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Effects of different suboptimal dietary protein levels on growth, nutrient utilization and physio-metabolic status of *Anabas testudineus* fingerlings in inland saline water

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Abstract

A 60-day feeding trial was conducted to illustrate the effect of suboptimal crude protein (CP) levels on growth and physio-metabolic status of *Anabas testudineus* fingerlings reared in inland saline water (ISW) at 8 g/L salinity. Six isoenergetic (16 MJ/kg) and isolipidic (60 g/kg) diets with 240 (T24), 260 (T26), 280 (T28), 300 (T30), 320 (T32) and 340 (T34) g suboptimal CP/kg diet were formulated. Weight gain (%) and specific growth rate were significantly higher ($p < .05$) in T32 and T34 groups. Feed conversion ratio was significantly lower ($p < .05$) in T30 and T32 group than the other groups. The protein utilizing efficiency was significantly ($p < .05$) decreased beyond 320 g CP/kg diet. The protease activity was significantly increased ($p < .05$) up to 320 g CP/kg diet, whereas amylase activity was significantly higher ($p < .05$) in 240–260 g CP/kg diets. The transaminase enzyme activities were significantly higher ($p < .05$), and energy demanding enzymes were significantly lower ($p < .05$) in 300–340 g CP/kg diets. Fish of T24 and T26 groups had lowest ($p < .05$) glycogen, erythrocyte count and total protein with highest ($p < .05$) glucose and antioxidant enzyme activities. In conclusion, feeding 320 g CP/kg diet is recommended for cost-effective growth of *A. testudineus* reared in ISW at 8 g/L salinity.

KEYWORDS
Anabas testudineus, dietary suboptimal protein, growth, Inland saline water, nutrient utilization, physio-metabolic status

1 | INTRODUCTION

Over the last decades, the tremendous demand-driven growth in aquaculture sector is possible due to the species diversification, intensification and high input farming. However, land and water scarcity are likely to affect the horizontal expansion of aquaculture due to increased urbanization and growing population. Nevertheless, sustainable and alternate use of abandoned land such as salt affected inland saline areas have immense potential for expanding the aquaculture activity. The problem of soil salinity and water logging affects more than 300-million-hectare land worldwide, which is not suitable for agriculture crop and expected to rise by 50% by the year 2050 (Jamil et al., 2011; Partridge et al., 2008). The salt affected areas with inland saline water (ISW) of salinity ranging from 3 to 15 g/L has been made use as a potential site for aquaculture practice for many euryhaline and salt tolerant fish species (Allan et al., 2009). The salinity of ISW in North Western part of India shows variation at both spatial and depth profile. This offers an opportunity for farmers to manage salinity in the culture pond depending upon the species stocked by pumping out water by pumps installed at different depths. Several brackishwater and freshwater fish species such as *Chanos*

chanos (Jana et al., 2006), *Lates calcarifer* and *Oncorhynchus mykiss* (Partridge et al., 2006), GIFT tilapia (Singha, Shamna, Sahu, Sardar, Hari Krishna, et al., 2020; Singha, Shamna, Sahu, Sardar, Harikrishna, et al., 2020) and *Mugil cephalus* (Talukdar, Deo, et al., 2020) have been cultured in ISW. Among the candidate species, culture of *L. vannamei* has emerged as a successful enterprise in salt affected region worldwide (Talukdar, Dharmendra Deo, et al., 2020; Roy et al., 2010). However, the large-scale usage of salt affected inland regions for commercial aquaculture is still constrained by the high infrastructure requirement and risks associated in shrimp farming. Therefore, culture of salinity tolerant fishes in ISW requiring minimal infrastructure could offer options to the resource poor farmers of salt affected inland regions. In this context, culture of salinity tolerant freshwater fishes in ISW could be another perspective towards expanding the aquaculture activities.

The climbing perch, *Anabas testudineus*, is a freshwater air-breathing fish commonly found in canals, lakes, ponds, paddy fields, swamps and estuaries and can tolerate adverse environmental conditions (Amornsakun et al., 2005; Talwar & Jhingran, 1991). *A. testudineus* fetches a very high market price with good consumer preference and distributed all across South-East Asia (Takagi et al., 2011). The fish is being considered as a potential candidate species for aquaculture diversification since the year 2000 (Sarkar et al., 2005). Generally, the culture of *A. testudineus* is carried out in freshwater, whereas it also inhabits in brackishwater. In context, several studies concluded that the survival and growth of *A. testudineus* is not affected in saline water and can be cultured up to 15 g/L salinity (Chotipuntu & Avakul, 2010; Chowdhury et al., 2014; Dubey et al., 2015). However, salinity tolerance and reorganization of the physiological functions to survive in the saline water will have a significant energetic cost to be used for osmoregulatory adaptation (Tseng & Hwang, 2008). Further, *A. testudineus* actively degrades protein and catabolizes amino acid to gain extra energy required for osmoregulation as an adaptive response to saline environment (Chang et al., 2007). Considering the ability to tolerate saline water and adverse environmental condition, *A. testudineus* could possibly be a candidate species to be culture in ISW.

For culture practice of any species, it is crucial to formulate nutritionally balanced along with economically feasible feed. Fish growth rate is a result of the equilibrium between protein synthesis and degradation that in turn relies on the dietary protein level and environmental condition (NRC, 2011). Feeding low protein in the fish diet results in reduced growth rate, whereas surplus protein can be catabolized to provide energy while excreting more ammonia into water (McGoogan & Gatlin, 2000; Yang et al., 2016). Besides, the protein requirement may differ in ISW-reared fish for the reorganization of physiological processes during salinity acclimatization that may alter growth (Tseng & Hwang, 2008; Wootton, 1990). Considerable energy saving is possible if optimum dietary protein is fed without compromising the growth and physio-metabolic status of fish. In this context, widely available ingredient such as leaf meal or plant protein source can be the alterative and inexpensive protein source for aquafeed (Adewolu et al., 2008).

Previous studies reported that 300–400 g crude protein (CP)/kg diet is optimum for the maximum growth of *A. testudineus* fry or fingerlings reared in freshwater (Chotipuntu & Avakul, 2010; Hossain et al., 2012; Patra et al., 2017). Yet contrary to the actual situation on field, feeding low protein (280–300 g CP/kg diet) feed is usually being practised by the farmers in lieu to reduce cost and due to unavailability of species-specific feed. Besides, higher inclusion of protein in the diet escalates the feed cost, which will not be economically viable for the culture of *A. testudineus* in ISW. Therefore, the present study was conducted to evaluate the effect of suboptimal dietary protein levels on growth, nutrient utilization, digestive enzymes activities and physio-metabolic status of *A. testudineus* fingerlings reared in ISW at 8 g/L salinity.

2 | MATERIALS AND METHODS

2.1 | Diet preparation

Six experimental diets were formulated to be isoenergetic (16 MJ/kg) and isolipidic (60 g/kg lipid) containing 240, 260, 280, 300, 320 and 340 g suboptimal crude protein (CP)/kg diet and designated as T24, T26, T28, T30, T32 and T34 treatment groups, respectively (Table 1). Defatted soybean meal, groundnut oil cake, fish meal and *Sesbania* leaf meal were used as main protein source, whereas de-oiled rice bran and wheat flour as an energy source to make diets isoenergetic. The α-cellulose, carboxymethyl cellulose (CMC) and butylated hydroxytoluene (BHT) were used as filler, binder and antioxidant in the diets, respectively. *Sesbania* leaves were collected from local village of Assam, India, and washed thoroughly before sun drying and stored in sealed container until use. All the practical ingredients were properly mixed with required amount of water to make dough and steam-cooked in a pressure cooker for 30 min. Additives, oil and vitamins mineral mixture (Premix Plus, India) were added into the dough after cooling, and the dough was pressed through a mechanical pelletizer (Uniextrude, S.B. Panchal & Company, India) of 2 mm diameter to prepare the pellets. The pellets were air-dried followed by drying in hot air oven at 50°C until the moisture level was below 10%, labelled according to the treatments and stored at 4°C until use.

