Aquaculture 525 (2020) 735306

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Feeding graded levels of protein to Genetically Improved Farmed Tilapia (GIFT) juveniles reared in inland saline water: Effects on growth and gene expression of IGF−I, IGF-IR and IGF-BPI

Krishna Pada Singha, N. Shamna*, Narottam Prasad Sahu, Parimal Sardar, V. HariKrishna, R. Thirunavukkarasar, Munish Kumar, Gopal Krishna

ICAR-Central Institute of Fisheries Education, Versova, Mumbai 400 061, India

ARTICLE INFO

Keywords:
Growth performance
IGF-I
Inland saline water
Optimum protein level
Tilapia (GIFT)

ABSTRACT

A 60-day feeding trial was conducted on Genetically Improved Farmed Tilapia (GIFT) juveniles in inland saline water (ISW) of 10 ppt salinity. Seven isoenergetic (400 kcal DE/100 g), isolipidic (6%) and hetero-nitrogenous (20, 25, 30, 35, 40, 45 and 50% CP) experimental diets were formulated and prepared using purified ingredients. Three hundred and fifteen GIFT juveniles (average body weight 2.68 ± 0.01 g) were distributed in seven experimental groups *viz.*, T20 (20% CP), T25 (25% CP), T30 (30% CP), T35 (35% CP), T40 (40% CP), T45 (45% CP) and T50 (50% CP) in triplicates following a completely randomized design (CRD) with the stocking density of 15 fish/tank (92 cm diameter X 45 cm height) with 200 l water volume. Fishes of different experimental groups were fed with respective experimental diets thrice daily at satiation level. Results indicated that dietary crude protein has significant ($p < .05$) overall, linear and also quadratic effect on final body weight, percent weight gain (WG%) and feed conversion ratio (FCR) with higher and lower value in T35, T40, T45 and T50 groups, respectively. The protein efficiency ratio (PER) and apparent net protein utilisation (ANPU%) values of fish were decreased with the increasing dietary CP level. The whole body crude protein content was affected linearly as well as quadratically ($p < .05$) with higher value in T30, T35 and T40 groups, whereas whole body ether extract content of the fish were significantly ($p < .05$) decreased with the increasing the dietary CP level. Among digestive enzymes, protease and amylase activities were significantly ($p < .05$) affected due to dietary crude protein linearly as well as quadratically. Though, the expression of insulin like growth factor binding protein I (IGF-BPI) did not show any defined trend, the expression of insulin like growth factor I (IGF−I) and Insulin like growth factor I receptor (IGF−IR) were highly correlated with WG% ($r = 0.92$ and $r = 0.97$, respectively). The expression of IGF−IR was also correlated with IGF-I expression ($r = 0.93$). According to broken-line and second-order polynomial regression analysis in relation to WG% and expression of hepatic IGF-I gene, the optimum dietary crude protein levels of GIFT juveniles reared in inland saline water of 10 ppt salinity were found to be 37.37, 37.72, 40.92 and 38% crude protein with isoenergetic of 400 kcal DE/100 g diet, respectively.

1. Introduction

Aquaculture has become the fastest growing sector among agriculture and the allied food production sectors (FAO, 2016). But, horizontal expansion of the coastal aquaculture has limited scope due to urbanization and industrialization. Hence, it is essential to utilize the underutilized resources like non-productive agriculture lands for aquaculture production in order to fulfill the increasing demand of fish. Moreover, around 380 million ha fertile agricultural land in the globe has become un-productive due to man-made and/or natural (ground saline water) salinization (Allan et al., 2001; Lambers, 2003; Boyd et al., 2009). Converting this land for aquaculture purpose can be a right strategy not only to reduce the salt content in underground water tables but also to generate income through enhanced production of euryhaline and marine fish species with high growth potential (Allan et al., 2009). Unlike the seawater, the ionic composition of inland saline water varies widely; the ionic profile may vary even if the water come from same aquifer. In most of the cases, the inland saline water is deficient in potassium ion (Allan et al., 2001) which plays an important role in physiology of the aquatic animals (Shiau and Hsieh, 2001). In this condition, tilapia being the wide salinity tolerant and fast-growing fish could be the suitable species for inland saline aquaculture and GIFT

* Corresponding author at: Fish Nutrition, Biochemistry & Physiology Division, ICAR-Central Institute of Fisheries Education, Versova, Mumbai 400 061, India.
E-mail address: shamna.sudhi@gmail.com (N. Shamna).

https://doi.org/10.1016/j.aquaculture.2020.735306
Received 26 December 2019; Received in revised form 30 March 2020; Accepted 30 March 2020
Available online 01 April 2020

Table 1
Formulation and proximate composition of different experimental diets with graded levels of crude protein fed to GIFT juveniles reared in ISW of 10 ppt for the period of 60 days.

Ingredients composition (%)	Diets						
	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
Dextrin[1]	10	10	10	10	10	10	10
Starch[1]	52.8	46.9	41	35.1	29.2	23.3	17.35
Cellulose[1]	3.45	3.45	3.45	3.45	3.44	3.45	3.45
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
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 fiber (%)	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] (kcal/100 g)	495.23	492.86	488.27	485.19	491.81	489.55	498.95
DE[9](kcal/100 g)	400.00	399.98	400.00	400.00	399.99	399.98	400.01
P:E[10] (mg protein/kcal DE)	51.15	63.58	76.10	88.53	101.20	113.56	126.20

[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 D3, 11,00,000 IU; Vitamin B2, 2000 mg; Vitamin E, 750 mg; Vitamin K, 1000 mg; Ascorbic acid, 2500 mg; Vitamin B6, 1000 mg; Vitamin B12, 6 mg; Calcium pantothenate, 2500 mg; Nicotinamide, 10 g; Mn, 27,000 mg; I, 1000 mg; Fe, 7500 mg; Zn, 5000 mg; Cu, 2000 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; P:E, protein to energy ratio.

strain from Nile Tilapia (*Oreochromis niloticus*) could be one of the most preferable and promising ones because of its adaptability to the wide range of environment. The effect of salinity on growth of tilapia is complex process and can be modified by the interactive effects of both non-osmoregulatory (territorial aggression) and osmoregulatory factors on metabolism (Kang'ombe and Brown, 2008). Though the species can grow and reproduce in higher salinities, it doesn't mean the condition is adequate or appropriate for proper development (El-Sayed, 2006). The salinity tolerance of tilapia varies with different factors like species, strain, age and size, adaptation time in salinity, method of acclimatization and environmental factors (Chervinski, 1982; Philippart and Ruwet, 1982; Suresh and Lin, 1992). The better growth and feed utilisation of GIFT tilapia compared to red tilapia was reported (Ng and Hanim, 2007). The more disease resistant and better growth are the key factors that can be taken as indicators of potentiality of GIFT tilapia in commercial farming practices.

For successful culture of any species, feeding with nutritionally balanced feed is the most important factor. Thus, the knowledge about the nutritional requirement of the species is crucial to formulate cost effective and nutritionally balanced quality feed. Among the macronutrients, protein being the costliest nutrient in the feed, needs to be optimized. The dietary protein requirement of different species of tilapia varies from 20 to 56% (Winfree and Stickney, 1981; Shiau and Huang, 1990; El-Sayed and Teshima, 1992; FAO, 2019) in relation to freshwater, brackish water or sea water culture conditions. According to NRC (2011), the protein requirement of < 20, 20–200, 200–600, 600–1500 and > 1500 g of Nile tilapia is 40, 34, 30, 28 and 26%, respectively. However, there is no available report on the requirement of GIFT in inland saline condition, although a single report on dietary protein requirement of all male Nile tilapia fingerlings in ISW is available (Mohammadi et al., 2014).

The nutrient composition of diet has effect on the digestive system of fish and macro-nutrients play a major role in adaptation of digestive system in response to different diets (Hofer, 1979a, 1979b; Melo et al., 2012). In protein requirement studies where there is wide variation in dietary protein level could modulate the intermediary metabolic pathways by influencing digestive and metabolic enzymes related to protein as well as other macro nutrients. The inverse relation between protease and amylase activity with increasing dietary protein levels has been reported (Bazaz and Keshavanath, 1993; Kumar et al., 2011; Jayant et al., 2018) where the activity of lipase did not show any changes.

In fish, the growth is the result of hyperplasic muscle development, which is likely to be controlled by insulin-like growth factor axis (Jones and Clemmons, 1995; Laron, 2001; Cruz et al., 2006). Insulin-like growth factor I (IGF-I), Insulin-like growth factor I receptor (IGF-IR) and Insulin-like growth factor binding protein I (IGF-BPI) are the mitogenic peptides, which are influenced by the nutritional composition especially protein content of the diet. IGF-I expression is one of the most suitable indicators of growth in tilapia (Brown et al., 2012) and rohu (Kumar et al., 2019). The application of gene expression data along with nutrient utilisation for optimization of protein requirement helps in understanding the molecular mechanism of protein on growth of fish. Hence, considering the above facts, the present study was planned and designed to optimize the dietary protein requirement of GIFT juveniles under inland saline water at 10 ppt condition based on both percent weight gain and expression of growth-related genes.

