



Efficacy of nucleotide related products on growth, blood chemistry, oxidative stress and growth factor gene expression of juvenile red sea bream, *Pagrus major*



Md. Sakhawat Hossain^{a,b,c,*}, Shunsuke Koshio^{a,b}, Manabu Ishikawa^{a,b}, Saichiro Yokoyama^b, Nadia Mahjabin Sony^b, Mahmoud A.O. Dawood^{a,b}, Md. Abdul Kader^{b,d}, Mahbuba Bulbul^{b,e}, Takeshi Fujieda^f

^a The United Graduate School of Agricultural Sciences, Kagoshima University, Korimoto 1-21-24, Kagoshima 890-0065, Japan

^b Laboratory of Aquatic Animal Nutrition, Faculty of Fisheries, Kagoshima University, Shimoarata 4-50-20, Kagoshima 890-0056, Japan

^c Department of Aquaculture, Faculty of Fisheries, Sylhet Agricultural University, Sylhet -3100, Bangladesh

^d School of Fisheries and Aquaculture Sciences, Universiti Malaysia Terengganu, Kuala Terengganu 21030, Malaysia

^e Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, 21030, Kuala Terengganu, Malaysia

^f Research Institute for Bioscience Products & Fine Chemicals, Ajinomoto Co., Inc., Kawasaki, Kanagawa 210-8681, Japan

ARTICLE INFO

Article history:

Received 15 April 2016

Received in revised form 1 June 2016

Accepted 2 June 2016

Available online 4 June 2016

Keywords:

Nucleoside by-product

Inosine

Oxidative stress

Growth

Blood chemistry

Red sea bream

ABSTRACT

A feeding trial was conducted to determine the efficacy of nucleotide related products on growth, blood chemistry, oxidative stress and growth factor gene expression of juvenile red sea bream. Five experimental diets were formulated to contain 49% protein, 11% lipid and the diet without nucleotide related product supplementation was the control (D1). Nucleotide related products like; nucleoside by-products (NBPs) and inosine were supplemented at 1, 3 and 0.03, 0.1% consecutively with basal ingredients of D1 and named as D2, D3 & D4 and D5 respectively. Experimental diets were fed to triplicate groups of fish for 60 days. Fish fed diet D5 showed significantly the highest final body weight and % weight gain followed by the diet groups D2 and D4. Fish fed control group showed the lowest growth performance and were not differed significantly with diet group D3. Feed conversion ratio and protein efficiency ratio were also significantly higher in diet group D5, whereas the other supplemented group showed intermediate value. A wide variation in some of the blood parameters was observed. In case of oxidative stress parameters, fish fed inosine supplemented diets showed the best conditions because they performed better under oxidative stress conditions as well as had the highest tolerance against oxidation. Among NBP supplemented groups, diet group D2 also showed acceptable conditions of oxidative stress tolerance. Stress resistance against low salinity exposure (LT_{50}) also increased with dietary supplementation and it was significantly the highest in fish fed diet group D5. Total serum protein, serum lysozyme activity and total peroxidase content tended to be higher ($P > 0.05$) in NBP and inosine supplemented diet groups. In numerically higher hepatic IGF-1, mRNA expression was found in diet groups D2 and D4. However, IGF-1 and IGF-2 mRNA expressions were not significantly altered by dietary supplementations in the present study. Considering overall performance of the present study, we concluded that inosine and low concentration of NBP (1%) could be effectively used as dietary supplements for better growth and health performance of *Pagrus major*.

Statement of relevance: In this study industrial by-products, nucleoside by-products and relatively low cost nucleoside, inosine has been evaluated as potential functional nutrients for marine fish such as red sea bream. Utilization of these functional supplements will help to reduce nucleotide administration cost in fish feed as well as to develop low fishmeal based functional aquafeed in the near future.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Nucleotides are low molecular weight intracellular compounds which play key roles in diverse essential physiological and biochemical

functions including encoding genetic information, mediating energy metabolism and signal transduction (Carver and Walker, 1995). Dietary nucleotides have been reported to be beneficial for humans and animals (Gil, 2002) since they positively influence lipid metabolism, immunity, and tissue growth, development and repair (reviewed in Gil, 2002). In aquatic animals both nucleotides and nucleosides have long been implicated as feed attractants in both vertebrate and invertebrate species (Mackie and Adron, 1978; Carr and Thompson, 1983; Person-Le Ruyet

* Corresponding author at: Laboratory of Aquatic Animal Nutrition, Faculty of Fisheries, Kagoshima University, Shimoarata 4-50-20, Kagoshima City, Kagoshima 890-0056, Japan.
E-mail address: fishsakhawat@yahoo.com (M.S. Hossain).

et al., 1983; Carr et al., 1984). However, world-wide heightened attention on nucleotide supplementation into potential growth and health benefits in aquaculture species was only instigated after 2000s. To date, research pertaining to nucleotide nutrition in fishes has shown rather consistent and encouraging beneficial results in fish health management.

Inosine, a purine nucleoside containing the base hypoxanthine and the sugar ribose, occurs in transfer RNAs, and is formed during the breakdown of adenosine by adenosine deaminase (Barankiewicz and Cohen, 1985). During the industrial preparation of inosine a liquid form of by-product is produced after the separation of inosine. This liquid nucleoside by-product (NBP) contains considerable portion of inosine nucleoside and small portion of some other nucleotides. Recently, in aquaculture research nucleotides and its related product has been paid attention promisingly as functional nutrients. In aquaculture dietary nucleotide and its related products supplementation has been shown to enhance growth of certain fish species (reviewed by Li and Gatlin, 2006) immune responses and disease resistance of all male hybrid tilapia (*Saratheradon niloticus* ♀ × *Saratheradon aureus* ♂) (Ramadan et al., 1994), Atlantic salmon (*Salmo salar* L.) (Burrells et al., 2001a), common carp (*Cyprinus carpio* L.) (Sakai et al., 2001), hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) (Li et al., 2004), grouper (*Epinephelus malabaricus*) (Lin et al., 2009), red drums (*Sciaenops ocellatus*) (Cheng et al., 2011), rainbow trout (*Oncorhynchus mykiss*) (Tahmasebi-Kohyani et al., 2012) and Japanese flounder (*Paralichthys olivaceus*) (Song et al., 2012). Supplementation of nucleotides was also reported to increase stress tolerance in Atlantic salmon (Burrells et al., 2001b), rainbow trout (Leonardi et al., 2003), red sea bream (Hossain et al., 2016) and even gastrointestinal physiology and morphology of tilapia (Ramadan et al., 1994), Atlantic salmon (Burrells et al., 2001b) and red drum (Cheng et al., 2011).

Red sea bream, *Pagrus major*, is one of the commercially important aquaculture species, whose production reaches the second largest in Japan (Koshio, 2002). Intensive culture of this species often exposed it to stressful conditions which impaired growth and immunity of the fish. Exposure to stress places additional demands on available nucleotides, and an additional exogenous supply of nucleotides provided by dietary supplementation may help to counter the immunosuppressive effects of stress (Low et al., 2003). Although, there are some research into potential growth and health benefits of dietary nucleotides in aquaculture species (Burrells et al., 2001a, 2001b; Li et al., 2004; Cheng et al., 2011; Song et al., 2012; Hossain et al., 2016). However, in most of cases, purified nucleotide mixtures were used and still, some gap exists about current knowledge of nucleotide supplementation in fish diets and its effects on physiology and immunity. Moreover, in aquaculture, nucleotides are more widely studied than relatively cheaper nucleosides. So far, study conducted on nucleosides has mainly focused on its feeding stimulatory properties rather than its functional properties. Until recently, there is also no research on the use of low cost industrial by-product, which can also be used as a source of nucleotide or nucleoside for red sea bream as well as other marine species. In this circumstance, studies on the efficacy of supplementing relative low cost nucleotide related products viz. NBP and inosine on growth and health performance are important for the effective use of these functional supplements. So, the aim of this study was to investigate the efficacy of utilizing nucleotide related products on growth, blood chemistry, oxidative stress and growth factor gene expression of red sea bream.

