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Optimum dietary crude protein for culture of genetically improved farmed tilapia (GIFT), *Oreochromis niloticus* (Linnaeus, 1758) juveniles in low inland saline water: Effects on growth, metabolism and gene expression

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ABSTRACT

A 60-day feeding trial was conducted to determine the effects of dietary crude protein (CP) on growth, metabolism and growth-related gene expression in genetically improved farmed tilapia (GIFT), *Oreochromis niloticus* juveniles reared in low inland saline water (ISW) of 5 g/l salinity. Three hundred and fifteen fish (initial weight 2.68 ± 0.01 g) were distributed (15 fish/tank in triplicates) in seven experimental groups (20–50% CP with 5% increment) following completely randomized design (CRD) and fed with seven isolipidic (6%), isoenergetic (16.74 MJ digestible energy/kg) and hetero-nitrogenous (20–50 % CP) purified diets, respectively. Results showed inverse relation between percent weight gain (WG%) and feed conversion ratio which were significantly ($p < 0.05$) varied in overall, linearly and quadratically due to varying dietary CP. Overall and linear trend of protein efficiency ratio and overall, linear and quadratic trend of apparent net protein utilisation were significantly ($p < 0.05$) decreased with increasing dietary CP. Overall, linear and quadratic trend of whole body CP along with overall and quadratic trend of whole body lipid contents were significantly ($p < 0.05$) increased and decreased, respectively with increasing dietary CP up to 35 % and then decreased further. Whereas, overall, linear and quadratic trend of whole body ash content was increased significantly ($p < 0.05$) with increasing dietary CP. Overall and quadratic trend of protease activity in 30–45 % CP fed groups was significantly higher ($p < 0.05$) than 20 and 50 % CP fed groups; but overall and linear trend of amylase activity in 20−25 % CP fed groups was significantly higher ($p < 0.05$) than 35–50 % fed groups. Overall, linear and quadratic trend of hepatic glutamate pyruvate transaminase activity in 40 % CP fed group was significantly lower ($p < 0.05$) than that of 20−25 and 45–50 % CP fed groups and similar to 30–35 % CP fed groups. However, these trends of hepatic lactate dehydrogenase activity of 40–45 % CP fed groups was significantly lower ($p < 0.05$) than 20–30 % fed groups and similar to 35 and 50 % CP fed groups. Whereas, overall, linear and quadratic trend of hepatic malate dehydrogenase in 35 % fed group was significantly lower ($p < 0.05$) than 20–30 % CP fed groups and similar to other groups. The hepatic insulin like growth factor-1 (IGF-1) and

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IGF-1 receptor expression showed high correlation (r=0.92 and 0.90, respectively) with WG%. The optimum dietary protein requirement of GIFT juveniles at 5 g/l salinity in ISW was found to be 34.53–38.10% based on both broken-line linear and second-order polynomial regression with respect to WG% and hepatic IGF-1 expression.

1. Introduction

The ever-growing human population needs sustainable protein sources to meet the increasing food demand. In this context, fisheries and aquaculture can play a pivotal role for global nutritional security of human being. However, stagnation of capture fisheries production necessitates the strengthening of aquaculture to fulfill the protein demands of ever-growing population (FAO, 2016). Though the scope of horizontal expansion of aquaculture is limited, there is a colossal opportunity to expand aquaculture in wasteland or unutilized salt affected inland saline areas. Globally, there is around 380 million ha of land, which has lost its productivity due to salinisation (Allan et al., 2001). In India, there is around 10.1 million ha land comprising 1.93 ha of ground saline water and 8.62 million ha of salt affected land (Lakra et al., 2014; Aklakur, 2017). These vast unutilized lands can be utilized in a judicial and sustainable way through aquafarming with suitable species that will not only help to reduce the underground salinity but also to uplift the livelihood of poor farmers (Allan et al., 2009). Thus, the euryhaline species can be the right choice for culturing in the inland saline water (ISW) regardless the deficiency of potassium ion (K^+) in this water (Allan et al., 2009). Accordingly, the Genetically Improved Farmed Tilapia (GIFT), a strain of *Oreochromis niloticus*, could be one of the most promising species for culture in ISW because of its ability to withstand a wide range of adverse environmental conditions including salinity. Moreover, GIFT is reported to be advantageous in terms of biological traits with reduced production cost by 20–30 % compared to the normal strain of Nile tilapia (Eknath et al., 1998; Dey et al., 2000).

The provision of a nutritionally balanced diet is a crucial prerequisites for sustainable production of any fish species. Therefore, the knowledge of nutritional requirement of the cultivable fish is the utmost criteria to formulate a nutritionally balanced and economically viable aquafeed. Protein, the most expensive macro nutrient of feed, needs to be optimized for economical aquaculture production. In general, Nile tilapia requires 25–45% dietary protein (Khattab et al., 2001; NRC, 2011). There is a linear correlation between growth and dietary protein up to the optimum level and further it may adversely impact the growth of fish (Carneiro et al., 2017; Singha et al., 2020). Because in a high protein diet the non-protein energy sources (carbohydrate and lipid) are less, thus fish catabolizes more absorbed amino acids for energy satiation in which ammonia produced is excreted through gill and keto-acid undergoes energy production *via* gluconeogenic pathway (Philips et al., 1979).

Besides, body indices, activities of digestive and metabolic enzymes are indicators of nutrient digestion and absorption, along with heath condition of the liver and gut (Singha et al., 2020). The dietary nutrient composition regulates the digestive system (Hofer, 1979; Melo et al., 2012) and varying dietary protein modulates the intermediary metabolic pathways by modifying enzymatic activities (Singha et al., 2020). Additionally, dietary protein level influences the activities of hepatic and muscle glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) to indicate the functioning of liver and muscle tissue as well as health status of animals including fish (Dabrowski and Guderley, 2003; Melo et al., 2006). In fish, higher activities of lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) enzymes are considered as outcome of oxidative stress mediated through either dietary or environmental factors (De Silva and Anderson, 1994; Guillaume et al., 2001; Dabrowski and Guderley, 2003). Along with these metabolic enzymes, enhanced activities of the oxidative stress enzymes (*e.g.,* superoxide dismutase, catalase *etc.*) are important index of stress in fish as a consequence of altered homeostasis mediated through dietary or environment factor (Martínez-Álvarez et al., 2005). So, to make the aquaculture venture economical with enhanced production, dietary protein needs to be optimized with optimum level of protein to energy ratio (P:E).

The mitogenic peptides like Insulin-like growth factor 1 (IGF-1) and IGF-1 receptor (IGF-1R) are greatly regulated by the dietary composition (Brown et al., 2012; Singha et al., 2020). Moreover, IGF-1 gene is a good indicator of growth especially for tilapia (Brown et al., 2012). Besides growth *i.e.*, cell proliferation and differentiation, IGF-1 also plays important role in functional modulation of fish immune system (Franz et al., 2016). IGF-1 expression was found to be regulated due to infection or exposure of stress in fish (Franz et al., 2016).

The knowledge and understanding of basic nutritional requirement of fish is undoubtedly important to establish a successful aquaculture practice. The ISW, which is not suitable for agriculture and drinking purpose, can be judicially utilized through aquaculture with production of protein food for nourishing the ever-growing human population. Considering salinity tolerance, GIFT can be preferable for culturing in ISW. However, the ionic imbalance in ISW adversely affects the energy metabolism, which may alter the nutrient requirement especially the protein requirement. Although, the optimum requirement of dietary protein of GIFT juveniles reared at 10 g/l salinity in ISW has been reported recently (Singha et al., 2020), its requirement needs to be optimized under different salinity conditions. The optimum nutrient requirement for growth of fish varies with the salinity probably due to re-orientation of physiological process under altered salinity condition (Tseng and Hwang, 2008). Moreover, there is a wide variation in salinity of ISW seasonally as well as spatially (Allan et al., 2001, 2009; Singha et al., 2020). Hence, it is imperative to study the optimum dietary protein requirement of GIFT juveniles reared under ISW in relation to the salinity. Considering the facts mentioned above, the current study was designed for optimization of dietary CP of GIFT juveniles in ISW at 5 g/l salinity with respect to growth, expression of growth gene, and the activities of digestive and metabolic enzymes.

2. Materials and methods

2.1. Experimental fish

The GIFT juveniles were obtained from Rajiv Gandhi Centre for Aquaculture (RGCA), Visakhapatnam, India, and brought to Rohtak Centre, ICAR-Central Institute of Fisheries Education (ICAR-CIFE), Haryana, India, and transferred to the cemented tanks (4mX3m X 1 m, 10,000 L capacity) containing freshwater with proper aeration. During first 15 days of freshwater acclimatization, a commercial diet (30 % CP) was fed to the fish thrice daily up to satiation level. After that, the salinity of the water was increased daily by 1 g/l to reach the target salinity of 5 g/l by adding ISW and fish were acclimated for 15 days before commencing the feeding trial. The handling and feeding trial of the fish were carried out as per the guidelines of the ethical committee of ICAR-CIFE, Mumbai, India.

2.2. Experimental diets

Seven isolipidic (6%), isoenergetic (16.74 MJ DE/kg), and hetero-nitrogenous purified diets with varying dietary crude protein (20–50 % CP with 5 % increment) were formulated. Casein and gelatin (4:1) were used as purified source of protein; whereas, starch and dextrin were the carbohydrate source. Fish oil and sunflower oil (1:1) was used as lipid source. Laboratory made vitamin-mineral mixture was used to prepare the diet. Other ingredients or additives like choline chloride, cellulose, butylated hydroxytoluene (BHT) and stay C were also used.