2. Material and methods

2.1. Experimental animal

The GIFT juveniles were procured from Rajiv Gandhi Centre for

Aquaculture (RGCA), Visakhapatnam, Andhra Pradesh, India and transported to the wet laboratory of Rohtak Centre of ICAR-Central Institute of Fisheries Education, Haryana, India. The fish were carefully transferred to previously disinfected cemented tanks of 10,000 l (4 m × 3 m × 1 m) capacity containing freshwater and left undisturbed for whole night. The fish were fed with commercial diet containing 30% crude protein thrice daily on satiation basis during the acclimatization period of 15 days. After 15 days of freshwater acclimatization, inland saline water was added slowly to raise the salinity up to 10 ppt by increasing 1 ppt salinity per day and acclimated for another 15 days. For the experiment, the inland saline water was collected from a borewell of Baniyani farm of Rohtak Centre which was nearby to the experimental site. Proper aeration was provided during salinity acclimatization period and fish were fed with same diet thrice daily on satiation basis.

2.2. Formulation and preparation of experimental diets

As per the experimental design, seven isoenergetic (400 kcal DE/100 g), isolipidic (6%) and hetero-nitrogenous purified diets with graded levels of dietary protein *viz.,* 20%, 25%, 30%, 35%, 40%, 45% and 50% CP were formulated and prepared (Table 1). Different purified ingredients were used to prepare the experimental diets. Casein and gelatin were used as protein sources and starch and dextrin were used as carbohydrate sources. The sources of lipid were fish oil and sunflower oil. All the ground ingredients were weighed as per formula and kept in a plastic tray. The gelatin crystals were first dissolved in lukewarm water to form a jelly like mass. Other ingredients except oil and additives were first mixed uniformly and then jelly like mass of gelatin was added to it. Required amount of water was added to this mixture for making dough, which was then put in a plastic bag and placed in the pressure cooker for steam cooking of 20 min. Then the dough was smashed to cool rapidly and powered well. Further, rest of the ingredients *viz.,* oil, BHT, choline chloride, vitamin-mineral mixture and stay C were mixed along with it and tried to mix it uniformly. The dough was pressed through a pelletizer with 1 mm diameter dye to make pellets, which was then dried under fan. Finally, the dried pellets were packed in airtight containers, labelled properly and stored at 4 °C until use.

2.3. Experimental design, set-up and fish maintenance

Three hundred and fifteen acclimated GIFT juveniles (average body weight 2.68 ± 0.01 g) were randomly distributed in seven treatment groups *viz.*, T20 (20% CP), T25 (25% CP), T30 (30% CP), T35 (35% CP), T40 (40% CP), T45 (45% CP) and T50 (50% CP) with triplicates following a completely randomized design (CRD). Water salinity was maintained at 10 ppt throughout the experimental period. Stocking density was 15 fish per circular tank (300 l capacity with 92 cm diameter and 45 cm height; 200 l water volume) which was placed in an indoor system where 12 h of photoperiod was maintained. The water temperature was maintained from 27.5 °C to 28.8 °C by using water thermostat. The GIFT juveniles were fed to satiation level with respective experimental diet thrice daily at 10 am, 2 pm and 6 pm throughout the experimental period of 60 days. The weight of fish was measured fortnightly to adjust the feeding rate and at the end of the trial the final body weight of fish was measured. Throughout the experiment, 30% water was exchanged from each experimental tank at every three days interval. The faeces were siphoned out everyday morning before starting the next day's feeding. Quantity of feed intake by fish was recorded accurately to calculate the dry matter intake (DMI).

2.4. Physico-chemical parameters of water

The water temperature, pH, and salinity of experimental tanks were monitored every day, whereas other water quality parameters like dissolved oxygen (DO), free carbon-dioxide, total hardness, total alkalinity, ammonia-N, nitrite-N, nitrate-N, water ions and osmolality were monitored every three days interval. The temperature of the water of all the experimental tanks was checked thrice in a day (8 am, 4 pm, and 10 pm) by using a water thermometer (MERCK, Germany). The water pH of all the experimental tanks was checked every day by using a pH probe (HI11310, HANNA Instruments, Singapore). Dissolved oxygen, free carbon-dioxide, total hardness, calcium ions and magnesium ions concentration were measured as per the standard methods (APHA, 2005). The water salinity of all the experimental tanks was checked every day by using a refractometer (Z741839, Merck Instruments, Germany). Ammonia-N and nitrite-N concentrations were determined using ammonia-nitrite Test Kit (Spectroquant NOVA-MERCK, Germany). The potassium ions concentration was measured using Microprocessor Flame Photometer (RS232, Electronics India, India). Besides the standard method, the DO probe (HI764080, HANNA Instruments, Singapore) was used to check the dissolved oxygen on daily basis. The osmolality of the water was measured using a vapor pressure osmometer (VAPRO®, Germany).

2.5. Sampling

Wet weight of fish at the start and end of the experiment was measured using electronic weighing balance for calculation of growth parameters. Fishes were starved overnight before taking the weight. For proximate composition of the carcass, five fish from each tank were taken and the body surface water was removed using tissue paper and placed in petri plate after taking the weight using an electronic weighing balance. Six fish from each tank were collected and anaesthetized with clove oil (50 µl/l). Among them, three fish were dissected; viscera (consists of intestine, intestinal glands, airbladder, heart, spleen excluding kidney) and liver was collected for determination body indices. Weighing was done for both liver and the viscera separately for each fish. Other three fish were dissected and the intestine was taken out to make the intestinal tissue homogenate for analysis of digestive enzyme activity. The liver tissues were collected in 1 ml screwcap cryotubes (Tarsons Products Pvt. Ltd., USA) using RNAlater™ solution (Qiagen, Netherlands) for gene expression study.

2.6. Growth and nutrient utilisation parameters

Growth parameters *viz.,* percent weight gain (WG%), feed intake, feed conversion ratio (FCR), protein efficiency ratio (PER), and apparent net protein utilisation (ANPU%) were calculated as follows:

$$\text{WG\%} = \frac{\text{Final wet weight (g)} - \text{Initial wet weight (g)}}{\text{Initial wet weight (g)}} \text{x } 100$$

$$\text{Feed intake (g/fish)} = \frac{\text{Total feed intake (g)}}{\text{Total number of fish}}$$

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

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

$$\text{ANPU (\%)} = \frac{\text{Final body protein (g wet weight)} - \text{Initial body protein (g wet weight)}}{\text{Protein intake (dry weight in g)}} \text{X100}$$

2.7. Percent survival

At the end of the experiment, the number of the experimental animals in each experimental tank was counted and the percent survival was calculated by the following formula,

$$\text{Survival (\%)} = \frac{\text{Total number of experimental fish harvested}}{\text{Number of experimental fish stocked}} \text{X } 100$$

2.8. Proximate composition of diets and carcass

Proximate analysis (AOAC, 1995) of the experimental diets were estimated by using micro-Kjeldahl method (Kelplus, PELICAN, India), Soxhlet extraction method (SOCS plus, SAS-AS 08, PELICAN, India), and muffle furnace (550 °C for 5 h), respectively. The crude fiber (*CF*) of the diets (fat free sample) was estimated by using Fiber Tech (Tulin equipment, India) apparatus followed by incineration in a muffle furnace at 550 °C for 5 h. The nitrogen free extract (NFE) of the diets and total carbohydrate (TC) of carcass were calculated using following formulae:

$$\text{NFE\%} = 100 - (\text{CP\%} + \text{EE\%} + \text{CF\%} + \text{TA\%})$$

$$\text{TC\%} = 100 - (\text{CP\%} + \text{EE\%} + \text{TA\%})$$

In case of carcass, finally the proximate composition other than moisture (%) was expressed on wet weight basis. Gross energy content of the experimental diets was measured using a Bomb calorimeter (Model- 5E-AC/PL, Changsha Kaiyuan Instruments Co., Ltd., China) by following manufacture's protocol. Digestible energy (DE) of the diet was calculated according to Halver (1976) as per the following formula:

Digestible Energy (kcal/100g)
= {Crude Protein (%) X4 + Ether Extract (%)
X9 + Nitrogen Free Extract (%) X4}

The protein to energy ratio was calculated by using following formula:

P: E (mg CP/kcal DE) = (CP%X 1000)/DE

2.9. Body indices

Viscerosomatic index (VSI) and hepatosomatic index (HSI) were calculated as follows:

$$\text{VSI (\%)} = \frac{\text{Wet weight of the viscera (g)}}{\text{Wet weight of the fish (g)}} \text{X } 100$$

$$\text{HSI (\%)} = \frac{\text{Wet weight of the liver (g)}}{\text{Wet weight of the fish (g)}} \text{X } 100$$

2.10. Enzyme assays

2.10.1. Preparation of tissue homogenate

A 5% intestinal tissue homogenate was prepared in chilled 0.25 M sucrose solution by Teflon coated mechanical homogenizer (REMI Equipments, Mumbai, India). The whole procedure was done in ice cold condition to preserve the enzymatic activity. The tissue homogenates were centrifuged at 5000 rpm for 10 min at 4 °C with a refrigerated centrifuge (Heraeus Megafuge 8R Centrifuge, ThermoFisher Scientific, Germany). The supernatant was collected in 2 ml eppendorf tubes and stored in a deep freezer (-20 °C) until used for enzyme assay (Gopan et al., 2019).

2.10.2. Determination of tissue protein

Quantification of protein in the intestinal tissue was carried out by Lowry's method (Lowry et al., 1951) where bovine serum albumin was used as standard to prepare the standard curve. From the standard curve, the protein concentration of different tissue samples was estimated. The resultant tissue protein values were used for calculating the digestive enzymes activity of the samples.

