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Effects of Three Dietary Growth Hormones on Growth Performance and Lysozyme Activity in Juvenile Olive Flounder, *Paralichthys olivaceus*

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In this study, tests were conducted to investigate the effects of three dietary growth hormones, administered in various amounts, on the growth performance and lysozyme activity in juvenile olive flounder, *Paralichthys olivaceus*. Three dietary growth hormones, recombinant human growth hormone (rHGH), recombinant bovine somatotropin A (rBST A) and recombinant bovine somatotropin B (rBST B) were tested at three different supplemental levels (10, 20 or 40 mg/kg body weight per week) by a 3×3 factorial design and a complete randomized design in comparison to a control group. Fish were fed one of the ten experimental diets (control, $rHGH_{10}$, $rHGH_{20}$, $rHGH_{40}$, rBST A_{10}, rBST A_{20}, rBST A_{40}, rBST B_{10}, rBST B_{20} and rBST B_{40}) for 6 weeks and afterward were analyzed for growth performance by measuring weight gain (WG), feed efficiency (FE), specific growth rate (SGR) and protein efficiency ratio (PER). Based on the factorial design analysis, fish fed rHGH diets demonstrated significantly higher growth performance than fish fed rBST A or rBST B diets. However there were no significant differences in WG, FE, SGR and PER between fish fed rBST A and rBST B diets. Neither hormone level nor the interaction between the different hormones and their various levels had a significant effect on WG, FE, SGR, PER, lysozyme activity or whole-body proximate composition. A complete randomized design analysis confirmed fish fed $rHGH_{10}$, $rHGH_{20}$, $rHGH_{40}$, rBST A_{10}, rBST A_{20}, rBST A_{40}, rBST B_{20} and rBST B_{40} diets for 6 weeks showed higher WG than fish fed the control diet ($P<0.05$). A higher FE was observed in fish fed $rHGH_{10}$, $rHGH_{20}$, $rHGH_{40}$, rBST A_{20} and rBST A_{40} diets in comparison to fish fed the control diet. Fish fed all graded rHGH, rBST A and rBST B supplemented diets showed a higher SGR than fish fed the control diet. Regarding PER, fish fed $rHGH_{10}$, $rHGH_{20}$, $rHGH_{40}$, rBST A_{10}, rBST A_{20}, rBST A_{40} and rBST B_{20} diets were higher than fish fed the control diet. Furthermore, the lysozyme activity of fish fed a diet of $rHGH_{20}$ was significantly higher than that of fish fed any other diet. The results measuring the growth and development of the fish clearly suggest the biopotency of dietary rHGH could be higher than those of both dietary rBST A and rBST B. Further implied is the probability that within the range of 10 to 40 mg/kg BW/week the dietary growth hormones could accelerate growth performance, and that 20 mg rHGH/kg BW/week could possibly enhance lysozyme activity in juvenile olive flounder, *Paralichthys olivaceus*.

Key words: Growth hormone, Lysozyme activity, Growth performance, Olive flounder, Recombinant bovine somatotropin, Recombinant human growth hormone, *Paralichthys olivaceus*

Introduction

Growth hormone (GH, somatotropin), produced by the somatotrophs in the anterior pituitary of animals, is a polypeptide of about 22 kD that has been suggested to enhance fish growth by stimulating appetite, improving feed efficiency and increasing protein conversion (Markert et al., 1977; Donaldson et al., 1979; Matty 1986). A number of studies have previously shown the effectiveness of human growth hormone (HGH) in promoting growth performance in a variety of animals, including catfish (Nayak et al., 2003), tropical Panaeids (Toullec et al., 1991), com-

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mon carp (Hertz et al., 1991), crucian carp (Xu et al., 1991) and lobster (Charmantier and Aiken, 1991). Moreover, there are numerous studies demonstrating the efficacy of both bovine and porcine somatotropin, known as (BST) and (PST) respectively, in the growth acceleration of carp (Adelman, 1977), Pacific salmon (Gill et al., 1985), American elver (Degani and Gallagher, 1985), rainbow trout (Rasmussen et al., 2001), channel catfish (Silverstein et al., 2000; Peterson et al., 2004; 2005), tilapia (Leedom et al., 2002), coho salmon (Higgs et al., 1975; 1976; McLean et al., 1990) and Korean rockfish (Yoo et al., 2007).