All the ingredients excluding oil and additives were weighed as per the formulation and mixed well using a blender to make dough by adding water. Lukewarm water was used to dissolve gelatin to make it a semi-liquid viscous substance and then mixed with other ingredients to make dough, which steam-cooked in a pressure cooker for 20 min. After cooking, the dough was cooled and spreaded and then rest of ingredients *i.e.*, oil and additives were mixed homogenously. A mechanical pelletizer (Uniextrude, S.B. Panchal and Company, India) (1 mm diameter) was used to make pellets from the dough and then air dried overnight and oven dried (at 50°C) further for 12 h and broken into pieces (4−6 mm). Finally, the pellets were stored at 4°C in airtight containers for few days until used for feeding trial.

2.3. Experimental design and feeding trial

The acclimated GIFT juveniles were allocated randomly in seven experimental groups (*i.e.*, CP20, CP25, CP30, CP35, CP40, CP45 and CP50) in triplicate tanks following completely randomized design (CRD) maintaining stocking density of 15 fish per tank (92 cm diameter X45 cm height; 200 l water volume). The average initial weight of fish was 2.68 ± 0.01 g. The fish were fed thrice daily (10:00, 14:00 and 18:00 h) to satiation level for a period of 60 days maintaining 12 h photoperiod. The fish were weighed at 15 days interval to check the growth, general health status and also to adjust the satiation level of feeding. The water exchange rate was 30 % in every three days and the fecal matters were siphoned every day morning.

2.4. Water quality

The water salinity was maintained near the targeted salinity (5 g/l) throughout the study period. The salinity of the water was adjusted during 30 % water exchange every three days interval. Water temperature, pH, dissolved oxygen (DO) and salinity were monitored daily by a thermometer (MERCK, Germany), pH probe (HANNA Instruments, Singapore), DO probe (HANNA Instruments, Singapore) and refractometer (Merck Instruments, Germany), respectively. Other water quality parameters were checked every three days interval. Total hardness, free carbon-dioxide, calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions concentration were determined as per the standard protocols (APHA, 2005). Commercial kit (Spectroquant NOVA-MERCK, Germany) was used to estimate the ammonia-N and nitrite-N level. The potassium ions (K^{+}) concentration of the experimental water was estimated by Flame Photometer (Electronics India, India).

2.5. Sampling

At the start and termination of the experiment, the GIFT juveniles were starved for overnight and weighed for calculating the growth indices. The clove oil (50 μL/l) was used to anaesthetize the fish at the end of feeding trial. All the fish were taken out using a hand-net to take the total body weight of fish using an electronic weighing balance. A total of five fish were collected from each tank (replicate) and oven dried for analysing the proximate composition. Rest of fish from each tank were collected and and three fish were dissected to collect the viscera (excluding kidney) and liver followed by weighing for calculation of body indices or biometric parameters. Gill, intestine, liver and muscle were dissected out from the remaining fish after giving anesthesia and prepared tissue homogenates for enzyme assays. For gene expression study, liver tissue was collected aseptically to store in RNAlater™ solution (Qiagen, Netherlands) in 1 mL cryo-tubes.

2.6. Growth, nutrient utilization and survival

The parameters like percent weight gain (WG%), feed conversion ratio (FCR), protein efficiency ratio (PER), apparent net protein utilisation (ANPU%) and percent survival were calculated by using the following formulae:

$$\text{WG\%} = \frac{\text{Final body weight (wet weight, g)} - \text{Initial body weight (wet weight, g)}}{\text{Initial body weight (wet weight, g)}} \text{X} \quad 100$$

$$\text{FCR} = \frac{\text{Feed intake (dry weight, g)}}{\text{Body weight gain (wet weight, g)}}$$

$$\text{PER} = \frac{\text{Body weight gain (wet weight, g)}}{\text{Protein intake (dry weight, g)}}$$

$$\text{ANPU (\%)} = \frac{\text{Final body protein (wet weight, g)} - \text{Initialbody protein (wet weight, g)}}{\text{Protein intake (dry weight, g)}} \text{X} \quad 100$$

$$\text{Survival (\%)} = \frac{\text{Total number of fish counted at end of feeding trial}}{\text{Number of experimental fish stocked}} \text{X} \quad 100$$

2.7. Biometric parameters

Biometric parameters (*e.g.*, viscerosomatic index, VSI; hepatosomatic index, HSI) were determined by the following formulae:

$$\text{VSI (\%)} = \frac{\text{Weight of the viscera (wet weight, g)}}{\text{Body weight of the fish (wet weight, g)}} \text{X } 100$$

$$\text{HSI (\%)} = \frac{\text{Weight of the liver (wet weight, g)}}{\text{Body weight of the fish (wet weight, g)}} \text{X } 100$$

Table 1
Formulation and proximate composition of different experimental diets.

Ingredients composition (%)	Diets (Experimental groups)						
	20 % CP	25 % CP	30 % CP	35 % CP	40 % CP	45 % CP	50 % CP
Casein[1]	19	24	28.5	33	38	42.5	47.5
Gelatin[1]	4.7	5.6	7	8.4	9.31	10.7	11.65
Starch[1]	52.8	46.9	41	35.1	29.2	23.3	17.35
Dextrin[1]	10	10	10	10	10	10	10
Fish oil[2]	3	3	3	3	3	3	3
Sunflower oil[3]	3	3	3	3	3	3	3
Vitamin–mineral mixture[4]	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Cellulose[1]	3.45	3.45	3.45	3.45	3.44	3.45	3.45
CMC[5]	1.5	1.5	1.5	1.5	1.5	1.5	1.5
BHT[6]	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Choline chloride	1	1	1	1	1	1	1
Stay C[7]	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 matter basis)							
Dry matter (%)	93.99	94.12	94.15	94.21	94.2	94.33	94.3
Crude protein (%)	20.46	25.43	30.44	35.41	40.48	45.42	50.48
Ether extract (%)	6.16	6.14	6.08	6.12	6.11	6.14	6.09
Crude fibre (%)	4.55	4.45	4.47	4.42	4.46	4.46	4.42
Nitrogen free extract (%)	65.68	60.75	55.88	50.82	45.77	40.76	35.82
Total ash (%)	3.15	3.22	3.13	3.23	3.18	3.21	3.19
GE[8] (MJ/kg)	20.72	20.62	20.42	20.30	20.58	20.48	20.88
DE[9](MJ/kg)	16.74	16.74	16.74	16.74	16.74	16.74	16.74
P:E[10] (mg protein/kJ DE)	12.23	15.20	18.19	21.16	24.19	27.14	30.16

[1] Purified ingredients procured from HiMedia Ltd., India.
[2] Procured from Seacod Oil by Sanofi India Ltd., India.
[3] Fortune Refined Sunflower Oil procured from DMart, Mumbai, India.
[4] Composition of the Vitamin-mineral mixture (quantity/kg): Vitamin A, 55,00,000 IU; Vitamin D_3, 11,00,000 IU; Vitamin E, 750 mg; Vitamin K, 1,000 mg; Ascorbic acid, 2500 mg; Vitamin B_2, 2,000 mg; Vitamin B_6, 1,000 mg; Vitamin B_{12}, 6 mg; Calcium pantothenate, 2,500 mg; Nicotinamide, 10 g; Mn, 27,000 mg; I, 1,000 mg; Fe, 7,500 mg; Zn, 5,000 mg; Cu, 2,000 mg; Co, 450 mg; Selenium, 125 mg.
[5] CMC, carboxymethyl cellulose.
[6] BHT, butylated hydroxytoluene.
[7] Stay C, ROVIMIX® STAY-C®35 (DSM in Animal Nutrition & Health).
[8] GE, gross energy.
[9] DE, digestible energy.
[10] P:E, protein to energy ratio.

2.8. Proximate composition of diets and whole body of fish

Proximate composition of the diets (Table 1) and whole body of fish was analyzed according to the AOAC (1995) standard protocols (moisture, 934.01; crude protein, 976.05; ether extract, 945.16; crude fibre, 978.10; total ash, 942.05) at Fish Nutrition Laboratory, ICAR-CIFE, Mumbai, India. The gross energy (GE) of the diets was determined with a bomb calorimeter (5E-AC/PL, Changsha Kaiyuan Instruments Co., Ltd., China) as per the manufacturer's protocol. The digestible energy (DE) (Halver, 1976) and protein to energy ratio (P:E) of the diets were calculated as follows:

$$\text{DE (MJ/kg)} = \frac{\{\text{Crude Protein (\%)X4 + Ether Extract (\%)X9 + Nitrogen Free Extract (\%)X4}\}}{100} \text{X } 4.184$$

$$\text{P : E (mg CP / kJ DE)} = \frac{\text{Crude protein (g/kg)}}{\text{Digestible energy (kJ/kg)}} \text{X } 1000$$

2.9. Enzyme assays

2.9.1. Tissue homogenate

250 mM sucrose solution was used to make 5% tissue homogenates using a tissue homogeniser (REMI Equipments, India) in ice cold condition. Then the homogenates of different tissues (*i.e.*, gill, intestine, liver and muscle) were centrifuged (ThermoFisher Scientific, Germany) at 5000 rpm for 10 min at 4 °C followed by collection of the supernatant in 2 mL eppendorf tubes to store at −20 °C until used.

2.9.2. Tissue protein concentration

The protein content of different tissue was estimated as per the Lowry's method (Lowry et al., 1951). The standard curve was made based on graded concentration of bovine serum albumin (BSA) and the tissue protein concentration was calculated as mg/mL.