2.10.3. Digestive enzymes activity

Protease activity of the intestinal tissue was determined by the casein digestion method as suggested by Drapeau (1974). The protease activity was expressed in millimole of tyrosine released/min/mg protein. Amylase activity was determined as the reducing sugars produced due to the action of gluco-amylase and α-amylase on carbohydrate using di-nitrosalicylic acid (DNS) method (Rick and Stegbauer, 1974). Maltose was used as the standard and amylase activity was expressed as mole of maltose released from starch per min at 37 °C temperature (micromole of maltose released/min/mg protein). The lipase activity was estimated by the method of Cherry and Crandall (1932), which is based on the determination of fatty acids released due to enzymatic hydrolysis of triglycerides from stabilized emulsion of olive oil and expressed in unit/h/mg protein.

2.11. IGF−I, IGF-IR and IGF-BPI expression

2.11.1. RNA extraction and synthesis of single-strand cDNA

100 mg of liver tissue was used for total RNA isolation by using 1 ml TRIzol (Invitrogen, USA) reagent following the manufacture's protocol. The concentration and purity (260/280) of RNA was checked using a Nano-Drop spectrophotometer (Thermo scientific, USA). The relative quantity and integrity of RNA were confirmed by electrophoresis. Before cDNA synthesis, the isolated total RNA was treated with RNase free DNase I (Invitrogen, USA). Reverse transcription was carried out using the commercial cDNA synthesis Kit (iScriptTM cDNA Synthesis Kit, BIO-RAD, USA) following the manufacturer's protocol. The concentration and purity (260/280) of product of the single-strand cDNA synthesis was checked using a Nano-Drop spectrophotometer (Thermo scientific, USA) and the product was stored at −80 °C until the quantitative RT-PCR (qRT-PCR) was performed.

2.11.2. Primer designing

GeneRunner (version 6.5.48 × 64 Beta) software was used to design the RT-qPCR primers and then the stability was assessed by checking of primer-dimer formation and self-binding. The β-actin was used as reference gene. The primers for β-actin, IGF−I, IGF-IR and IGF-BPI were designed from reported sequence of Nile tilapia (Table 2). All the designed RT-qPCR primers were purchased from Eurofins Genomics India

Table 2
Primers used for gene expression study in real-time PCR for GIFT juveniles reared in ISW of 10 ppt and fed with graded levels of dietary crude protein for the period of 60 days.

Target gene	Accession number	Primer sequence	Amplicon size
IGF-I	EU272149.1	FP 5'-GGACGAGTGCTGCTTCCAAAGC-3' RP 5'-TGCTCTTGGCATGTCTGTGTGC-3'	121
IGF-IR	KC506777.1	FP 5'-GCGACCCAAAGAGCAACAGTGG-3' RP 5'-TGCCAGATCTCGGTGGACAAAC-3'	130
IGF-BPI	XM_003438121.3	FP 5'-CCACTGGCGTTTCCTCAATGG-3' RP 5'-GATGAGCAACCCATCCCAAACC-3'	128
β-actin	KJ126772.1	FP 5'-AATCCTGCGGAATCCACGAAAC-3' RP 5'-CTCCTTCTGCATCCTGTCAGCG-3'	140

Pvt. Ltd. (Bangalore, India).

2.11.3. Quantitative real time PCR for mRNA expression

Quantification of IGF−I, IGF-IR and IGF-BPI genes of liver tissue was done in an AriaMx Real-Time PCR System (Model no. G8830A, Agilent Technologies, USA). A reaction volume of 10 μl containing 5 μl SYBR Green qPCR Master Mix (BIO-RAD, USA), 1 μl (1.25 pmole for IGF−I, IGF-BPI, and β-actin; and 5.0 pmole for IGF−IR) of each gene specific primer, 1 μl of cDNA and 3 μl of nuclease free water were mixed for the quantification. The RT-qPCR was programmed for 40 cycle's reactions each of which includes denaturation at 95 °C for 15 s, annealing at 59.3 °C for 15 s and extension at 59.3 °C for 60 s. The quantified value of mRNA was expressed in terms of CT (threshold cycle) value and melting curve analysis of the amplified products was performed at the end of each PCR reaction. The relative expression of IGF-I, IGF-IR and IGF-BPI mRNA was calculated by following $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) where β-actin was used as reference gene.

ΔCT = target gene CT value − reference gene CT value

$\Delta\Delta CT$ value
= ΔCT value of treatment group − ΔCT value of control group*

*The lowest protein fed group (T20) was used as control group in our study.

2.12. Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA) using General Linear Model procedure of IBM SPSS Statistics (version 22). The overall treatments effects were determined and polynomial contrasts were used to test linear and quadratic effects of graded levels of dietary crude protein. Duncan's multiple range test (DMRT) under Post-hoc was used for observing significant differences among the mean values at 5% probability level ($p < .05$). The optimum dietary crude protein requirement of GIFT tilapia juveniles reared at 10 ppt salinity was determined by fitting the percent weight gain (WG%) and IGF-I expression data in both broken line and second-order polynomial regression analysis model to determine optimum dietary protein requirement level according to Robbins et al. (2006) and Jobling (1994). In second-order polynomial regression analysis model, the optimum dietary crude protein is considered to be that dietary crude protein level which promotes 95% of the maximum growth response (Jobling, 1994).

3. Results

3.1. Physico-chemical parameters of water

The water temperature, pH and salinity were ranged from 27.5 °C to 28.8 °C, 7.7 to 8.5 and 9.4–10.6 ppt, respectively. Dissolved oxygen, total alkalinity and total hardness were ranged from 4.5 to 6.3 mg/l, 240 to 281 mg/l and 2795 to 3012 mg/l, respectively. The free CO_2 was not detectable during the entire experimental period. The ammonia-N, nitrite-N and nitrate-N levels were within permissible range. The Ca^{2+}, Mg^{2+} and K^{+} ion concentrations were ranged from 320 to 351 mg/l, 479 to 520 mg/l and 8.4 to 12 mg/l, respectively. The osmolality of 10 ppt water was ranged from 295 to 305 mOsmole/kg.

3.2. Proximate composition of the experimental diets and whole body of fish

The crude protein contents of different experimental diets *viz.*, T20, T25, T30, T35, T40, T45 and T50 were 20.46, 25.43, 30.44, 35.41, 40.48, 45.42 and 50.48%, respectively and the ranges of gross energy (GE), digestible energy (DE) and ether extract (EE) values were 485.19–498.95 kcal/100 g, 399.98–400.01 kcal/100 g and 6.08–6.16%, respectively (Table 1). Therefore, the experimental diets were heteronitrogenous (20–50% CP) and isocaloric (GE and DE of around 485 and 400 kcal/100 g) and isolipidic (around 6% EE). The P:E ratio of different experimental diets *viz.*, T20, T25, T30, T35, T40, T45 and T50 were 51.15, 63.58, 76.10, 88.53, 101.20, 113.56 and 126.20 mg protein/kcal DE, respectively.

The proximate composition of whole body of the juveniles is given in Table 4. The moisture, crude protein and crude fat were significantly ($p < .05$) affected in overall and same was followed in linear and quadratic trend. Whereas, total carbohydrate and total ash content did not show any significant ($p > .05$) effect. The higher values of moisture were recorded in T45 and T50 group and the lower values were found in T25, T30 and T35 groups. Conversely, in the case crude fat the higher values were found in T20, T25 and T30 group and the lower values were in T45 and T50 group. The whole body crude protein showed higher value above 35% crude protein fed group (T35) *i.e.,* T35, T40, T45 and T50 group, though the highest crude protein was recorded in T35 group.

3.3. Growth, nutrient utilisation and survival of fish

All the parameters related to growth and nutrient utilisation *i.e.,* final body weight, feed intake, WG%, FCR, PER and ANPU% were found to be significantly ($p < .05$) affected by the dietary crude protein in overall as well as linearly and quadratically (Table 3). There was an increasing trend of final body weight and WG% with increasing dietary crude protein up to 35% dietary crude protein and then it showed plateauing trend. The highest and lowest values of final body weight and WG% were recorded in T40 and T20 groups, respectively. Feed intake showed increasing trend up to 35% CP and then there was no significant ($p > .05$) variation among the groups above 35% CP. The FCR value followed just opposite trend to the WG%; so, the lowest and highest values of FCR were found in T40 and T20 groups, respectively. Both PER and ANPU% showed a decreasing trend with increasing dietary crude protein. There was no mortality of fish in any experimental group during the experimental period of 60 days.

3.4. Body indices

The body indices *i.e.,* VSI and HSI are given in the Table 3. There was no significant ($p > .05$) effect on VSI among the groups fed with graded level of dietary crude protein. Whereas, there was an overall significant ($p < .05$) effect on HSI and the same was being seen linearly and quadratically. The HSI value followed an increasing trend with increasing dietary crude protein level.

3.5. Digestive enzymes activity

Among digestive enzymes, protease and amylase activity were significantly ($p < .05$) affected due to feeding on graded levels of crude protein; whereas, lipase activity did not show significant effect ($p > .05$) (Table 5). The protease activity was found to be increased with increasing dietary crude protein up to 40%. The groups fed with 40 and 45% crude protein showed highest protease activity and the lowest value was found in 20% crude protein fed group. The amylase activity showed a decreasing trend with increasing dietary crude protein.

3.6. Expression of hepatic IGF−I, IGF-IR and IGF-BPI genes

Expression of hepatic IGF−I, IGF-IR and IGF-BPI genes are given in the Table 6. The expression of hepatic IGF-I gene was significantly ($p < .05$) differ among the treatments. Hepatic IGF-I expression was increased with increasing dietary crude protein up to 35% and then showed a decreasing trend.

