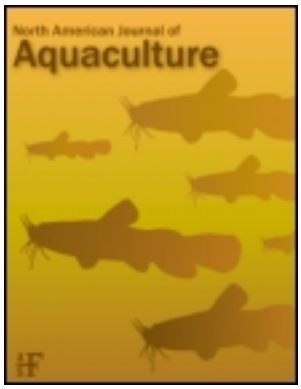

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ARTICLE

Effects of Inactivated *Enterococcus faecalis* and Mannan Oligosaccharide and Their Combination on Growth, Immunity, and Disease Protection in Rainbow Trout

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Abstract

We examined the effects of the following seven experimental diets that varied in the concentration of inactivated cells of *Enterococcus faecalis* (*Ef*) and mannan oligosaccharides (MOS), on Rainbow Trout *Oncorhynchus mykiss*: control (C) diet (no *Ef* and no MOS), diet E0.25% (2.5 g/kg *Ef*), diet E0.5% (5 g/kg *Ef*), diet M0.25% (2.5 g/kg MOS), diet M0.5% (5 g/kg MOS), diet EM0.25% (2.5 g/kg *Ef* + 2.5 g/kg MOS) and diet EM0.5% (5 g/kg *Ef* + 5 g/kg MOS). Rainbow Trout, initially weighing 36.27 ± 0.42 g (mean ± SD) were distributed into fourteen 60-L glass tanks at a stocking density of 35 fish per tank. Each diet was hand-fed to duplicate groups of fish twice daily for a 12-week period. After the feeding test, an intraperitoneal injection challenge test of *Aeromonas salmonicida* was conducted over 14 d. Resulting data were submitted to a multivariate analysis of variance. Weight gain increased significantly ($P < 0.05$) in E0.25%, M0.25%, and EM0.5% experimental groups compared with the control. Specific growth rate was significantly higher ($P < 0.05$) in fish fed E0.25%, M0.25%, and EM0.5% diets compared with fish fed the C diet. Feed gain ratio and protein efficiency ratio were significantly improved ($P < 0.05$) in fish fed the EM0.5% diet compared with fish fed the C diet. Feed intake, protein efficiency ratio, protein retention, and the apparent digestibility coefficient recorded slight differences within experimental groups. Hematocrit value and phagocytic activity were significantly higher ($P < 0.05$) in fish fed E0.25%, E0.5%, M0.5%, EM0.25%, and EM0.5% diets compared with fish fed the C diet. Except for fish in the E0.25% group, fish in all other experimental groups showed a significantly higher ($P < 0.05$) mucus weight compared with those in the C group. After the challenge test, cumulative mortality and frequency of *A. salmonicida* were significantly decreased ($P < 0.05$) in all experimental groups compared with the C group. In conclusion, dosage and single or combined supplementation of *Ef* and MOS are factors that significantly affect fish performance.

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In the last few years, the use of dietary components that enhance the functional attributes of fish has grown significantly (Merrifield et al. 2010). The use of natural origin stimulants offers a wide range of attractive methods to enhance the fish immune system and to promote the growth of cultured organisms (Anderson 1992). Among these additives, the most effective and widely used are components derived from microorganisms. Bacteria with probiotic properties elicit positive effects when supplemented in either a viable (Balcázar et al. 2006, 2007a, 2007b; Kim et al. 2006; Aly et al. 2008; Díaz-Rosales et al. 2009) or nonviable form (Villamil et al. 2002; Irianto and Austin 2003; Panigrahi et al. 2005; Díaz-Rosales et al. 2006; Salinas et al. 2006; Choi et al. 2008; Pan et al. 2008). Prebiotics confer favorable growth performance and immune stimulation benefits when supplemented in the diets of aquatic farmed organisms (Li and Gatlin 2004, 2005; Mahious et al. 2006; Rairakhwada et al. 2007; Sealey et al. 2007; Staykov et al. 2007; Torrecillas et al. 2007; Zhou et al. 2007; Burr et al. 2008; Grisdale-Helland et al. 2008; Gupta et al. 2008; Dimitroglou et al. 2010; Ringø et al. 2010). Furthermore, different types of probionts (Robertson et al. 2000; Nikoskelainen et al. 2001; Balcázar et al. 2007b) and prebiotics (Li and Gatlin 2004, 2005; Sink et al. 2007; Sink and Lochmann 2008; Li and Mai 2009) can reduce mortality induced by certain pathogens that affect cultured organisms. Factors, such as the supplementation level (Nikoskelainen et al. 2001, 2003; Panigrahi et al. 2004; Torrecillas et al. 2007; Yilmaz et al. 2007; Bagheri et al. 2008; Sang et al. 2009) or the use of single or combined forms of immunostimulants and growth promoters (Salinas et al. 2008a; Hai and Fotedar 2009; Rishi et al. 2009; Kiron 2012) are fundamental for achieving enhanced outcomes.