2. Materials and methods

2.1. Test fish and experimental system

Juvenile red sea bream were obtained from a local hatchery, in Kagoshima prefecture, Japan, and transported to the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The fish were maintained in 500 L tank with continuous aeration and

flow through sea water and fed a commercial formulated diet (54% crude protein, Higashimaru Foods, Kagoshima Japan) for one week to acclimatize with the laboratory facilities. The feeding trial was carried out in 100 L polycarbonate tanks (filled with 80 L of water) in a flow through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. The tanks were maintained with natural light/dark regime. The seawater was pumped from the deep basin of Kagoshima bay, Japan; gravel filtered and supplied to the system. A flow rate of 1.5 L min⁻¹ was maintained throughout the experimental period.

2.2. Ingredients and test diets

Tables 1, 2, 3 and 4 summarize the formulation and chemical composition, total and free amino acid composition of the experimental diets, respectively. Five experimental diets were formulated to contain 49% protein, 11% lipid, without nucleotide related products supplementation considered as control (D1). Nucleotide related products like; nucleoside by-products (NBPs) and inosine were supplemented at 1, 3 and 0.03, 0.1% consecutively with basal ingredients of D1 and named as D2, D3 & D4 and D5 respectively. The diets were prepared by thoroughly mixing all the dry ingredients in a food mixer for 10 min. Liquid NBP were also simultaneously added with dry ingredients of the respective diets. Pollack liver oil and soybean lecithin were premixed with a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan), added to the dry ingredients and mixed for another 10 min. Water was added gradually (30–40% of the dry ingredients), to the premixed ingredients and mixed for another 10 min. The pH of the diets was adjusted to the range of 7.0–7.5 with 4 N sodium hydroxide. The mixture was then passed through a meat grinder with an appropriate diameter (1.2–2.2 mm) to prepare pellets, which were then dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 55 °C for about 150 min. The test diets were stored at –28 °C in a freezer until use.

Table 1
Ingredients and formulation of the experimental diets (% of the dry ingredients).

Ingredients	Diet groups				
	D1 ^a	D2	D3	D4	D5
Fishmeal ^b	46.53	46.15	45	46.53	46.53
Soybean meal ^c	20	20	20	20	20
Pollack liver oil ^d	3.65	3.68	3.73	3.65	3.65
Soybean lecithin ^e	2.5	2.5	2.5	2.5	2.5
Vitamin mixture ^f	3	3	3	3	3
Mineral mixture ^g	3	3	3	3	3
Stay-C ^h	0.3	0.3	0.3	0.3	0.3
Wheat flour	11	11	11	11	11
Activated gluten ⁱ	5	5	5	5	5
α-Cellulose	5.02	4.37	3.47	4.99	4.92
NBP ^j	0	1	3	0	0
Inosine ⁱ	0	0	0	0.03	0.1

^a According to Kader et al. (2012).

^b Nippon Suisan Co. Ltd., Tokyo, Japan.

^c J. Oil Mills, Japan.

^d Riken Vitamin, Tokyo, Japan.

^e Kanto Chemical Co., Inc. Tokyo, Japan.

^f Vitamin mixture (g kg⁻¹ diet): β-carotene, 0.10; Vitamin D₃, 0.01; Menadione NaHSO₃·3H₂O (K₃), 0.05; DL-α-Tocopherol Acetate (E), 0.38; Thiamine-Nitrate (B₁), 0.06; Riboflavin (B₂), 0.19; Pyridoxine-HCl (B₆), 0.05; Cyanocobalamin (B₁₂), 0.0001; Biotin, 0.01; Inositol, 3.85; Niacine (Nicotinic acid), 0.77; Ca Panthothenate, 0.27; Folic acid, 0.01; Choline chloride, 7.87; ρ-Aminobenzoic acid, 0.38; cellulose, 1.92.

^g Mineral mixture (g kg⁻¹ diet): MgSO₄, 5.07; Na₂HPO₄, 3.23; K₂HPO₄, 8.87; Fe citrate, 1.10; Ca lactate, 12.09; Al (OH)₃, 0.01; ZnSO₄, 0.13; CuSO₄, 0.004; MnSO₄, 0.03; Ca (IO₃)₂, 0.01; CoSO₄, 0.04.

^h Stay-C 35.

ⁱ Glico Nutrition Company Ltd. Osaka, Japan. Commercial name "A-glu SS".

^j NBP (nucleoside by-products) Ajinomoto Co., Inc., Tokyo, Japan.

^k Tokyo Chemical Industry Co., Ltd. Tokyo, Japan.

Table 2
Chemical composition of the nucleoside by-products (NBPs), and formulated experimental diets (% dry matter basis).

Parameters	NBP	Diet groups				
		D1	D2	D3	D4	D5
Moisture	66.2	7.3	6.2	6.3	6.7	6.5
Protein	25.4	48.5	49.1	49.0	48.9	49.2
Lipid	0.2	11.57	10.78	11.10	10.40	10.30
Ash	6.8	11.22	11.20	11.37	11.18	11.25
5'-IMP (%)	0.01	0.04	0.05	0.05	0.02	0.02
Inosine (%)	3.46	0.16	0.19	0.25	0.21	0.27
Gross energy (KJ g ⁻¹) ^a	–	20.96	20.82	20.85	20.72	20.71

Carbohydrate was calculated by the difference: 100 – (protein + lipid + ash).

^a Calculated using combustion values for protein, lipid and carbohydrate of 236, 395 and 172 KJ kg⁻¹, respectively.

2.3. Feeding protocol

After being acclimatized to the laboratory environment, similarly sized juveniles were sorted. Triplicate groups of fish were assigned to each dietary treatment. Fifteen fish, having a mean initial body weight of approximately 2.28 g were randomly allocated to previously prepared fifteen tanks. Fish were fed the experimental diets for 60 days by hand, twice a day to visual satiation at 9.00 and 16.00 h. The daily feed supplied was recorded, and the uneaten feed was collected 1 h after feeding, followed by drying, weighing and finally subtracted from the total amount of supplied diets to calculate the actual feed intake. All fish were weighed in bulk fortnightly to determine growth and visually check their health condition. The monitored water quality parameters (mean ± S.D.) were: water temperature 18.8 ± 1.9 °C; pH 8.1 ± 0.7 and salinity 34.5 ± 0.5 during the feeding trial.

2.4. Sample collection

The initial sample of 40 fish for whole body analysis was stored at –20 °C. At the end of the feeding trial, fish were starved for 24 h prior to final sampling. All the fish were anesthetized with Eugenol (4-allylmethoxyphenol, Wako Pure Chemical Ind., Osaka, Japan) at 50 mg L⁻¹. Then the total number, individual body weight and length of fish from each tank were measured. Three fish from each replicate tank were randomly collected and stored at –20 °C for a final whole body analysis. Using heparinized syringes, blood was collected from the caudal vein of five fish in each replicate tank and pooled for plasma

Table 3
Total amino acid content of the experimental diets (g, 100 g⁻¹, dry matter basis).^a

Amino acids	Diet groups				
	D1	D2	D3	D4	D5
Aspartic acid + Asparagine	4.13	4.17	4.21	4.24	4.20
Threonine	1.86	1.87	1.88	1.89	1.87
Serine	2.06	2.07	2.06	2.05	2.03
Glutamic acid + Glutamine	8.09	8.23	8.28	8.34	8.20
Glycine	2.48	2.55	2.58	2.57	2.56
Alanine	2.57	2.62	2.62	2.62	2.59
Valine	2.06	2.15	2.20	2.26	2.24
Isoleucine	1.79	1.88	1.91	1.98	1.97
Leucine	3.41	3.49	3.49	3.56	3.50
Tyrosine	1.51	1.56	1.56	1.55	1.55
Phenylalanine	2.01	2.06	2.07	2.08	2.07
Histidine	1.43	1.47	1.47	1.50	1.50
Lysine	3.04	3.11	3.12	3.14	3.12
Arginine	2.66	2.72	2.76	2.78	2.74
Proline	2.32	2.38	2.43	2.42	2.38
Cystine	0.54	0.55	0.54	0.54	0.54
Methionine	1.04	1.02	1.07	1.09	1.09
Tryptophan	0.599	0.603	0.602	0.602	0.590

^a Values are means of triplicate measurements.