Lysozyme is a major component of non-specific humoral defense in fish that possesses a direct antibacterial effect by splitting peptidoglycan layers in the cell wall. It further behaves as an opsonin by promoting phagocytosis activity in other immune cells (Yano, 1996). Lysozyme activity had a notable and significant effect in the plasma of brown trout (*Salmo trutta*) in which there was a positive correlation with the plasma GH level (Marc et al., 1995). In rainbow trout, a single injection of GH increased plasma lysozyme levels (*Oncorhynchus mykiss*) (Yada et al., 2001).

Regarding the application of GH in fish culture, oral administration is considered to be the most practical method. However, GH is mostly a protein based hormone and therefore would be hydrolyzed within the gastrointestinal tract when it is orally administrated to the animal. Some studies even showed that epithelial cells of the teleost's intestinal tract possess the ability to engulf protein molecules (Ash, 1985; McLean et al., 1990).

Olive flounder, *Paralichthys olivaceus*, is one of the most commercially important fish species in Rep. of Korea (Kim et al., 2005). Its production is consistently among the top of Korean mariculture finfish species. Culture of olive flounder in Rep. of Korea has rapidly increased in the last two decades, from an annual production of 1,037 metric tons (M/T) in 1990 to 41,171 M/T in 2007 (Statistical year book of Maritime Affairs and Fisheries, 2008). Although a great deal of research investigating the nutrient requirements and useful additives has been conducted for this species in Rep. of Korea and Japan, there still remains research to be performed that focuses on the improvement of flounder culture production that possess sufficient immune responses.

Considering these questions, the purpose of this study was therefore to test the effects of three dietary growth hormones on the growth performance and lysozyme activity in juvenile olive flounder, *Paralichthys olivaceus*.

Materials and Methods

Experimental design and diets

Both a 3×3 factorial design (Bai and Gatlin III 1992) and a complete randomized design were employed in combination with a control group in order to examine the effects of three dietary growth hormones in juvenile olive flounder. Composition and proximate analysis of the basal diet are shown in Table 1. Ten experimental diets controlled for both nitrogen and calorie content, were formulated to contain 50.0% crude protein and an available energy level of 17.6 kJ/g (Kim et al., 2002) while being supplemented either with or without dietary recombinant human growth hormone (expressed in yeast, rHGH, EBT NETWORKS CO., LTD., Korea), recombinant bovine somatotropin (expressed in yeast, rBST A, EBT NETWORKS CO., LTD., Korea) and rBST B (expressed in the bacteria, EBT NETWORKS CO., LTD., Korea). rHGH, rBST A and rBST B were all absent from the control diet (Control), each at a level of 0%. The remaining nine diets included increasing amounts (10, 20 and 40 mg) of either rHGH, rBST A or rBST B/kg body weight/week (Control, $rHGH_{10}$,

Table 1. Composition and proximate analysis of the basal diet (% of DM basis)

Ingredient	%
White Fish Meal[1]	57
Gelatin[2]	2.5
Casein[3]	2
Wheat meal[4]	15.4
Fish oil[5]	13.4
EPA-DHA (45%)[6]	0.5
Vitamin premix[7]	3
Mineral premix[8]	3
Alanine	1.5
Corn oil	1.1
Cellulose[9]	0.6
Proximate analysis (% of dry matter basis)	
Crude protein	50.2
Crude lipid	18.8
Crude ash	8.8