2.2 | Procurement and salinity acclimatization of experimental fish

For the experiment, ground ISW having a salinity of 12 g/L from bore well was pumped out into four cemented tanks (10,000 L) following filtration through 100-μm filter bag to remove any unwanted debris (Raizada et al., 2015). After settlement for a week,

TABLE 1 Ingredients and proximate composition of the experimental diets (g/kg dry matter basis)

	Treatments					
Composition	**T24**	**T26**	**T28**	**T30**	**T32**	**T34**
Ingredients (g/kg)						
Fish meal[b]	50	50	50	50	50	50
GNOC[b]	120	150	180	210	240	270
DSBM[c]	140	170	200	230	260	290
SLM[a]	100	100	100	100	100	100
DORB[b]	120	120	120	120	120	120
Wheat flour[b]	340	280	220	160	100	40
Fish oil: Veg oil (1:1)[b]	50	50	50	50	50	50
Vit-Min mix[d]	15	15	15	15	15	15
Cellulose[c]	44.8	44.8	44.8	44.8	44.8	44.8
CMC[c]	15	15	15	15	15	15
Betaine[c]	5	5	5	5	5	5
BHT[c]	0.2	0.2	0.2	0.2	0.2	0.2
Total	1000	1000	1000	1000	1000	1000
Proximate composition (g/kg; on dry matter basis; mean of triplicates)						
Moisture	76.2	76.5	75.0	76.4	74.4	75.2
Crude protein	242.8	262.7	283.2	303.1	322.2	342.9
Ether extract	62.5	62.7	63.0	63.2	63.4	63.7
Total ash	84.1	83.6	83.1	82.6	82.5	82.8
Crude fibre	74.3	73.9	73.3	73.1	72.3	71.6
Nitrogen free extract	536.4	517.1	497.4	477.4	459.7	439.1
Digestible energy (MJ/kg)	16.07	16.05	16.07	16.04	16.10	16.05
P/E ratio (mg protein/kJ DE)	15.61	16.86	18.15	19.41	20.60	21.91

Note: vitamin A, 55,00,000 IU; vitamin D3, 11,00,000 IU; vitamin B2, 2000 mg; vitamin E, 750 mg; vitamin K, 1,000 mg; vitamin B6, 1,000 mg; vitamin B12, 6 mcg; calcium pantothenate, 2,500 mg; nicotinamide, 10 g; choline chloride, 150 g; Mn, 27,000 mg; I, 1,000 mg; Fe, 7,500 mg; Zn, 5,000 mg; Cu, 2000 mg; Co, 450 mg; L-lysine, 10 g; DL-methionine, 10 g; selenium,125 mg; vitamin C, 2500 mg.

SLM: Sesbania leaf meal, DSBM: Defatted soybean meal, DORB: Deoiled rice bran, GNOC: Groundnut oil cake, CMC: Carboxymethyl cellulose, BHT: Butylated hydoxytoluene.

[a]Collected from local village, Assam, India.

[b]Procured from local market, India.

[c]Procured from HImedia ltd., India.

[d]Composition of vitamin mineral mix (Premix Plus) (quantity/2 kg).

ISW was diluted to 8 g/L salinity using freshwater and used as per the experimental needs. The fingerlings of *A. testudineus* were procured form local fish farm of West Bengal, India, and were safely transported to wet laboratory of Rohtak Centre, ICAR-Central Institute of Fisheries Education (ICAR-CIFE), Rohtak, Haryana, India, in airtight oxygen filled containers. The fish were randomly distributed into previously disinfected circular tanks of 1000-L capacity having freshwater and left overnight. The fish were fed with commercial fish feed twice a day to satiation level during the acclimatization period of 7 days. After 7 days of acclimatization, ground ISW of 8 g/L salinity was added gradually into the tank to achieve the desired salinity of 8 g/L by increasing 1 g/L salinity per day and acclimatized for another 7 days to the saline culture condition.

2.3 | Ethical statement

The feeding trial and subsequent handling and sampling of the experimental fish were carried out as per the guidelines of Ethical committee of ICAR-CIFE, Mumbai, India.

2.4 | Experimental set-up, feeding trial and maintenance

The salinity acclimatized fingerlings of *A. testudineus* (average initial body weight 2.02 ± 0.3 g) were randomly stocked into triplicate tanks (300-L capacity) in six treatment groups, *viz*., T24, T26, T28, T30, T32 and T34 following a completely randomized design (CRD)

with 15 fish per tank maintaining 40/m^2 stocking density. Based on the initial observation of feed consumption, all groups of fish were hand fed twice a day (09:00 and 18:00 h) to satiation level for 60 days. The excreta were siphoned every day morning before commencing feeding and equal volume of well-aerated ISW of 8 g/L salinity were added to maintain constant water volume. Water salinity was maintained at 8 g/L throughout the experimental period as per the experimental design. The tanks were kept inside the wet laboratory maintaining 12 h of photoperiod, and aeration was provided with submerged air diffusers to each experimental tank throughout the experimental period. At every 15-day interval, all the fish from each tank were weighed to determine growth and to observe the general health condition.

Water quality parameters including water temperature, pH and salinity were recorded every day, whereas dissolved oxygen concentration, total alkalinity, total hardness, ammonia-N, nitrite-N, nitrate-N, potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}) ion levels and osmolality were recorded at 7-day interval. The water temperature was measured using a thermometer (MERCK, Germany), water pH by pH probe (HANNA Instruments, Singapore) and salinity with a refractometer (Atago, Tokyo, Japan). Dissolved oxygen concentration, total hardness, Ca^{2+} and Mg^{2+} concentrations were measured as per the standard methods (APHA, 2005). Ammonia-N, nitrite-N and nitrate-N were determined using a commercial test kit (Spectroquant NOVA-MERCK, Germany). The K^+ concentration was measured using Microprocessor flame photometer (Model 1382, ESICO, India).

2.5 | Sampling and sample preparation

Before starting of the trial, 20 fish were randomly sampled and kept for the initial whole-body proximate composition analysis. At the end of the experimental period, fish were counted and not given feed overnight before taking total body weight from each tank using electronic weighing balance. Three fish from each tank were anaesthetized with clove oil (50 μl/L) for the collection of blood. Blood samples were drawn from the caudal vein using a 1-ml hypodermic syringe (without anticoagulant) and transferred immediately into Eppendorf tubes and kept for an hour at room temperature for clotting and centrifuged (4850 *g* for 10 min at 4°C) to obtain serum. Then, remaining blood sample was transferred to an Eppendorf tube containing EDTA, gently shaken and kept for analysis of haematological parameters. Subsequently, six fish from each tank were anaesthetized with clove oil (50 μl/L) for the analysis of enzyme assays (three fish per tank) and whole-body composition (three fish per tank). Dissected tissues of different organs (intestine, liver, muscle and gill) were immediately homogenized with ice-cold 0.25 M sucrose solution in a glass tube using Teflon-coated mechanical tissue homogenizer (MICCRA D-9, ART Prozess & Labortechnik, Germany) to prepare a 5% tissue homogenate. The homogenate samples were centrifuged (2800 *g* for 10 min at 4°C) using a refrigerated-centrifuge (Thermo Scientific, USA), and the supernatant was collected in sample vials and stored at −20°C until use. Muscle sample from these fish was separately kept for the analysis of glycogen content. For the calculation of body indices, liver and intestine from three fish per tank were dissected out and weighed. Quantification of the protein of different tissues was carried out by Lowry et al. (1951) method.

2.6 | Growth, nutrient utilization, survival and body indices

The parameters related to growth (per cent weight gain, WG%; specific growth rate, SGR), nutrient utilization (feed conversion ratio, FCR; protein efficiency ratio, PER; apparent net protein utilization, ANPU; protein growth rate, PGR), survival and body indices (hepatosomatic index, HSI; intestinal somatic index, ISI) were calculated as follows-

$$\text{WG (\%)} = \{(\text{Final body weight in g} - \text{Initial body weight in g}) / \text{Initial body weight in g}\} \times 100$$

$$\text{SGR} = [\{\text{Log}_e(\text{Final body weight in g}) - \text{Log}_e(\text{Initial body weight in g})\} / \text{Number of days cultured}] \times 100$$

$$\text{FCR} = \text{Feed consumed (in g on dry weight basis)} / \text{Net weight gain (in g on wet weight basis)}$$

$$\text{PER} = \text{Net weight gain (in g on wet weight basis)} / \text{Protein intake (in g on dry weight basis)}$$

$$\text{ANPU (\%)} = \{(\text{Final body protein} - \text{Initial body protein}) / \text{total protein fed}\} \times 100$$

$$\text{PGR (\%/day)} = [\{\text{Log}_e(\text{Final body protein}) - \text{Log}_e(\text{Initial body protein})\} / \text{Number of days cultured}] \times 100$$

$$\text{Survival (\%)} = (\text{Final number of fish harvested} / \text{Initial number of fish stocked}) \times 100$$

$$\text{HSI (\%)} = (\text{wet weight of liver in g} / \text{whole body weight of fish in g}) \times 100$$

$$\text{ISI (\%)} = (\text{wet weight of intestine in g} / \text{whole body weight of fish in g}) \times 100$$