Table 4
Free amino acid content of the experimental diet (g, 100 g⁻¹).^a

Free amino acids	Diet groups				
	D1	D2	D3	D4	D5
Taurine	0.28	0.29	0.28	0.28	0.29
Aspartic acid	0.03	0.03	0.03	0.03	0.03
Threonine	0.03	0.03	0.03	0.03	0.03
Serine	0.02	0.02	0.02	0.02	0.02
Glutamic acid	0.06	0.06	0.06	0.06	0.06
Glycine	0.03	0.03	0.03	0.03	0.03
Alanine	0.10	0.10	0.10	0.10	0.10
Valine	0.03	0.03	0.03	0.03	0.03
Isoleucine	0.02	0.02	0.02	0.02	0.02
Leucine	0.05	0.05	0.05	0.05	0.05
Tyrosine	0.02	0.03	0.03	0.02	0.02
Phenylalanine	0.03	0.03	0.04	0.03	0.03
Tryptophan	0.01	0.01	0.01	0.01	0.01
Lysine	0.05	0.06	0.07	0.05	0.05
Histidine	0.33	0.33	0.33	0.33	0.33
Arginine	0.07	0.07	0.07	0.07	0.07
Asparagine	0.01	0.01	0.02	0.01	0.01
Proline	0.03	0.03	0.03	0.03	0.03
Total free AA	1.21	1.24	1.24	1.21	1.23

^a Values are means of triplicate measurements.

analysis. In addition non-heparinized disposable syringes were used to collect blood for serum analysis. A small fraction of the heparinized blood was used to analyze the hematocrit level. Plasma samples were obtained by centrifugation at 4000 × g for 15 min using a high-speed refrigerated microcentrifuge (MX-160; Tomy Tech USA Inc., Tokyo, Japan) and kept at –80 °C. Liver and viscera were dissected out from three fish in each replicate tank, weighed individually to get hepatosomatic index and viscerasomatic index.

2.5. Biochemical analysis

The ingredients, diets and fish whole body were analyzed for moisture, crude protein, crude lipid and ash, in triplicate, using standard methods (AOAC, 1995). Total amino acids (TAA) and free amino acids (FAA) in diets were analyzed using high performance liquid chromatography (HPLC, Shimadzu Corp. Kyoto, Japan) as described previously (Kader et al., 2012). Inosine and IMP contents of feeds were also analyzed by High Performance Liquid Chromatography (HPLC). The inosine and IMP contents of the samples were determined by acidic extraction of ~1 g dry weight samples. In brief, for extraction process 25 mL of 5% perchloric acid was added to the feed sample (1 g), shaking for 10 min. After that deionized water was added to make constant volume up to 50 mL. Samples were sonicated for 10 mins, filtered and 5 mL sample solution were prepared. Finally, 0.4 mL potassium hydroxide (3 Mol/L) was added to neutralize the sample solution and stored in 4 °C. After extraction, for inosine quantification samples were loaded on a CAP CELL PAK C-18 column (Shisheido Co., Ltd. Japan), column temperature was 40 °C. The mobile phase was the mixture of 200 mmol/L phosphate buffer solution (pH 2.5) and methanol (95:5 v/v) contained 0.6% sodium heptanes sulphonate. Measurement wavelength was 260 nm. For IMP quantification samples were loaded on a MCI CDR-10 column (Mitsubishi Chemical Co. Ltd.). Column temperature was 40 °C. The mobile phase was 1 mol/L acetate buffer (pH 3.3). Measurement wavelength was 260 nm. The minimum amount of nucleotides detectable by HPLC is ~3 ng (in 50 µL injection volume). Total serum protein and plasma chemical parameters such as total albumin, total bilirubin, glucose, glutamic-pyruvate transaminase (GPT), blood urea nitrogen (BUN), triglycerides and total cholesterol (T-Cho) were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan). Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were also measured spectrophotometrically from blood plasma with an automated

analyzer FRAS4, Diacron International s.r.l., Grosseto, Italy by following the method described previously (Morganti et al., 2002). Briefly for BAP measurement, 50 μL of R2 reagent (ferric salt) was transferred to R1 reagent (a particular thiocyanate derivative), mixed gently and read on a photometer at a wavelength of 505 nm. Then, 10 μL plasma sample was added to the cuvette, mixed gently and read on the previous photometer. In case of d-ROMs, 20 μL plasma was added to 1 mL R2 reagent (a buffer, pH 4.8) and mixed gently. Then, 10 μL R1 reagent (an alkylamine) was added and incubated for 1 min at 37 °C. The resulting deep red coloring was photometrically detected at a wavelength of 505 nm. Free radicals were expressed in Carratelli Units (U.Carr). One Carratelli Unit is equal to a hydrogen peroxide concentration of 0.08% mg. For the analysis of plasma cortisol, 100 μL plasma was mixed with 1 mL diethylether by using a vortex mixture and allow to separate the organic phase. The diethylether was evaporated under a gentle stream of nitrogen. The extract was then analyzed for cortisol concentration using an enzyme-linked immunosorbent assay (Cortisol EIA Kit, product number EA65, Oxford Biomedical Research Inc., Oxford, MI).

2.6. Evaluation of non specific immune parameters

Serum lysozyme activity was measured with turbidimetric assays (Lygren et al., 1999). Ten microliters of samples was put into well of microplate, then added 190 μL of substrate (0.2 mg *Micrococcus lysodeikticus*, lyophilized cell, Sigma, USA)/mL PBS, pH 7.4, their absorbance were measured after 1 and 5 min, which were incubated with gentle shaking at constant room temperature, at 450 nm with ImmunoMini NJ-2300 (System Instruments, Tokyo, Japan). A unit of enzyme activity was defined as the amount of enzyme that causes a decrease in absorbance of 0.001/min.

The total peroxidase content in serum was measured according to Salinas et al. (2008), with some modifications. Briefly, 15 μL of serum were diluted with 35 μL of Hank's Buffered salt solution (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. Then, 50 μL of peroxidase substrate (3, 3', 5, 5'-tetramethylbenzidine hydrochloride) (TMB; Thermo Scientific Inc., USA) was added. The serum mixture was incubated for 15 min. The color-developing reaction in serum samples was stopped by adding 50 μL of 2 M sulphuric acid and the OD (450 nm) was measured in a plate reader. PBS was used as a blank instead of serum.

2.7. Fresh water stress test

Fresh water stress test was conducted to determine the lethal time of 50% mortality (LT_{50}) in fresh water. After the feeding trial, four fish from each rearing tank (total 12 fish per treatment) were randomly selected and transferred as duplicate groups in to a blue colored 20 L rectangular glass aquarium with 15 L tap water which was aerated for 24 h. The glass aquaria for stress test were equipped with continuous aeration and kept under ambient temperature during the stress test. The time duration for 50% mortality of red sea bream juvenile was calculated according to Moe et al. (2004) as follows: time to death (min) of a juvenile was converted to log₁₀ values. When the juvenile were transferred to the fresh water at first time, they were still alive so that the survival rate was assumed to be 100% (log value was $\log_{10} 100 = 2$). The calculation was then conducted every 5 min after the first exposure. The values of log survival rate were then plotted against every minutes to determine the time needed for 50% mortality of the juveniles in each treatment. The equation is as follows:

$Y = aX + b$ where $Y = \log_{10}(\text{survival})$, $X = \text{time for individual juvenile death (min)}$.

$LT_{50}(X)$ was obtained when $Y = 1.7$ since $\log_{10}(50) = 1.7$. Each value obtained from the equation above was compared statistically.

2.8. Growth factor gene expression (IGF-1 and IGF-2)

After the completion of the feeding trials, three fish from each tank were removed and immediately anesthetized with Eugenol (4-allylmethoxyphenol, Wako Pure Chemical Ind., Osaka, Japan) at 50 mg L^{-1} . Liver samples were obtained, placed in four volumes of RNAlater (Ambion; Applied Biosystems, Foster City, CA, USA) and stored at -80 °C until analysis. For the RNA extraction the RNeasy Mini Kit (Qiagen) was used. Liver samples of 15 mg inserted in a tube (SARSTEDT A.200.01S), homogenized and centrifuged at 5000 $\times g$ for 15 s. The supernatant were collected and mixed with 70% ethanol. After extraction of RNA, cDNA was synthesized using the Prime Script RT Reagent Kit (Takara, Japan) following manufacturer's protocol. Real time PCR was done using SYBR select Master Mix kit (Thermo Fisher Scientific, Japan) using following primers as shown in Table 5. Elongation factor (ef1) was used as housekeeping gene (Table 5). Amplification was performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems) with the protocol as follows: Initial 2 min denaturation at 95 °C, 40 cycles of 95 °C for 15 s, and 65 °C for 30 s. each assay was done in triplicate.