Gram-positive *Enterococcus faecalis* (*Ef*) belongs to the lactic acid bacteria (LAB) group. It is a heat-killed commercial preparation (FK-23) and was isolated from the intestines of healthy humans. Immune stimulation and adjuvant benefits have been reported when farmed animal diets were supplemented with *Ef* (Shimada et al. 2004; Rodriguez-Estrada et al. 2009; T. Shimada and colleagues [abstract presented at the *Enterococcus faecalis* FK-23 Conference, 2006]). Mannan oligosaccharides (MOS) is a commonly used prebiotic derived from the cell wall of *Saccharomyces cerevisiae* and has recently been used in both poultry husbandry and aquaculture as an additive (Torrecillas 2007). In finfish farming, MOS can promote growth (Staykov 2007; Yilmaz et al. 2007; Refstie et al. 2010), stimulate the immune system (He et al. 2003; Welker et al. 2007), and protect the fish from certain pathogens (Samrongpan et al. 2008; Rodriguez-Estrada et al. 2009; Peterson et al. 2010). This research aims to evaluate the effectiveness of *Ef* and MOS for enhancing growth performance, feed utilization, immune response, and disease resistance against *Aeromonas salmonicida*, the causal agent of furunculosis (Bullock et al. 1983), when supplemented at different dosages and in single or combined forms in the diets of Rainbow Trout *Oncorhynchus mykiss*.

METHODS

Experimental diets.—A commercial preparation of inactivated cells of *Ef* (Nichinichi Pharmaceutical, Iga-city, Mie, Japan) and MOS (Bio-Mos; Alltech, Nicholasville, Kentucky) were used. These additives were supplemented in seven isonitrogenous experimental diets: control (C) diet (no *Ef* and MOS), diet E0.25% (2.5 g/kg *Ef*), diet E0.5% (5 g/kg *Ef*), diet M0.25% (2.5 g/kg MOS), diet M0.5% (5 g/kg MOS), diet EM0.25% (2.5 g/kg *Ef* + 2.5 g/kg MOS), and diet EM0.5% (5 g/kg *Ef* + 5 g/kg MOS). Additionally, all experimental diets were supplemented with 0.5% chromium oxide as an inert ingredient used as an indicator for the protein digestibility analysis (Table 1). The experimental diets were analyzed for moisture and crude ash by standard methods (AOAC 1990). Crude protein was determined by the Kjedahl method. Total lipid contents were determined gravimetrically after extraction by chloroform and methanol according to Folch et al. (1957) (Table 2).

Experimental fish and feeding.—Juvenile Rainbow Trout were obtained from Oizumi Research Station, Tokyo University of Marine Science and Technology (TUMSAT), Yamanashi, Japan, and kept in the wet laboratory of the fish nutrition laboratory of TUMSAT. Fish were fed a commercial diet (Nippon Formula Feed, Yokohama, Japan) until the beginning of the experiment. A total of 490 fish (weight, 36.3 ± 0.42 g [mean ± SD]) were equally distributed into fourteen 60-L glass tanks. Based on previous studies (Perera et al. 1995; Taylor et al. 2005; Shoemaker et al. 2006; Rodriguez-Estrada et al. 2009), duplicate groups received one of the seven experimental diets. Fish were fed to satiation twice daily six times per week during a 12-week period. The water temperature (mean ± SD) was 16 ± 2°C.

