX® STAY-C®35 purchased from DSM Animal Nutrition and Health, India; [9]Nitrogen free extract; [10]Gross energy; [11]Digestible energy; [12]Protein to energy ratio

acids taking part for synthesis and accretion of somatic tissue protein at lower CP level. However, instead of higher protein utilization, accretion of body protein resulted from lower dietary CP probably could not be sufficient to attribute maximum growth whereas amino acids derived from optimum dietary CP level with optimum P:E value probably could synthesize sufficient body protein for accretion leading to maximum growth of fish. While, feeding higher dietary CP with less non-protein energy sources (carbohydrate and lipid) failed to improve the growth further due to catabolism of excess dietary protein derived amino acids to fulfil energy satiation of body by producing energy rather than taking part in synthesis and accretion of tissue protein attributing lower value of ANPU.

Similar observation was demonstrated by Mohammadi *et al.* (2014) in Nile tilapia reared in low saline IGSW. No mortality was found in any of the experimental groups suggesting neither water quality parameters nor the experimental diets were detrimental for the GIFT juveniles reared in IGSW. The activities of digestive enzymes positively influence the growth of fish probably by enhancing nutrient utilization and secretion also activities of digestive enzymes are dependent on the presence of macronutrients in the digestive tract of fish (Talukdar *et al.,* 2020). In this study, protease and amylase activity (Table 4) exhibited high quadratic (R^2=0.74 and 0.98, respectively) relations with the dietary crude protein in the experimental diet whereas protease activity significantly ($p<0.05$) increased by feeding graded level of dietary protein up to 40% (CP_{40} groups), which might be due to consumption of variable level of crude protein and starch, although activities of protease enzyme was non-significant ($p>0.05$) with Cp_{30}, CP_{35} and Cp_{45} groups. Similar findings were demonstrated by Mohanta *et al.* (2008), Jayant *et al.* (2018) and Talukdar *et al.* (2020) in *Puntius gonionotus, Pangasiodon pangasius* and *Mugil cephalus*, respectively. On the other hand, the intestinal amylase activity significantly ($p<0.05$) reduced in relation to graded level of dietary protein up to 40% (CP_{40} groups) probably due to consumption of comparatively less digestible carbohydrate while increasing of crude protein level in the diet,

Table 2: Whole body proximate composition (% wet weight basis) of GIFT juveniles cultured in 15 ppt inland saline water and fed with heteronitrogenous experimental diets for 60 days

Experimental groups[1]	Moisture	Crude protein	Crude lipid	Total ash	Total carbohydrate
IBC[2]	76.41	13.99	3.98	3.75	1.87
FBC[3]					
CP_{20}	75.04	13.73	5.43^{c}	2.88^{a}	2.92^{a}
CP_{25}	75.18	13.80	5.05^{bc}	2.99^{ab}	2.99^{a}
CP_{30}	74.16	13.88	4.92^{bc}	3.08^{abc}	3.96^{b}
CP_{35}	74.65	14.02	4.32^{ab}	3.22^{bc}	3.79^{b}
CP_{40}	74.59	14.38	3.96^{a}	3.33^{c}	3.74^{b}
CP_{45}	74.89	14.03	5.23^{c}	2.89^{a}	2.96^{a}
Cp_{50}	74.65	13.95	4.42^{ab}	2.97^{ab}	4.01^{b}
SEM[4]	0.12	0.07	0.13	0.04	0.12
Contrast *P* value					
Overall	0.421	0.326	0.005	0.016	0.001
Linear	0.451	0.128	0.011	0.468	0.008
Quadratic	0.247	0.168	0.026	0.003	0.051
Regression equation and R^2 value					
Equation[5]	y = -0.0094x	y = 0.0116x	y = -0.0259x	y = 0.0023x	y = 0.0214x
Linear	+ 75.067	+ 13.565	+ 5.6689	+ 2.9714	+ 2.7339
R^2	0.09	0.35	0.27	0.02	0.21
Equation[5]	$y = 0.0017x^2$	$y = -0.0012x^2$	$y = 0.0025x^2$	$y = -0.0014x^2$	$y = -0.0017x^2$
Quadratic	- 0.1294x +	+ 0.0936x +	- 0.2036x +	+ 0.0976x +	+ 0.1417x +
R^2	76.996 0.32	12.247 0.62	8.5243 0.47	1.4393 0.56	0.8 0.32