2.7 | Quantification of RNA and RNA/ DNA ratio

Quantification of nucleic acids was performed by pentose analysis following the method of Schneider (1957). DNA and RNA concentrations in the tissue were calculated based on the following formulae-

$$\mu\text{g/DNA/ml} = ¼ \text{ OD at 600 nm} \times 0.019$$

$$\mu\text{g/RNA/ml} = \{(\text{OD at 660 nm} + 0.0081) - \mu\text{g DNA/ml} \times 0.013\} / 0.116$$

$$\text{RNA/DNA} = \mu\text{g/DNA/ml} / \mu\text{g/RNA/ml}$$

2.8 | Proximate composition analysis

The proximate composition of the experimental diets and fish was determined using the standard methods of AOAC (1995) for

moisture, crude protein (CP), ether extract (EE), crude fibre (CF) and total ash (TA). Moisture was determined by drying samples in an oven at 102°C till a constant weight. The CP and EE content were determined using an automated Kjeldahl (Kelplus, PELICAN, India) and Soxhlet apparatus (Model SD2, 1045, PELICAN, India), respectively. The TA content was determined by burning the samples in a muffle furnace (WIT; C & L Tetlow, Australia) at 550°C for 6 h. The CF of the diets was determined by Fibre tech (Tulin equipment, India). The nitrogen-free extract (NFE) and digestible energy (DE) (Halver, 1976) of the diets were calculated based on the following formulas:

$$\text{Nitrogen free extract (NFE)} = 1000 - (\text{g/kg CP} + \text{g/kg EE} + \text{g/kg CF} + \text{g/kg TA})$$

$$\text{Digestible energy (MJ/kg)} = \{(\text{CP g/kg} \times 17) + (\text{EE g/kg} \times 37) + (\text{NFE g/kg} \times 17)\} / 100$$

2.9 | Enzyme assays

2.9.1 | Digestive enzyme assays

The protease activity was assayed following the method of Drapeau (1974) using casein as substrate. The reaction mixture was prepared by adding 1% (w/v) casein as substrate to 0.05 M Tris-phosphate buffer (pH 7.8) and incubated for 5 min at 37°C. Subsequently 100 µl tissue homogenate was added to the reaction mixture, and the reaction was stopped after 10 min by adding 10% trichloroacetic acid (TCA) followed by filtration. Protease activity was expressed as millimole of tyrosine released/min/g protein at 37°C. The amylase activity was assayed following the dinitro-salicylic-acid (DNS) method of Rick and Stegbauer (1974). The reaction mixture consists of 0.1 M phosphate buffer (pH 7.0), substrate (2% w/v starch) and tissue homogenate (100 µl) and incubated for 30 min at 37°C. The reaction was stopped by adding DNS to the reaction mixture and kept for 5 min in boiling water bath. Following cooling of the reaction mixture, 10 ml of distilled water was added and absorbance was recorded at 540 nm. The amylase activity was expressed as the micromole of maltose released from starch/min/mg protein at 37°C. Lipase activity was determined as described by Cherry and Crandall (1932) method. The reaction mixture was prepared with distilled water, sample, phosphate buffer solution (pH 7.0) and olive oil emulsion and incubated for 37°C for 24 h. The reaction mixture was then titrated against 0.05 N NaOH until it showed a permanent pink colour. The milli-equivalent of alkali consumed was the activity of the enzyme and expressed as unit/mg protein.

2.9.2 | Metabolic enzyme assays

The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were assayed following the method of Wooten (1964). The α-ketoglutarate and DL-alanine were used as the substrate for ALT activity and α-ketoglutarate and DL-aspartic acid for AST activity. The substrate and tissue homogenate reaction mixture were incubated at 37°C for 1 h, and the reaction was stopped by adding of 2, 4-dinitrophenylhydrazine (DNPH). Following incubation at room temperature for 20 min, 0.4 N NaOH (5 mL) was added and the absorbance was recorded at 540 nm. The activity of the enzyme was expressed as nanomoles oxaloacetate formed/min/mg protein at 37°C. The lactate dehydrogenase (LDH) activity was estimated by the method of Wrobleiuski and Laude (1955). The reaction was started by adding 100 µl sodium pyruvate to the reaction mixture containing 0.1 M phosphate buffer (pH 7.5), NADH solution (2 mg NADH dissolved in 1 ml of phosphate buffer) and 100 µl of tissue homogenate. The absorbance was recorded at 340 nm at 30-s intervals to 3 min and enzymatic activity was expressed as unit/mg protein/min at 37°C, where one unit was equal to Δ0.01 OD/min. The malate dehydrogenase (MDH) activity was assayed by the method of Ochoa (1955) and the specific enzymatic activity was expressed as unit/mg protein/min at 37°C, where one unit was equal to Δ0.01 OD/min.

2.10 | Determination of serum glucose concentration, osmolality and glycogen content

The serum glucose was measured following the method of Trinder (1969) using glucose kit (ERBA G-250) and value expressed as mg/dl. The serum osmolality was measured using the cryoscopic osmometer (Osmomat® 030, Gonotec GmbH, Berlin, Germany) and expressed as mOsm/kg. The glycogen concentration of muscle was measured with the method of Dmors et al. (1956). Briefly, muscle homogenate was prepared in the 5% trichloroacetic acid (TCA) buffer for 2 min at 5000 rpm. After supernatant separation, the glycogen was dissolved by 0.5 ml of distilled water, and then, 5 ml of concentrated sulphuric acid and phenol (5%) was added and mixed followed by reading absorbance at 490 nm. The glycogen content was expressed as mg glycogen per g wet weight (mg/g).

2.11 | Oxidative stress-related enzymes and haematological parameters

The superoxide dismutase (SOD) was assayed following the method of Mishra and Fridovich (1972) based on the oxidation of epinephrine-adrenochrome transition by the enzyme and absorbance was measured at 480 nm for 3 min. The activity of one unit of SOD was expressed as the amount of protein required for 50% inhibition of epinephrine auto-oxidation. The catalase (CAT) activity was assayed according to the method of Takahara et al. (1960) using phosphate buffer (50 mM, pH 7.0), and the reaction was started by the addition of H_2O_2 solution and the decreasing absorbance was measured at 240 nm. One unit of CAT activity was the amount of protein required to decompose H_2O_2.

The red blood cell (RBC) count was done using Neubauer's counting chamber of a haemocytometer as described by Hendricks (1952). A solution having 20 µl of blood with 3980 µl of erythrocyte

TABLE 2 Growth, nutrient utilization, nucleic acid content and survival of *A. testudineus* fingerlings fed diets containing different dietary suboptimal CP levels

Parameters	Treatments[1]						SEM	(*p* values)	Regression analysis (*p*, r^2)	
	T24	T26	T28	T30	T32	T34		Overall	Linear	Quadratic
WG (%)	118.27^{a}	142.09^{b}	158.68^{c}	196.62^{d}	209.51^{de}	220.55^{e}	9.07	0.000	0.000, 0.97	.089, 0.98
SGR (%/day)	1.30^{a}	1.47^{b}	1.58^{c}	1.81^{d}	1.87^{de}	1.94^{e}	0.05	0.000	0.000, 0.96	.015, 0.98
FCR	2.53^{d}	2.19^{c}	1.99^{bc}	1.70^{a}	1.62^{a}	1.80^{ab}	0.08	0.000	0.000, 0.77	.001, 0.96
PER	1.65^{a}	1.76^{ab}	1.80^{ab}	1.96^{b}	1.94^{b}	1.64^{a}	0.04	0.035	0.312, 0.06	.005, 0.69
ANPU (%)	26.91^{a}	28.80^{ab}	29.56^{abc}	32.54^{bc}	33.90^{c}	28.34^{ab}	0.77	0.038	0.067, 0.27	.022, 0.70
PGR (%/day)	0.14^{a}	0.15^{a}	0.17^{ab}	0.18^{ab}	0.24^{b}	0.23^{b}	0.01	0.031	0.002, 0.89	.058, 0.89
DNA/ml	0.60	0.60	0.56	0.58	0.56	0.58	0.01	0.974	0.609, 0.02	.673, 0.03
RNA/ml	2.46^{a}	2.54^{ab}	2.98^{b}	3.04^{b}	3.61^{c}	3.73^{c}	0.12	0.000	0.000, 0.95	.622, 0.95
RNA/DNA	4.11^{a}	4.26^{a}	5.31^{ab}	5.37^{ab}	6.47^{b}	6.51^{b}	0.26	0.005	0.000, 0.93	.924, 0.93
Survival (%)	100	100	100	100	100	100	–	–	–	–

Note: Data expressed as mean (n = 3); Mean values in the same row with different superscripts differ significantly ($p < .05$).

Abbreviations: ANPU, Apparent net protein utilization; FCR, Feed conversion ratio; PER, Protein efficiency ratio; PGR (%/day), Protein growth rate; SEM, Standard error means; SGR, Specific growth rate; WG (%), Weight gain %.