Growth performance.—Growth performance and feeding efficiency were determined based on weight gain (WG), specific growth rate (SGR), feed gain ratio (FGR), feed intake, protein efficiency ratio, and protein retention. Weight gain was calculated as WG = final body weight − initial body weight. Specific growth rate (SGR) was calculated as SGR = [($\log_e$ final body weight − $\log_e$ initial body weight) / number of rearing days] × 100 (Steffens 1989).

Feed utilization.—Feed gain ratio (FGR) was calculated as FGR = feed intake as dry matter (g) / weight gain (g) (Steffens 1989). The amount of feed consumed by the experimental fish during the feeding test was calculated as feed intake (g/fish) = total feed consumed per tank / number of fish per tank. The protein efficiency ratio (PER) is defined as the nutrient intake needed to increase the body weight of fish (Takeuchi 1988) and is calculated as PER = body weight gain (g) / protein intake (g). Protein retention (PR) is calculated as PR = [final body weight (g) × feed intake (g)] − [initial body weight (g) × initial fish protein] × 100/feed intake (g/fish) × feed protein.

Protein apparent digestibility.—One week before the end of the feeding test, a feces collection process was performed

TABLE 1. Formulation (as % of total) of experimental diets for Rainbow Trout with two inclusion levels (0.25% and 0.5%) of *Ef* and MOS supplemented in a single (E or M) or combined (EM) form; C = control.

		Supplementation level					
		Single				Combined	
Ingredient	C	E0.25%	E0.5%	M0.25%	M0.5%	EM0.25%	EM0.5%
Anchovy meal	60	60	60	60	60	60	60
Pollock liver oil	8	8	8	8	8	8	8
Soybean oil	3	3	3	3	3	3	3
Dextrin	5	5	5	5	5	5	5
Pregelatinized starch	10	10	10	10	10	10	10
Mineral premixture[a]	1	1	1	1	1	1	1
Vitamin premixture[b]	3	3	3	3	3	3	3
Choline chloride	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin E (50%)[c]	0.1	0.1	0.1	0.1	0,1	0.1	0.1
Chromium oxide (Cr_2O_3)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Cellulose	8.9	8.7	8.4	8.7	8.4	8.4	7.9
Enterococcus faecalis		0.25	0.5			0.25	0.5
Mannan oligosaccharide				0.25	0.5	0.25	0.5
Total	100	100	100	100	100	100	100

[a]Composition (as g/100 g): NaCI (1), $MgSO_4 \cdot 7H_2O$ (15), $NaH_2PO_4 \cdot 2H_2O$ (25), KH_2PO_4(32), $Ca(H_2PO_4)2H_2O$ (20), $FeC_6H_5O_7 \cdot nH_2O$ (2.5), $C_6H_{10}O_6Ca.5H_2O$ (1), $ZnSO_4 \cdot 7H_2O$ (1.2), $MnSO_4 \cdot 5H_2O$ (0.6), $CuS0_4 \cdot 5H_2O$ (0.1), $CoCI_3 \cdot 6H_20$ (0.0035), KIO_3 (0.0105), cellulose (1.586).

[b]Composition (as g/100 g premix): thiamine hydrochloride (0.72), riboflavin (1.21), pyridoxine hydrochloride (0.48), cyanocobalamin (0.06), ascorbic acid (60.40), niacin (4.83), calcium pantothenate (1.21), inositol (24.15), biotin (3.62), folic acid (0.18), *P*-aminobenzoic acid (0.60), vitamin A acetate (0.97), vitamin D_3 (0.97), vitamin K_3 (0.60).

[c]Vitamin E as DL-α-tocopherol acetate, purity 50%.

according to the method described by Takeuchi (1988). In brief, during the 5 d before the final feeding any feed remaining in the rearing tanks was removed, and a feces collector was attached to each experimental tank and left in place overnight. Feces collectors were removed the following morning, and the collected feces were separated from the accompanying water by centrifuging at 250 × *g* for 10 min and stored at −20°C until a considerable amount of solids accumulated. The feces were then freeze-dried in a Kyowac RLE II-206 vacuum freeze-dryer (Tokyo, Japan) for 72 h, and then powdered and stored at −80°C for further analysis. The apparent digestibility coefficient (ADC) of protein (%) was calculated according to Takeuchi (1988): %ADC = 100 − [100 × (Cr_2O_3 diet/Cr_2O_3 feces) × (protein in feces/protein in diet)].