[1]Han Chang Fishmeal Co., Busan, Korea.
[2,3,9]United States Biochemical, Cleveland, Ohio 44122.
[4]Young Nam Flour Mills Co., Busan, Korea.
[5,6] E-Wha oil Co., Ltd., Buasn Korea.
[7]Vitamin premix (mg/kg feed unless indicated otherwise): vit. A, 3000IU; vit.D_3, 2400IU; vit.E, 120 IU; menadione sodium bisulfate, 6; vit. B_1-HCl, 15; vit. B_2, 30; vit. B_6-HCl, 15; vit.B_{12}, 0.06; vit.C, 300; calcium pantothenate, 150; nicotinamide, 150; inositol, 150; d-biotin, 1.5; choline chloride, 3000; pancreatin, 12.5.
[8]Mineral premix (mg/kg feed): $MnSO_4$, 320; $ZnSO_4$, 270; $FeSO_4$, 750; $CuSO_4$, 60; $CoSO_4$, 7; $MgSO_4$, 17.25; K_2SO_4, 212.24; NaCl, 51.88; K_2HPO_4, 136.09; $NaSeO_3$, 0.013; KI, 0.15.

$rHGH_{20}$, $rHGH_{40}$, rBST A_{10}, rBST A_{20}, rBST A_{40}, rBST B_{10}, rBST B_{20} and rBST B_{40}). Procedures for diet preparation and storage were followed as previously described by Bai and Kim (1997). In order to develop the experimental diets, a thorough mixture of the dry ingredients was made, including squid liver oil, eicosapentaenoic aicd (EPA) and docosahexaenoic acid (DHA) with 30% well water. Finally, experimental diets were pelleted by using a laboratory-pelleting machine and afterwards were stored at -20°C until used.

Fish and feeding trial

Fish were fed a basal control diet for 1 week to acclimate them to the experimental diet and conditions before the feeding trials began. Juvenile olive flounder averaging 0.60±0.01 g (Mean±SD) were divided into ten groups of 25 fish each of which was assigned one of the experimental diets. Groups were randomly distributed into separate 60 L aquariums with a water flow rates of 1-2 L/min. Water temperature was maintained at constant 21±1.3°C, while each aquarium was aerated by a diffusion stone during the 6 week period in which the feeding trial was conducted. The experimental diets were fed twice daily at 1000 and 1600 hrs to triplicate groups of fish at a fixed rate of 3% (dry-mass) of total body weight per day. The feeding rate was adjusted according to the total weight of fish in each tank, which was measured every 2 weeks.

Sample collection and analyses

After the 6-week feeding trial, fish were anestheticzed with 200 ppm MS 222, counted and then weighed for calculations of weight gain, feed efficiency, specific growth rate, protein efficiency ratio and survival rate. Fish were stored at -20°C prior to whole-body composition analyses. Ten randomly selected fish per aquarium were used for whole-body proximate analyses of experimental diets and fish body composition, performed by the standard methods of AOAC (1995). Diet and fish samples were dried to a temperature of 105°C and the constant weight was measured to determine moisture content. Ash was determined by incineration at 550°C; protein by Kjeldahl method (N×6.25) after acid digestion; and crude lipid content by soxhlet extraction using Soxtec system 1,046 (Tacator AB, Sweden) after freeze-drying samples for 20 hrs.

Lysozyme activity

To determine the lysozyme activity of fish, test serum (0.1 mL) was added to 2 mL of a suspension of *Micrococcus lysodeikticus* (0.2 mg/mL) in a 0.05 M sodium phosphate buffer (pH 6.2) and turbidity levels were measured according to the method by Parry et al. (1965). The reactions were carried out at 20°C and absorbance at 530 nm was measured between 0.5 and 4.5 min by a spectrophotometer. A lysozyme activity unit was defined as the amount of enzyme producing a 0.001/min decrease in absorbance.