Data are expressed as mean (n=3); Mean values in the same column with different superscripts differ significantly ($p<0.05$); [1]CP_{20}-CP_{50}: 20-50% dietary crude protein; [2]IBC: Initial body composition; [3]FBC: Final body composition; [4]SEM: Average standard error of means and [5]In the equation, 'x' and 'y' represent dietary crude protein levels and respective parameters, respectively

Table 3: Growth, nutrient utilisation and survival of GIFT juveniles cultured in 15 ppt inland saline water and fed with heteronitrogenous experimental diets for 60 days

Experimental groups[1]	WG[2] (%)	SGR[3] (%/day)	FCR[4]	ANPU[5] (%)	Survival (%)
CP_{20}	367.88^{a}	2.57^{a}	1.93^{d}	35.19^{d}	100.00
CP_{25}	461.01^{b}	2.87^{b}	1.66^{c}	33.01^{d}	100.00
CP_{30}	528.87^{c}	3.06^{c}	1.52^{b}	30.26^{c}	100.00
CP_{35}	566.43^{d}	3.16^{d}	1.40^{ab}	28.51^{c}	100.00
CP_{40}	667.59^{e}	3.40^{e}	1.30^{a}	27.79^{c}	100.00
CP_{45}	601.11^{d}	3.25^{d}	1.47^{b}	21.04^{b}	100.00
Cp_{50}	575.03^{d}	3.18^{d}	1.51^{b}	18.35^{a}	100.00
SEM[6]	20.65	0.06	0.04	1.29	0.00
Contrast *p* value					
Overall	<0.001	<0.001	<0.001	<0.001	-
Linear	<0.001	<0.001	<0.001	<0.001	-
Quadratic	<0.001	<0.001	<0.001	0.015	-
Regression equation and R^2 value					
Equation[7]	y = 7.4312x	y = 0.0209x	y = -0.0133x	y = -0.5495x	-
Linear	+ 278.18	+ 2.3375	+ 2.0064	+ 46.968	
R^2	0.67	0.68	0.50	0.95	-
Equation[7]	$y = -0.5431x^2$	$y = -0.0016x^2$	$y = 0.0015x^2$	$y = -0.0098x^2$	-
Quadratic	+ 45.45x -	+ 0.1299x +	- 0.118x +	+ 0.1335x +	
R^2	332.83 0.94	0.5857 0.96	3.6886 0.96	35.991 0.97	-

Data are expressed as mean (n=3); Mean values in the same column with different superscripts differ significantly ($p<0.05$); [1]CP_{20}-CP_{50}, 20-50% dietary crude protein; [2]WG: Weight gain; [3]SGR: Specific growth rate; [4]FCR: Feed conversion ratio; [6]ANPU: Apparent net protein utilization; [6]SEM: Average standard error of means and [7]In the equation, 'x' and 'y' represent dietary crude protein levels and respective parameters, respectively

Table 4: Digestive enzymes activities in the intestine of GIFT juveniles cultured in 15 ppt inland saline water and fed with heteronitrogenous experimental diets for 60 days