The expression of hepatic IGF−IR gene was significantly ($p < .05$) differ among the treatments as like hepatic IGF−I. Similar to the hepatic

Table 3
Growth, nutrient utilisation, survival and body indices of GIFT juveniles reared in ISW of 10 ppt and fed with graded levels of dietary crude protein for the period of 60 days.

Treatments[1]	Fn.wt.[2] (g)	WG[3]%	FI[4] (g/fish)	FCR[5]	PER[6]	ANPU[7]%	Survival (%)	VSI[8](%)	HSI[9](%)
T20	13.23[a]	393.56[a]	14.56[a]	1.38[d]	3.62[g]	50.33[e]	100.00	8.48	0.89[a]
T25	14.53[b]	442.96[b]	15.55[b]	1.31[c]	3.05[f]	44.14[d]	100.00	8.39	0.90[a]
T30	16.35[c]	501.23[c]	16.19[bc]	1.19[b]	2.81[e]	43.24[d]	100.00	8.26	0.92[a]
T35	17.57[d]	553.92[d]	16.64[c]	1.12[a]	2.56[d]	42.77[d]	100.00	9.40	0.95[ab]
T40	17.51[d]	556.50[d]	16.30[bc]	1.10[a]	2.28[c]	36.48[c]	100.00	9.22	1.02[b]
T45	17.39[d]	551.99[d]	16.78[c]	1.14[ab]	1.95[b]	30.40[b]	100.00	8.55	1.18[c]
T50	17.28[d]	547.57[d]	16.85[c]	1.15[ab]	1.74[a]	27.03[a]	100.00	9.37	1.24[c]
SEM	0.36	13.49	0.19	0.02	0.14	1.74	0.00	0.16	0.03
Overall (p value)	0.000	0.000	0.001	0.000	0.000	0.000	–	0.196	0.000
Linear (p value, R^2)	0.000, 0.722	0.000, 0.758	0.000, 0.617	0.000, 0.617	0.000, 0.975	0.000, 0.908	–	0.072, 0.158	0.000, 0.806
Quadratic (p value, R^2)	0.000, 0.970	0.000, 0.982	0.017, 0.730	0.000, 0.911	0.000, 0.982	0.032, 0.924	–	0.825, 0.160	0.000, 0.910

Mean values (n = 3) in the same column with different superscripts differ significantly (p < .05).
[1]T20-T50, 20–50% crude protein.
[2]Fn.wt., final body weight; [3]WG, weight gain; [4]FI, feed intake; [5]FCR, feed conversion ratio; [6]PER, protein efficiency ratio; [7]ANPU, apparent net protein utilisation; [8]VSI, viscerosomatic index; [9]HSI, hepatosomatic index.

Table 4
Whole body proximate composition (on % wet weight basis) of GIFT juveniles reared in ISW of 10 ppt and fed with graded levels of dietary crude protein for the period of 60 days.

	Moisture	CP[1]	EE[2]	TC[3]	TA[4]
Initial fish	75.12	14.73	4.30	2.24	3.61
Treatments[5],*					
T20	73.23[bc]	14.06[a]	8.51[c]	1.50	2.69
T25	72.71[ab]	14.53[ab]	8.44[c]	1.41	2.92
T30	72.97[ab]	15.30[cd]	7.78[bc]	1.38	2.57
T35	72.14[a]	16.41[d]	6.90[b]	1.72	2.84
T40	73.69[bcd]	15.81[cd]	7.18[b]	1.07	2.26
T45	74.34[d]	15.49[bcd]	5.61[a]	1.77	2.79
T50	74.13[cd]	15.45[bcd]	5.55[a]	2.06	2.80
SEM	0.20	0.20	0.27	0.11	0.07
Overall (p value)	0.003	0.003	0.000	0.238	0.124
Linear (p value, R^2)	0.002, 0.306	0.002, 0.300	0.000, 0.778	0.152, 0.003	0.787, 0.099
Quadratic (p value, R^2)	0.015, 0.463	0.002, 0.599	0.507, 0.783	0.152, 0.053	0.271, 0.198

Mean values (n = 3) in the same column with different superscripts differ significantly (p < .05).
[1]CP, crude protein; [2]EE, ether extract or crude fat; [3]TC, total carbohydrate; [4]TA, total ash.
[5]T20-T50, 20–50% crude protein.
* Only treatments were considered for statistical analysis.

Table 5
Digestive enzyme activities in the intestine of GIFT juveniles reared in ISW of 10 ppt and fed with graded levels of dietary crude protein for the period of 60 days.

Treatments[1]	Protease[2]	Amylase[3]	Lipase[4]
T20	0.23[a]	39.78[c]	0.17
T25	0.32[b]	37.70[c]	0.18
T30	0.36[c]	33.51[b]	0.19
T35	0.38[cd]	31.54[ab]	0.18
T40	0.41[d]	29.53[a]	0.21
T45	0.41[d]	28.97[a]	0.18
T50	0.30[b]	28.19[a]	0.15
SEM	0.01	1.01	0.01
Overall (p value)	0.000	0.000	0.797
Linear (p value, R^2)	0.000, 0.242	0.000, 0.794	0.682, 0.010
Quadratic (p value, R^2)	0.000, 0.849	0.045, 0.845	0.162, 0.138

Mean values (n = 3) in the same column with different superscripts differ significantly (p < .05).
[1]T20-T50, 20–50% crude protein.
[2]Protease activity is expressed as millimole of tyrosine released/min/mg protein; [3]Amylase activity is expressed as micromole of maltose released /min/mg protein; [4]Lipase activity is expressed in unit/h/mg protein.

IGF-I expression, the expression of hepatic IGF-IR showed an increasing trend with increasing dietary crude protein up to 35%.

Though the expression of IGF-BPI was significantly (p < .05) differ among the treatment, but did not show any clear trend as like hepatic IGF-I or IGF—IR.

3.7. Correlation among growth and expression of hepatic IGF-I, IGF-IR and IGF-BPI genes

The correlation among growth (WG%) and expression of hepatic IGF—I, IGF-IR and IGF-BPI genes is given in Table 7. The results showed that WG%, hepatic IGF-I and IGF—IR expression have positive correlation among themselves. WG% showed to be highly correlated with hepatic IGF-I and IGF-IR expression with correlation coefficient (r) values of 0.92 and 0.97, respectively. The expression of hepatic IGF-IR highly correlated with the hepatic IGF-I expression (r = 0.93). However, the expression of hepatic IGF-BPI did not show any correlation with other parameters.

3.8. Dietary protein requirement

According to Broken line regression analysis, in relation to WG% and hepatic IGF-I expression the optimum dietary crude protein requirement of GIFT juveniles reared in ISW of 10 ppt salinity and fed with graded levels of dietary crude protein was found to be 37.86 (Fig. 1) and 37.72% (Fig. 2), respectively. Moreover, according to Second-order polynomial regression analysis, in relation to WG% and hepatic IGF-I expression the optimum dietary crude protein was found to be 40.92% (Fig. 1) and 38% (Fig. 2), respectively.

4. Discussion

The present study was conducted to determine the optimum dietary crude protein requirement of GIFT juveniles under inland saline water

Table 6
Hepatic IGF−I, IGF-IR and IGF-BPI expression of GIFT juveniles reared in ISW of 10 ppt and fed with graded levels of dietary crude protein for the period of 60 days.

Treatments[1]	IGF-I	IGF-IR	IGF-BPI
T20	1.01[a]	1.06[a]	1.00[a]
T25	1.28[a]	2.22[b]	3.00[e]
T30	1.78[b]	3.62[c]	1.87[c]
T35	3.11[e]	7.07[f]	1.50[b]
T40	3.02[e]	5.56[d]	2.70[d]
T45	2.51[d]	6.16[e]	1.36[b]
T50	2.17[c]	5.91[de]	1.11[a]
SEM	0.13	0.36	0.13
Overall (p value)	0.000	0.000	0.000
Linear (p value, R^2)	0.000, 0.430	0.000, 0.684	0.000, 0.042
Quadratic (p value, R^2)	0.000, 0.817	0.000, 0.852	0.000, 0.302

Mean values (n = 3) in the same column with different superscripts differ significantly ($p < .05$).
[1]T20-T50, 20–50% crude protein.

Table 7
Correlation (r = correlation coefficient) among growth and expression of hepatic IGF−I, IGF-IR and IGF-BPI genes.

	WG%	IGF-I	IGF-IR	IGF-BPI
WG%	1			
IGF-I	0.92	1		
IGF-IR	0.97	0.93	1	
IGF-BPI	−0.01	0.04	−0.14	1

at 10 ppt salinity. Inland saline water (ISW) has a great opportunity for the farmers to utilize their salt affected unproductive land for the purpose of aquaculture where GIFT could be one of the most suitable species as it can tolerate adverse conditions of ISW. The gene expression study was performed along with the growth study to get a better insight picture about the molecular mechanism of growth of the GIFT juveniles reared in ISW of 10 ppt salinity and fed with graded level of crude protein.