Statistical analysis

All data were subjected to a factorial analysis of variance in order to test whether there was any interaction between the three dietary hormones and their levels versus a one-way ANOVA test using Statistix 3.1 (Analytical Software, St. Paul, MN, USA). If a significant treatment effect was observed, a Least Significant Difference (LSD) test was further used in order to compare means. Treatment effects were considered significant at $P<0.05$.

Results and Discussion

Previously, there has been an absence of research comparing biopotency of the growth hormones rHGH and rBST in fish. However, a factorial analysis of variance (Table 2) of fish fed rHGH diets indicates weight gain (WG), feed efficiency (FE), specific growth rate (SGR) and protein efficiency ratio (PER) were all significantly higher than those of fish fed rBST A and rBST B diets. Conversely, there were no significant differences in WG, FE, SGR and PER between fish fed rBST A and rBST B diets. Effects seemed not to be dose-dependent as both hormone levels and the interactions between the dietary hormones and their levels had no significant effects on WG, FE, SGR, PER, lysozyme activity or whole-body proximate composition. From the results of this study, it could be assumed that rHGH has a higher potency to increase growth performance than either rBST A or rBST B. However, exactly what causes juvenile olive flouner to utilize rHGH more efficiently than rBST is still unknown.

Results of the complete randomized design analysis of WG, FE, SGR, PER and lysozyme activity are shown in Table 3. Fish fed $rHGH_{10}$, $rHGH_{20}$, $rHGH_{40}$, rBST A_{10}, rBST A_{20}, rBST A_{40}, rBST B_{20} and rBST B_{40} diets demonstrated a significantly higher WG than that of fish fed a control diet ($P<0.05$). Fish fed a diet consisting of $rHGH_{10}$, $rHGH_{20}$, $rHGH_{40}$, rBST A_{20} and rBST A_{40} showed a higher FE than did fish fed a control diet. A higher SGR was observed in all fish fed a diet supplemented with any amount of rHGH, rBST A or rBST B compared to fish fed a control diet. Likewise, fish fed $rHGH_{10}$, $rHGH_{20}$, $rHGH_{40}$, rBST A_{10}, rBST A_{20}, rBST A_{40} and rBST B_{20}

diets showed higher PER than did fish fed a control diet. Prior evidence has supported a role of growth hormone in increasing fish growth. Supporting this, it formerly has been demonstrated administration of rHGH improved growth performances of catfish (Nayak et al., 2003), tropical Panaeids (Toullec et al., 1991), common carp (Hertz et al., 1991), crucian carp (Xu et al., 1991) and lobster (Charmantier et al., 1989). Intraperitoneal injection or dietary administration of rBST has previously led to acceleration of the growth of carp (Adelman, 1977), Pacific salmon (Gill et al., 1985), American elver (Degani and Gallagher, 1985), rainbow trout (Rasmussen et al., 2001), channel catfish (Silverstein et al., 2000; Peterson et al., 2004, 2005), tilapia (Leedom et al., 2002) and coho salmon (Higgs et al., 1975; 1976; McLean et al., 1990). Similarly, improved performance has been recorded by the immersion of abalone in growth hormone (Moriyama and Kawauchi, 2004). Interestingly, the highest WG increase in fish fed rHGH was obtained from fish fed a diet of $rHGH_{20}$, which was significantly higher than values obtained from fish fed a diet of $rHGH_{40}$.