Immunology.—At the end of the feeding experiment, four fish from each tank (eight fish per treatment) were randomly selected for blood tests after a 24-h fasting period (Panigrahi et al. 2004; Puangkaew et al. 2004). Blood was obtained from the caudal vein of individual fish that had been anesthetized with 300 ppm 2-phenoxyethanol (Wako Pure Chemical Industries, Tokyo, Japan). Approximately 2 mL of blood were collected using heparinized syringes and needles. To collect leucocytes from the head kidney (HK), the organ was aseptically removed after partial decapitation of the fish to expose the trunk kidney area. Leukocytes were prepared and enriched according to the techniques of Chung and Secombes (1988). Skin mucus was collected in a separate sampling from eight fish per experimental group following the method described by Staykov et al. (2007).

TABLE 2. Proximate composition (mean ± SD) of experimental diets for Rainbow Trout with two inclusion levels (0.25% and 0.5%) of *Ef* and MOS supplemented in a single (E or M) or combined (EM) form; C = control.

		Supplementation level					
		Single				Combined	
Component	C	E0.25%	E0.5%	M0.25%	M0.5%	EM0.25%	EM0.5%
Moisture	4.8 ± 0.0	3.4 ± 0.7	3.5 ± 0.1	4.6 ± 0.0	3.0 ± 0.0	2.6 ± 0.3	3.1 ± 0.3
Crude protein	40.5 ± 0.3	41.1 ± 0.4	40.7 ± 0.7	41.1 ± 0.1	40.3 ± 0.6	41.4 ± 0.2	40.4 ± 0.0
Crude lipid	14.7 ± 0.6	14.7 ± 0.8	15.1 ± 11.5	14.6 ± 0.6	14.7 ± 5.6	14.0 ± 1.2	16.8 ± 1.5
Crude ash	11.0 ± 7.0	11.3 ± 0.1	11.2 ± 0.0	11.0 ± 0.1	11.2 ± 0.1	11.3 ± 0.1	11.0 ± 0.2

Briefly, each experimental organism was carefully sampled using identical treatments for each individual. Fish were removed from rearing tanks and placed in a pail containing 300 ppm 2-phenoxyethanol. To imitate the rearing water temperature and avoid stressful conditions that could trigger the over production of mucus, fish were placed on a flat area previously cooled with ice. Thereupon, the skin surface was scraped with a glass slide (2 × 5 cm) following a 10-cm straight line path from the base of the operculum. Each mucus collection was individually weighed using an analytical balance.

The phagocytic activity of leucocytes was determined according to the methods of Puangkaew et al. (2004) and Panigrahi et al. (2004), with minor modifications. After collection, the HK of eight fish (ca. 0.5 g) per treatment were homogenized, filtered through a nylon membrane (100 μL mesh size), diluted in L-15 medium (Sigma, Tokyo, Japan), and sterile-filtered with glutamine in a petri dish. The HK cells obtained from this process were centrifuged twice at 250 × *g* for 5 min. The resulting pellet was diluted with L-15 medium, which was layered onto Percoll. This new solution was centrifuged at 400 × *g* for 20 min. The resulting layered white blood cells were transferred to a new microcentrifuge tube and centrifuged at 250 × *g* for 5 min. The resulting leukocyte suspension was counted using a hemocytometer (Hausser Scientific, Horsham, Pennsylvania) with 4.25% NaCl (10 μL) and 0.25% Tripan blue (40 μL) and adjusted to 2×10^7 cells/mL. The opsonization process was performed by incubating 1 mL of the adjusted cells in a 25% zymosan solution (0.5 mg zymosan + 1 mL L-15 + 60 μL serum from the corresponding fish) for 1 h at 16°C in duplicate chambers (1.7 cm diameter, 2 cm depth) of a 24-chamber slide (Corning, Corning, New York). The final opsonized cells were centrifuged at 40 × *g* for 3 min using an SC-2 chamber (Tomy Seiko, Tokyo, Japan). The cells were then fixed with methanol (100%) and stained using the Giemsa staining method (Clark 1973). Cell counting was conducted using a light microscope (Nikon, Tokyo, Japan).