4.1. Physico-chemical parameters of water

The water quality parameters are very crucial factor for the aquatic animals as they are directly depending on the environmental condition to maintain their physiological homeostasis. In the present study, the water quality was within the optimum range of fish culture at low salinity in inland saline aquaculture system. Among different water quality parameters, temperature plays an important role to control the metabolic process in poikilothermic fish. The optimum range of temperature for rearing different tilapia species varies from 22 to 29 °C in freshwater condition (Sarig, 1969; Morgan, 1972; and Mires, 1995). In the present study, the temperature was maintained at an optimum level of 27.5 to 28.8 °C. Tilapia can tolerate wide range of pH from 3.7 to 11 (Ross, 2000) and the optimum level remains within the range of 7 to 9. The pH was ranged from 7.8 to 8.5 during the experimental period which lies under the normal range. The salinity tolerance of tilapia depends on the species and strain. Among different species, mozambique, blue, and redbelly tilapia are the most salt tolerant. Optimum performance of tilapia can be observed from 0 to 19 ppt salinity, although it can tolerate the salinity up to 36 ppt (El-Sayed, 2006). Nile tilapia can reproduce at 13.5 to 29 ppt saline water and its upper limit of salinity tolerance is about 36 ppt (El-Sayed, 2006). The present study was conducted to determine the optimum dietary protein requirement of GIFT at 10 ppt salinity prevailing in ISW. The dissolved oxygen (DO) level in the present study was maintained at an average value of 5.25 mg/l throughout the experimental period, which lies on the optimum DO level for different tilapia culture condition *i.e.*, > 3 mg/l (Magid and Babiker, 1975; Ross, 2000). The optimum total alkalinity level in freshwater culture system of tilapia is ranged from 20 to 200 mg/l (Setiadi et al., 2018); however, there are many reports suggesting that the culture of fish can be done in inland saline water with above 200 mg/l total alkalinity (Allan et al., 2009). The total hardness of 2800 to 3000 mg/l is well within the range and supported by the reports of Lakra et al. (2014) and Jahan et al. (2018). The water ammonium-N (0.02–0.07 mg/l), nitrite (0.001–0.005 mg/l), and nitrate (0.03–0.07 mg/l) values obtained within the safe level of tilapia culture as suggested by Mjoun et al. (2010). The water ions *viz.*, Ca^{2+}

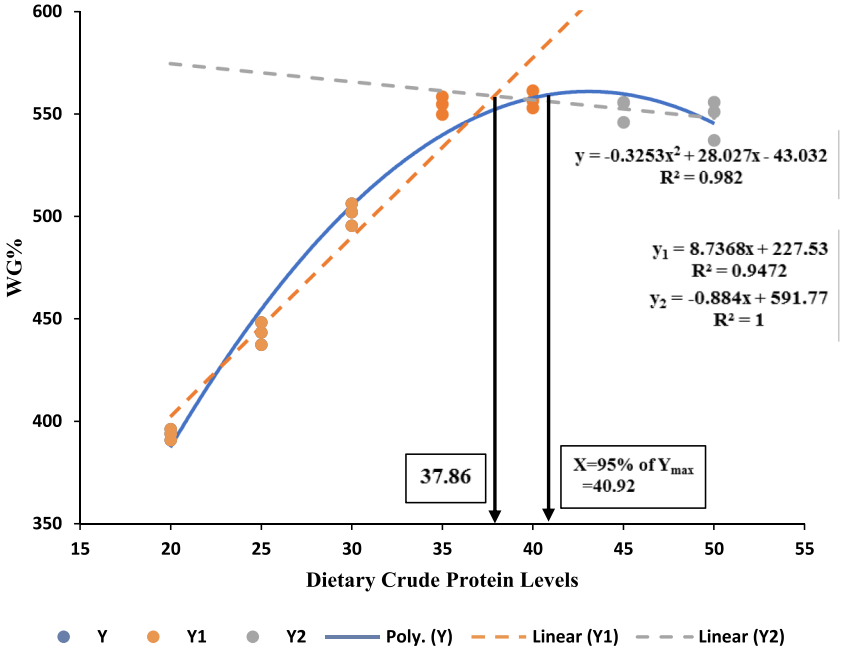

Fig. 1. The broken-line linear (dash line) and second-order polynomial (solid line) regression to the optimize dietary crude protein requirement in relation to weight gain percentage (WG%) of GIFT juveniles reared in ISW of 10 ppt and fed with graded levels of dietary crude protein for the period of 60 days.

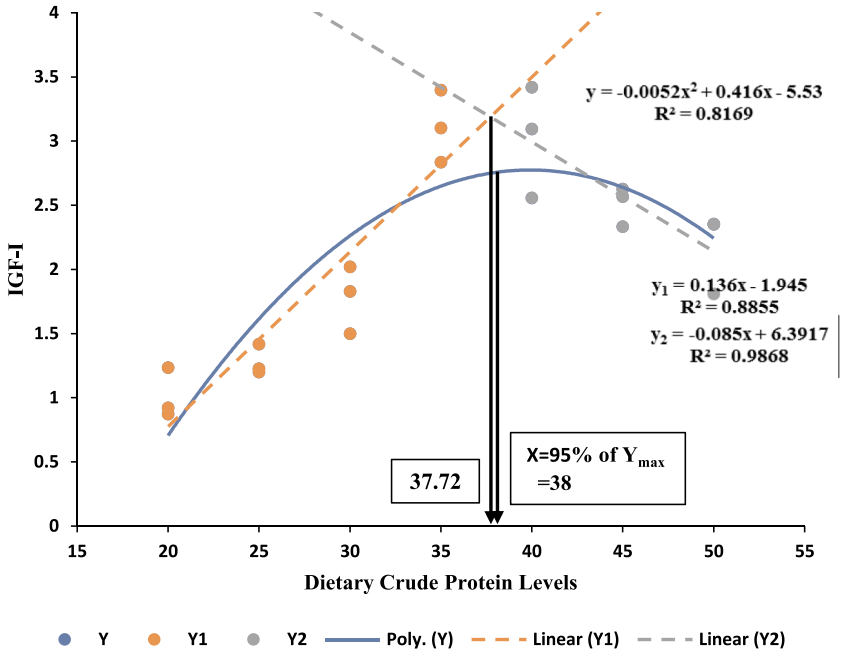

Fig. 2. The broken-line linear (dash line) and second-order polynomial (solid line) regression to the optimize dietary crude protein requirement in relation to hepatic IGF-I gene expression of GIFT juveniles reared in ISW of 10 ppt and fed with graded levels of dietary crude protein for the period of 60 days.

(320–350 mg/l), Mg^{2+} (480–520 mg/l), and K^{+} (8.5–12 mg/l) concentrations during the present experiment were within the range of normal ISW (Allan et al., 2009). The water osmolality of 295–305 mOsmole/kg at 10 ppt in the present experiment was supported by Jahan et al. (2018).

4.2. Proximate composition of the diets

The estimated crude protein contents of different experimental diets *viz.,* T20, T25, T30, T35, T40, T45 and T50 were found to be 20.46, 25.43, 30.44, 35.41, 40.48, 45.42 and 50.48% with P:E values of 51.15, 63.58, 76.10, 88.53, 101.20, 113.56 and 126.20 mg protein/kcal DE, respectively. The values of ether extract or crude fat, gross energy and digestible energy (Halver, 1976) of different experimental diets indicated that the diets were heteronitrogenous, isolipidic as well as isoenergetic to support the basis of protein requirement studies (Wilson and Halver, 1986).

4.3. Growth and nutrient utilisation

Growth is a consequence of accumulation of nutrients which have been ingested through diet. In case of fish, protein is the most important nutrient as it not only takes part in the growth but also supplies energy through amino acid oxidation. In the present study, the WG% of GIFT was found to be increased with the increasing the dietary crude protein levels up to 40% and further increment of dietary crude protein caused decrease in growth. This indicates that feeding of excess dietary protein did not contribute extra benefit for growth due to the reason that excess dietary amino acids beyond the optimal level with higher P:E value could be utilized for energy production purpose instead of synthesis and accretion of body protein. According to Philips (1979), the increase in dietary protein could cause increasing energy demand to catabolize the excess amino acids. Growth recorded in the present study corroborated the observation of several protein requirement studies on Nile tilapia, where Nile tilapia fry fed with graded level of protein from 20 to 50% CP with 5% of increment isocaloric diet in freshwater condition, the 35% CP fed group exhibited best growth (Santiago et al., 1982). Similarly, Nile tilapia fry (0.838 g) and juveniles (40.0 g) showed best growth at 40% and 30% CP, respectively when fed with four graded level of diet (20, 30, 40 and 50% CP) (Siddiqui et al., 1988). Rearing in inland saline water (approximately 8 ppt salinity), Mohammadi et al. (2014) reported that the growth of all-male Nile tilapia (13.85 ± 1.01 g) was highest at 29% CP fed group when fed with 15, 22, 29 or 36% crude protein for a period of 8 weeks.

The FCR, PER and ANPU of the feed is a reflection of how efficiently the fish utilize the feed and its protein content for growth purpose. The FCR value of the present study decreased significantly ($p < .05$) with the increasing dietary crude protein levels up to 40% and further increment of dietary protein level caused increase in FCR of fish. Fish fed 35 and 40% dietary crude protein with P:E values of 88.53 and 101.20 mg protein/kcal DE, respectively exhibited higher PER than the fish fed higher dietary protein with higher P:E values (T45 and T50). This finding indicated that optimum dietary protein and P:E value might be the important dietary factor for the efficient utilisation of the protein leading to better growth of fish. Similar results were also reported by Santiago et al. (1982), Siddiqui et al. (1988) in the experimental conditions as mentioned above in the growth section. Moreover, Mohammadi et al. (2014) found a lower FCR in Nile tilapia under inland saline water (approximately 8 ppt salinity) due to feeding of 36% crude protein than 29% crude protein fed group. Both PER and ANPU% were found to be decreased with the increasing dietary protein level, suggesting that at lower dietary protein level almost all dietary amino acids might be directed to synthesis and accretion of body protein for growth, but at higher dietary protein level a considerable proportion of dietary amino acids might be utilized for energy production instead of body protein synthesis leading to decreased PER and ANPU. Similar kind of result was reported by Shiau and Huang (1990) for hybrid tilapia reared in seawater. In the present study, high value of ANPU (50.33%) in 20% dietary protein fed group indicates very efficient utilisation of dietary protein for growth but at the same time comparatively lower growth of this group indicates insufficient supply of dietary amino acids to support optimum growth of fish. Similarly, Mohammadi et al. (2014) observed a higher ANPU value (48.28 ± 2.26%) in all male Nile tilapia due to feeding of 29% dietary crude protein under inland saline water (approximately 8 ppt salinity).