2.9. Evaluation of performance parameters

The following variables were evaluated:

Weight gain (%) = (final weight – initial weight) \times 100 / initial weight.

Specific growth rate (SGR, % day^{-1}) = {Ln (final weight) – Ln (initial weight)}/duration \times 100.

Survival (%) = 100 \times (final no. of fish / initial no. of fish).

Feed intake ($\text{g fish}^{-1} 60 \text{ days}^{-1}$) = (dry diet given – dry remaining diet recovered) / no of fish.

Feed conversion efficiency (FCE) = live weight gain (g) / dry feed intake (g).

Protein efficiency ratio (PER) = live weight gain (g) / dry protein intake (g).

Condition factor (CF, %) = weight of fish / (length of fish)³ \times 100.

Hepatosomatic index (HSI, %) = weight of liver / weight of fish \times 100.

2.10. Statistical analysis

All data were subjected to statistical verification using one-way ANOVA (Package Super ANOVA 1.11, Abacus Concepts, Berkeley, California, USA). Probabilities of $P < 0.05$ were considered significant. Significance differences between means were evaluated using the Turkey Kramer test.

3. Results

3.1. Fish performance parameters

Growth performance, nutrient utilization and survival of fish are presented in Table 6. Fish fed diet D5 showed significantly highest final body weight (FBW) and % weight gain (%WG) followed by diet

Table 5
Forward (F) and reverse (R) primers used for real-time quantitative RT-PCR.

Name	Database name	ID	Primer sequence: 5'-3'	Product size
igf1(F)	miseq db	2564	TAAACCCACACCCGAGTGACA	90
igf1(R)			CGCATGAAGAAAAGCTACGG	
igf2(F)	miseq db	1567	CGGCAACTAGTGTATGAGCA	97
igf2(R)			CAGTGTCAAGGGGAAGTGT	
ef1a(F)		666	TGTGGGTGCAGTTTGACAAT	204
ef1a(R)			CTTCAACGCTCAGGTCATCA	

Table 6
Growth performance and feed utilization in red sea bream fed test diets for 60 days.^a

Parameters	Diet groups				
	D1	D2	D3	D4	D5
IBW ^b	2.29 ± 0.00	2.28 ± 0.02	2.29 ± 0.01	2.29 ± 0.0	2.29 ± 0.01
FBW ^c	13.66 ± 0.5a	14.44 ± 0.1 ab	13.87 ± 0.2a	14.41 ± 0.4ab	15.58 ± 0.3b
WG ^d	491.1 ± 26.4a	532.3 ± 3.6ab	506.3 ± 11.4 a	527.0 ± 16.0ab	578.8 ± 13.4b
SGR ^e	2.96 ± 0.07 a	3.07 ± 0.01ab	3.00 ± 0.03a	3.06 ± 0.04ab	3.19 ± 0.03b
FI ^f	12.1 ± 0.4b	11.2 ± 0.5ab	10.2 ± 0.3a	10.9 ± 0.3ab	12.6 ± 0.4b
FCE ^g	0.89 ± 0.10a	1.04 ± 0.05ab	1.04 ± 0.02ab	1.06 ± 0.01ab	1.10 ± 0.02 b
PER ^h	1.83 ± 0.2a	2.11 ± 0.1ab	2.15 ± 0.04ab	2.17 ± 0.0ab	2.25 ± 0.0b
Sur ^k	76.7 ± 3.4	80.0 ± 0.0	86.7 ± 3.8	80.0 ± 0.0	82.2 ± 2.2

^a Values are means of triplicate groups ± SEM. Within a row, means with different letters are significantly different ($P < 0.05$), and means with the same letters are not significantly different ($P > 0.05$). The absence of letters indicates no significant difference between treatments.

^b IBW: initial body weight (g).

^c FBW: final body weight (g).

^d WG: percent weight gain (%).

^e SGR: specific growth rate (% day⁻¹).

^f FI: feed intake (g fish⁻¹ 60 days⁻¹).

^g FCE: feed conversion efficiency.

^h PER: protein efficiency ratio.

^k Sur: survival (%).

groups D2 and D4. Fish fed control group showed lowest growth performance and it was not differed significantly with diet group D3. Feed conversion ratio and protein efficiency ratios were also significantly higher in diet group D5, whereas the other supplemented group showed intermediate value. Feed intake (FI) was significantly higher in fish fed diet D1 followed by D5, D2, D4 and D3. Significantly lower growth performance and feed utilization were observed in control diet group (D1). Survival was not significantly influenced by dietary supplementation.

3.2. Hematological parameters

Table 7 represents the hematological parameters of red sea bream after 60 days of feeding trial. Overall, dietary treatments had no significant effect on blood chemical parameters of fish among different treatments. However, plasma glucose level showed decreasing trend numerically with the dietary supplementation of NBP and inosine. Hematocrit content was significantly highest in fish fed diet group D5, whereas the other supplemented groups showed intermediate values. Supplementation free control group showed the lowest value ($P < 0.05$).

3.3. Oxidative stress parameters

Oxidative status of fish was analyzed from plasma and significant differences were found in BAP and d-ROM values among different

treatments (Table 8). Inosine supplemented groups showed significantly lowest d-ROM values. Whereas control and 1% NBP supplemented groups showed intermediate value. At high supplementation level of NBP (3%), there was significantly higher d-ROM value. On the other hand, inosine supplemented groups showed significantly highest BAP values and it was not differed significantly with control. NBP supplementation of 1% (D2) showed intermediate value and the 3% NBP supplemented group showed lowest BAP value. Fig. 1 shows the pattern of combined effects of d-ROMs and BAP. Fish fed diet groups D4 and D5 were located in Zone A, where it is categorized as lower intensity of oxidative stress and higher tolerance ability against oxidative stress. Diet groups D1 and D3 were located in zone B and zone C, respectively. On the other hand, fish fed diet group containing the higher level of NBP (D3) was located in Zone D, which is categorized as higher intensity of oxidative stress and lower tolerance ability against oxidative stress. Dietary supplementations of inosine and NBP also reduce the plasma cortisol levels (%) compared to non supplemented control group but not at a significant level (Table 8).

3.4. Immunological assays

Non-specific immune parameters viz. total serum protein (TSP), peroxidase activity and lysozyme activity are shown in Table 8. Numerically increasing non-specific immune responses were observed in dietary supplemented groups compared to control but not at a significant level.

Table 7
Blood chemistry of juvenile red sea bream fed test diets for 60 days.^a

Parameters	Diet groups				
	D1	D2	D3	D4	D5
Hematocrit (%)	28.7 ± 0.7a	31.3 ± 0.3ab	31.3 ± 0.9ab	31.0 ± 2.3ab	35.0 ± 0.6b
Glucose (mg dL ⁻¹)	60.7 ± 3.8	56.7 ± 2.9	54.0 ± 4.0	53.0 ± 14.0	50.7 ± 2.7
T-Cho ^b	202.7 ± 6.4	223.7 ± 19.3	205.3 ± 5.9	179.0 ± 11.0	220.3 ± 19.2
BUN ^c	<5	<5	<5	<5	<5
T-Bill ^d	<2	2.50	3.33	<2	<2
GPT ^e	22.3 ± 9.1	24.3 ± 7.3	28.3 ± 3.3	42.0 ± 7.0	26.3 ± 15.8
Amylase (IU L ⁻¹)	12.0 ± 2.0	14.0 ± 4.0	17.3 ± 4.7	10.5 ± 0.5	11.0 ± 0.6
TG ^f	104.3 ± 7.7	122.0 ± 5.7	117.3 ± 10.8	98.5 ± 0.5	126.7 ± 12.8

^a Values are means ± SE of triplicate groups. Within a row, means with the same letters are not significantly different ($P > 0.05$).

^b T-Cho: total cholesterol (mg dL⁻¹).

^c BUN: blood urea nitrogen (mg dL⁻¹).