[1]Treatment groups fed with graded levels of dietary suboptimal crude protein (CP), *that is* T24, 240 g CP/kg; T26, 260 g CP/kg; T28, 280 g CP/kg; T30, 300 g CP/kg; T32, 320 g CP/kg and T34, 340 g CP/kg.

diluting fluid (Qualigens, Mumbai, India) was taken in a clean test tube and shaken gently. The white blood cell (WBC) count was performed following the method of Shaw (1930). The haemoglobin (Hb) content was estimated following the cyanmethemoglobin method (Van Kampen & Zijlstra, 1961) using Drabkins Fluid (Qualigens, GlaxoSmithKline Pharmaceutical Ltd, India). The optical density (OD) was measured at 540 nm, and the final concentration was calculated based on the following formulas:

$$\text{Haemoglobin (mg/dl)} = [\{\text{OD (Test)}/\text{OD (Standard)}\} \times (251/1000) \times 60]$$

2.12 | Statistical analysis

Statistical analysis of data was performed by using the software program SPSS 22.0 (IBM Inc., Chicago, USA) for Windows 10 and then was expressed as means (n = 3) and standard error of means (SEM). All data were subjected to one-way ANOVA along with orthogonal-polynomial contrast analysis. Post hoc comparison of mean among treatment groups was carried out by Duncan's multiple range test (DMRT) at a 5% level of probability ($p < .05$). The regression analysis (both linear and quadratic) was performed to see the best fit-model (R^2) of the responses with the dietary suboptimal protein levels. Finally, parameters with high ($R^2 \geq 0.7$) R^2 value (both linear and quadratic) were selected to see the strength of association (correlation coefficient, r) with growth (per cent weight gain, WG%).

3 | RESULTS

3.1 | Physiochemical parameters of water

Water quality parameters such as water temperature, dissolved oxygen concentration, pH, salinity, total alkalinity, ammonia-N, nitrite-N, nitrate-N, calcium, magnesium and potassium were found in the range from 27–30°C, 5.6–6.2 mg/L, 7.7–8.3, 7.8–8 g/L, 254–262 mg/L, 0.013–0.033 mg/L, 0.001–0.003 mg/L, 0.03–0.05 mg/L, 210–223 mg/L, 442–527 mg/L and 92–98 mg/L, respectively, throughout the experimental period.

3.2 | Growth, feed utilization and survival

The effects of dietary suboptimal protein levels on the growth and survival of *A. testudineus* fingerlings reared in ISW are presented in Table 2. The WG% and SGR were significantly higher ($p < .05$) in the T34 group than the other treatment groups except T32 group, whereas significantly lower ($p < .05$) WG% and SGR were found in the T24 group as compared to other groups. The regression analysis of WG% and SGR showed both high linear (R^2 = 0.97 and 0.96, respectively) and quadratic (R^2=0.98 and 0.98, respectively) relation to feeding suboptimal CP levels. The overall, linear and quadratic trends of FCR were significantly decreased ($p < .05$) up to feeding

320 g CP/kg diet (T32 group), whereas quadratic relation ($R^2 = 0.96$) of FCR was higher than linear ($R^2 = 0.77$) with respect to increasing suboptimal CP levels. Significantly lower ($p < .05$) FCR was found in T30 and T32 groups than that of other groups except T34 group. The per cent survival remained similar ($p > .05$) among all the treatment groups.

3.3 | Protein utilization and nucleic acid content

The overall and quadratic trend of PER and ANPU% were significantly affected ($p < .05$) by the dietary suboptimal CP levels in which significantly higher ($p < .05$) values found in T30 and T32 groups than that of T24 and T34 groups except T26 and T28 groups (Table 2). However, the regression analysis of PER and ANPU% showed very low linear (R^2 = 0.06 and 0.27, respectively) and high quadratic (R^2 = 0.69 and 70, respectively) relations with the suboptimal CP levels. The overall and linear trends of PGR were affected significantly ($p < .05$) and regression analysis showed high linear (R^2 = 0.89) and quadratic (R^2 = 0.89) relations with the suboptimal CP levels (Table 2). Significantly higher ($p < .05$) PGR value was found in the T32 group than that of T24 and T26 groups but similar to T28, T30 and T34 groups with significantly lower ($p < .05$) value observed in T24 and T26 groups. The overall and linear trend of RNA concentration and RNA/DNA ratio were affected significantly ($p < .05$) and showed both high linear (R^2 = 95 and 93, respectively) and quadratic (R^2 = 95 and 93, respectively) (Table 2). The RNA concentration and RNA/DNA ratio were significantly higher ($p < .05$) in T32 and T34 groups as compared to other groups.

3.4 | Whole-body proximate composition and body indices

The overall and linear trends of whole-body protein and HSI of *A. testudineus* fingerlings were significantly affected ($p < .05$) due to dietary suboptimal CP levels (Table 3). The whole-body protein was gradually increased up to 320 g CP/kg diet (T32 group), which was similar ($p > .05$) to 340 g CP/kg diet (T34 group), whereas significantly lower ($p < .05$) whole-body protein content was found in T24 and T26 groups as compared to other groups. Besides, the whole-body protein content showed moderate linear ($R^2 = 0.62$) and high quadratic ($R^2 = 0.88$) relation to suboptimal CP levels. Whole body moisture, lipid and ash content remain similar ($p > .05$) among the different treatment groups. The HSI value was significantly lower ($p < .05$) in the T24 group than the other groups except T26 group (Table 3). The regression analysis showed higher quadratic relation ($R^2 = 0.96$) than the linear ($R^2 = 0.82$) relation to increasing suboptimal CP level, where HSI value increased only up to feeding 30% dietary CP (T30 group), whereas HSI value did not differ significantly ($p > .05$) among T30 to T34 groups. The ISI value was found to be similar ($p > .05$) among the different treatment groups.

3.5 | Enzyme assays

3.5.1 | Digestive enzymes activities

The overall, linear and quadratic trends of protease activity was significantly affected ($p < .05$) and showed high quadratic relation ($R^2 = 0.91$) to dietary suboptimal CP levels (Table 4). The proteases activity was significantly higher ($p < .05$) in T32 and T34 groups than that of other treatment groups. However, significantly lower ($p > .05$) value was found in T24 and T26 group as compared to other treatment groups. The overall and quadratic trends of amylase activity was significantly affected ($p < .05$) and showed high quadratic (R^2=0.91) relation to suboptimal CP levels (Table 4). The amylase activity showed a decreasing trend in response to dietary protein levels with a significantly higher ($p < .05$) activity in T24 and T26 groups than that of T32 and T34 groups except T28 and T30 groups. The lipase activity remains similar ($p > .05$) among the different treatment groups.

TABLE 3 Whole body proximate composition (% wet weight) and body indices of *A. testudineus* fingerlings fed diets containing different dietary suboptimal CP levels

	Treatments[1]							(*p* values)	Regression analysis (p, r^2)	
Parameters	T24	T26	T28	T30	T32	T34	SEM	Overall	Linear	Quadratic
Moisture (%)	74.52	74.21	74.39	74.26	74.12	74.33	0.06	0.624	0.365, 0.05	.401, 0.10
Crude protein (%)	15.38^{a}	15.50^{a}	15.63^{ab}	15.76^{ab}	16.23^{b}	16.10^{b}	0.10	0.030	0.001, 0.62	.740, 0.88
Crude fat (%)	4.75	4.83	4.91	4.94	4.81	4.64	0.06	0.814	0.664, 0.80	.036, 0.95
Total Ash (%)	4.23	4.10	4.28	4.27	4.13	4.11	0.08	0.986	0.969, 0.11	.745, 0.29
HIS	1.33^{a}	1.42^{ab}	1.61^{bc}	1.74^{c}	1.72^{c}	1.75^{c}	0.05	0.001	0.000, 0.82	.067, 0.96
ISI	2.50^{a}	2.57^{ab}	2.62^{ab}	2.70^{ab}	2.78^{ab}	2.81^{b}	0.03	0.121	0.007, 0.98	.955, 0.99

Note: Data expressed as mean ($n = 3$); Mean values in the same row with different superscripts differ significantly ($p < .05$).

Abbreviations: HSI, Hepatosomatic index; ISI, Intestinal somatic index; SEM, Standard error means.

[1]Treatment groups fed with graded levels of dietary suboptimal crude protein (CP), *that is* T24, 24% dietary CP; T26, 26% dietary CP; T28, 28% dietary CP; T30, 30% dietary CP; T32, 32% dietary CP; and T34, 34% dietary CP.