4.4. Whole body proximate composition of fish

The whole body crude protein level of GIFT juveniles was found to be higher in 35% dietary crude protein fed group than other groups, however, further increment of dietary crude protein level beyond 35% (40, 45 and 50%) could not change body crude protein significantly. Similar trend was also found by Siddiqui et al. (1988) in Nile tilapia when fed with four graded level of diet in freshwater condition. The increase in whole body protein content of fish with increasing dietary crude protein up to optimum level with optimum P:E value indicates the efficient utilisation of dietary protein to maximize the growth, but further increment of dietary protein beyond optimum level with higher P:E value cannot enhance growth further even reduces the growth in some cases because excess dietary amino acids instead of synthesis and accretion of body protein are utilized for energy production in fish leading to either no change or decrease the whole body crude protein level. Our finding was supported by the observation of Mohammadi et al. (2014) who also demonstrated that in Nile tilapia under inland saline condition (approximately 8 ppt salinity), the increase in dietary protein level up to 29% increased the whole body crude protein level which was similar to 36% crude protein fed group.

Feeding of 35 and 40% dietary crude protein caused lower whole body lipid content in GIFT juveniles than the 20 and 25% protein fed groups, however, increment of dietary crude protein beyond 40% further decreased the body lipid. Therefore, the highest lipid value was observed in 20 and 25% dietary protein fed groups. Whereas, the lowest value was found in 45 and 50% dietary protein fed groups. The decrease in whole body lipid with the increasing dietary CP level is supported by the report of Jauncey (1982). According to Jauncey (1982), *Serotherodon mossambicus* showed highest whole body lipid content at 8% crude protein fed group and further increase in dietary crude protein (16, 24, 32, 40, 48 and 56% CP) significantly ($p < .05$) decreased the whole body lipid content. Similarly, Mohammadi et al. (2014) reported highest whole body lipid content in 15% crude protein fed all-male Nile tilapia group under inland saline water (approximately 8 ppt salinity) and with the increase in dietary crude protein level (22, 29 and 36% CP) gradually decreased the whole body lipid content. The decrease in amount of body lipid with the increasing protein levels might be due to use of lipid for energy production required for osmoregulation of fish in ISW. In corroboration with our result, Bahnasawy (2009) reported the significant ($p < .05$) decrease in the whole body lipid content with the increasing dietary protein level up to 30% CP in juvenile monosex Nile tilapia reared in water fertilized with premix of superphosphate and urea at a dose of 6.8 mg/l.

In most of treatments there was inverse relation between whole body moisture and lipid content (Jauncey, 1982). The whole body total ash content of GIFT juveniles did not vary significantly ($p > .05$) among the different protein fed groups which is similar to the reports of Phillips et al. (1966) and Jauncey (1982) in *Salvelinus fontinalis* and *Serotherodon mossambicus*, respectively.

4.5. Body indices

The body indices (*e.g.,* VSI and HSI) provide information about metabolism of fish related to digestion, absorption, associated enzyme secretion, condition of the liver *etc.* VSI was not significantly ($p > .05$) varied among the experimental groups and the values in the present study were within the range reported by Kaushik et al. (1995) in Nile tilapia. Similar to our results, Huang et al. (2016) also observed non-significant variation in VSI values of *Cyprinus carpio*.

4.6. Digestive enzymes activity

Protease activity increased significantly ($p < .05$) with the increasing dietary protein level up to 45% might be due to availability of more protein as substrate; however, feeding of 50% dietary crude protein reduced the protease activity for the reason not known. The present result of protease activity corroborated the observations of Bazaz and Keshavanath (1993), Kumar et al. (2011), and Jayant et al. (2018) in *Tor khudree*, *Labeo rohita* and *Pangasiodon pangasius*, respectively. A reverse trend was observed in case of amylase activity of GIFT juveniles *i.e.*, the decrease in amylase activity with the increasing dietary protein level might be due to lower starch availability and *vice versa*. Similar kind of report was also demonstrated by Mohapatra et al. (2003) and Jayant et al. (2018) in *Labeo rohita* and *Pangasiodon pangasius*, respectively. There was no significant ($p > .05$) effect of dietary crude protein level on the lipase activity, which is also in agreement with the report of Jayant et al. (2018) in *Pangasiodon pangasius*.

4.7. Expression of IGF−I, IGF-IR and IGF-BPI genes in the liver tissue

The insulin-like growth factor I (IGF−I) is a mitogenic peptide, which plays a role in regulating growth of vertebrates (Humbel, 1990; Jones and Clemmons, 1995; Reinecke and Collet, 1998). Insulin-like peptides (including IGF−I) are supposed to be regulated by organism's nutritional status and nutrient metabolism to influence growth, development, reproduction and ageing of animals as the conserved features of the insulin-like peptides (Tatar et al., 2003). The membrane receptors of insulin-like growth factor *i.e.*, IGF−IR helps to activate both IGF-I and IGF-II, which are modulated by multiple IGF-binding proteins (IGF-BPs) (Pierce et al., 2011). Hepatic IGFs as the primary mediator of growth hormone-dependent growth play an essential role in animals.

The increasing expression of hepatic IGF-I with the increasing dietary protein up to 40% CP indicated the role of IGF-I on growth of fish. The further decrease in hepatic IGF-I expression in GIFT juveniles due to feeding of 45 and 50% dietary CP probably due to the fact that excess dietary protein might have produced metabolic stress on fish leading to decreased IGF-I expression and growth. In agreement with our finding, Qiang et al. (2012) reported that dietary protein level had significant impact on the serum IGF-I and hepatic IGF-I expression in tilapia juveniles. Jiang et al. (2010) also reported that dietary protein level could significantly affect the hepatic IGF-I expression which was significantly ($p < .05$) correlated with the specific growth rate in *Cirrhinus molitorella*. The high correlation ($r = 0.92$) between hepatic IGF-I expression and WG% supports the use of IGF-I expression as an indicator of growth. According to Kumar et al. (2018), the expression of IGF-I in *Labeo rohita* was found to be similar with the trend of WG% when fed with diet containing de-oiled rice bran up to 43% inclusion level.

The expression of hepatic IGF−IR gene in GIFT juveniles was found to be highly correlated ($r = 0.93$) with the expression of IGF-I gene probably due to the fact that the membrane receptor (IGF−IR) helps to activate the IGF−I. The result was supported by the observation of Pierce et al. (2011) in Mozambique tilapia. The high correlation ($r = 0.97$) between hepatic IGF-IR expression and WG% as like with IGF-I expression indicates its close association with the activity of IGF-I to support growth.

The expression of IGF-BPI did not show any clear correlation with IGF-I expression as well as IGF-IR expression might be due to the presence of various types and paralogs of IGF-BPs (Garcia de la Serrana and Macqueen, 2018) as Macqueen, and Garcia de la serrana, D., Johnston, I.A. (2013) reported that salmonids contain at least 19 unique IGF-BP genes, with salmonid-specific paralogs of IGF-BP (1a, *−1b*, *−2b*, *−3a*, *−3b*, *−5b*, *−6a*, and *-6b*). Alzaid et al. (2016) reported different response of the paralogs IGF-BP1 (IGF-BP1*a1* and IGF-BP1*a2*), where the expression of IGF-BP1*a1* was upregulated during infection serves a role in linking growth to innate immunity and also downregulation of growth; whereas, IGF-BP1*a2* was unaffected by infection which indicates IGF-BP1*a2* is not correlated with growth.

4.8. Dietary protein requirement based on SGR and IGF-I expression

In the nutritional requirement study, it is very important to note the magnitude of increase and decrease in growth rate relative to per unit increase in dietary protein before and after the point of maximum growth is achieved, respectively (De Silva et al., 1989). There may be overestimation of dietary protein requirement due to the partial use of dietary protein for energy production rather than for growth through amino acid oxidation (Millikin, 1982) and this phenomenon is much important in ISW as fish need to spend energy for osmoregulation. Differences in the experimental conditions (*e.g.,* species, size and age, stocking density, source and quality of protein, P:E ratio of the diet, rearing temperature *etc.*) cause considerable variation in the optimum dietary protein requirement (Hafedh, 1999).

In the present study, according to broken-line and second-order polynomial regression analysis in relation to WG%, the optimum dietary crude protein requirement of GIFT juvenile reared in ISW of 10 ppt was found to be 37.86 and 40.92%, respectively. To the best of our knowledge, the present study on dietary protein requirement of GIFT juveniles under inland saline water condition is the first report of its kind. However, Mohammadi et al. (2014) had attempted the optimization of dietary protein level in all male Nile tilapia fingerlings reared in inland saline water (8 ppt) in Iran using practical diets with four graded level of crude protein (15, 22, 29 and 36%).