Experimental groups[1]	Protease[2]	Amylase[3]	Lipase[4]
CP_{20}	0.20[a]	15.96[c]	0.28
CP_{25}	0.21[ab]	15.40[c]	0.27
CP_{30}	0.23[abcd]	14.15[b]	0.25
CP_{35}	0.24[bcd]	14.00[b]	0.25
CP_{40}	0.27[d]	12.91[a]	0.24
Cp_{45}	0.25[cd]	12.51[a]	0.26
Cp_{50}	0.23[abc]	12.32[a]	0.24
SEM[5]	0.30	0.01	0.01
Contrast *p* value			
Overall	<0.001	0.010	0.560
Linear	<0.001	0.010	0.110
Quadratic	0.100	0.010	0.340
Regression equation and R^2 value			
Equation[6]	y = 0.0015x	y = -0.1281x	y = -0.0011x
Linear	+ 0.1804	+ 18.378	+ 0.2932
R^2	0.47	0.96	0.59
Equation[6]	$y = -0.0001x^2$	$y = 0.002x^2$	$y = 6E-05x^2$
Quadratic	+ 0.0118x +	- 0.2688x +	- 0.0054x +
R^2	0.0143 0.81	20.639 0.98	0.3629 0.73

Data are expressed as mean (n=3); Mean values in the same column with different superscripts differ significantly ($p<0.05$); [1]CP_{20}-CP_{50}: 20-50% dietary crude protein; [2]Protease activity is expressed in millimole of tyrosine released/min/mg protein; [3]Amylase activity is expressed in micromole maltose released min^{-1} mg^{-1} protein; [4]Lipase activity is expressed in $unit^{-1}$ h^{-1} mg^{-1} protein; [5]SEM: Average standard error of means and [6]In the equation, 'x' and 'y' represent dietary crude protein levels and respective parameters, respectively

Table 5: DNA and RNA contents and RNA-DNA ratio in the muscle of GIFT juveniles cultured in 15 ppt inland saline water and fed with heteronitrogenous experimental diets for 60 days

Experimental groups[1]	DNA[2] (µg ml^{-1})	RNA[3] (µg ml^{-1})	RNA/DNA[4]
CP_{20}	21.41	8.26[a]	0.39[a]
Cp_{25}	21.12	8.42[a]	0.40[a]
CP_{30}	20.63	9.83[b]	0.48[ab]
CP_{35}	20.81	9.95[b]	0.48[ab]
CP_{40}	21.30	10.52[b]	0.49[b]
CP_{45}	21.86	9.42[ab]	0.43[ab]
CP_{50}	20.98	8.51[a]	0.40[ab]
SEM[5]	0.19	0.22	0.01
Contrast *p* value			
Overall	0.760	0.010	0.070
Linear	0.770	0.130	0.400
Quadratic	0.570	<0.001	<0.001
Regression equation and R^2 value			
Equation[6]	y = 0.0061x	y = 0.0246x	y = 0.0007x
Linear	+ 20.944	+ 8.4129	+ 0.4136
R^2	0.02	0.09	0.03
Equation[6]	$y = 0.0014x^2$	$y = -0.0081x^2$	$y = -0.0004x^2$
Quadratic	- 0.0912x +	+ 0.5912x -	+ 0.03x -
R^2	22.508 0.13	0.6943 0.83	0.0579 0.83

Data are expressed as mean (n=3); Mean values in the same column with different superscripts differ significantly ($p<0.05$); [1]CP_{20}-CP_{50}: 20-50% dietary crude protein; [2]DNA: Deoxyribonucleic acid; [3]RNA: Ribonucleic acid; [4]RNA/DNA, RNA-DNA ratio; [5]SEM: average standard error of means and [6]In the equation, 'x' and 'y' represent dietary crude protein levels and respective parameters, respectively

Table 6: Expression of hepatic IGF-I and IGF-IR genes in GIFT juveniles cultured in 15 ppt inland saline water and fed with heteronitrogenous experimental diets for 60 days