TABLE 4 Digestive enzymes activities of *A. testudineus* fingerlings fed diets containing different dietary suboptimal CP levels

	Treatments[1]							(*p* values)	Regression analysis (p, r^2)	
Parameters	T24	T26	T28	T30	T32	T34	SEM	Overall	Linear	Quadratic
Proteases[2]	0.85^{a}	0.92^{a}	1.22^{b}	1.31^{b}	2.28^{c}	2.31^{c}	0.14	0.000	0.000, 0.87	.004, 0.91
Amylase[3]	7.69^{b}	7.65^{b}	5.28^{ab}	5.15^{ab}	4.16^{a}	4.11^{a}	0.43	0.034	0.207, 0.68	.037, 0.91
Lipase[4]	6.83	6.61	8.05	7.86	7.54	8.11	0.59	0.737	0.173, 0.56	.959, 0.62

Note: Data expressed as mean ($n = 3$); Mean values in the same row with different superscripts differ significantly ($p < .05$).

SEM, Standard error means.

[1]Treatment groups fed with graded levels of dietary suboptimal crude protein (CP), *that is* T24, 240 g CP/kg; T26, 260 g CP/kg; T28, 280 g CP/kg; T30, 300 g CP/kg; T32, 320 g CP/kg and T34, 340 g CP/kg.

[2]Protease activity is expressed as millimole of tyrosine released/min/g protein.

[3]Amylase activity is expressed as the micromole of maltose released from starch/min/mg protein.

[4]Lipase activity is expressed as units/mg protein.

3.5.2 | Metabolic enzymes

The activities of muscle and liver transaminase enzymes and metabolic enzymes were significantly affected ($p < .05$) by the dietary suboptimal CP levels (Table 5). The muscle AST activity remains significantly similar ($p > .05$) among the treatment groups. The overall and linear trends of liver AST activity were significantly affected ($p < .05$) and showed high linear (R^2=0.96) relation to the suboptimal CP levels. Significantly higher ($p < .05$) value of liver AST was found in T30, T32 and T34 groups than that of other groups. The overall and linear trends of muscle and liver ALT activity were significantly affected ($p < .05$) by the suboptimal CP levels. Both linear ($R^2 = 0.96$ and 94, respectively) and quadratic ($R^2 = 0.88$ and 93, respectively) relation were high with the CP levels. Significantly higher ($p < .05$) value of muscle and liver ALT activity was observed in T32 and T34 group than that of T24, T26 and T28 group except T30 group for muscle ALT, whereas significantly lower ($p < .05$) muscle and liver ALT activity was found in T24 and T26 groups as compared to other groups.

The overall and linear trends of muscle and liver LDH activity were significantly affected ($p < .05$) by the dietary suboptimal CP levels with significantly higher ($p < .05$) values found in T24 group than the other groups except T26 group (Table 5), whereas significantly lower ($p < .05$) muscle and liver LDH activity were found in T30, T32 and T34 groups as compared to other groups. The overall and linear trends of muscle MDH activity were significantly affected ($p < .05$) by the suboptimal CP levels. The muscle MDH activity was gradually decreased in response to increasing dietary protein levels with significantly higher ($p < .05$) value found in T24 group than the other groups except T26 group. The overall, linear and quadratic trends of liver MDH activity were significantly affected ($p < .05$) with high quadratic ($R^2 = 0.87$) relation in relative to suboptimal CP levels. The liver MDH activity was found to be significantly higher ($p < .05$) in T24 and T26 groups than the other groups, whereas liver

TABLE 5 Metabolic enzymes activities of *A. testudineus* fingerlings fed diets containing different dietary suboptimal CP levels

		Treatments[1]							(*p* values)	Regression analysis (p, r^2)	
Parameters		T24	T26	T28	T30	T32	T34	SEM	Overall	Linear	Quadratic
AST	Muscle	3.15	3.25	3.60	4.12	4.28	4.22	0.15	0.084	0.005, 0.90	.603, 0.92
	Liver	6.99^{a}	8.02^{ab}	10.13^{b}	12.57^{c}	13.61^{c}	14.16^{c}	0.71	0.000	0.000, 0.96	.349, 0.97
ALT	Muscle	4.78^{a}	5.60^{ab}	8.14^{bc}	10.87 cd	12.60^{d}	13.02^{d}	0.84	0.000	0.000, 0.96	.561, 0.88
	Liver	6.64^{a}	7.99^{a}	13.15^{b}	14.29^{b}	17.37^{c}	17.73^{c}	1.11	0.000	0.000, 0.94	.139, 0.93
LDH	Muscle	10.67^{c}	9.37^{bc}	8.50^{ab}	7.35^{a}	6.90^{a}	6.77^{a}	0.38	0.003	0.000, 0.61	.503, 0.74
	Liver	13.66^{b}	12.64^{b}	9.97^{ab}	8.63^{a}	8.09^{a}	8.24^{a}	0.65	0.019	0.001, 0.56	.202, 0.62
MDH	Muscle	1.79^{c}	1.61^{bc}	1.51^{ab}	1.43^{ab}	1.38^{a}	1.39^{a}	0.04	0.004	0.000, 0.64	.072, 0.73
	Liver	0.70^{b}	0.65^{b}	0.42^{a}	0.40^{a}	0.44^{a}	0.40^{a}	0.03	0.002	0.000, 0.71	.025, 0.87

Note: Data expressed as mean ($n = 3$); Mean values in the same row with different superscripts differ significantly ($p < .05$).

SEM, Standard error means.

AST = aspartate transaminase activity is expressed as nano moles oxaloacetate released/min/mg protein, ALT = alanine transaminase activity is expressed as nano moles Na pyruvate released/min/mg protein, LDH = lactate dehydrogenase activity is expressed as units/min/mg protein, MDH = malate dehydrogenase activity is expressed as units/min/mg protein.

[1]Treatment groups fed with graded levels of dietary suboptimal crude protein (CP), *that is* T24, 240 g CP/kg; T26, 260 g CP/kg; T28, 280 g CP/kg; T30, 300 g CP/kg; T32, 320 g CP/kg and T34, 340 g CP/kg.

MDH activity was found to be similar ($p > .05$) when fed with 28% CP and more.

3.6 | Serum glucose, osmolality and liver glycogen content

The overall, linear and quadratic trends of serum glucose were significantly affected ($p < .05$) with both high linear ($R^2 = 0.95$) and quadratic ($R^2 = 0.97$) relation with the suboptimal CP levels (Table 6). Significantly higher ($p < .05$) value of serum glucose found in T24 group than the other groups except T26 group, whereas significantly lower ($p < .05$) value was found in T30, T32 and T34 groups as compared to other groups. The overall and linear trends of liver glycogen content were significantly affected ($p < .05$) with high linear ($R^2 = 0.77$) and quadratic ($R^2 = 0.84$) relations (Table 6). The liver glycogen content was significantly lower ($p < .05$) in the T24 and T26 groups than the other groups except T28 group, whereas significantly higher ($p < .05$) values were found in T32 and T34 groups than the other groups except T30 group. The serum osmolality remains similar ($p > .05$) among the different groups with value range from 333–356 mOsm/kg (Table 6).

3.7 | Oxidative stress-related enzymes and haematological parameters

The overall and linear trends of gill SOD and CAT activities were significantly affected ($p < .05$) by the dietary suboptimal CP levels (Table 7). The regression analysis of gill SOD activity showed a high linear ($R^2 = 0.77$) and moderate quadratic ($R^2 = 0.56$) relation, whereas gill CAT activity exhibited high linear ($R^2 = 0.79$) and moderate quadratic ($R^2 = 0.66$) relations with the suboptimal CP levels. The SOD and CAT activities in gill were found to be significantly higher ($p < .05$) in T24 group than that of other groups except T26 group. The liver SOD and CAT activities were found to be similar ($p > .05$) among the treatment groups regardless of the suboptimal CP levels.

The overall and linear trends of RBC count were significantly affected ($p < .05$) by the dietary suboptimal CP levels with high linear ($R^2 = 0.77$) and quadratic ($R^2 = 0.91$) relations with dietary suboptimal CP levels (Table 7). The RBC count significantly ($p < .05$) increases with the increased dietary CP level up to T32 group. The WBC and Hb content showed poor linear ($R^2 = 0.43$ and 0.35, respectively) and quadratic ($R^2 = 0.35$ and 0.36, respectively) relations with the suboptimal CP levels (Table 7). However, no clear trends of WBC and Hb content were observed among the different groups. The overall, linear and quadratic trends of serum total protein were significantly affected ($p < .05$) by the suboptimal CP levels with moderate linear ($R^2 = 0.61$) and high quadratic ($R^2 = 0.85$) relations. Significantly higher ($p < .05$) total protein value was found in the T32 and T34 groups as compared to other groups except T30 group, whereas significantly lower ($p < .05$) value was observed in the T24 group as compared to others groups except T28 group.

3.8 | Correlation of nutrient utilization and physio-metabolic responses with growth

Among the different selected parameters, PER, ANPU, RNA/DNA ratio, whole body crude protein, HSI, protease, AST and ALT activities, glycogen content and red blood cell count were positively correlated with growth (WG%) (Table 8), whereas parameters such as FCR, amylase activity, LDH activity, MDH activity, SOD and CAT activity and serum glucose were negatively correlated with growth.