The use of molecular tool for nutritional requirement study in animals can pave the way of better understanding about the molecular mechanism of growth due to feeding of graded level of dietary nutrients. Thus, in the present study, according to broken-line and second-order polynomial regression analysis in relation to the hepatic IGF-I expression, the optimum dietary crude protein requirement of GIFT juveniles reared in ISW of 10 ppt was found to be 37.72 and 38%, respectively. Narrow difference between both requirement values indicates the very high correlation between the growth and IGF-I expression in fish. Moreover, IGF-I gene expression is a suitable indicator for growth assessment of animals including fish as it directly stimulates the growth hormones (Brown et al., 2012).

5. Conclusion

In conclusion, the knowledge of nutritional requirement of any species is vital for formulation and preparation of cost-effective and eco-friendly feed to optimize the production. According to broken-line and second-order polynomial regression analysis in relation to WG% and expression of hepatic IGF-I gene, the optimum dietary crude protein levels of GIFT juveniles reared in ISW of 10 ppt salinity were found to be 37.86, 37.72, 40.92 and 38% with protein to energy ratio of 93.43, 94.30, 102.3, and 95 mg CP/kcal DE, respectively. These marginal differences indicate that the hepatic IGF-I expression may be considered for the nutrient requirement study as an alternate to classical growth experiment. Hence, the range of optimum dietary crude protein of GIFT juveniles reared in ISW of 10 ppt salinity were from 37.72 to 40.92% crude protein with isoenergetic of 400 kcal DE/100 g diet. These observations will provide baseline for further nutritional study on GIFT in ISW.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to express their gratitude to the Director, ICAR-Central Institute of Fisheries Education, Mumbai for providing well-equipped laboratories for the research work and the World Bank and the Govt. of India funded ICAR-National Agricultural Higher Education Project (NAHEP) for financial support to conduct the research.

References

Allan, G.L., Banens, B., Fielder, S., 2001. Developing sand saline aquaculture in Australia: part 2. Resource inventory and assessment. In: NSW fisheries final report series. 31. pp. 116.

Allan, G.L., Fielder, D.S., Fitzsimmons, K.M., Applebaum, S.L., Raizada, S., 2009. Inland saline aquaculture. In: New technologies in aquaculture. Woodhead publishing, pp. 1119–1147. https://doi.org/10.1533/9781845696474.6.1119.

Alzaid, A., Castro, R., Wang, T., Secombes, C.J., Boudinot, P., Macqueen, D.J., Martin, S.A., 2016. Cross talk between growth and immunity: coupling of the IGF axis to conserved cytokine pathways in rainbow trout. Endocrinology. 157 (5), 1942–1955. https://doi.org/10.1210/en.2015-2024.

American Public Health Association, APHA, 2005. Standard Methods for the Examination of Water and Wastewater, 21st ed. American Public Health Association, Washington DC 1220p.

AOAC, 1995. Animal feed. In: Cunniff, P.A. (Ed.), Official Methods of Analysis of AOAC International. AOAC international, Arlington, USA, pp. 5–15.

Bahnasawy, M.H., 2009. Effect of dietary protein levels on growth performance and body composition of monosex Nile tilapia, *Oreochromis niloticus* L. reared in fertilized tanks. Pak. J. Nutr. 8 (5), 674–678.

Bazaz, M.M., Keshavanath, P., 1993. Effect of feeding different levels of sardine oil on growth, muscle composition and digestive enzyme activities of mahseer, *Tor khudree*. Aquaculture. 115 (1–2), 111–119. https://doi.org/10.1016/0044-8486(93)90362-3.

Boyd, C.A., Chaney, P.L., Boyd, C.E., Rouse, D.B., 2009. Distribution of ground water suitable for use in saline-water aquaculture in central and west-Central Alabama. J. Appl. Aquac. 21 (4), 228–240. https://doi.org/10.1080/10454430903114048.

Brown, C.L., Cruz, E.M.V., Bolivar, R.B., Borski, R.J., 2012. Production, growth, and insulin-like growth factor-i (igf-i) gene expression as an instantaneous growth indicator in Nile tilapia *Oreochromis niloticus*. In: Saroglia, M., Liu, Z. (Eds.), Functional Genomics in Aquaculture. Wiley-Blackwell, Oxford, UK, pp. 79–89. https://doi.org/10.1002/9781118350041.ch3.

Cherry, I.S., Crandall, L.A., 1932. The specificity of pancreatic lipase: its appearance in the blood after pancreatic injury. Am. J. Physiol. Legacy Content. 100 (2), 266–273.

Chervinski, J., 1982. Environmental physiology of tilapias, in: the biology and culture of Tilapia. In: Proceedings of the 7th ICLARM conference, Manila, Philippines. The International Center for Living Aquatic Resources Management, pp. 119–128.

Cruz, E.M.V., Brown, C.L., Luckenbach, J.A., Picha, M.E., Bolivar, R.B., Borski, R.J., 2006. Insulin-like growth factor-I cDNA cloning, gene expression and potential use as a growth rate indicator in Nile tilapia, *Oreochromis niloticus*. Aquaculture. 251 (2–4), 585–595. https://doi.org/10.1016/j.aquaculture.2005.06.039.

De Silva, S.S., Gunasekera, R.M., Atapattu, D., 1989. The dietary protein requirements of young tilapia and an evaluation of the least cost dietary protein levels. Aquaculture. 80 (3–4), 271–284. https://doi.org/10.1016/0044-8486(89)90175-0.

Drapeau, G., 1974. Protease from *Staphylococcus aureus*. Methods Enzymol. 45, 469–475.

El-Sayed, A.F.M., 2006. Tilapia culture in salt water: environmental requirements, nutritional implications and economic potentials. In: Eighth symposium on advances in nutritional aquaculture, pp. 15–17.

El-Sayed, A.F.M., Teshima, S.I., 1992. Protein and energy requirements of Nile tilapia, *Oreochromis niloticus*, fry. Aquaculture. 103 (1), 55–63. https://doi.org/10.1016/0044-8486(92)90278-S.

FAO, 2016. Yearbook, F.A.O., Fishery and aquaculture statistics 2014. FAO, Rome.

FAO, 2019. Aquaculture feed and fertilizer resources information system. http://www.fao.org/fishery/affris/species-profiles/nile-tilapia/nile-tilapia-home/en/ (assessed 10 August 2018).

Garcia de la Serrana, D., Macqueen, D.J., 2018. Insulin-like growth factor-binding proteins of teleost fishes. Front. Endocrinol. 9, 80. https://doi.org/10.3389/fendo.2018.00080.

Gopan, A., Sahu, N.P., Varghese, T., Sardar, P., Maiti, M.K., 2019. Karanj protein isolate prepared from karanj seed cake: effect on growth, body composition and physio-metabolic responses in Labeo rohita fingerlings. Aquac. Nutr. https://doi.org/10.1111/anu.13033.

Hafedh, Y.A., 1999. Effects of dietary protein on growth and body composition of Nile tilapia, *Oreochromis niloticus* L. Aquac. Res. 30 (5), 385–393. https://doi.org/10.1046/j.1365-2109.1999.00343.x.

Halver, J.E., 1976. The Nutritional Requirements of Cultivated Warmwater and Coldwater Fish Species. In: FAO Technical Conference on Aquaculture, Kyoto (Japan).

Hofer, R., 1979a. The adaptation of digestive enzymes to temperature, season and diet in roach, *Rutilus rutilus* and Rudd *Scardinius erythrophthalmus*. Proteases. J. Fish Biol. 15 (4), 373–379. https://doi.org/10.1111/j.1095-8649.1979.tb03619.x.

Hofer, R., 1979b. The adaptation of digestive enzymes to temperature, season and diet in roach, *Rutilus rutilus* and Rudd *Scardinius erythrophthalmus*. Proteases. J. Fish Biol. 15 (4), 373–379. https://doi.org/10.1111/j.1095-8649.1979.tb03619.x.

Huang, J.F., Xu, Q.Y., Chang, Y.M., 2016. Effects of temperature and dietary protein on the growth performance and IGF-I mRNA expression of juvenile mirror carp (*Cyprinus carpio*). Aquac. Nutr. 22 (2), 283–292. https://doi.org/10.1111/anu.12254.

Humbel, R.E., 1990. Insulin-like growth factors I and II. Eur. J. Biochem. 190 (3), 445–462. https://doi.org/10.1111/j.1432-1033.1990.tb15595.x.

Jahan, I., Reddy, A.K., Sudhagar, S.A., Harikrishna, V., Singh, S., Varghese, T., Srivastava,

P.P., 2018. The effect of fortification of potassium and magnesium in the diet and culture water on growth, survival and osmoregulation of Pacific white shrimp, *Litopenaeus vannamei* Reared in Inland Ground Saline Water. Turk. J. Fish. Aquat. Sc. 18 (10), 1235–1243. https://doi.org/10.4194/1303-2712-v18_10_10.

Jauncey, K., 1982. The effects of varying dietary protein level on the growth, food conversion, protein utilization and body composition of juvenile tilapias (*Sarotherodon mossambicus*). Aquaculture. 27 (1), 43–54. https://doi.org/10.1016/0044-8486(82)90108-9.

Jayant, M., Muralidhar, A.P., Sahu, N.P., Jain, K.K., Pal, A.K., Srivastava, P.P., 2018. Protein requirement of juvenile striped catfish, *Pangasianodon hypophthalmus*. Aquacult. Int. 26, 375–389. https://doi.org/10.1007/s10499-017-0216-0.

Jiang, J., Zhang, D., Qiu, L., Lin, H., Jiang, S., 2010. Research on assessing effects of diets of mud carp (*Cirrhinus molitorella*) using IGF-I mRNA expression level. South China Fisheries Science. 6 (2), 66–72.