2.9.3. The activities of digestive, metabolic and oxidative stress enzymes

The digestive enzymes *viz.,* protease, amylase and lipase activities of intestinal homogenate were estimated by the methods of Drapeau (1974), Rick and Stegbauer (1974), Cherry and Crandell (1932), respectively. Briefly, the protease activity assay of Drapeau (1974) is based on the casein digestion method, whereas, the di-nitrosalicylic acid (DNS) method of Rick and Stegbauer (1974) to assay the amylase activity was used to measure the reducing sugars generated by the action of gluco-amylase and α-amylase on carbohydrate (starch). The lipase activity assay of Cherry and Crandell (1932) was used to determine the fatty acids released due to the enzymatic breakdown of triglycerides from the stabilized olive oil emulsion.

The activities of metabolic enzymes *viz.,* glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) were determined for tissue homogenates of liver and muscle. The GOT and GPT activities were measured by the method of Wooten (1964) where the D, L-aspartic acid and L-alanine were used as substrate, respectively. The lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) activities were determined following Wroblewski and Ladue (1955) and Ochoa (1955) where sodium pyruvate and oxaloacetate solution were used as substrates, respectively.

The enzymes related to oxidative stress *viz.*, superoxide dismutase (SOD) and catalase (CAT) activities were assayed for the tissue homogenate of gill and live. The SOD activity was determined by the method of Misra and Fridovich (1972) based on the oxidation of epinephrine-adrenochrome transition by the enzyme. The CAT activity was determined by following the method of Takahara et al. (1960) where 3% hydrogen peroxide (H_2O_2) solution was used as substrate.

2.10. Gene expression

2.10.1. RNA isolation and cDNA synthesis

Total RNA content of 100 mg liver tissue was isolated by TRIzol reagent (1 mL) (Invitrogen, USA) as per the manufacturer's

Table 2
Primers for expression study of targeted genes in GIFT juveniles reared in low inland saline water of 5 g/l salinity and fed with graded levels of dietary crude protein for 60 days.

Gene	Accession number	Primer sequence	Amplicon size
IGF-1[1]	EU272149.1	FP 5′-GGACGAGTGCTGCTTCCAAAGC-3′ RP 5′-TGCTCTTGGCATGTCTGTGTGC-3′	121
IGF-1R[2]	KC506777.1	FP 5′-GCGACCCAAAGAGCAACAGTGG-3′ RP 5′-TGCCAGATCTCGGTGGACAAAC-3′	130
β-actin[3]	KJ126772.1	FP 5′-AATCCTGCGGAATCCACGAAAC-3′ RP 5′-CTCCTTCTGCATCCTGTCAGCG-3′	140

[1] IGF-1, insulin like growth factor 1.
[2] IGF-1R, insulin like growth factor 1 receptor.
[3] β-actin was used as reference or housekeeping gene.

Table 3
Ranges of physico-chemical parameters of inland saline water in different experimental units during 60 days.

Experimental groups[1]}	Temp[2]}	pH}	Salinity[3]}	DO[4]}	CO_2[5]}	TA[6]}	TH[7]}	NH_3-N[8]}	NO_2-N[9]}	NO_3-N[10]}	Ca^{2+} [11]}	Mg^{2+} [12]}	K^+ [13]}
CP20	28.20 ± 0.38	7.87 ± 0.20	4.93 ± 0.30	5.17 ± 0.46	ND	263.00 ± 10.79	1984.00 ± 77.39	0.05 ± 0.01	0.003 ± 0.001	0.04 ± 0.01	220.00 ± 4.62	286.33 ± 8.41	8.03 ± 0.74
CP25	28.07 ± 0.38	7.90 ± 0.21	4.97 ± 0.26	5.47 ± 0.49	ND	260.00 ± 12.17	1955.67 ± 82.68	0.04 ± 0.02	0.003 ± 0.001	0.06 ± 0.01	222.67 ± 4.33	279.67 ± 4.98	8.13 ± 0.74
CP30	28.27 ± 0.39	7.77 ± 0.18	4.87 ± 0.32	5.17 ± 0.43	ND	261.67 ± 9.82	1926.00 ± 31.53	0.05 ± 0.02	0.003 ± 0.001	0.05 ± 0.01	221.33 ± 2.03	287.00 ± 6.81	8.30 ± 0.70
CP35	28.13 ± 0.38	7.97 ± 0.15	4.83 ± 0.34	5.27 ± 0.43	ND	257.33 ± 10.53	1918.33 ± 57.76	0.05 ± 0.01	0.003 ± 0.001	0.04 ± 0.01	219.33 ± 5.81	282.67 ± 7.84	8.30 ± 0.67
CP40	28.03 ± 0.39	7.93 ± 0.22	5.07 ± 0.30	5.33 ± 0.47	ND	263.33 ± 12.17	1920.00 ± 47.70	0.04 ± 0.01	0.003 ± 0.001	0.06 ± 0.01	225.33 ± 3.48	285.00 ± 8.14	8.43 ± 0.61
CP45	28.23 ± 0.38	8.00 ± 0.26	4.93 ± 0.35	5.57 ± 0.55	ND	260.67 ± 10.27	1933.33 ± 61.94	0.05 ± 0.02	0.002 ± 0.001	0.06 ± 0.01	221.33 ± 3.48	285.33 ± 8.67	8.40 ± 0.62
CP50	28.20 ± 0.38	7.73 ± 0.24	5.07 ± 0.30	5.27 ± 0.49	ND	262.67 ± 12.47	1902.33 ± 57.78	0.04 ± 0.02	0.003 ± 0.001	0.05 ± 0.01	217.33 ± 4.84	280.00 ± 7.23	8.30 ± 0.52
p value	0.999	0.963	0.997	0.995	–	1.000	0.972	0.996	0.996	0.967	0.874	0.984	0.999

All values are expressed as Mean ± SE (n=3); Mean values in the same row with different superscripts differ significantly at 5% probability level ($p < 0.05$).

[1] CP20, 20 % crude protein; CP25, 25 % crude protein; CP30, 30 % crude protein; CP35, 35 % crude protein; CP40, 40 % crude protein; CP45, 45 % crude protein; CP50, 50 % crude protein.

[2] Temp, temperature in ^oC.

[3] Salinity is expressed as g/l.

[4] DO, dissolved oxygen, is expressed as mg/l.

[5] CO_2, free carbon dioxide, is expressed as mg/l (ND, not detected).

[6] TA, total alkalinity, is expressed as mg/l.

[7] TH, total hardness, is expressed as mg/l.

[8] NH_3-N, ammonia nitrogen, is expressed as mg/l.

[9] NO_2-N, nitrite nitrogen, is expressed as mg/l.

[10] NO_3-N, nitrate nitrogen, is expressed as mg/l.

[11] Ca^{2+}, calcium ion, is expressed as mg/l.

[12] Mg^{2+}, magnesium ion, is expressed as mg/l.

[13] K^+, potassium ion, is expressed as mg/l.

Table 4
Growth, nutrient utilisation, survival and biometric parameters of GIFT juveniles reared in low inland saline water of 5 g/l salinity and fed with graded levels of dietary crude protein for 60 days.

Parameters	Experimental groups[1]							SEM[2]	Contrast analysis			Regression analysis			
									p values			Linear		Quadratic	
	CP20	CP25	CP30	CP35	CP40	CP45	CP50		Overall	Linear	Quadratic	Equation*	R^2	Equation*	R^2
WG[3]%	384.97^{a}	472.72^{b}	540.78^{c}	565.71^{c}	563.81^{c}	552.79^{c}	540.47^{c}	13.15	0.000	0.000	0.000	$y = 4.6405x + 354.9$	0.543	$y = -0.4521x^2 + 36.286x - 153.68$	0.929
FCR[4]	1.43^{c}	1.25^{b}	1.14^{a}	1.12^{a}	1.12^{a}	1.15^{a}	1.17^{ab}	0.025	0.000	0.000	0.000	$y = -0.0074x + 1.4543$	0.422	$y = 0.0008x^2 - 0.0655x + 2.3894$	0.827
PER[5]	3.49^{g}	3.20^{f}	2.92^{e}	2.55^{d}	2.24^{c}	1.94^{b}	1.72^{a}	0.138	0.000	0.000	0.548	$y = -0.0609x + 4.7113$	0.978	$y = 0.0002x^2 - 0.0722x + 4.8942$	0.979
ANPU[6]%	49.74^{f}	48.35^{ef}	46.34^{e}	42.79^{d}	35.64^{c}	30.52^{b}	27.01^{a}	1.895	0.000	0.000	0.002	$y = -0.8182x + 68.69$	0.932	$y = -0.0159x^2 + 0.293x + 50.832$	0.958
Survival%	100	100	100	100	100	100	100	0.000	–	–	–	$y = 100$	#N/A	$y = -9E\text{-}16x^2 + 4E\text{-}14x + 100$	#N/A
VSI[7]	8.36^{a}	8.02^{a}	8.61^{ab}	9.19^{ab}	9.69^{b}	8.68^{ab}	8.09^{a}	0.166	0.042	0.411	0.010	$y = 0.0111x + 8.2745$	0.022	$y = -0.0045x^2 + 0.3247x + 3.2352$	0.297
HSI[8]	0.91^{a}	0.93^{a}	0.96^{a}	0.99^{a}	1.20^{b}	1.11^{ab}	1.19^{b}	0.032	0.015	0.001	0.932	$y = 0.0102x + 0.6859$	0.506	$y = 2E\text{-}05x^2 + 0.0085x + 0.7121$	0.506

All values are expressed as Mean (n=3); Mean values in the same row with different superscripts differ significantly at 5% probability level ($p < 0.05$).