4 | DISCUSSION

Several studies demonstrated that the survival and growth of *A. testudineus* is not affected in saline water and can be cultured up to 15 g/L salinity (Chotipuntu & Avakul, 2010; Chowdhury et al., 2014; Dubey et al., 2015). Therefore, culture of *A. testudineus* in ISW having 8 g/L salinity would be a promising avenue for income generation by the farmers of salt affected regions. Among the major nutrients, protein is the most vital nutrient which takes part in protein synthesis and provide energy through oxidation of amino acid. Besides, the protein level in diet is major determinant of feeding cost and overall profitability. Considering the actual field situation, several farmers prefer to use suboptimal protein in lieu to reduce feeding cost depending on the market value of the produced fish. This study therefore investigated the potentiality of culturing *A. testudineus* in ISW at 8 g/L salinity when fed with the dietary suboptimal protein levels. After 60 days of feeding, the maximum WG (%) and SGR of *A. testudineus* fingerlings were observed in the T34 group (340 g suboptimal CP/kg diet) when reared in ISW of 8 g/L salinity. Although higher value of WG (%) and SGR were observed with the T34 group, non-significant ($p > .05$) growth between T32 and T34 groups could be related to the suboptimal dietary protein content in the both T32 and T34 groups (320 and 340 g suboptimal CP/kg diet) that let protein to be available for protein synthesis and together satisfying the physiological energy needs (Tucker, 1992). Besides, *A. testudineus* preferably catabolize amino acid to maintain the osmotic balance at higher salinities (Chang et al., 2007). In line, fish of T34 group could have actively catabolized the available protein for better osmotic acclimatization which resulted in insignificant growth with the T32 group. Mostly, the growth of fish increases when fed with optimum dietary protein level and beyond which growth remains unaffected (Kim & Lall, 2000). The significantly lower ($p < .05$) growth with the T24 group is due to the low protein content of the diet, which cannot meet the energy and nutrient requirement for metabolism and tissue building (Lee et al., 2001). Further, inferior growth in the T24 and T26 groups could be due to a lower P/E ratio in the diet that cannot support the tissue demand for protein synthesis (Moore et al., 1988). Previous studies reported that 300–400 g CP/kg diet is optimum for the maximum growth of *A. testudineus* fingerlings reared in freshwater (Chotipuntu & Avakul, 2010; Hossain et al., 2012; Patra et al., 2017). However, we could not come across any study exploring the optimal dietary protein requirement of *A. testudineus* reared either

TABLE 6 Serum osmolality, glucose and glycogen content of *A. testudineus* fingerlings fed diets containing different dietary suboptimal CP levels

	Treatments[1]							(*p* values)	Regression analysis (*p*, r^2)	
Parameters	T24	T26	T28	T30	T32	T34	SEM	Overall	Linear	Quadratic
Serum osmolality (mOsm/kg)	351	355	352	333	340	356	3.87	0.194	0.353, 0.05	0.187, 0.32
Water osmolality (mOsm/kg)	278	285	279	275	283	275	6.34	0.998	0.862, 0.00	0.922, 0.00
Glucose (mg/dl)	91.32^{c}	82.8^{bc}	74.38^{b}	58.74^{a}	53.80^{a}	52.16^{a}	3.60	0.000	0.000, 0.95	0.030, 0.97
Glycogen (mg/g)	1.42^{a}	1.45^{a}	1.77^{ab}	2.33^{bc}	2.30^{c}	2.17^{c}	0.11	0.040	0.000, 0.77	0.604, 0.84

Note: Data expressed as mean ($n = 3$); Mean values in the same row with different superscripts differ significantly ($p < .05$).

Abbreviation: SEM, Standard error means.

[1]Treatment groups fed with graded levels of dietary suboptimal crude protein (CP), *that is* T24, 240 g CP/kg; T26, 260 g CP/kg; T28, 280 g CP/kg; T30, 300 g CP/kg; T32, 320 g CP/kg and T34, 340 g CP/kg.

in brackishwater or ISW. In the present study, 340 g suboptimal CP/kg diet resulted in the maximum growth of *A. testudineus* fingerlings reared in 8 g/L salinity. Nevertheless, from economical perspective feeding diet having 320 g suboptimal CP/kg diet (T32 group) would be more practical for rearing *A. testudineus* fingerling when considering the growth performance of T32 and T34 groups. In context, 320 g suboptimal CP/kg diet (T32 group) gives slightly slower growth but less FCR as compared to 340 g suboptimal CP/kg diet. This indicates the efficient utilization of feed and nutrients in the T32 group resulting in low cost of feeding. Mohammadi et al. (2014) and Singha, Shamna, Sahu, Sardar, Harikrishna, et al. (2020) reported that 290 and 300 g CP/kg diet is optimum for the maximum growth of GIFT tilapia reared in ISW at 8 and 5 g/L salinity, respectively. Talukdar, Deo, et al. (2020) observed better growth of *Mugil cephalus* fed with 300 g CP/kg diet when reared in ISW at 8 g/L salinity.

In fish, often better growth is observed when freshwater fish is reared in intermediate salinity (8–20 g/L) due to increased feed efficiency (Boeuf & Payan, 2001). Besides, fish would consume more feed to overcome the deficient nutrient in the diet and as a result digestibility reduces with the low protein diets (Sá et al., 2014). This could be the reason for the higher FCR value observed with the T24 and T26 groups. The lower FCR value in T30 and T32 groups implies that the feed consumed was efficiently utilized for growth thus reducing the catabolism of protein for energy production. However, further increase in dietary protein beyond 320 g suboptimal CP/kg diet does not improves the feed utilization probably due to the preferential catabolism of protein. Singha, Shamna, Sahu, Sardar, Hari Krishna, et al. (2020), Singha, Shamna, Sahu, Sardar, Harikrishna, et al. (2020) and Siddiqui et al. (1988) have reported similar trend of FCR with the increasing dietary protein level in tilapia reared in ISW and saline water, respectively. The RNA/DNA ratio is a reliable parameter of protein synthesis hence describe fish growth (Steinhart & Eckman, 1992). DNA is necessary for protein synthesis that remains constant in tissue (Mitra & Mukhopadhyay, 2002) as found in this study. The RNA concentration and RNA/DNA ratios were corroborating to the good growth observed in T32 or T34 group. Similar results of positive relation of RNA/DNA ratio with growth performance was also observed in *Labeo rohita* (Gangadhar et al., 1997; Kumar et al., 2010). Therefore, results indicate that further increase in protein level is required to achieve maximum growth potential of fish *A. testudineus* reared in ISW; however, the suboptimal level of protein gives better growth and feed utilization efficiency.

The higher protein utilization efficiency such as PER, ANPU and PGR in T30 and T32 groups can be attributes to the sufficient non-energy nutrient content of the diet, which aids to deposition of protein for maximizing growth. There is a high quadratic relation of PER and ANPU ($r^2 = 0.69$, 0.70, respectively) with the increasing dietary CP levels where protein efficiency was significantly reduced ($p < .05$) in group fed 340 g suboptimal CP/kg diet (T34 group). It has been reported that feeding higher protein beyond optimum resulted in lower protein efficiency due to the catabolism of protein, whereas lower dietary protein increases the protein utilizing efficiency (Kaushik & Seiliez, 2010). In this study, the increasing suboptimal CP level beyond 320 g suboptimal CP/kg diet did not provided the incremental growth per unit of dietary protein fed as protein efficiency greatly reduced beyond T32 group (Figure 1). Therefore, this suggest that even if growth is higher in the T34 group, T32 group provides better cost-effective growth and protein utilizing efficiency. Usually, dietary protein is preferably catabolized to provide energy in fish acclimating to adverse environment such as higher salinity (Cara et al., 2007). It has been reported that *A. testudineus* actively degrade protein and catabolize amino acid for the osmoregulatory acclimation during salinity stress (Chang et al., 2007). Lower protein utilizing efficiency in T34 group is possibly due to some portion of dietary protein being catabolized to provide energy for the osmoregulatory purpose preferably due to the insufficient energy nutrient content of the diet. Similarly, lower protein utilizing efficiency in T24 and T26 groups is due to the lower dietary protein content of the diets, which is insufficient to meet the nutrient requirement of *A. testudineus* fingerlings reared in ISW. It appears that the non-protein energy source is adequately utilized in T30 and T32 groups thus satisfying the energy need for various physiological processes and consequently channelizing dietary protein for growth. However, higher protein efficiency in T30 group does not corroborate to higher growth probably because of the low protein content of the diet. Similar result has been reported in *A. testudineus* (Hossain et al., 2012) and tilapia (Singha, Shamna, Sahu, Sardar, Hari Krishna, et al., 2020; Singha, Shamna, Sahu, Sardar, Harikrishna, et al., 2020). No