Jobling, M., 1994. Fish Bioenergetics, first ed. Chapman & Hall, London.

Jones, J.I., Clemmons, D.R., 1995. Insulin-like growth factors and their binding proteins: biological actions. Endocr. Rev. 16 (1), 3–34. https://doi.org/10.1210/edrv-16-1-3.

Kang'ombe, J., Brown, J.A., 2008. Effect of salinity on growth, feed utilization, and survival of *Tilapia rendalli* under laboratory conditions. J. Appl. Aquac. 20 (4), 256–271. https://doi.org/10.1080/10454430802498229.

Kaushik, S.J., Doudet, T., Médale, F., Aguirre, P., Blanc, D., 1995. Protein and energy needs for maintenance and growth of Nile tilapia (*Oreochromis niloticus*). J. Appl. Ichthyol. 11 (3–4), 290–296. https://doi.org/10.1111/j.1439-0426.1995.tb00029.x.

Kumar, R.V., Ramesh, K.S., Patil, P., Kumar, B., Manissery, J.K., 2011. Dietary protein requirement of stunted fingerlings of rohu, *Labeo rohita* (Hamilton) during grow-out stage. Indian J. Fish. 58 (4), 49–53.

Kumar, S., Sahu, N.P., Ranjan, A., Gupta, S., Sardar, P., Kumar, M., Gupta, G., 2019. Optimization of de-oiled rice bran inclusion level in the diet of Labeo rohita: effect on growth performance, nutrient utilization, insulin-like growth factors I and II gene expression. Aquac. Nutr. 25 (2), 477–485. https://doi.org/10.1111/anu.12873.

Lakra, W.S., Reddy, A.K., Harikrishna, V., 2014. Technology for commercial farming of Pacific white shrimp *Litopenaeus vannamei* in inland saline soils using ground saline water. CIFE Technical Bulletin 1 (2014), 1–28.

Lambers, H., 2003. Introduction, dryland salinity: a key environmental issue in southern Australia. Plant Soil 257 (2), 5–7. https://doi.org/10.1023/B:PLSO.0000003909.80658.d8.

Laron, Z., 2001. Insulin-like growth factor 1 (IGF-1): a growth hormone. Mol. Pathol. 54 (5), 311. https://doi.org/10.1136/mp.54.5.311.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method. Methods. 25 (4), 402–408. https://doi.org/10.1006/meth.2001.1262.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Macqueen, D.J., Garcia de la serrana, D., Johnston, I.A., 2013. Evolution of ancient functions in the vertebrate insulin-like growth factor system uncovered by study of duplicated salmonid fish genomes. Mol. Biol. Evol. 30 (5), 1060–1076. https://doi.org/10.1093/molbev/mst017.

Magid, A.M.A., Babiker, M.M., 1975. Oxygen consumption and respiratory behaviour of three Nile fishes. Hydrobiologia. 46 (4), 359–367.

Melo, J.F.B., Lundstedt, L.M., Moraes, G., Inoue, L.A.K.A., 2012. Effect of different concentrations of protein on the digestive system of juvenile silver catfish. Arq. Bras. Med. Vet. Zootec. 64 (2), 450–457. https://doi.org/10.1590/S0102-09352012000200027.

Millikin, M.R., 1982. Qualitative and quantitative nutrient requirement of fishes: a review. Fish. Bull. 80, 655–696.

Mires, D., 1995. The tilapias. In: Nash, C.E., Novotny, A.J. (Eds.), Production of aquatic animals, pp. 133–152.

Mjoun, K., Rosentrater, K., Brown, M.L., 2010. Tilapia: environmental biology and nutritional requirements. In: Fact sheets. South Dakota State University, pp. 164.

Mohammadi, M., Sarsangi, A.H., Haghighi, T.D., Webster, C., Rajabipour, F., Mashaii, N., Bitaraf, A., Hafeziyeh, M., 2014. Optimization of dietary protein in all male Nile Tilapia (*Oreochromis niloticus*) reared in inland saline water. Anim. Nutr. Feed Techn. 14 (1), 91–99.

Mohapatra, M., Sahu, N.P., Chaudhari, A., 2003. Utilization of gelatinized carbohydrate in diets of *Labeo rohita* fry. Aquac. Nutr. 9 (3), 189–196. https://doi.org/10.1046/j.1365-2095.2003.00243.x.

Morgan, P.R., 1972. Causes of mortality in the endemic tilapia of Lake Chilwa (Malawi). Hydrobiologia. 40 (1), 101–119. https://doi.org/10.1007/BF00123596.

Ng, W.K., Hanim, R., 2007. Performance of genetically improved Nile tilapia compared with red hybrid tilapia fed diets containing two protein levels. Aquac. Res. 38 (9), 965–972. https://doi.org/10.1111/j.1365-2109.2007.01758.x.

NRC, 2011. In: National Research Council (Ed.), Nutrient Requirements of Fish and Shrimp. The National Academies Press, Washington, DC, USA, pp. 70. https://doi.org/10.17226/13039.

Philippart, J.C., Ruwet, J.C., 1982. Ecology and distribution of tilapias: the biology and culture of tilapias. International Center for Living Aquatic Resources Management, Manila, pp. 15–59.

Philips, A.M., 1979. Calorie and energy requirement. In: Halver, J.E. (Ed.), Fish Nutrition. Academic Press, Inc., New York, pp. 1–28.

Phillips, A.M., Livingston, D.L., Poston, H.A., 1966. The effect of changes in protein quality, calorie sources and calorie levels upon the growth and chemical composition of brook trout. Fish. Res. Bull NY 29, 6–14.

Pierce, A.L., Breves, J.P., Moriyama, S., Hirano, T., Grau, E.G., 2011. Differential regulation of Igf1 and Igf2 mRNA levels in tilapia hepatocytes: effects of insulin and cortisol on GH sensitivity. J. Endocrinol. 211 (2), 201–210. https://doi.org/10.1530/JOE-10-0456.

Qiang, J., Yang, H., Wang, H., Kpundeh, M.D., Xu, P., 2012. Growth and IGF-I response of juvenile Nile tilapia (*Oreochromis niloticus*) to changes in water temperature and dietary protein level. J. Therm. Biol. 37 (8), 686–695. https://doi.org/10.1016/j.jtherbio.2012.07.009.

Reinecke, M., Collet, C., 1998. The phylogeny of the insulin-like growth factors. Int. Rev. Cytol. 183, 1–94. https://doi.org/10.1016/S0074-7696(08)60142-4.

Rick, W., Stegbauer, H.P., 1974. α-Amylase: measurement of reducing groups. Methods of Enzymatic Analysis. 2, 885–915.

Robbins, K.R., Saxton, A.M., Southern, L.L., 2006. Estimation of nutrient requirements using broken-line regression analysis. J. Anim. Sci. 84, E155–E165. https://doi.org/10.2527/2006.8413_supplE155x.

Ross, L.G., 2000. Environmental physiology and energetics. In: Beveridge, M.C.M., McAndrew, B.J. (Eds.), Tilapias: Biology and Exploitation. Springer, Dordrecht, pp. 89–128. https://doi.org/10.1007/978-94-011-4008-9_4.

Santiago, C.B., Bañes-Aldaba, M., Laron, M.A., 1982. Dietary crude protein requirement of *Tilapia nilotica* fry. Kalikasan Philipp. J. Biol. 11 (2–3), 255–265.

Sarig, S., 1969. Winter storage of Tilapia. FAO Fish Culture Bulletin 2, 8–9.

Setiadi, E., Widyastuti, Y.R., Prihadi, T.H., 2018. Water quality, survival, and growth of red tilapia, Oreochromis niloticus cultured in aquaponics system, in: E3S web of conferences. EDP Sciences 47, 02006. https://doi.org/10.1051/e3sconf/20184702006.

Shiau, S.Y., Hsieh, J.F., 2001. Quantifying the dietary potassium requirement of juvenile hybrid tilapia (*Oreochromis niloticus* × *O. aureus*). Br. J. Nutr. 85 (2), 213–218. https://doi.org/10.1079/BJN2000245.

Shiau, S.Y., Huang, S.L., 1990. Optimal dietary protein level for hybrid tilapia (*Oreochromis niloticus* × *O. aureus*) reared in seawater. Aquaculture. 81 (2), 119–127. https://doi.org/10.1016/0044-8486(89)90237-8.

Siddiqui, A.Q., Howlader, M.S., Adam, A.A., 1988. Effects of dietary protein levels on growth, feed conversion and protein utilization in fry and young Nile tilapia, *Oreochromis niloticus*. Aquaculture. 70 (1–2), 63–73. https://doi.org/10.1016/0044-8486(88)90007-5.

Suresh, A.V., Lin, C.K., 1992. Tilapia culture in saline waters: a review. Aquaculture. 106 (3–4), 201–226. https://doi.org/10.1016/0044-8486(92)90253-H.

Tatar, M., Bartke, A., Antebi, A., 2003. The endocrine regulation of aging by insulin-like signals. Science. 299 (5611), 1346–1351. https://doi.org/10.1126/science.1081447.

Wilson, R.P., Halver, J.E., 1986. Protein and amino acid requirements of fishes. Annu. Rev. Nutr. 6 (1), 225–244. https://doi.org/10.1146/annurev.nu.06.070186.001301.

Winfree, R.A., Stickney, R.R., 1981. Effects of dietary protein and energy on growth, feed conversion efficiency and body composition of *Tilapia aurea*. J. Nutr. 111 (6), 1001–1012. https://doi.org/10.1093/jn/111.6.1001.