[1] CP20, 20 % crude protein; CP25, 25 % crude protein; CP30, 30 % crude protein; CP35, 35 % crude protein; CP40, 40 % crude protein; CP45, 45 % crude protein; CP50, 50 % crude protein.

[2] SEM, average standard error of means.

[3] WG, weight gain.

[4] FCR, feed conversion ratio.

[5] PER, protein efficiency ratio.

[6] ANPU, apparent net protein utilization.

[7] VSI, viscerosomatic index.

[8] HSI, hepatosomatic index.

[*] In the equation, 'x' represents graded levels of dietary crude protein and 'y' represents respective parameters.

protocol. RNA purity (260/280) and concentration was checked in Nano-Drop (Thermo scientific, USA) along with its integrity by electrophoresis. After DNase I (Invitrogen, USA) treatment, the purity of RNA was re-checked and the concentration of RNA was adjusted to 1000 ng/μl. Then the RNA was subjected to reverse transcription for cDNA synthesis using cDNA synthesis kit (iScriptTM, BIO-RAD, USA) as per the manufacturer's protocol and stored at −80 °C until used.

2.10.2. Primer designing

Primers of β-actin, IGF-1 and IGF-1R for qRT-PCR were designed using GeneRunner (version 6.5.48 × 64 Beta) based on the sequences of the NCBI Database (Table 2) and procured from Eurofins Genomics Pvt. Ltd. (Bangalore, India).

2.10.3. Quantitative real time PCR

A 10 μl reaction mixture (5 μl SYBR® Green Master Mix (BIO-RAD, USA), 1 μl gene specific primer (in case of IGF-1 and β-actin 1.25 pmole; and for IGF-1R 5.0 pmole), 1 μl cDNA and 3 μl nuclease free water) was used for qRT-PCR (AriaMx Real-Time PCR System, Agilent Technologies, USA). Forty cycles of reactions were programmed where each cycle comprised 15 s of denaturation (95 °C) with 15 s of annealing (59.3 °C) and 60 s of extension (59.3 °C). The CT (threshold cycle) value was used to express the quantified value of mRNA and the relative gene expression was estimated following Livak and Schmittgen, 2001 (*i.e.,* $2^{-\Delta\Delta CT}$ method) considering the CT value of β-actin as reference gene.

$$\Delta CT = \text{CT value of target gene} - \text{CT value of reference gene}$$

$$\Delta\Delta CT = \Delta CT \text{ value of treatment group} - \Delta CT \text{ value of control group}*$$

*The lowest CP fed group *i.e.*, CP20.

2.11. Statistical analysis

The normality and homogeneity of variance were checked by examining the residual plots. All the data were subjected to one-way analysis of variance (ANOVA) with contrast analysis (SPSS, version 22) where along with overall treatments effects, the polynomial contrast was used to test both linear and quadratic effects of dietary CP on different parameters. The significant variation among the mean values at 5 % probability level ($p < 0.05$) was observed by Duncan's multiple range test (DMRT) under Post-hoc. Regression analysis (linear and quadratic regression) were performed to identify the best fit model (R^2) to represent the data. The parameters with high R^2 value were used to determine the correlation coefficient (r) with growth (percent weight gain). The broken line (Robbins et al., 2006) and second-order polynomial (Jobling, 1994) regression analysis model were fitted to percent weight gain (WG%) and expression of hepatic IGF-1 for determining the optimum dietary CP requirement. Besides, the water quality parameters were subjected to one-way ANOVA and expressed as mean ± SE (standard error).

3. Results

3.1. Water quality parameters

The water temperature, pH, dissolved oxygen (DO), total alkalinity, total hardness, ammonia-N, nitrite-N and nitrate-N in experimental tanks were within the range of 28.07 ± 0.38–28.23 ± 0.38 °C, 7.73 ± 0.24–8.00 ± 0.26, 5.17 ± 0.43–5.57 ± 0.55, 257.33 ± 10.53–263.33 ± 12.17, 1902.33 ± 57.78–1984.00 ± 77.39, 0.04 ± 0.02 to 0.05 ± 0.02, 0.002 ± 0.001 to 0.003 ± 0.001 and 0.04 ± 0.01 to 0.06 ± 0.01 mg/l, respectively (Table 3). The water salinity was within the range of 4.83 ± 0.34–5.07 ± 0.30 g/l. The water ions like Ca^{2+}, Mg^{2+} and K^{+} concentration ranged from 217.33 ± 4.84 to 225.33 ± 3.48, 279.67 ± 4.98 to 287.00 ± 6.81 and 8.03 ± 0.74 to 8.43 ± 0.61 mg/l, respectively.

3.2. Growth, nutrient utilization and survival of fish

Overall, linear and quadratic trends of percent weight gain (WG%) in fish were affected significantly ($p < 0.05$) due to graded levels of dietary CP. But, the regression analysis revealed that quadratic relation ($R^2 = 0.929$) of WG% was more than linear ($R^2 = 0.543$) one with respect to increasing dietary CP level, where it increased significantly up to 30 % dietary CP (CP30); beyond which no further significant change ($p > 0.05$) of WG% was observed (Table 4). The overall, linear and quadratic trends of the FCR values were significantly decreased ($p < 0.05$) with the increasing dietary CP level up to 30 % (CP30 group). The quadratic relation ($R^2 = 0.827$) of FCR with the increasing CP in diet was higher in fish. Overall and linear trends of PER and overall, linear and quadratic trends of ANPU % were affected significantly ($p < 0.05$) in which both PER and ANPU% were significantly ($p < 0.05$) decreased due to increasing dietary CP with higher linear (R^2, 0.978 and 0.932, respectively) and quadratic (R^2, 0.979 and 0.958, respectively) relations (Table 4). No mortality was found in any group during the entire experimental period.

3.3. Biometric parameters

Although VSI of fish was affected significantly ($p < 0.05$) in overall and quadratic trends due to feeding of increasing dietary CP,

Table 5
Whole body proximate composition (on % wet weight basis) of GIFT juveniles reared in low inland saline water of 5 g/l salinity and fed with graded levels of dietary crude protein for 60 days.

Proximate composition	Experimental groups[1]							SEM[2]	Contrast analysis			Regression analysis			
									p values			Linear		Quadratic	
	CP20	CP25	CP30	CP35	CP40	CP45	CP50		Overall	Linear	Quadratic	Equation*	R^2	Equation*	R^2
Moisture	73.99^{b}	74.15^{b}	74.55^{bc}	74.85^{c}	73.20^{a}	74.04^{b}	72.88^{a}	0.156	0.000	0.000	0.000	$y = -0.0349x + 75.171$	0.252	$y = -0.004x^2 + 0.2417x + 70.727$	0.494
Crude protein	14.35^{a}	15.03^{b}	15.68^{c}	16.47^{d}	15.72^{c}	15.57^{bc}	15.59^{bc}	0.147	0.000	0.000	0.000	$y = 0.0345x + 14.281$	0.276	$y = -0.005x^2 + 0.382x + 8.6963$	0.704
Crude fat	6.32^{d}	5.41^{bc}	5.06^{b}	4.07^{a}	6.71^{d}	4.97^{b}	5.93^{cd}	0.202	0.000	0.757	0.001	$y = -0.003x + 5.6002$	0.001	$y = 0.0046x^2 - 0.3244x + 10.766$	0.194
Total carbohydrate	2.52^{b}	2.65^{b}	1.85^{ab}	1.70^{ab}	1.45^{a}	2.18^{ab}	2.43^{b}	0.130	0.073	0.313	0.008	$y = -0.0113x + 2.5084$	0.038	$y = 0.0038x^2 - 0.2801x + 6.8289$	0.363
Total ash	2.82^{ab}	2.76^{a}	2.86^{bc}	2.91^{c}	2.91^{c}	3.24^{d}	3.17^{d}	0.038	0.000	0.000	0.001	$y = 0.0147x + 2.4392$	0.738	$y = 0.0005x^2 - 0.0191x + 2.9826$	0.797

All values are expressed as Mean (n=3); Mean values in the same row with different superscripts differ significantly at 5% probability level ($p < 0.05$).
[1] CP20, 20 % crude protein; CP25, 25 % crude protein; CP30, 30 % crude protein; CP35, 35 % crude protein; CP40, 40 % crude protein; CP45, 45 % crude protein; CP50, 50 % crude protein.
[2] SEM, average standard error of means.
[*] In the equation, 'x' represents graded levels of dietary crude protein and 'y' represents respective parameters.

Table 6
Digestive, metabolic and oxidative stress enzyme activities of GIFT juveniles reared in low inland saline water of 5 g/l salinity and fed with graded levels of dietary crude protein for 60 days.