Immediately after collection, blood samples were stored in 1.5-mL microcentrifuge tubes. Blood was taken directly from these tubes using heparinized capillary tubes (length, 75 mm; diameter, 1.45–1.65 mm) (Shibuya, Tokyo, Japan) and centrifuged at 250 × *g* for 5 min using a high-speed centrifuge (MC-150, Tomy Seiko). The hematocrit value was measured with a hematocrit scale (Tomy Seiko).

Challenge test.—To perform a pathogen challenge test, 224 fish from the feeding experiment were randomly selected and distributed among fourteen 60-L glass tanks in the closed recirculating system at TUMSAT, Shinagawa campus. Each group of fish was fed the same diet that it had been administered during the feeding experiment. Before commencing the test, head kidneys from four experimental groups were plated on tryptic soy agar (TSA; 20°C, 14 h) to ensure that experimental fish were not infected with any bacteria.

At the beginning of the test, all of the fish were intraperitoneally injected with 0.1 mL of 2.4×10^3 CFU/mL of *A. salmonicida*. Water temperature was maintained at 21°C, and the photoperiod was adjusted to 12 h light : 12 h dark. The mortality was recorded daily for a 14-d period. To confirm any internal damage caused by the inoculated pathogen, all dead fish were submitted to a necropsy, and internal furunculosis symptoms were compared with the descriptions of those given in Bullock et al. (1983). To confirm deaths and the presence or absence of this pathogen in survivors, samples from HK were obtained and plated in TSA (20°C, 14 h). The resulting colonies were identified by their morphology and then submitted to molecular identification using 16S RNA sequencing with forward primer 5′-AGTTTGATCCTGGCTAG-3′ and reverse primer 5′GTTACCTTGTTACGACTTC-3′. The amplification of the 16S rRNA gene was conducted via PCR using a Takara rTaq gene amplification PCR kit (Takara Bio, Shiga, Japan). After amplification, PCR products were first analyzed by electrophoresis in a 1% (w/v) agarose gel and then purified using the polyethylene glycol (PEG) method. The PCR products were sequenced using a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, California). Sequencing reactions were analyzed using an Applied Biosystems 310 genetic analyzer (Applied Biosystems). The resulting sequences were compared with known sequences using the basic local alignment search tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST/).

Statistical analysis.—Statistical analysis was performed with STATISTICA software (version 6.0, StatSoft, Tulsa, Oklahoma). To assess the effects of each treatment together with their interactions (multiple-variables experimental design) and to obtain precise results using a small number of replicates (two) per experimental group, a multivariate ANOVA (MANOVA) was used. Pillai's, Hotelling's, and Roy's tests of significance were applied. Data analysis was submitted to sigma-restricted parameterization and effective hypothesis decomposition methods. Differences between means were analyzed by Duncan's multiple range test (5% level of significance) to discriminate homogeneous groups.

RESULTS

Results of the MANOVA for growth, nutrient utilization, and protein apparent digestibility showed that the $P < 0.05$ value was recorded in Roy's test (Table 3). While Pillais, Hotelling's,

TABLE 3. Multivariate tests (Pillai's, Hotelling's, and Roy's) of significance ($P < 0.05$) of growth, nutrient utilization, and protein apparent digestibility of Rainbow Trout fed diets with two inclusion levels (0.25% and 0.5%) of *Ef* and MOS supplemented in a single (E or M) or combined (EM) form. Data analyses were submitted to sigma-restricted parameterization and effective hypothesis decomposition processes ($P < 0.05$).

Test	Value	*F*-value	*P*-value
Pillai's	4.00	1.30	0.23
Hotelling's	81.00		
Roy's	48.00	41.00	0.00

TABLE 4. Multivariate tests (Pillai's, Hotelling's, and Roy's) of significance ($P < 0.05$) of hematocrit value, phagocytic activity, mucus weight, cumulative mortality, and frequency of *Aeromonas salmonicida* in head kidney of Rainbow Trout fed diets with two inclusion levels (0.25% and 0.5%) of *Ef* and MOS supplemented in a single (E or M) or combined (EM) form. Data analyses were submitted to sigma-restricted parameterization and effective hypothesis decomposition processes ($P < 0.05$).