The increase of growth performance among the three hormones and their varying levels showed no dose-dependent manner. In fish fed rHGH diets, WG increased only when the dietary hormone level increased from 10 to 20 mg/kg BW/week and then dropped with the hormone level beyond this level. Furthermore, fish fed rBST A and rBST B diets ranging from 10 to 40 mg/kg BW/week showed no significant increase in WG. Recently, Yoo et al. (2007) showed the WG of Korean rockfish fed a diet containing 10-50 mg rBST/kg BW/week was not

Table 2. Factorial analysis on growth performance, lysozyme activity and whole-body proximate analysis of fish fed the experimental diets for 6 weeks[1]

Diets	WG[2] (%)	FE[3] (%)	SGR[4] (%)	PER[5]	Lysozyme activity (U/mL)	Crude protein[6]	Crude lipid[7]	Ash[8]
rHGH	251[a]	106[a]	2.99[a]	2.26[a]	362	69.7	15.3	12.4
rBST A	224[b]	97.3[b]	2.80[b]	2.02[b]	353	68.1	15.8	12.1
rBST B	222[b]	94.8[b]	2.78[b]	1.95[b]	344	67.8	16.9	12.4
Analysis of variance								
Hormones	0.00059	0.0016	0.0007	0.0002	NS	NS	NS	NS
Hormone levels	NS[10]	NS	NS	NS	NS	NS	NS	NS
Hormones · Levels	NS	NS	NS	NS	NS	NS	NS	NS
Pooled SEM[11]	3.93	1.34	0.03	0.04	14	0.06	0.17	0.26

[1]Values are means from groups (n=9) of fish where the means in each column with a different superscript are significantly different ($P<0.05$). [2]Weight gain (%)=(final weight - initial weight)×100/initial weight. [3]Feed Efficiency (%)=wet weight gain (g)×100 / dry feed intake (g). [4]Specific growth rate (%)=($\log_e$ final wt. - $\log_e$ initial wt.)/days. [5]Protein efficiency ratio=wet weight gain/protein intake. [6,7,8]% of dry matter basis. [9]Probability of significance. [10]Not significant different ($P>0.05$). [11]Pooled standard error of mean: $SD/\sqrt{n}$.

Table 3. Growth performance and non-specific immune response parameters based on the complete randomized design in juvenile olive flounder fed the experimental diets for 6 weeks[1]

Dietary factors		WG (%)	FE (%)	SGR (%)	PER	Lysozyme activity (U/mL)
Hormone	Level					
Control		195[d]	88.2[e]	2.57[d]	1.77[e]	322[b]
rHGH	10	245[ab]	105.1[ab]	2.95[ab]	2.22[ab]	341[b]
	20	266[a]	118.9[a]	3.09[a]	2.39[a]	392[a]
	40	242[b]	102.9[abc]	2.93[ab]	2.18[abc]	352[b]
rBST A	10	219[c]	95.4[cde]	2.76[c]	1.98[cd]	359[b]
	20	227[bc]	97.6[bcd]	2.81[bc]	2.03[bcd]	362[b]
	40	228[bc]	99.1[bcd]	2.82[bc]	2.05[bcd]	337[b]
rBST B	10	217[cd]	92.6[de]	2.74[c]	1.87[de]	363[b]
	20	225[bc]	96.7[bcde]	2.80[bc]	2.02[bcd]	323[b]
	40	223[bc]	95.3[cde]	2.79[bc]	1.96[de]	345[b]
Pooled SEM[2]		3.93	1.34	0.03	0.04	14

[1]Values are means from triplicate groups of fish where the means in each column with a different superscript are significantly different ($P<0.05$). [2]Pooled standard error of mean: $SD/\sqrt{n}$.

significantly increased compared to a control diet, contrary to fish fed a diet above 10 mg rBST/kg BW/week, in which a higher WG was indeed observed. These results indicated that the growth-promoting effects of GH resulted from the absorption of its intact form at the physiologically active site in the digestive tracts of fish (Yoo et al., 2007), and morever that the absorption rate of the GHs were not dose-dependent in manner beyond a certain concentration level in the diets.