Enzyme activities		Experimental groups[1]							SEM[2]	Contrast analysis			Regression analysis			
										p values			Linear		Quadratic	
		CP20	CP25	CP30	CP35	CP40	CP45	CP50		Overall	Linear	Quadratic	Equation*	R^2	Equation*	R^2
Protease[3]		0.26^{a}	0.33^{bc}	0.39^{c}	0.39^{c}	0.37^{c}	0.36^{c}	0.30^{ab}	0.012	0.001	0.074	0.000	$y = 0.0013x + 0.2994$	0.061	$y = -0.0005x^2 + 0.0368x - 0.2712$	0.742
Amylase[4]		39.34^{c}	39.35^{c}	34.18^{bc}	28.74^{ab}	24.51^{a}	23.18^{a}	23.60^{a}	1.599	0.000	0.000	0.185	$y = -0.6372x + 52.718$	0.794	$y = 0.0113x^2 - 1.4259x + 65.392$	0.813
Lipase[5]		0.23^{cd}	0.19^{bc}	0.26^{d}	0.18^{bc}	0.21^{bc}	0.17^{ab}	0.13^{a}	0.010	0.001	0.000	0.018	$y = -0.0028x + 0.2932$	0.356	$y = -0.0002x^2 + 0.0102x + 0.0852$	0.473
GOT[6]	Liver	11.78	12.62	13.36	13.69	12.46	12.43	12.16	0.280	0.618	0.974	0.097	$y = -0.001x + 12.674$	6E-05	$y = -0.006x^2 + 0.4172x + 5.9542$	0.171
	Muscle	25.58^{c}	21.21^{abc}	20.96^{abc}	16.79^{a}	22.41^{bc}	23.27^{bc}	18.85^{ab}	0.748	0.014	0.074	0.079	$y = -0.1044x + 24.948$	0.097	$y = 0.0118x^2 - 0.9328x + 38.262$	0.191
GPT[7]	Liver	20.61^{d}	18.10^{cd}	13.22^{ab}	13.44^{ab}	12.28^{a}	15.51^{bc}	15.78^{bc}	0.688	0.000	0.001	0.000	$y = -0.1473x + 20.717$	0.229	$y = 0.0246x^2 - 1.8697x + 48.398$	0.708
	Muscle	24.44^{ab}	25.82^{b}	28.47^{b}	24.39^{ab}	25.85^{b}	25.29^{b}	20.06^{a}	0.711	0.053	0.055	0.016	$y = -0.1202x + 29.11$	0.143	$y = -0.0181x^2 + 1.1464x + 8.7534$	0.386
LDH[8]	Liver	0.164^{d}	0.144^{c}	0.140^{bc}	0.122^{ab}	0.117^{a}	0.116^{a}	0.124^{ab}	0.004	0.000	0.000	0.005	$y = -0.0014x + 0.182$	0.605	$y = 8E-05x^2 - 0.0073x + 0.2761$	0.763
	Muscle	7.62	5.36	6.36	5.70	6.30	6.16	6.13	0.232	0.236	0.350	0.149	$y = -0.0208x + 6.9605$	0.040	$y = 0.0038x^2 - 0.2867x + 11.233$	0.140
MDH[9]	Liver	0.39^{d}	0.35^{cd}	0.32^{bc}	0.23^{a}	0.28^{ab}	0.28^{ab}	0.29^{abc}	0.012	0.001	0.000	0.001	$y = -0.0034x + 0.4242$	0.385	$y = 0.0003x^2 - 0.0263x + 0.792$	0.648
	Muscle	0.71^{c}	0.60^{abc}	0.57^{ab}	0.49^{a}	0.59^{ab}	0.61^{bc}	0.52^{ab}	0.019	0.013	0.010	0.050	$y = -0.004x + 0.7244$	0.226	$y = 0.0003x^2 - 0.0272x + 1.0975$	0.344
SOD[10]	Liver	25.29^{b}	24.88^{b}	25.63^{b}	17.07^{a}	18.47^{a}	18.35^{a}	20.84^{ab}	0.919	0.005	0.001	0.060	$y = -0.2398x + 29.899$	0.341	$y = 0.0143x^2 - 1.2422x + 46.009$	0.432
	Gill	21.62^{b}	19.47^{ab}	20.42^{b}	17.22^{a}	19.10^{ab}	19.81^{ab}	19.86^{ab}	0.398	0.105	0.237	0.030	$y = -0.0421x + 21.119$	0.056	$y = 0.0095x^2 - 0.7074x + 31.811$	0.270
CAT[11]	Liver	128.89^{c}	125.82^{bc}	121.19^{bc}	94.97^{a}	95.94^{a}	105.21^{ab}	112.21^{abc}	3.641	0.018	0.008	0.018	$y = -0.8321x + 141.16$	0.261	$y = 0.083x^2 - 6.6394x + 234.49$	0.456
	Gill	36.46^{b}	30.62^{ab}	30.80^{ab}	28.09^{a}	28.11^{a}	31.63^{ab}	35.76^{b}	0.965	0.077	0.810	0.002	$y = -0.0197x + 32.326$	0.002	$y = 0.0343x^2 - 2.4198x + 70.9$	0.475

All values are expressed as Mean (n=3); Mean values in the same row with different superscripts differ significantly at 5% probability level ($p < 0.05$).

[1] CP20, 20 % crude protein; CP25, 25 % crude protein; CP30, 30 % crude protein; CP35, 35 % crude protein; CP40, 40 % crude protein; CP45, 45 % crude protein; CP50, 50 % crude protein.

[2] SEM, average standard error of means.

[3] Protease activity is expressed as millimole of tyrosine released/min/mg protein (equivalent to 1.67^{-05} katal/mg protein or, 10^{3} U/mg protein).

[4] Amylase activity is expressed as micromole of maltose released/min/mg protein (equivalent to 1.67^{-08} katal/mg protein or, 1 U/mg protein).

[5] Lipase activity is expressed as unit/hour/mg protein (equivalent to 2.78^{-4} katal/mg protein or, 1.67^{4} U/mg protein).

[6] GOT, glutamate oxaloacetate transaminase, activity is expressed as nanomoles of oxaloacetate released/min/mg protein (equivalent to 1.67^{-11} katal/mg protein or, 1.00^{-03} U/mg protein).

[7] GPT, glutamate pyruvate transaminase, activity is expressed as nanomoles of sodium pyruvate released/min/mg protein (equivalent to 1.67^{-11} katal/mg protein or, 1.00^{-03} U/mg protein).

[8] LDH, lactate dehydrogenase, activity is expressed in unit/mg protein/min (equivalent to 1.67^{-08} katal/mg protein or, 1 U/mg protein).

[9] MDH, malate dehydrogenase, activity is expressed in unit/mg protein/min (equivalent to 1.67^{-08} katal/mg protein or, 1 U/mg protein).

[10] SOD, superoxide dismutase, activity is expressed as 50 % inhibition of epinephrine auto-oxidation/mg protein/min (equivalent to 1.67^{-05} katal/mg protein or, 10^{3} U/mg protein); [11]CAT, catalase, activity is expressed as nanomoles H_2O_2 decomposed/min/mg protein (equivalent to 1.67^{-11} katal/mg protein Or, 1.00^{-03} U/mg protein).

* In the equation, 'x' represents graded levels of dietary crude protein and 'y' represents respective parameters.

there was no clear-cut trends of VSI among the experimental groups (Table 4). On the other hand, overall and linear trends of HSI values were significant ($p < 0.05$) in which CP40 and CP50 groups exhibited significantly higher HSI values, which were non-significant ($p > 0.05$) to the value of CP45 group (Table 4).

3.4. Proximate composition of the diets and whole body of fish

The CP level of the experimental diets *viz.*, CP20, CP25, CP30, CP35, CP40, CP45 and CP50 were 20.46, 25.43, 30.44, 35.41, 40.48, 45.42 and 50.48 %, respectively with digestible energy (DE), gross energy (GE), ether extract (EE) and P:E ranges of 16.74 MJ/kg, 20.30–20.88 MJ/kg, 6.08–6.16 %, 12.23–30.16 mg protein/kJ DE, respectively (Table 1).

Overall, linear as well as quadratic trend of moisture, CP and total ash (TA), overall and quadratic trend of crude fat and only quadratic trend of total carbohydrate (TC) were affected significantly ($p < 0.05$) with varying dietary CP levels. The whole body moisture level of fish was gradually increased with the increasing dietary CP level with significantly ($p < 0.05$) higher value in 35 % CP fed group (CP35), which was non-significant ($p > 0.05$) to CP30 group (Table 5). The whole body CP content was affected significantly ($p < 0.05$) in overall, linear and quadratically where the highest CP value was recorded in CP35 group. Additionally, the whole body CP content showed poor linear ($R^2 = 0.276$) and high quadratic relation ($R^2 = 0.704$) with dietary CP level. Though the whole body crude fat content was affected significantly ($p < 0.05$), but it didn't show any significant relation with the dietary CP level in regression analysis. However, significantly ($p < 0.05$) low whole body crude fat was recorded in CP35 group. Though the whole body TC content showed poor quadratic relation ($R^2 = 0.363$) with the dietary CP level, contrast analysis revealed significant ($p < 0.05$) quadratic effect of whole body TC. Overall, linear and quadratic trends of the whole body TA content significantly ($p < 0.05$) varied with ascending manner in relation to graded level of dietary CP. Moreover, whole body TA showed high linear ($R^2 = 0.738$) as well as quadratic ($R^2 = 0.797$) relations with the dietary CP levels (Table 5).

3.5. Enzymes activities

3.5.1. Digestive enzymes activities

The protease activity was varied significantly ($p < 0.05$) with overall and quadratic trends and showed high quadratic ($R^2 = 0.742$) relation with varying dietary CP (Table 6). Protease activity was increased following dietary CP level up to 30 %; however, further increment up to 45 % dietary CP showed no significant variation; moreover, it decreased in CP50 fed group. The overall and linear trends of amylase activity was affected significantly ($p < 0.05$) and exhibited both high linear ($R^2 = 0.794$) and quadratic ($R^2 = 0.813$) relation due to feeding graded level of dietary CP (Table 6). With the opposite trend of protease activity, the amylase activity was reduced significantly ($p < 0.05$) with the increasing CP level up to 35 % and further increment of dietary CP exhibited non-significant ($p > 0.05$) difference of this enzyme activity with CP35 group. The overall, linear and quadratic trends of lipase activity was affected significantly ($p < 0.05$) and showed poor linear ($R^2 = 0.356$) and quadratic ($R^2 = 0.473$) relations with varying dietary CP (Table 6). However, in contrast to protease or amylase activity, there was unclear trend in lipase activity observed due to graded level of dietary CP.