^d T-Bill: Total bilirubin (mg dL⁻¹).

^e GPT: glutamic pyruvate transaminase (IU L⁻¹).

^f TG: triglyceride (mg dL⁻¹).

Table 8Non specific immunity and oxidative stress response of red sea bream fed test diets for 60 days.^a

Parameters	Diet groups				
	D1	D2	D3	D4	D5
T Pro ^b	2.47 ± 0.2	2.53 ± 0.1	2.57 ± 0.0	2.60 ± 0.1	2.70 ± 0.1
LA ^c	80.0 ± 11.6	90.0 ± 0.0	93.3 ± 12.0	83.3 ± 8.8	103.3 ± 8.8
PA ^d	1.74 ± 0.03	1.86 ± 0.1	1.78 ± 0.1	1.89 ± 0.2	1.91 ± 0.02
d-ROMs ^e	19.0 ± 1.0ab	18.0 ± 1.0ab	35.0 ± 8.8b	13.0 ± 0.0a	12.0 ± 1.0a
BAP ^f	3387.5 ± 130.5b	3109.7 ± 48.5ab	2934.0 ± 80.2a	3386.5 ± 24.5b	3421.7 ± 46.8b
CORT ^g	61.1 ± 0.2	58.8 ± 0.6	60.4 ± 0.5	59.4 ± 0.7	59.3 ± 0.9

^a Values are means ± SE of triplicate groups. Within a row, means with the same letters are not significantly different ($P > 0.05$).

^b T-Pro: Total protein (g dL⁻¹).

^c LA: Lysozyme activity of serum (unit mL⁻¹).

^d PA: Peroxidase activity of serum (OD 450 nm).

^e d-ROMs: reactive oxygen metabolites (μmol L⁻¹).

^f BAP: biological antioxidant potential (U.Carr.).

^g CORT: relative value of cortisol (%).

3.5. Low salinity stress test

The results of lethal stress test against fresh water shock on LT₅₀ obtained by regression analysis are shown in Fig. 2. Significantly the highest LT₅₀ was obtained in diet group D5. Although, there was no significance among the other dietary groups (D2 to D4), there was notable numerical increase in LT₅₀ values compared to the control group.

3.6. Hepatic IGF-1 and IGF-2 mRNA expressions

Hepatic IGF-1 and IGF-2 growth factor gene expressions were presented in Fig. 3a,b. Numerically higher hepatic IGF-1 mRNA expression was found in diet groups D2 and D4. However, overall hepatic insulin like growth factor (IGF-1, IGF-2) mRNA expression was not significantly influenced by the dietary treatment.

3.7. Whole body proximate composition and biometric indices

Initial and final whole body proximate compositions of juvenile red sea bream were shown in Table 9. All the fish showed a change in the analyzed parameters compared to those of the initial values. However, whole body proximate composition and (%) and biometric indices were not influenced significantly ($P > 0.05$) by the dietary treatments except in hepatosomatic index (HSI) where, diet group D3 showed significantly highest HSI value and it was not differed significantly with inosine supplemented diet groups (D4 and D5). Diet group D2 showed

the intermediate value, whereas control group showed significantly the lowest value.

4. Discussion

Increasing feed cost is the major limitation of the nucleotide as well as the immunostimulant administration in aquaculture diets. One of the most important alternative to reducing feed cost in nucleotide administration in fish feed is to search for relatively low cost industrial by-products obtained through recycling of industrial waste which contain considerable portion of nucleotides or nucleosides. In the present study a nucleoside inosine and a liquid NBP obtained through the production process of inosine were evaluated on growth, blood chemistry, oxidative stress and hepatic IGF-1 and IGF-2 mRNA expression of juvenile red sea bream.

In the present study, significantly highest FBW, %WG and SGR (% day⁻¹) of juvenile red sea bream were observed in fish fed diet D5 followed by fish fed the diets with D2, D4, D3 and D1. The highest growth performance in diet D5 and improved growth performance in diet D2 to D4 might be due to supplementation of nucleoside inosine and NBP, which contains considerable portion of inosine and small portion of some other nucleotides. Unlike our present results, the growth enhancing and feeding stimulating properties of nucleosides inosine have been reported earlier in some studies (Ishida and Hidaka, 1987; Kumazawa and Kurihara, 1990; Yamaguchi, 1991). Person-Le Ruyet et al. (1983) reported dietary inosine enhanced growth and survival of turbot larvae. In subsequent research, this group showed that 10 or 20 days of feeding a diet supplemented with 0.77% inosine also significantly increased weight gain of turbot larvae. So far, there is no exact

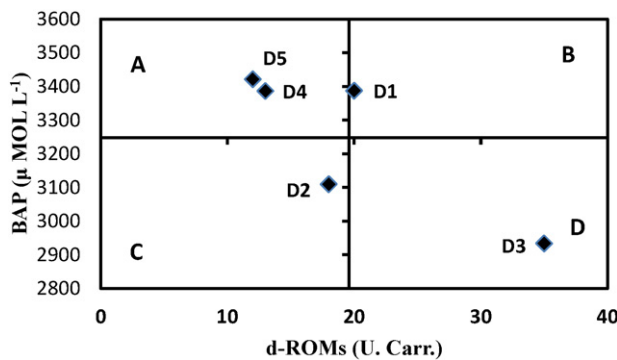


Fig. 1. Oxidative stress parameters in red sea bream fed test diets for 60 days. (Values are means of triplicate groups. Central axis based on mean values of d-ROM and BAP from each treatment). Zone A: high antioxidant potential and low reactive oxygen metabolites (good condition). Zone B: high antioxidant potential and high reactive oxygen metabolites (acceptable condition). Zone C: low antioxidant potential and low reactive oxygen metabolites (acceptable condition). Zone D: low antioxidant potential and high reactive oxygen metabolites (stressed condition).

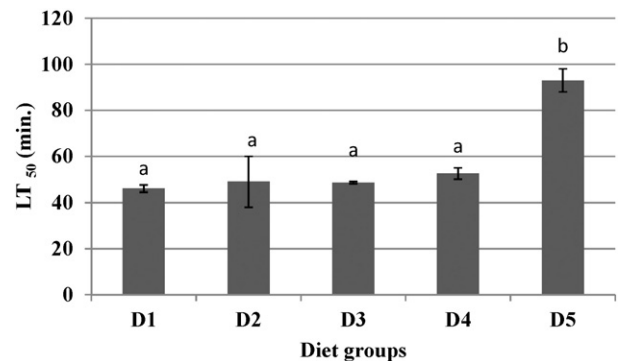


Fig. 2. LT₅₀ (min) calculated from the lethal time of red sea bream exposed to fresh water (see the text). Data were expressed as mean ± S.E.M. from duplicate groups. Values with different letters are significantly different ($P < 0.05$).

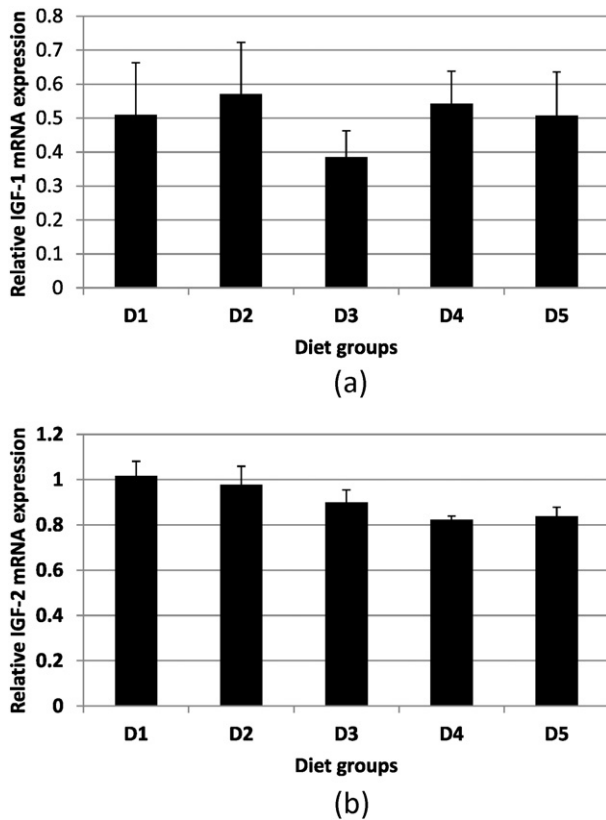


Fig. 3. (a, b): Hepatic insulin like growth factor gene expression (IGF-1 and IGF-2) of red sea bream fed experimental diet for 60 days.

explanation how dietary nucleotide and nucleosides work to enhance growth. However, it is assumed that the growth-enhancing effect of inosine and other nucleotide in the present study resulted from improved feed intake at the beginning of weaning, promoting more rapid food intake that reduced nutrient leaching into the water or possibly playing roles in metabolism (Metailler et al., 1983; Hossain et al., 2016). It is also well known that growth response was strongly related to improve feed utilization. Our results suggested that among the tested fish that utilized experimental diets effectively, there were significantly higher feed efficiency ratio (FER) and protein efficiency ratio (PER) in fish fed diet D5. Other supplemented diet groups also showed moderately improved feed utilization (FCE, PER) values. The improved FER and PER would explain the increased growth performance in supplemented groups in the present study.