Experimental groups[1]	IGF-I[2]	IGF-IR[3]
CP_{20}	1.01[a]	1.00[a]
CP_{25}	1.45[b]	2.05[b]
CP_{30}	1.91[c]	2.97[c]
CP_{35}	2.48[d]	3.88[d]
CP_{40}	3.16[f]	5.49[f]
CP_{45}	2.70[e]	4.55[e]
Cp_{50}	2.38[d]	4.50[e]
SEM[4]	0.11	0.23
Contrast *p* value		
Overall	<0.001	<0.001
Linear	<0.001	<0.001
Quadratic	<0.001	<0.001
Regression equation and R^2 value		
Equation[5]	y = 0.0561x	y = 0.1287x
Linear	+ 0.1907	- 1.0136
R^2	0.66	0.78
Equation[5]	$y = -0.0039x^2$	$y = -0.0064x^2$
Quadratic	+ 0.3288x -	+ 0.5754x -
R^2	4.1914 0.90	8.1921 0.93

Data are expressed as mean (n=3); Mean values in the same column with different superscripts differ significantly ($p<0.05$); [1]CP_{20}-CP_{50}: 20-50% dietary crude protein; [2]IGF-I: Insulin like growth factor-I; [3]IGF-IR: Insulin like growth factor-I receptor; [4]SEM: Average standard error of means and [5]In the equation, 'x' and 'y' represent dietary crude protein levels and respective parameters, respectively

however, activity of intestinal amylase of this group did not show any significant ($p>0.05$) variation with CP_{45} and CP_{50} groups. To corroborate our finding, Mohanta *et al.* (2008) and Jayant *et al.* (2018) did not found in significant variation in amylase activity due to feeding of varying dietary protein level in *P. gonionotus* and *P. pangasius*, respectively. The activity of intestinal lipases did not show significant variation ($p>0.05$) with moderate linear and quadratic (R^2=0.59 and 0.73) relation among the groups as all the experimental diets contain similar levels of dietary lipid (Talukdar *et al.*, 2020).

Muscle RNA content and RNA/DNA of fish shows positive correlation with the nutritional status to influence tissue protein synthesis and accretion in the body as well as growth of fish, however, muscle DNA content usually remains constant (Kumar *et al.,* 2017). In this study, muscle RNA concentration with high quadratic (R^2=0.83) relation with graded level of dietary protein was significantly higher ($p<0.05$) in 30, 35 and 40% protein fed group with probable influence of more protein synthesis and growth, however, RNA content of these groups were similar ($p>0.05$) to 45% protein fed group. However, muscle RNA/DNA was insignificantly higher ($p<0.05$) with high quadratic (R^2=0.83) relation in CP_{40} group than CP_{20} and CP_{25} groups, but similar ($p>0.05$) to other groups. This finding is positively correlated with growth (WG% and SGR) and hepatic IGF-I and IGF-IR expression. In corroboration with the present findings, Mohanta *et al.* (2008) observed that RNA/DNA ratio increased in *P. gonionotus* with increasing the dietary protein. The DNA content of muscle of fish showed poor linear and quadratic (R^2=0.02 and R^2=0.13) relation with dietary CP levels with non-significant ($p>0.05$) variation among the dietary groups.

This finding is well corroborated with the observations of Mohanta *et al.* (2008) and Kumar *et al.* (2017) in *P. gonionotus* and *Labeo rohita*, respectively (Table 5). Molecular tools provide better insight towards growth and nutrient utilization of fish. Growth hormone induced expression of hepatic IGF-I is commonly influenced by diet quality and nutritional status and nutrient metabolism of animals, including fish (Kumar *et al.,* 2017). The circulatory IGF-I is combined with IGF-binding proteins (IGF-BPs) and transported to targeted tissue where it is activated by IGF-IR to influence the growth of animals including fish (Pierce *et al.,* 2011). The mRNA expression of hepatic IGF-I and IGF-IR (Table 6) with higher quadratic (R^2 =0.90 and 0.93) relations with graded level of dietary CP significantly increased ($p<0.05$) with enhancing dietary protein up to 40% and decreased significantly ($p<0.05$) beyond that.