3.5.2. Metabolic enzymes activities

3.5.2.1. Activities of protein metabolic enzymes. The hepatic GOT activity exhibited no significant ($p > 0.05$) variation among different groups (Table 6). Although the overall trend of the muscle GOT activity was affected significantly ($p < 0.05$) due to graded levels of dietary CP, both linear ($R^2 = 0.097$) and quadratic ($R^2 = 0.191$) relations of this enzyme activity was very poor. Significantly lower ($p < 0.05$) activity of muscle GOT was observed in CP35 group, which was non-significant ($p > 0.05$) with CP25, CP30 and CP50 groups.

The hepatic GPT activity was varied significantly ($p < 0.05$) among the different experimental groups with high quadratic ($R^2 = 0.708$) relation to dietary CP level (Table 6). While, only quadratic trend of the muscle GPT activity of experimental groups was significantly ($p < 0.05$) affected by graded levels of dietary CP. However, poor linear ($R^2 = 0.143$) and quadratic ($R^2 = 0.386$) relations of the muscle GPT activity was observed with dietary CP levels.

3.5.2.2. Activities of carbohydrate metabolic enzymes. The contrast analysis showed significant ($p < 0.05$) overall, linear and quadratic effect of dietary CP level on the hepatic LDH activity with moderate linear ($R^2 = 0.605$) and high quadratic ($R^2 = 0.763$) relation between them (Table 6). The hepatic LDH activity was significantly ($p < 0.05$) high in 20 % dietary CP fed group (CP20) followed by CP25 and CP30 groups, however, higher dietary CP fed groups (CP35, CP40, CP45 and CP50) displayed significantly ($p < 0.05$) lower LDH activity. On the other hand, muscle LDH activity was non-significant ($p > 0.05$) among the experimental groups.

Both the hepatic and muscle MDH activities were reduced significantly ($p < 0.05$) with the increasing dietary CP with the overall, linear and quadratic trends in which poor linear ($R^2 = 0.385$) and high quadratic ($R^2 = 0.648$) relations were observed in case of hepatic MDH activity, but both linear ($R^2 = 0.226$) and quadratic ($R^2 = 0.344$) relations were poor in case of muscle MDH activity.

3.5.3. Oxidative stress enzymes activities

. The overall and linear trend of hepatic SOD activity and quadratic trend of gill SOD activity in fish were varied significantly ($p < 0.05$) due to graded level of dietary CP (Table 6). A poor linear ($R^2 = 0.341$) and quadratic ($R^2 = 0.432$) and very poor linear

Table 7
Hepatic IGF-1 and IGF-1R expressions of GIFT juveniles reared in low inland saline water of 5 g/l salinity and fed with graded levels of dietary crude protein for 60 days.

Genes	Experimental groups[1]							SEM[2]	Contrast analysis			Regression analysis			
									p values			Linear		Quadratic	
	CP20	CP25	CP30	CP35	CP40	CP45	CP50		Overall	Linear	Quadratic	Equation*	R^2	Equation*	R^2
IGF-1[3]	1.00^{a}	1.64^{b}	2.04^{c}	3.18^{f}	2.82^{e}	2.44^{d}	2.24^{cd}	0.154	0.000	0.000	0.000	$y = 0.0435x + 0.6743$	0.400	$y = -0.0053x^2 + 0.4132x - 5.2664$	0.843
IGF-1R[4]	1.04^{a}	2.08^{b}	2.93^{c}	5.04^{e}	4.89^{e}	3.82^{d}	3.20^{c}	0.306	0.000	0.000	0.000	$y = 0.0851x + 0.3085$	0.386	$y = -0.0107x^2 + 0.8326x - 11.705$	0.842

All values are expressed as Mean (n=3); Mean values in the same row with different superscripts differ significantly at 5 % probability level ($p < 0.05$).

[1] CP20, 20 % crude protein; CP25, 25 % crude protein; CP30, 30 % crude protein; CP35, 35 % crude protein; CP40, 40 % crude protein; CP45, 45 % crude protein; CP50, 50 % crude protein.

[2] SEM, average standard error of means.

[3] IGF-1, insulin like growth factor 1.

[4] IGF-1R, insulin like growth factor 1 receptor.

[*] In the equation, 'x' represents graded levels of dietary crude protein and 'y' represents respective parameters.

($R^2 = 0.056$) and poor quadratic ($R^2 = 0.270$) relations of hepatic and gill SOD activities were recorded, respectively with the dietary CP level. The CP35, CP40, and CP45 groups displayed significantly ($p < 0.05$) lower hepatic SOD activity than the CP20, CP25 and CP30 groups; however, the enzyme activity of CP50 group was non-significant ($p > 0.05$) with any other group. However, no clear trend was encountered for the gill SOD activity of fish.

On the other hand, the overall, linear and quadratic trend of hepatic CAT and only quadratic trend of gill CAT activities of fish were affected significantly ($p < 0.05$) due to graded level of dietary CP in which hepatic CAT activity showed poor linear ($R^2 = 0.261$) and quadratic ($R^2 = 0.456$) relationship. The CP35 and CP40 groups exhibited significantly ($p < 0.05$) reduced hepatic CAT activity than the CP20, CP25 and CP30 groups; whereas, CP35, CP40 and CP50 groups displayed significantly ($p < 0.05$) lower gill CAT activity than the CP20 group.

3.6. Expression of hepatic IGF-1 and IGF-1R genes

The expression of hepatic IGF-1 and IGF-1R were affected significantly ($p < 0.05$) due to dietary CP in overall, linear and quadratic analysis (Table 7). There was high quadratic relation of IGF-1 ($R^2 = 0.843$) and IGF-1R ($R^2 = 0.842$) expression with the dietary CP. However, expression of both the genes showed poor linear ($R^2 = 0.400$ and 0.386, respectively) relation with dietary CP level. The IGF-1 expression was increased following dietary CP levels up to 35 % and further increase in dietary CP caused a decreasing trend of this gene expression. Similarly, the hepatic IGF-1R expression also displayed an increasing trend following dietary CP up to 35 % which was non-significant ($p > 0.05$) to the CP40 group. However, lower expression of IGF-1R was encountered due to further increase in dietary CP.

3.7. Correlation of nutrient utilisation, physio-metabolic and molecular response with growth

Among the different parameters selected, whole body CP and TA, protease activity, expression of hepatic IGF-1 and IGF-1R exhibited positive correlation with growth (WG%) (Table 8). Whereas, FCR, PER, ANPU%, amylase, liver GPT and LDH activities showed negative correlation with growth.

3.8. Dietary protein requirement

According to the broken-line linear regression analysis, the optimum dietary CP requirement of GIFT juveniles reared in low inland saline water of 5 g/l salinity was 34.53 (Fig. 1) and 35.35 % (Fig. 2) with respect to WG% and expression of hepatic IGF-1, respectively.

Table 8
Correlation (r = correlation coefficient) of nutrient utilization, physio-metabolic and molecular response with growth in GIFT juveniles reared in low inland saline water of 5 g/l salinity and fed with graded levels of dietary crude protein for 60 days.

Parameters[1]	Correlation[2] (r) with growth (WG[3]%)	Interpretation as strength of association[4]
FCR[5]	−0.99	Negatively high
PER[6]	−0.76	Negatively high
ANPU[7]%	−0.62	Negatively moderate
WBCP[8]%	0.92	Positively high
WBTA[9]%	0.49	Positively low
Protease[10]	0.81	Positively high
Amylase[11]	−0.79	Negatively high
Liver GPT[12]	−0.92	Negatively high
Liver LDH[13]	−0.93	Negatively high
IGF-1[14]	0.92	Positively high
IGF-1R[15]	0.90	Positively high

[1] Parameters which showed high (≥0.7) regression coefficient (R^2) in with dietary crude protein.
[2] Only the mean (n = 3) values are considered for correlation analysis.
[3] WG, weight gain.
[4] Strength of association (both for positive and negative), high, ≥0.7; moderate, ≥0.5 to <0.7; low, ≤0.5.
[5] FCR, feed conversion ratio.
[6] PER, protein efficiency ratio.
[7] ANPU, apparent net protein utilization.
[8] WBCP, whole body crude protein.
[9] WBTA, whole body total ash.
[10] Intestinal protease activity.
[11] Intestinal amylase activity.
[12] GTP, glutamate pyruvate transaminase.
[13] LDH, lactate dehydrogenase.
[14] IGF-1, insulin like growth factor 1.
[15] IGF-1R, insulin.

However, the second-order polynomial regression analysis showed that the optimum dietary CP requirement of GIFT juveniles under same rearing condition was 38.10 (Fig. 1) and 37.03 % (Fig. 2) with respect to WG% and expression of hepatic IGF-1, respectively.