Hematological parameters are being used as an effective and sensitive index for the physiological and pathological condition as well as

welfare of fish (Hossain et al., 2016). Blood parameters obtained in the present experiment are considered to be within the normal range for juvenile red sea bream, compared to those of the previous findings (Aoki et al., 1998; Hossain et al. 2016). Hematocrit, which is used as an assessment for general health in fish based on different nutritional strategies, was enhanced with supplementation of dietary NBP and inosine in juvenile red sea bream. In the present experiment, hematocrit content was significantly highest in fish fed diet group D5 and the other supplemented groups showed intermediate values, whereas control group (D1) showed significantly lowest value. This indicated that dietary NBP and inosine elevated the health status of fish. Similarly, Song et al. (2012) and Hossain et al. (2016) reported the enhanced hematocrit level with the supplementation of nucleotides in Japanese olive flounder and red sea bream diets, respectively. Interestingly, dietary NBP and inosine affected glucose levels in red sea bream ($P > 0.05$). Plasma glucose is commonly considered to be one stress indicator in fish, high glucose levels often indicate the higher stress status of fish (Eslamloo et al., 2012). The lower glucose contents in fish fed supplemented diet groups, indicated that red sea bream fed supplemented diets displayed optimal physiological conditions compared to the control group. Blood glucose levels of the present study ($50.7\text{--}60.7\text{ mg dL}^{-1}$) were comparable to the values ($56.50\text{--}83.50\text{ mg dL}^{-1}$) reported previously for juvenile red sea bream (Hossain et al., 2016) fed nucleotide supplemented diet.

Stress is one of the emerging factors in aquaculture activities which may affect hormonal secretion rates, intermediary metabolism, immunity and nutrient utilization (Li et al., 2009). Plasma or serum cortisol concentration is a reliable biological indicator of stress response in fish and terrestrial animals (Li et al., 2009; Small and Davis, 2002). In the present study, the relative values of plasma cortisol concentrations were decreased compared with control, with the supplementation of inosine and NBP but not at a significant level. Reduced plasma cortisol levels of red sea bream fed inosine and NBP supplemented diet in the present study are in agreement with what has been previously reported in rainbow trout (Leonardi et al., 2003; Tahmasebi-Kohyani et al., 2012) and beluga sturgeon (*Huso huso*) Yousefi et al. (2011). Oxidative stress can be generated at high level of reactive oxygen species (ROS) and/or decreased efficacy of antioxidant system, which is another health risk factor in humans or other mammals (Pasquini et al., 2008). Oxidative stress was measured using the free radical analytical system assessing the derivatives of oxidative stress by measuring reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) in plasma samples (Gao et al., 2012). Hossain et al. (2016) reported that, fish with higher value of d-ROMs confirm a more oxidative condition, therefore, higher BAP value indicate they have stronger tolerance against oxidation. Using these parameters, our study showed that fish fed diets D4 and D5 were more tolerant of oxidative stress indicating a higher health status, which indicated high BAP and low d-ROMs values. On the contrary, fish fed diet D3 were located in Zone D, which is categorized as higher intensity of oxidative stress and lower tolerance ability against oxidative stress. To date, there is a lack of explanation about how

Table 9
Whole body proximate analysis (%) and biometric indices in juvenile red sea bream fed test diets for 60 days.^a

Parameters	Initial ^b	Diet groups				
		D1	D2	D3	D4	D5
Moisture	19.6	75.9 ± 0.3	76.0 ± 0.5	76.2 ± 0.5	76.1 ± 0.4	76.3 ± 0.4
Crude protein	12.7	13.7 ± 0.2	14.0 ± 0.4	13.6 ± 0.2	13.9 ± 0.3	13.9 ± 0.1
Crude lipid	3.18	6.06 ± 0.1	5.92 ± 0.3	5.80 ± 0.1	6.02 ± 0.1	6.01 ± 0.0
Crude ash	3.42	3.63 ± 0.1	3.73 ± 0.1	3.74 ± 0.1	3.79 ± 0.1	3.41 ± 0.1
CF ^c	–	1.76 ± 0.0	1.79 ± 0.0	1.74 ± 0.1	1.84 ± 0.1	1.85 ± 0.0
HSI ^d	–	1.19 ± 0.5a	1.23 ± 0.02ab	1.50 ± 0.08c	1.47 ± 0.07bc	1.47 ± 0.05bc

^a Values are means of triplicate groups ± S.E.M. Within a row, means with the same letters are not significantly different ($P > 0.05$). Crude protein, crude lipid and ash are expressed on a wet weight basis.

^b Initial values are not included in the statistical analysis.

^c CF: condition factor (%).

^d HSI: hepatosomatic index (%).

these additives work to affect these parameters, so more studies are needed.

The lethal stress test is used to assess health status by measuring the lethal time of 50% mortality (LT50) in fresh water of the fish (Hossain et al., 2016). It is well known that stress affects the survival and growth of fish, since stress responses tend to increase the energy demand at the expense of anabolic processes (Kubilyay and Ulukoy, 2002). The higher value of LT50 in fish fed diet group D5 indicated a higher tolerance of the red sea bream against low-salinity stress. Fish antioxidant status is strongly related to its immune system, contributing to enhanced resistance towards different stressors (Tovar-Ramirez et al., 2010). In the light of the previous findings, results of the current study confirmed a higher tolerance against low-salinity stress in fish under less oxidative stress conditions.

In the present study, whole-body proximate composition and biometric indices were not significantly influenced by dietary inosine supplementation except HSI (Table 9). However, whole body compositions were within the normal ranges that have been reported previously (Hossain et al., 2016). No significant effects of dietary nucleotides on red sea bream and red drum whole body and biometric indices were also observed by Hossain et al. (2016) and Li et al. (2007), respectively which are similar to our study. The liver size is relative to the nutritional status of the fish (Shoemaker et al., 2003; Sridee & Boonanuntanasarn, 2012). In the present study, increased HSI with NBP and inosine supplementation indicates proper storage of macro and micronutrients, healthy condition of liver and clinically healthy signs of fish.

Molecular tools have been increasingly utilized in aquaculture research to complement existing husbandry techniques and to examine the responses of fish to culture stress and environmental changes (Stone et al., 2008; Panserat and Kaushik, 2010). Molecular technology (gene expression of insulin-like growth factors IGF- I and II) has been used as the most promising molecular marker to date as a rapid indicator of growth in teleost fish (Picha et al., 2008) and to evaluate the efficacy of diets for commercial purpose within very short time. Previous research investigating hepatic IGF-I in relation to nutritional status and growth in cultured finfish has focused on feed deprivation or feed restriction, rather than on the manipulation of a selected dietary component such as protein or lipid (Picha et al., 2006; Bower et al., 2008; Hagen et al., 2009). In the present study, an attempt has been made to investigate hepatic IGF-1, IGF-2 in relation to supplementation of functional nutrients (nucleotide related products) in red sea bream diets. IGF-1 and IGF-2 mRNA expression were not significantly altered by dietary supplementations in the present study. However, numerically higher hepatic IGF-1, mRNA expression was found in diet groups D2 and D4 and with some small exception, in most of the dietary treatments, gene expressions correlates with the growth rate of red sea bream. The positive relationship between IGF-I, IGF-2 and growth rate were further confirmed in other fishes (Uchida et al., 2003; Dyer et al., 2004; Picha et al., 2006). This unexpected correlation might be due to large variation in gene expression between individual fish. Pedroso et al. (2009) demonstrated substantial variation in hepatic IGF-1 mRNA in the congeneric Japanese yellowtail over a 21-day sampling period in response to feed restriction, with sampling intervals of 3 days. Another important cause of this unexpected correlation of IGF-1 with some dietary treatments might be due to the lower temperature (less than 18 °C) during the rearing period which could disturb the IGF-1 and IGF-2 expression of red sea bream. Similar temperature related drawbacks of IGFs study were also reported by Beckman et al. (2004 a, b).