TABLE 7 Oxidative stress enzymes and haematological parameters of *A. testudineus* fingerlings fed diets containing different dietary suboptimal CP levels

Parameters		Treatments[1]							(*p* values)	Regression analysis (p, r^2)	
		T24	T26	T28	T30	T32	T34	SEM	Overall	Linear	Quadratic
SOD	Gill	2.59^{b}	2.16^{ab}	1.93^{ab}	1.71^{a}	1.65^{a}	1.57^{a}	0.11	0.041	0.003, 0.77	.156, 0.56
	Liver	5.56	5.19	4.77	4.27	3.78	3.81	0.24	0.203	0.014, 0.40	.694, 0.41
CAT	Gill	0.94^{d}	0.70^{c}	0.64^{c}	0.58^{b}	0.37^{a}	0.32^{a}	0.06	0.020	0.001, 0.79	.561, 0.66
	Liver	4.67	4.38	4.19	4.09	3.85	3.77	1.12	0.106	0.005, 0.48	.956, 0.48
RBC ($\times 10^6$/μl)		2.29^{a}	2.37^{ab}	2.41^{abc}	2.48^{c}	2.55^{d}	2.51^{d}	0.03	0.002	0.000, 0.77	.638, 0.91
WBC ($\times 10^3$/μl)		131.03	133.77	130.71	128.73	125.27	127.43	1.51	0.136	0.009, 0.43	.676, 0.44
Hb (mg/dl)		7.72	7.78	7.97	8.41	8.80	9.10	0.20	0.294	0.024, 0.35	.660, 0.36
Total protein (g/dl)		3.11^{a}	3.24^{ab}	3.27^{b}	3.38^{bc}	3.48^{c}	3.43^{c}	0.13	0.010	0.001, 0.61	.025, 0.85

Note: Data expressed as mean (n = 3); Mean values in the same row with different superscripts differ significantly ($p < .05$).

Abbreviations: CAT, Catalase; EM, Standard error means; Hb, Haemoglobin; RBC, Red blood cell; SOD, Superoxide dismutase; WBC, White blood cell.

[1]Treatment groups fed with graded levels of dietary suboptimal crude protein (CP), *that is* T24, 240 g CP/kg; T26, 260 g CP/kg; T28, 280 g CP/kg; T30, 300 g CP/kg; T32, 320 g CP/kg and T34, 340 g CP/kg.

mortality occurred during the experimental period, which suggest the suitability of *A. testudineus* fingerlings as a potential fish species for culture in ISW at 8 g/L salinity.

The change in whole-body proximate composition of fish is related to changes in their synthesis and deposition rate, diet composition and culture environment (Shearer, 1994; Abdel-Tawwab et al., 2010). Higher whole-body protein content in the T32 (320 g suboptimal CP/kg diet) group is possibly due to higher protein accretion, synthesis and nutrient utilization. However, feeding dietary protein beyond 320 g suboptimal CP/kg diet to *A. testudineus* fingerlings does not improves the protein accretion which could be due to the preferential catabolism of protein. Significantly lower ($p < .05$) whole-body protein in T24 and T26 groups implies that dietary protein below 260 g suboptimal CP/kg diet is not enough to support protein accretion for growth. This also corroborates to the lower protein utilizing efficiency observed in the same treatment groups. Similar results were also observed in *A. testudineus* (Hossain et al., 2012) under freshwater and Mohammadi et al. (2014) under ISW at 8 g/L salinity and Singha, Shamna, Sahu, Sardar, Hari Krishna, et al. (2020), Singha, Shamna, Sahu, Sardar, Harikrishna, et al. (2020) under ISW at 5 and 10 g/L salinities. Further, it has been reported that when fish are exposed to salinity deviation, protein is catabolized to have sufficient amino acid which is required for the physiological adaptation to salinity stress (Ballantyne, 2001). Generally, omnivorous fishes are well known to utilize a higher level of carbohydrate which in excess is stored as glycogen and lipid (Hemre et al., 2002). The lowest HSI in the T24 group can be related to mobilization of energy reserve to satisfy high energy demand for osmoregulation as carbohydrate is the immediate source for energy during physiological stress (Tseng & Wang, 2008). Comparatively higher HSI value in fish fed 300–340 g suboptimal CP/kg diet could be an adaptive strategy to have energy reserve during the osmoregulatory adaptation in ISW and prioritizing protein for growth.

The activities of digestive enzymes influence the nutrient utilization and growth performance of fish that in turn depends upon the availability of dietary nutrients in intestine (Sagada et al., 2017). The protease activity showed an increasing positive response to the increasing dietary protein levels up to 320 g suboptimal CP/kg diet. This suggests that fish try to maximize the utilization of available protein up to optimum level by increasing the digestive capacity that might have driven maximum protein synthesis in T32 group as supported by the higher RNA/DNA ratio and growth. The increased protease activity at optimum protein level and no further increase with the increasing dietary protein is supported by Kumar et al. (2019) in *L. rohita*, Jayant et al. (2018) in *Pangasiodon pangasius* and Singha, Shamna, Sahu, Sardar, Hari Krishna, et al. (2020), Singha, Shamna, Sahu, Sardar, Harikrishna, et al. (2020) in GIFT tilapia. The amylase activity was decreased with the increase in dietary protein levels, which is mainly due to the low content of digestible carbohydrate (NFE) level in the higher protein fed groups (Mohanta et al., 2008). The high positive and negative correlation of protease ($r = 0.90$) and amylase ($r = -0.68$) activities, respectively with growth suggest improved utilization of available nutrient (protein or carbohydrate) in

Parameters	Correlation coefficient (*r*) with weight gain %[a]	Interpretation as strength of association[b]
Food conversion ratio (FCR)	−0.93	Negatively high
PER	0.35	Positively low
ANPU	0.51	Positively moderate
RNA/DNA ratio	0.94	Positively high
Whole body crude protein content	0.68	Positively moderate
Hepatosomatic index (HSI)	0.94	Positively high
Protease	0.90	Positively high
Amylase	−0.68	Negatively moderate
Liver aspartate transaminase (AST)	0.89	Positively high
Liver alanine transaminase (ALT)	0.94	Positively high
Muscle lactate dehydrogenase (LDH)	−0.95	Negatively high
Muscle malate dehydrogenase (MDH)	−0.86	Negatively high
Superoxide dismutase (SOD)	−0.94	Negatively high
Catalase (CAT)	−0.96	Negatively high
Glucose	−0.37	Negatively low
Glycogen content	0.59	Positively moderate
Red blood cell count	0.82	Positively high

[a]Only the mean ($n = 3$) values are considered for correlation analysis.

[b]Strength of association is depicted as high, ≥0.7; moderate, ≥0.5 to 0.7; low, ≥0.5.

TABLE 8 Correlation of nutrient utilization and physio-metabolic responses with growth of *A. testudineus* fingerlings fed diets containing different dietary suboptimal CP levels

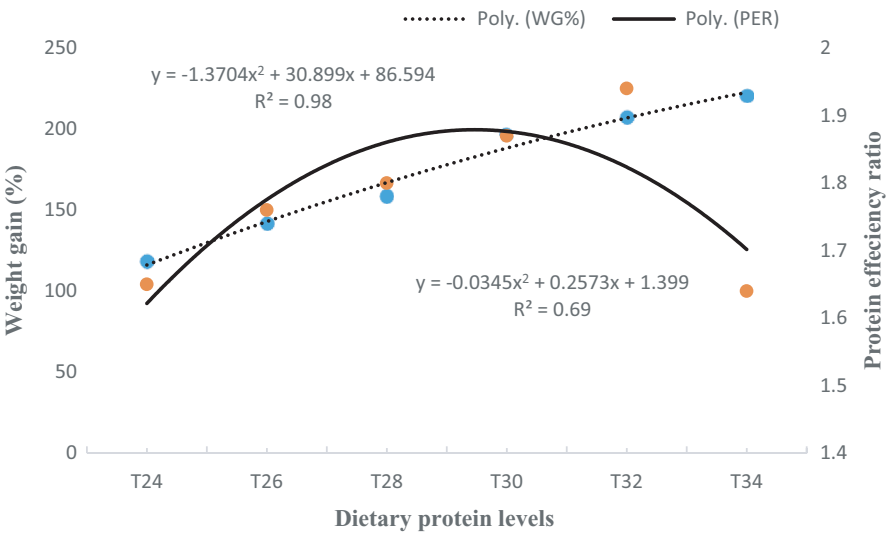

FIGURE 1 Polynomial trend of weight gain (%) and protein efficiency ratio (PER) in response to suboptimal dietary CP levels

the diet. Lipase activity was found to be similar among the treatment groups, which could be due to the similar lipid content of the diets.