An increase in lysozyme activity was found to be specific to fish fed a diet containing 20 mg/kg BW/week rHGH, with a significantly higher activity measurement compared to that of fish fed the other diets, in which there were no significant differences observed. Yada et al. (2001) reported that stimulation of plasma lysozyme activity of rainbow trout (*Oncorhynchus mykiss*) was observed by the intraperitoneal injection of 0.25 ug chum salmon GH/g BW. Yada et al. (2004) also showed a dose-dependent increase of plasma lysozyme levels in rainbow trout after the implantation of the cholesterol pellet containing 50 and 500 ng chum salmon GH/g BW. Different results , however, were observed in studies conducted using a different GH. In the same study, euryhaline tilapia (*Oreochromis mossambicus*) injected with recombinant bovine growth hormone at doses of 100 and 1,000 ug/g once a week for 4 weeks failed to show any significant increase in lysozyme activity. The lack of lysozyme activation by rBST in this prior study, when considered with our own results, therefore indicate a varying degree of effectiveness of GH on immune cells, and that only 20 mg rHGH/kg BW/week could increase lysozyme activity in juvenile olive flounder.

Results of the whole-body proximate analyses are shown in Table 4. Whole-body protein (WBP) content of fish fed graded rHGH, rBST A and rBST B diets, with the exception of rBST B_{10}, was signify-cantly higher than that of fish fed a control diet. Whole-body lipid (WBL) content showed a more selective increase, in which only fish fed graded rHGH and rBST A diets had a significantly higher WBL content than that of fish fed a control diet. Lastly, no significant differences were demonstrated in either moisture or whole-body ash content of fish fed any of the experimental diets, which suggests weight gains were due to tissue increase and not simply water content. Increases in both the rate of protein systhesis and fat decomposition have been demonstrated by GH in various tissues of rainbow trout (Foster et al., 1991) and by administration of graded rBST in Korean rockfish (Yoo et al., 2007). It has further been determined that GH stimulates the synthesis and secretion of insulin-like growth factor-I (IGF-I), which belongs to a family of polypeptide hormones (for review, see Humbel, 1984; Nissley and Rechler, 1984; Peterson et al., 2005) called somatomedins, thus reflecting their role as mediators of certain anabolic effects elicited by GH (Sumpter, 1992). The direct role of GH in promoting growth is further seen in how IGF-I is responsible for suflate uptake, increased synthesis of collagen and other proteins by cartilage, RNA synthesis and promotion of DNA synthesis.

Table 4. Whole-body proximate composition of olive flounder fed the experimental diets for 6 weeks (% of dry matter basis)[1]

Diets	Moisture	Crude Protein	Crude fat	Ash
Control	75.3	67.4^{de}	17.0^{a}	12.1
$rHGH_{10}$	74.6	68.8^{b}	16.4^{bc}	12.5
$rHGH_{20}$	75.1	72.0^{a}	14.2^{f}	12.6
$rHGH_{40}$	75.3	68.4^{bc}	15.4^{e}	12.1
$rBSTA_{10}$	75.2	68.0^{cd}	16.2^{cd}	11.9
$rBSTA_{20}$	75.1	68.1^{bc}	15.5^{e}	12.4
$rBSTA_{40}$	74.8	68.2^{bc}	15.8^{de}	12.1
$rBSTB_{10}$	75.2	66.7^{e}	16.8^{ab}	12.3
$rBSTB_{20}$	75.4	68.2^{bc}	16.8^{ab}	12.4
$rBSTB_{40}$	75.1	68.4^{bc}	17.1^{a}	12.6
Pooled SEM[2]	0.11	0.06	0.17	0.26

[1]Values are means from triplicate groups of fish where the means in each column with different superscripts are significantly different ($P<0.05$).
[2]Pooled standard error of mean: SD / $\sqrt{n}$.

These results conclusively showed that the biopotency of dietary rHGH could be higher than that of dietary rBST A and rBST B, as observed by direct comparison. Overall growth performance was accelerated by dietary GHs within the range of 10 to 40 mg/kg BW/week. Finally, only 20 mg rHGH/kg BW/week could enhance lysozyme activity in juvenile olive flounder, *Paralichthys olivaceus*.

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