4. Discussion

4.1. Water quality parameters

Water quality parameters are the critical factors to maintain the general homeostasis of the aquatic organisms. In this study, the experimental water temperature, pH, DO were within the normal range for tilapia culture (Ross, 2000). The salinity of the ISW was maintained around 5 g/l throughout the feeding trial. Due to its wide range of salinity (0–36 g/l) tolerance (El-Sayed, 2006), tilapia is the most preferable species for rearing in ISW where salinity fluctuates tremendously. However, nutrient requirement of tilapia may vary with the changes of salinity in the rearing water to maintain its homeostasis. The values of total alkalinity and hardness were within the normal range of ISW (Lakra et al., 2014; Singha et al., 2020). Though the total alkalinity is higher than reported optimum range (20–200 mg/l) (Setiadi et al., 2018), but tilapia can be reared in higher total alkalinity (Allan et al., 2009). The ranges of ammonium-N, nitrite, and nitrate were found to be within normal level (Mjoun et al., 2010). The water ions *viz.,* Ca^{2+}, Mg^{2+} and K^{+} concentrations were found to be within their normal ranges in ISW (Allan et al., 2009).

4.2. Growth, nutrient utilization and survival of fish

Growth of animals including fish is a phenotypic expression of muscle hyperplasia that is controlled by both nutritional and environmental factors (Singha et al., 2020). Among the nutritional factors, optimum dietary protein is crucial to maximize the growth of fish. The ISW with low salinity encompasses a variety of differences with freshwater, brackishwater as well as marine water in terms of physico-chemical parameters, may play prime role in regulating the growth of fish. In our study, the non-significant ($p > 0.05$) changes in WG% of GIFT juveniles beyond 30 % dietary CP indicates that the excess dietary CP did not support growth probably as the amino acids derived from excess protein were catabolized for energy production and energy demand of fish was consequently increased due to excretion of more ammonia (Philips et al., 1979). Similarly, the highest growth in all-male Nile tilapia in inland saline water (8 g/l salinity) was reported at 29 % dietary CP fed group (Mohammadi et al., 2014). However, Santiago et al. (1982) found the best growth of Nile tilapia fry at 35 % dietary CP in freshwater. The result of WG% with respect to dietary protein is supported by our recent study on GIFT juveniles at 10 g/l salinity in ISW (Singha et al., 2020).

The inverse trend of FCR values with the WG% and no further significant change ($p > 0.05$) in FCR beyond 30 % CP indicates that increasing dietary CP up to optimum level (30 % CP in this case) could improve the feed utilization, but further increment of dietary CP could not change the feed utilization probably due to the fact that amino acids absorbed from excess dietary CP were not directed towards body protein synthesis, rather catabolized for production of energy. Santiago et al. (1982) and Siddiqui et al. (1988) also reported similar trend of FCR in tilapia with the increasing dietary CP. Moreover, the negative correlation (r=−0.99) between growth and FCR indicates the maximize utilization of diet for growth purpose in group fed with protein near to optimum level. The decreasing trend of both PER and ANPU values with respect to increasing dietary CP indicating maximum utilisation of dietary CP for synthesis of body protein at lower feeding level. However, accretion of synthesized body protein from amino acids derived from lower dietary CP was insufficient to attribute maximum growth, though protein utilization (PER and ANPU) was higher, whereas amino acids derived from optimum dietary CP with optimum P:E may result sufficient synthesis and deposition of protein in body to maximum growth of

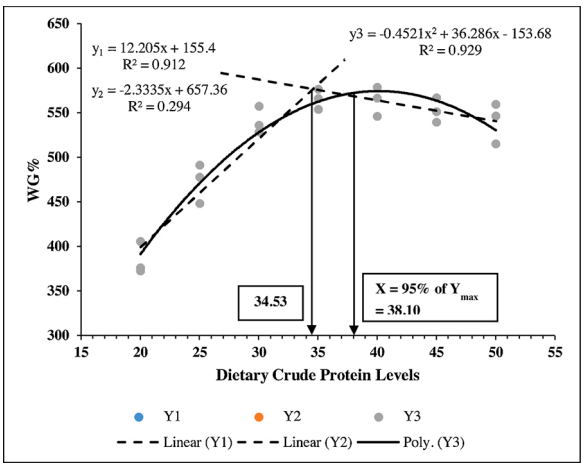

Fig. 1. The broken-line linear (dash line) and second-order polynomial (solid line) regression to optimise the dietary crude protein requirement in relation to percent weight gain (WG%) of GIFT juveniles reared in low inland saline water of 5 g/l salinity and fed with graded levels of dietary crude protein for 60 days.

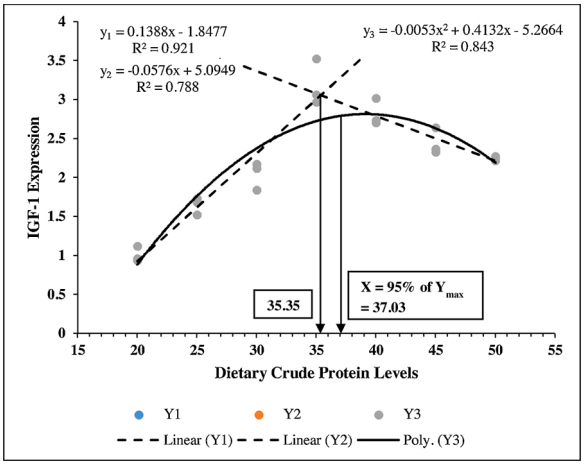

Fig. 2. The broken-line linear (dash line) and second-order polynomial (solid line) regression to optimise the dietary crude protein requirement in relation to hepatic IGF-1 gene expression of GIFT juveniles reared in low inland saline water of 5 g/l salinity and fed with graded levels of dietary crude protein for 60 days.

fish. On the other hand, feeding of higher dietary CP with less non-protein energy source (carbohydrate and lipid) could not able to improve growth further because amino acids derived from excess dietary protein probably did not participate in body protein synthesis, rather catabolized for meeting the energy demand causing lower values of PER and ANPU in fish. Additionally, the negative correlation of PER (r=−0.76) and ANPU (r=−0.62) with growth could be due to maximum utilisation of dietary protein by lower protein fed groups, but due to lower dietary CP the growth cannot be obtained as like groups fed with protein near to optimum level. Shiau and Huang (1990) and Singha et al. (2020) also reported similar findings in case of hybrid tilapia and GIFT juvenile under seawater and ISW condition, respectively. No mortality during the entire experiment period suggested the suitability of GIFT strains as a candidate species for culture in inland saline water.

4.3. Biometric parameters

The biometric parameters provide information about general condition of different organs in fish. The VSI values recorded in different experimental groups were within the accepted range of Nile tilapia (Kaushik et al., 1995). Whereas, the higher HSI values with enhanced liver size and weight among higher dietary CP fed groups probably mediated through excess glycogen and lipid deposition in liver and/or excessive metabolic activity of liver to catabolise amino acids derived from excess dietary CP (Jayant et al., 2018; Singha et al., 2020).

4.4. Proximate composition of whole body of fish

The whole body proximate composition not only provides information about nutritional quality (Njinkoue et al., 2016) but also gives the information about the nutrient utilization efficiency and health status of fish. The increased whole body CP following the increasing dietary CP level up to 35 % followed by significant decrease of whole body CP might be due to the fact that 35 % dietary CP being optimum level, which supported the maximum growth of fish *via* synthesis and accretion of sufficient body protein from dietary CP derived amino acid, however, amino acids derived from excess dietary CP probably could participate in the catabolism resulting no further change of growth in fish as observed for the growth parameters. Similar observations were also reported in different strains of Nile tilapia due to feeding of grade levels of dietary CP under freshwater (Siddiqui et al., 1988) and ISW of 8 g/l (Mohammadi et al., 2014) and 10 g/l (Singha et al., 2020) salinity. The positive correlation (r = 0.92) of whole body CP with growth may be due maximum body protein accretion and synthesis in the groups showed improved growth which fed with near to optimum protein level. The significant ($p < 0.05$) increase of whole body ash content due to feeding of increasing levels of dietary CP is also supported by Nwanna et al. (2014). However, in contrast to our observation, Ahmed and Maqbool (2017) reported that the whole body ash content of Nile tilapia significantly ($p < 0.05$) enhanced following the increasing dietary CP level. But, Nwanna et al. (2014) and Jauncey (1982) could not find any significant ($p > 0.05$) change of whole body ash content due to graded levels of dietary CP. However, exact reason for these variable observations under similar feeding regime remains unclear. In addition to this, we did not get proper reason for positive correlation (r=0.49) of whole body TA with growth.

4.5. Enzymes activities

4.5.1. Digestive enzymes activities

There is a positive correlation between digestive enzymes activities and the growth of fish (Mozanzadeh et al., 2018). Moreover,

secretion of digestive enzymes is associated with the presence of macronutrients in the digestive system of animals including fish (Melo et al., 2012) for their utilization to the maximum extent. The inverse relation between protease and amylase activities probably due to feeding of variable levels of dietary CP and NFE (digestible carbohydrate). Whereas, lipase activity did not show any significant relations with the dietary CP level might be due to the reason that the experimental diets were isolipidic. The increase in protease activity following the increasing dietary CP level up to 30 % and no further change of this enzyme activity beyond this level was supported by the reports on *Tor khudree* (Bazaz and Keshavanath, 1993), *Labeo rohita* (Kumar et al., 2011), and *Pangasiodon hypopthalmus* (Jayant et al., 2018). Whereas, the amylase activity displayed a decreasing fashion both linearly and quadratically in relation to increasing dietary CP level which may be explained by the lower availability of carbohydrate (starch) in the digestive tract of higher dietary CP fed groups. Similarly, lowered amylase activity in *Labeo rohita* (Mohapatra et al., 2003), *Pangasiodon pangasius* (Jayant et al., 2018) and GIFT juvenile (Singha et al., 2020) of higher protein fed groups was reported when fed with varying dietary CP. Moreover, the positive and negative correlation of protease ($r = 0.81$) and amylase ($r=-0.79$) activities with growth, respectively is due to improved protease activity with growth and higher activity of amylase in lower protein fed groups because of higher carbohydrate content in these groups. In contrast to other reports (Kumar et al., 2011; Jayant et al., 2018), the significant ($p < 0.05$) changes in lipase activity of GIFT juveniles was observed in the current study.