According to the result of the study, it is clear that low concentration of NBP (1%) supplemented group (D2) and inosine supplemented group improves the growth, feed utilization and some of the hematological parameters of red sea bream. Further, supplementation also improves non specific immune response like, total serum protein, lysozyme activity and peroxidase activity ($P > 0.05$). Blood chemical parameters and growth factor gene expression (IGF1 and IGF-2) were not significantly

affected by dietary treatments. This might be due to the inappropriate dosages or other causes. Interestingly, inosine supplementation showed best oxidative stress condition compared with NBP. Finally, it can be concluded that, inosine and low dosage of NBP (1%) could be effectively used in red sea bream diet for improved growth and health performance of this species. However, further study is needed to optimize the inclusion level of these nucleotide related products and to discover the underlying reasons for the growth-promoting effects and the improved health features found in the present study.

Acknowledgments

The first author would like to thank the Ministry of Education, Culture, Sports, Science and Technology (MONBUKAGAKUSHO), Japan for the scholarship provided to perform this research. The research was partially supported by the United Graduate School of Agricultural Sciences, Kagoshima University Grant and research funds from Ajinomoto Co., Inc. offered to Dr. Shunsuke Koshio.

References

- AOAC, 1995. Official Methods of Analysis of the Association of Analytical Chemistry. 16th ed. American Association of Official Analytical Chemists, Washington, DC.
- Aoki, H., Furuichi, M., Viyakarn, V., Yamagata, Y., Watanabe, T., 1998. Feed protein ingredients for red sea bream. *Suisanzoshoku* 46, 121–127.
- Barankiewicz, J., Cohen, A., 1985. Purine nucleotide metabolism in resident and activated rat macrophages in vitro. *Eur. J. Immunol.* 15, 627–631.
- Beckman, B.R., Shimizu, M., Gadberry, B.A., Cooper, K.A., 2004a. Response of the somatotrophic axis of juvenile coho salmon to alterations in plane of nutrition with an analysis of the relationships among growth rate and circulating IGF-I and 41 kDa IGFBP. *Gen. Comp. Endocrinol.* 135, 334–344.
- Beckman, B.R., Shimizu, M., Gadberry, B.A., Parkins, P.J., Cooper, K.A., 2004b. The effect of temperature change on the relations among plasma IGF-I, 41-kDa IGFBP, and growth rate in post-smolt coho salmon. *Aquaculture* 241, 601–619.
- Bower, N.L., Li, X., Taylor, R., Johnston, I.A., 2008. Switching to fast growth: the insulin-like growth factor (IGF) system in skeletal muscle of Atlantic Salmon. *J. Exp. Biol.* 211, 3859–3870.
- Burrells, C., Williams, P.D., Forno, P.F., 2001a. Dietary nucleotides: a novel supplement in fish feeds. 1. Effects on resistance to disease in salmonoids. *Aquaculture* 199, 159–169.
- Burrells, C., Williams, P.D., Southgate, P.J., Wadsworth, S.L., 2001b. Dietary nucleotides: a novel supplement in fish feeds. 2. Effects on vaccination, salt water transfer, growth rates and physiology of Atlantic salmon (*Salmo salar* L.). *Aquaculture* 199, 171–184.
- Carr, W.E.S., Netherton, J.C., Milstead, M.L., 1984. Chemo-attractants of the shrimp, *Palaemonetes pugio*: variability in responsiveness and the stimulatory capacity of mixtures containing amino acids, quaternary ammonium compounds, purines and other substances. *Comp. Biochem. Physiol.* 77A (3), 469–474.
- Carr, W.E.S., Thompson, H.W., 1983. Adenosine 5'-monophosphate, an internal regulatory agent, is a potent chemoattractant for a marine shrimp. *J. Comp. Physiol.* 153, 47–53.
- Carver, J.D., Walker, W.A., 1995. The role of nucleotides in human nutrition. *J. Nutr. Biochem.* 6, 58–72.
- Cheng, Z., Buentello, A., Gatlin III, D.M., 2011. Dietary nucleotides influence immune responses and intestinal morphology of red drum *Sciaenops ocellatus*. *Fish Shellfish Immunol.* 30, 143–147.
- Dyer, A.R., Barlow, C.G., Bransden, M.P., Carter, C.G., Glencross, B.D., Richardson, N., Thomas, P.M., Williams, K.C., Carragher, J.F., 2004. Correlation of plasma IGF-1 concentrations and growth rate in aquacultured finfish: a tool for assessing the potential of new diets. *Aquaculture* 236, 583–592.
- Eslamlou, K., Falahatkar, B., Yokoyama, S., 2012. Effects of dietary bovine lactoferrin on growth, physiological performance, iron metabolism and non-specific immune responses of Siberian sturgeon *Acipenser baeri*. *Fish Shellfish Immunol.* 32, 976–985.
- Gao, J., Koshio, S., Ishikawa, M., Yokoyama, S., Mamauag, R.E.P., Han, Y., 2012. Effects of dietary oxidized fish oil with vitamin E supplementation on growth performance and reduction of lipid peroxidation in tissues and blood of red sea bream *Pagrus major*. *Aquaculture* 356–357, 73–79.
- Gil, A., 2002. Modulation of the immune response mediated by dietary nucleotides. *Eur. J. Clin. Nutr.* 56 (Suppl. 3), S1–S4.
- Hagen, Ø., Fernandes, J.M.O., Solberg, C., Johnston, I.A., 2009. Expression of growth-related genes in muscle during fasting and refeeding of juvenile Atlantic halibut, *Hippoglossus hippoglossus* L. *Comp. Biochem. Physiol. Part B Biochem. Mol. Biol.* 152, 47–53.
- Hossain, M.S., Koshio, S., Ishikawa, M., Yokoyama, S., Sony, N.M., 2016. Dietary nucleotide administration influences growth, immune responses and oxidative stress resistance of juvenile red sea bream (*Pagrus major*). *Aquaculture* 455, 41–49.
- Ishida, Y., Hidaka, I., 1987. Gustatory responses profiles for amino acids, glycinebetaine and nucleotides in several marine teleosts. *Nippon Suisan Gakkaishi* 53, 1391–1398.
- Kader, M.A., Bulbul, M., Koshio, S., Ishikawa, M., Yokoyama, S., Nguyen, B.T., Komilus, C.F., 2012. Effect of complete replacement of fishmeal by dehulled soybean meal with crude attractants supplementation in diets for red sea bream, *Pagrus major*. *Aquaculture* 350–353, 109–116.