This finding was positively correlated with the growth pattern of GIFT juveniles where IGF-I in association with IGF-IR probably attributed optimum growth of fish at 40% dietary crude protein level might be through synthesis of tissue protein and accretion at optimum level, however, dietary crude protein level beyond that probably caused metabolic stress to suppress the expression of these genes resulting in reduced growth (Pierce *et al.,* 2011). However, amino acids derived from less than 40%

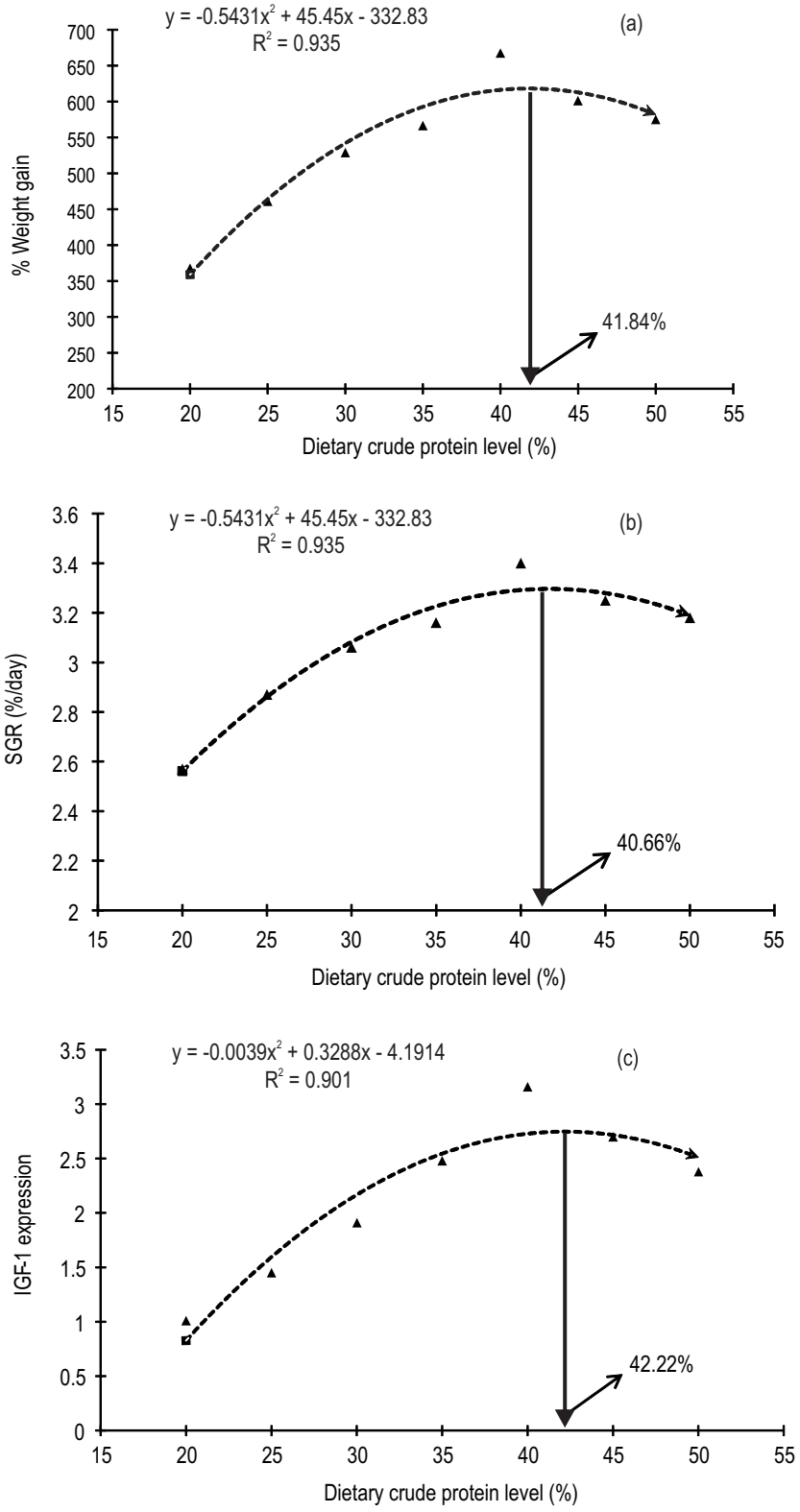

Fig. 1: Second order polynomial regression model indicating the optimum dietary crude protein requirement of GIFT juveniles reared in IGSW of 15 ppt for 60 days. (a) In relation to percentage weight gain (WG%); (b) In relation to specific growth rate (SGR) and (c) In relation to mRNA expression of hepatic IGF-I gene.

dietary protein probably were insufficient to synthesize body protein for exhibiting optimum growth of fish. This result corroborates with the findings of Cruz *et al.* (2006) in Nile tilapia who suggested that hepatic IGF-I expression could be significantly affected by dietary protein level. Moreover, high correlation between hepatic IGF-I and WG% (r=0.99), IGF-IR and WG% (r=0.99) and IGF-I and IGF-IR (r=0.99) indicated that expression of hepatic IGF-I and IGF-IR genes could be good indicators for growth of fish (Kumar *et al.,* 2017) and activation of IGF-I expression probably could be mediated through IGF-I membrane receptor (IGF-IR) to influence growth of fish (Pierce *et al.,* 2011).

In optimization of dietary protein, graded level of dietary protein is tested to ascertain the optimum level in relation to growth because excess amount of protein in the diet does not give extra growth benefit to fish as extra protein derived amino acids are catabolized to satiate the energy demand rather than synthesis and accretion of body tissue protein (De Silva *et al.,* 1989). Among different factors, dietary P: E ratio is the most crucial factor to ensure optimum dietary protein requirement of the animal (Jayant *et al.,* 2018). Moreover, consideration of molecular tool such as hepatic IGF-I expression