4.5.2. Metabolic enzymes activities

4.5.2.1. Activities of protein metabolic enzymes. The GOT and GPT activities of liver and muscle are associated with both synthesis and breakdown of non-essential amino acids in animals including fish depending on the availability of dietary non-protein energy (Ye et al., 2017). In the current study, only hepatic GPT activity exhibited high quadratic relation ($R^2 = 0.708$) with the dietary CP where higher activity was found in low (20 and 25 % CP) and high (45 and 50 % CP) protein fed groups might be due to the need of more body protein synthesis in presence of sufficiently higher non-protein energy and more energy production in presence of sufficiently lower non-protein energy for low and high protein fed groups, respectively. However, the fish of 30–40 % dietary CP fed groups exhibited the moderate hepatic GPT activity, which was non-significantly ($p > 0.05$) among themselves probably due to feeding of the diets with optimum level of dietary P:E. Due to these reasons, low and high protein fed group exhibited higher and lower protein utilization efficiency, respectively in terms of PER and ANPU values. This corroborates with the report that the higher activity of hepatic GPT in Nile tilapia fed with varying dietary CP (Gaye-Siessegger et al., 2006).

4.5.2.2. Activities of carbohydrate metabolic enzymes. Under oxidative stress condition the fish tissue becomes hypoxic, thus the pyruvate, the end product of glycolysis cannot be catabolized further *via* TCA cycle for production of energy, which is rather produced through catabolizing the pyruvate to lactate by LDH enzyme (Murray et al., 2000). In the present study, the higher hepatic LDH activity was found in low dietary protein (20 and 25 % CP) fed groups might be due to high dietary carbohydrate mediated oxidative stress in fish. Moreover, less availability of free amino acids (organic osmolytes) probably could not maintain the optimum osmoregulation leading to osmotic stress to aggravate the situation in fish of these groups. Due to the same reasons, hepatic MDH activity was higher in low dietary CP fed groups in which hepatic MDH probably could convert cytosolic oxaloacetate to malate leading to higher gluconeogenesis (Hemre et al., 2002) to satisfy the stress mediated high energy demand of these groups. However, except hepatic MDH, the hepatic and muscle LDH and muscle MDH activities didn't show significant ($p > 0.05$) variation in *Mugil cephalus* fingerlings fed with varying protein levels in ISW of 8 g/l salinity where the lower activity of hepatic MDH was found in lower protein fed groups (Talukdar et al., 2020).

4.5.3. Oxidative stress enzymes activities

Higher activities of SOD and CAT enzymes are the indicators of oxidative stress in animals including fish which act as first line antioxidant defense. SOD catalyzes reactive oxygen species (ROS) to hydrogen peroxide (H_2O_2), which is subsequently neutralized by CAT with the production of molecular oxygen and water to prevent ROS mediated cellular damage (Martínez-Álvarez et al., 2005; Ighodaro and Akinloye, 2018). The higher SOD and CAT activities in both liver and gill in the case of low protein fed groups might be due to osmotic stress along with high dietary carbohydrate mediated stress. In corroboration to our finding, the activities of hepatic SOD and CAT in Siberian sturgeon (*Acipenser baerii*) was higher in case of lower protein fed groups (Babaei et al., 2017).

4.6. Expression of hepatic IGF-1 and IGF-1R genes

IGF-1, being the most suitable molecular marker for growth of tilapia (Brown et al., 2012), was found to be influenced by nutritional health status and metabolism of organism (Tatar et al., 2003). The significant ($p < 0.05$) changes in hepatic IGF-1 expression with high quadratic relation ($R^2 = 0.843$) to dietary CP level supported its positive relation with growth of GIFT juveniles. The significant ($p < 0.05$) increase of hepatic IGF-1 expression linearly following the increasing dietary CP up to 35 % followed by significant ($p < 0.05$) decrease due to further increment of dietary CP indicated that dietary CP higher than optimum level did not exhibit any added benefit to fish in terms of growth probably due to catabolism of amino acids derived from extra dietary CP instead of participating in synthesis of body protein to reflect the growth of fish (Jiang et al., 2010; Qiang et al., 2012; Singha et al., 2020). Similarly, the expression of hepatic IGF-1R exhibited high quadratic relation ($R^2 = 0.842$) to dietary CP level in which expression of this gene was increased significantly ($p < 0.05$) with the increasing dietary CP up to 35–40 % followed by significant ($p < 0.05$) reduction in expression due to further increment of dietary CP level. The similar trend of expression between IGF-1 and IGF-1R genes in

GIFT juveniles might be due to the fact that the IGF-1R gene being a membrane receptor probably activates the IGF-1 gene for its better expression (Pierce et al., 2011).

4.7. Correlation of nutrient utilisation, physio-metabolic and molecular response with growth

The negative correlation between growth and FCR indicates the maximize utilisation of diet for growth purpose in group fed with protein near to optimum level. However, the negative correlation of PER and ANPU with growth could be due to maximum utilisation of dietary protein by lower protein fed groups, but due to lower dietary CP the growth cannot be obtained as like groups fed with protein near to optimum level. The positive correlation of whole body CP with growth may be due maximum body protein accretion and synthesis in the groups showed improved growth which fed with near optimum protein level as already discussed above (section *4.4*). However, we did not get proper reason for positive relation of whole body TA with growth. The positive and negative correlation of protease and amylase activities with growth, respectively is due to improved protease activity with growth and higher activity of amylase in lower protein fed groups due to relatively high carbohydrate content in these groups. The positive correlation of expression of hepatic IGF-1 and IGF-1R with growth clearly indicates that these mitogenic peptides are good indicators of growth.

4.8. Optimum dietary protein requirement

These narrow difference in dietary protein requirement with respect to WG% (34.53 and 38.10 %) and IGF-1 expression (35.35 and 37.03 %) indicates that growth and growth related gene expression are closely related. Moreover, narrow overall range of protein requirement value (34.53–38.10 %) indicated that there was little overestimation of optimum requirement that might have happened because of partial utilisation of protein for energy production instead of growth (Millikin, 1982). The use of protein for production of energy is very common in aquatic animals because fish utilize dietary protein for energy production more efficiently than the terrestrial animals. Moreover, in ISW where the need of extra energy for maintaining osmoregulation of fish may be satisfied through the oxidation of protein derived amino acids.

Besides, water salinity affects the dietary protein requirement of fish (Boeuf and Payan, 2001) where higher requirement of dietary protein in higher salinity as compared to freshwater mostly due to excess energy need for osmoregulation (Sardar et al., 2019). Nile tilapia fry fed diet with graded levels of protein (20, 30, 40 and 50 %) and reared in four salinities (0, 15, 20 and 25 g/l) showed best growth at 30 % and 40 % dietary CP reared up to 15 and 20−25 g/l salinity, respectively (Larumbe-Morán et al., 2010). Similarly, we found 37.37–40.92 % dietary protein required for GIFT juveniles of similar size (2.68 ± 0.01 g) reared in ISW of 10 g/l salinity (Singha et al., 2020) which is higher than the present study.

5. Conclusion

In conclusion, it can be stated that most of the parameters studied (*i.e.,* WG%, FCR, PER, ANPU, whole body CP and total ash content, protease and amylase activities, hepatic GPT and LDH activities, expression of hepatic IGF-1 and IGF-1R genes) exhibited significantly ($p < 0.05$) high quadratic relation ($R^2 \geq 0.7$) with the dietary CP level, although some parameters (*i.e.,* PER, ANPU, whole body total ash and amylase activity) showed high linear relation ($R^2 \geq 0.7$). The high quadratic relation ($R^2 \geq 0.7$) between dietary CP level and different parameters suggests that the dietary CP is optimally utilized in fish with the expression of most of the studied parameters in a specific trend. Finally, within the range of 34.53–38.10% dietary CP is found to be optimum for GIFT juveniles reared in ISW of 5 g/l salinity based on both the broken-line linear and second-order polynomial regression analysis with respect to WG% and expression of hepatic IGF-1 gene. This baseline information will be utilized for future nutritional study and feed development for GIFT juveniles in ISW condition.

CRediT authorship contribution statement

Krishna Pada Singha: Conceptualization, Data curation, Formal analysis, Methodology, Software, Writing - original draft, Writing - review & editing. **Naseemashahul Shamna:** Conceptualization, Data curation, Supervision, Validation, Writing - original draft, Writing - review & editing. **Narottam Prasad Sahu:** Conceptualization, Data curation, Writing - original draft, Writing - review & editing. **Parimal Sardar:** Conceptualization, Data curation, Writing - original draft, Writing - review & editing. **Vungarala Harikrishna:** Methodology, Writing - original draft, Writing - review & editing. **Rajasekaran Thirunavukkarasar:** Formal analysis, Software, Writing - original draft, Writing - review & editing. **Dilip Kumar Chowdhury:** Formal analysis, Software, Writing - original draft, Writing - review & editing. **Manas Kumar Maiti:** Formal analysis, Software, Writing - original draft, Writing - review & editing. **Gopal Krishna:** Conceptualization, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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