The activities of AST and ALT are related to synthesis and breakdown of amino acid derived from diet or tissue, which in turn depend upon availability of energy nutrients (Ye et al., 2017). In the present study, the transaminase activities increased with the increasing dietary protein levels with maximum activities found in higher protein fed groups (300–340 g suboptimal CP/kg diet). The reason could be the enhanced protein metabolism or increasing turnover of the amino acids in the body of *A. testudineus* fingerlings required for various physiological activities. This preferably resulted in better availability of amino acid for growth and osmoregulatory purpose in the higher protein fed groups, which correspond to better protein utilization and growth. Chang et al. (2007) reported that *A. testudineus* increased its protein metabolism in order to sustain its osmoregulatory capacity for adaptation to brackishwater or seawater. However, low transaminase activity in the T24 or T26 group is due to deficient in protein to form keto acid, thereby reducing the transaminase enzymes in muscle and liver (Lin & Luo, 2011). Moreover, the high positive correlation of liver AST ($r = 0.89$) and ALT ($r = 0.94$)

activities with growth suggests the enhanced protein metabolism with the increasing dietary suboptimal CP levels. Therefore, dietary protein above 300 g suboptimal CP/kg diet can enhance protein availability and promote growth of *A. testudineus* fingerlings reared in ISW at 8 g/L salinity. Further, it indicates anabolic synthesis of amino acids which increased protein efficiency at the suboptimal protein level and increased feed efficiency at given protein level compared to close suboptimal higher level (T34 group).

The activity of LDH and MDH is related to anaerobic metabolism that occurs during energy demanding condition where pyruvate is converted to lactate and malate into oxaloacetate (or vice versa), respectively for providing energy (Hemre et al., 2002; Murray et al., 2000). The higher muscle and liver LDH activity in T24 and T26 groups indicates that the rate of glycolysis exceeded the aerobic metabolism thus favouring formation of lactate to derive extra energy required during stress condition. Besides, low availability of free amino acid and high dietary carbohydrate (as osmolytes) in T24 and T26 groups could not support the osmoregulatory adaptation which leads to osmoregulatory mediated stress in *A. testudineus* fingerlings. Similarly, hepatic MDH activity in lower protein fed groups is due to the high energy demand of *A. testudineus* that could be satisfied by the production of ATP through gluconeogenesis. Besides, reduced LDH and MDH activities above 280 g suboptimal CP/kg diet fed groups could be due to the fact that the higher energy demand was satisfied through the dietary nutrients. Singha, Shamna, Sahu, Sardar, Harikrishna, et al. (2020) reported similar result of increased LDH and MDH activity with low dietary CP level in the diet of GIFT tilapia reared in ISW at 5 g/L salinity. This suggests that *A. testudineus* increased its energy metabolism, which could be a physiological response to satisfy the high energy demand for osmoregulation occurring in ISW at 8 g/L salinity.

Glycogen reserve provides immediate and additional source of energy required during any unfavourable condition (Bacca et al., 2005). Carbohydrate plays a major role in energy metabolism for osmoregulation by the oxidation of glucose or lactate (Morgan & Iwama, 1991; Tseng & Hwang, 2008). Several studies have reported that upon exposure to salinity fluctuations, fish mobilize its glycogen reserve in order to derive extra energy required for gill and other high energy consuming organs (Chang et al., 2007; Guo et al., 2020; Polakof et al., 2006; Sangiao-Alvarellos et al., 2005). In the present study, lower liver glycogen in the T24 and T26 groups indicates the mobilization of glycogen reserve to satisfy the high energy demand occurred mediated by salinity stress. The glucose in the circulation thus can be metabolized in gill and high energy demanding organs to maintain constant supply of energy required during the osmoregulatory process. This along with higher carbohydrate content of the diet could be the reason for higher glucose value found in T24 and T26 groups, which reflects the high energy demanding condition of fish (Manush et al., 2005). Therefore, *A. testudineus* when reared in ISW ensures the availability of energy nutrient in the circulation to satisfy the prompt energy required for the osmoregulatory process. Similar result has been observed by Wang et al. (2016) in *E. akaara* and Yang et al. (2016) in *L. macrochirus*.

Continuous cellular metabolic activity leads to the production of free radicals, which in excess cause oxidative stress and damage biomolecules (Storey, 1996). The SOD and CAT are two endogenous antioxidant enzymes that convert the free radicals to more degradable form and act as a biomarker for oxidative stress and immunity in fish (Martínez-Álvarez et al., 2005). Dietary protein and plant-based diet are known to affect endogenous antioxidant enzyme activities in fish (Olsvik et al., 2011; Pérez-Jiménez et al., 2009). In the present study, higher activities of gill SOD and CAT in the T24 or T26 groups indicate the production of free radicals possibly as a result of excessive energy metabolism. The quadratic trend of SOD ($R^2 = 0.77$) and CAT ($R^2 = 0.89$) activities and negative correlation ($r = -0.94$, 96, respectively) with growth indicates that higher level of dietary suboptimal protein (300–340 g suboptimal CP/kg diet) improves the oxidative stress condition of *A. testudineus* in saline environment. The rise in endogenous antioxidant enzymes in lower protein fed groups (240–280 g suboptimal CP/kg diet) could be an adaptive response of *A. testudineus* to ensure its survival at salinity stress. Euryhaline fish maintain their osmolality between 300 and 400 mOsmol/kg in brackishwater or seawater and act as hypo-osmotic osmoregulators (Lange & Fugelli, 1965; Yancey, 2001). In the present study, the serum osmolality value ranges between 340 and 356 mOsmol/kg and remains similar among the different groups because osmolality is more a function of water salinity, which was constant (8 g/L) in the present experiment. Previous study shows that common carp at hyper-saline environment maintain its internal osmolality higher than the water osmolality in order to allow an influx of water for osmotic balance (De Boeck et al., 2020).

Changes in blood haematological parameters indicate the adaptability of fish to different dietary compositions and environmental conditions, which reflects the immunological potential of fish (Bahmami et al., 2001; De et al., 2018; Kumar et al., 2005). In the present study, the RBC count increases with the increase in dietary protein levels, which could be due to the early release of erythrocytes from spleen as dietary protein does affect spleen activity (Abdel-Tawwab, 2012). A similar increase in RBC value with the increasing dietary protein level was observed in Nile tilapia (Abdel-Tawwab et al., 2010) and *Megalobrama amblycephala* (Habte-Tsion et al., 2013). The WBC and Hb values remain unchanged due to the dietary protein levels, which was consistent with the findings of Kumar et al. (2005) and Baruah et al. (2009) in *L. rohita*. Further, in hypersaline condition, fish would loss water passively thus increasing the concentration of RBC and Hb value in blood (Plaut, 1998). However, subsequently on long-term exposure the values return to normal by compensatory influx of water as a result of re-establishment of the extracellular volume (Martínez-Álvarez et al., 2005; Shahkar et al., 2015). Total protein constitutes of nutritive component that play important role in immune functions (Kumar et al., 2010). In the present study, higher value of serum total protein in the higher protein fed groups suggests the dietary composition or the environmental condition does not influence the immune potential of *A. testudineus* fingerlings as no apparent changes were observed in the haematological parameters.

5 | CONCLUSION

In summary, it can be concluded that most of the parameters such as growth response, activities of digestive and metabolic enzymes and health status of *A. testudineus* fingerlings were better with diet having 320 or 340 g suboptimal CP/kg diet. Besides, PER and ANPU have poor positive correlation ($r \leq 0.7$) with growth where protein efficiency decreased beyond 320 g suboptimal CP/kg diet. Better protein utilizing efficiency and feed utilization were found with 320 g suboptimal CP/kg as compared to 340 g suboptimal CP/kg diet. As there was insignificant ($p < .05$) difference in growth between T32 and T34 groups, use of T32 diet will be more economical than T34 diet considering the cost of ingredients as indicated by lowest FCR and higher protein efficiency found in the same group. Feeding lower dietary protein below 300 g suboptimal CP/kg diet to *A. testudineus* fingerlings resulted in energy demanding condition and oxidative stress, which have high negative correlation ($r \geq -0.7$) with growth. Further, *A. testudineus* actively mobilized carbohydrate source and catabolize available protein to satisfy the physiological needs for adaptation to the salinity stress when reared in physiologically stressed condition. Based on the results, 320 g CP/kg diet could be suggested for the cost-effective growth and better health status of *A. testudineus* fingerlings reared in ISW at 8 g/L salinity, when fed with the suboptimal CP diets. This preliminary study will help for further nutritional studies and to develop cost-effective practical feed for *A. testudineus* reared in ISW.

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DATA ACCESSIBILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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