helps to achieve more accurate dietary protein requirement value as hepatic IGF-I expression and growth of animals including fish are positively correlated. Based on WG%, SGR, hepatic IGF-I expression, second order polynomial regression analysis revealed that optimum dietary crude protein requirement of GIFT juveniles in IGSW of 15 ppt ambient salinity could be 41.84%, 40.66% and 42.22% (Fig. 1a,b,c) with the corresponding P:E value of 105.25, 102.28 and 106.20 mg protein $kcal^{-1}$ DE, respectively. This small difference indicated high correlation among WG%, SGR and hepatic IGF-I expression of GIFT tilapia juveniles. However, Brown *et al.* (2012) reported that IGF-I gene directly stimulates the growth hormones, thus expression of this gene is a specific molecular marker for assessing growth performance of the animal including fish.

The present study concluded that optimum dietary crude protein requirement of GIFT juveniles reared in IGSW with 15 ppt salinity ranged between 40.66 and 42.22% with 400 kcal DE 100 g^{-1} diet to maximize the growth performance. These findings will help to develop an economic and environment-friendly diet for culturing GIFT in IGSW.

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Add-on Information

Authors' contribution: **M. Paul:** Conceptualization; Data curation; Formal analysis; Methodology; Software; Roles/Writing - original draft; Writing-review and editing; **P. Sardar:** Conceptualization; Data curation; Supervision; Validation; Roles/Writing - original draft; Writing-review and editing ; **N.P. Sahu, T. Varghese, N. Shamna, V. Harikrishna:** Conceptualization; Data curation; Roles/Writing - original draft; Writing - review & editing; **A.D. Deo:** Methodology; Set up & monitoring of trial, formal analysis, editing of manuscript; **P. Jana:** Methodology, Formal analysis; Software; Data curation, Writing-review and editing of manuscript; **K.P. Singha, G. Gupta, M. Kumar:** Formal analysis; Software; Writing-review and editing **and G. Krishna:** Conceptualization, Data curation; Roles/Writing-original draft, Writing-review and editing.

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