- Koshio, S., 2002. Red Sea bream, *Pagrus major*. In: Webster, C.D., Lim, C.E. (Eds.), *Nutrient Requirements and Feeding of Finfish for Aquaculture*. CABI Publishing, 10E 40th Street, Suite 3203, New York, NY 10016, USA, pp. 51–63.
- Kubilay, A., Ulukoy, G., 2002. The effects of acute stress on rainbow trout (*Oncorhynchus mykiss*). *Turk. J. Zool.* 26 (2), 249–254 (2002).
- Kumazawa, T., Kurihara, K., 1990. Large synergism between monosodium glutamate and 5'-nucleotides in canine taste nerve responses. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 259, 420–426.
- Leonardi, M., Sandino, A.M., Klempau, A., 2003. Effect of a nucleotide-enriched diet on the immune system, plasma cortisol levels and resistance to infectious pancreatic necrosis (IPN) in juvenile rainbow trout (*Oncorhynchus mykiss*). *Bull. Eur. Assoc. Fish Pathol.* 23, 52–59.
- Li, P., Gatlin III, D.M., Neill, W.H., 2007. Dietary supplementation of a purified nucleotide mixture transiently enhanced growth and feed utilization of juvenile red drum, *Sciaenops ocellatus*. *J. World Aquacult. Soc.* 38, 281–286.
- Li, P., Gatlin, D.M.I.I.I., 2006. Nucleotide nutrition in fish: current status and future applications. *Aquaculture* 251, 141–152.
- Li, P., Lewis, D.H., Gatlin, D.M.I.I.I., 2004. Dietary oligonucleotides from yeast RNA influence immune responses and resistance of hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) to *Streptococcus iniae* infection. *Fish Shellfish Immunol.* 16, 561–569.
- Li, P., Ray, B., Gatlin, I.I.I.D.M., Sink, T., Chen, R., Lochmann, R., 2009. Effect of handling and transport on cortisol response and nutrient mobilization of golden shiner, *Notemigonus crysoleucas*. *J. World Aquacult. Soc.* 40, 803–809.
- Lin, Y.H., Wang, H., Shiau, S.Y., 2009. Dietary nucleotide supplementation enhances growth and immune responses of grouper, *Epinephelus malabaricus*. *Aquac. Nutr.* 15, 117–122.
- Low, C., Wadsworth, S., Burrells, C., Secombes, C.J., 2003. Expression of immune genes in turbot (*Scophthalmus maximus*) fed a nucleotide-supplemented diet. *Aquaculture* 221, 23–40.
- Lygren, B., Sveier, H., Hjeltnes, B., Waagbo, R., 1999. Examination of the immunomodulatory properties and the effect on disease resistance of dietary bovine lactoferrin and vitamin C fed to Atlantic salmon (*Salmo salar*) for a short-term period. *Fish & Shellfish Immunology*, 9, 95–107.
- Mackie, A.M., Adron, J.W., 1978. Identification of inosine and inosine 5'-monophosphate as the gustatory feeding stimulants for the turbot, *Scophthalmus maximus*. *Comp. Biochem. Physiol.* 60A, 79–83.
- Metailler, R., Cadena-Roa, M., Person-Le Ruyet, J., 1983. Attractive chemical substances for the weaning of Dover sole (*Solea vulgaris*): qualitative and quantitative approach. *J. World Maric. Soc.* 14, 679–684.
- Moe, Y.Y., Koshio, S., Teshima, S., Ishikawa, M., Matsunaga, Y., Panganiban, A.J., 2004. Effect of vitamin C derivatives on the performance of larval kuruma shrimp, *Marsupenaeus japonicus*. *Aquaculture* 242, 501–512.
- Morganti, P., Bruno, C., Guarneri, F., Cardillo, A., Del Ciotto, P., Valenzano, F., 2002. Role of topical and nutritional supplement to modify the oxidative stress. *Int. J. Cosmet. Sci.* 24 (6), 331–339.
- Panserat, S., Kaushik, S., 2010. Regulation of gene expression by nutritional factors in fish. *Aquac. Res.* 41, 751–762.
- Pasquini, A., Luchetti, E., Marchetti, V., Cardini, G., 2008. Analytical performances of d-ROMs test and BAP test in canine plasma. Definition of the normal range in healthy Labrador dogs. *Vet. Res. Communi.* 32, 137–143.
- Pedroso, F.L., Fukada, H., Masumoto, T., 2009. Molecular characterization, tissue distribution patterns and nutritional regulation of IGFBP-1, -2, -3 and -5 in yellowtail, *Seriola quinqueradiata*. *Gen. Comp. Endocrinol.* 161, 344–353.
- Person-Le Ruyet, J., Menu, B., Cadena-Roa, M., Me'tailler, R., 1983. Use of expanded pellets supplemented with attractive chemical substances for the weaning of turbot (*Scophthalmus maximus*). *J. World Maric. Soc.* 14 (676–67), 8.
- Picha, M.E., Silverstein, J.T., Borski, R.J., 2006. Discordant regulation of hepatic IGF-I mRNA and circulating IGF-I during compensatory growth in a teleost, the hybrid striped bass (*Morone chrysops* × *Morone saxatilis*). *Gen. Comp. Endocrinol.* 147, 196–205.
- Picha, M.E., Turano, M.J., Beckman, B.R., Borski, R.J., 2008. Endocrine biomarkers of growth and applications to aquaculture: a minireview of growth hormone, insulin-like growth factor (IGF)-I, and IGF-binding proteins as potential growth indicators in fish. *N Am. J. Aquacult.* 70, 196–211.
- Ramadan, A., Afifi, N.A., Moustafa, M., Samy, A.M., 1994. The effect of ascogen on the immune response of tilapia fish to *Aeromonas hydrophila* vaccine. *Fish Shellfish Immunol.* 5, 159–165.
- Sakai, M., Taniguchi, K., Mamoto, K., Ogawa, H., Tabata, M., 2001. Immunostimulant effects of nucleotide isolated from yeast RNA on carp, *Cyprinus carpio* L. *J. Fish Dis.* 24, 433–438.
- Salinas, I., Abelli, L., Bertoni, F., Picchietti, S., Roque, A., Furones, D., Cuesta, A., Meseguer, J., Esteban, M.A., 2008. Monospecies and multispecies probiotic formulations produce different systemic and local immunostimulatory effects in the gillhead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol.* 25, 114–123.
- Shoemaker, C.A., Klesius, P.H., Lim, C., Yildirim, M., 2003. Feed deprivation of channel catfish, *Ictalurus punctatus* (Rafinesque), influences organosomatic indices, chemical composition and susceptibility to *Flavobacterium columnare*. *J. Fish Dis.* 25, 558–561.
- Small, B.C., Davis, K.B., 2002. Validation of a time-resolved fluoroimmunoassay for measuring plasma cortisol in channel catfish *Ictalurus punctatus*. *J. World Aquacult. Soc.* 33, 184–187.
- Song, J.W., Lim, S.J., Lee, K.J., 2012. Effects of dietary supplementation of inosine monophosphate on growth performance, innate immunity and disease resistance of olive flounder (*Paralichthys olivaceus*). *Fish Shellfish Immunol.* 33, 1050–1054.
- Sridee, N., Boonanutanasarn, S., 2012. The effects of food deprivation on hematological indices and blood indicators of liver function in *Oxyleotris marmorata*. *World Acad. Sci. Eng. Technol.* 6 (5), 248–252.
- Stone, D.A.J., Gaylord, T.G., Johansen, K.A., Overturf, K., Sealey, W.M., Hardy, R.W., 2008. Evaluation of the effects of repeated fecal collection by manual stripping on the plasma cortisol levels, TNF-[alpha] gene expression, and digestibility and availability of nutrients from hydrolyzed poultry and egg meal by rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquaculture* 275, 250–259.
- Tahmasebi-Kohyani, A., Keyvanshokoo, S., Nematollahi, A., Mahmoudi, N., Pashazanoosi, H., 2012. Effects of dietary nucleotides supplementation on rainbow trout (*Oncorhynchus mykiss*) performance and acute stress response. *Fish Physiol. Biochem.* 38, 431–440.
- Tovar-Ramírez, D., Mazurais, D., Gatesoupe, J.F., Quazuguel, P., Cahu, C.L., Zambonino-Infante, J.L., 2010. Dietary probiotic live yeast modulates antioxidant enzyme activities and gene expression of sea bass (*Dicentrarchus labrax*) larvae. *Aquaculture* 300 (1–4), 142–147.
- Uchida, K., Kajimura, S., Riley, L.G., Hirano, T., Aida, K., Grau, E.G., 2003. Effects of fasting on growth hormone/insulin-like growth factor I axis in the tilapia, *Oreochromis mossambicus*. *Comp. Biochem. Physiol.* 134A, 429–439.
- Yamaguchi, S., 1991. Basic properties of umami and effects on humans. *Physiol. Behav.* 49, 833–841.
- Yousefi, M., Abtahi, B., Kenari, A., 2011. A. Hematological, serum biochemical parameters, and physiological responses to acute stress of beluga sturgeon (*Huso huso*, Linnaeus 1785) juveniles fed dietary nucleotide. *Comp. Clin. Pathol.* 1–6. <http://dx.doi.org/10.1007/00580-011-1225-4>.