Test	Value	*F*-value	*P*-value
Pillai's	3.05	1.82	0.04
Hotelling's	72.54	3.38	0.05
Roy's	64.23	74.94	0.00

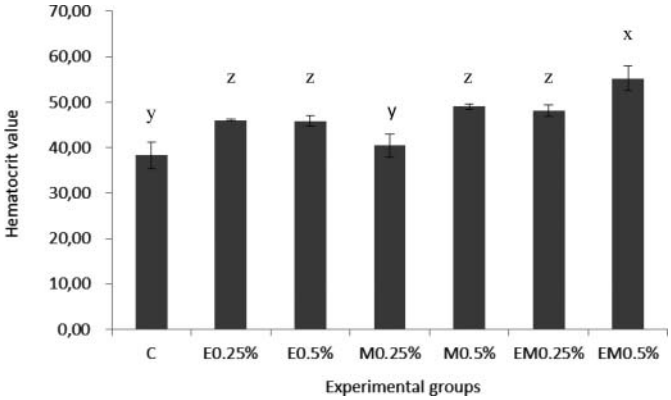

FIGURE 1. Hematocrit values (mean ± SD, $n = 8$) after a 12-week feeding test of Rainbow Trout fed diets with two inclusion levels (0.25% and 0.5%) of *Ef* and MOS supplemented in a single (E or M) or combined (EM) form. Different letters accompanying bars denote significant differences ($P < 0.05$).

and Roy's tests showed a value of $P < 0.05$ in hematocrit, phagocytic activity, mucus weight, cumulative mortality, and frequency of *Aeromonas salmonicida* in the HK of Rainbow Trout fed diets with two inclusion levels (0.25% and 0.5%) of *Ef* and MOS supplemented in a single (*Ef* or MOS only) or combined (*Ef* plus MOS) form (Table 4).

Feeding Experiment

During the complete feeding test, nonsignificant mortality was recorded. Neither *Ef* nor MOS, supplemented in a single or combined form at two different levels, significantly affected the final fish whole-body composition following the 12-week feeding experiment (Table 5).

Growth Performance

Fish fed a single, lower *Ef* dosage registered a significantly higher ($P < 0.05$) final body weight, weight gain, and SGR, compared with fish fed a single higher dosage. In contrast, no significant difference ($P > 0.05$) was recorded between the two single dosages of MOS. A combined 0.5% supplementation of *Ef* and MOS resulted in higher ($P < 0.05$) final body weight, weight gain, and SGR compared with a combined 0.25% diet. Compared with the control, fish fed E0.25%, M0.25%, and EM0.5% diets exhibited significantly higher ($P < 0.05$) SGR (Table 6).

Nutrient Utilization

The FGR was significantly higher ($P < 0.05$) in fish fed EM0.5% compared with fish fed the C diet. This parameter did not show significant differences ($P > 0.05$) between two single doses of *Ef*. A similar result was recorded between the M0.25% and M0.5% diets. Feed intake was significantly higher ($P < 0.05$) in fish fed the E0.25% diet compared with fish fed the E0.5% diet. There was no significant difference ($P > 0.05$) between the two single dosages of MOS. The PER was significantly higher ($P < 0.05$) in the fish fed the EM0.5% diet compared with the rest of the experimental groups. This parameter was not significantlydifferent ($P > 0.05$) between the E0.25% and E0.5% diets. Also, two single doses of MOS recorded similar values. Protein retention and protein apparent digestibility were not significantly affected by experimental diets ($P > 0.05$) (Table 6).

Immunological Response

Except for the M0.25% experimental group, Rainbow Trout fed diets supplemented with *Ef* and MOS tended to have higher hematocrit values over the 12-week feeding period (Figure 1). Fish fed two single supplementation levels of *Ef* did not show differences between groups. In contrast, a higher supplementation level of MOS resulted in a higher hematocrit value compared with the value at a lower level of this prebiotic ($P < 0.05$). Hematocrit values were significantly higher ($P < 0.05$) in fish

TABLE 5. Whole-body proximate composition (% wet basis, mean ± SD,) of Rainbow Trout fed experimental diets with two inclusion levels (0.25% and 0.5%) of *Ef* and MOS supplemented in a single (E or M) or combined (EM) form; C = control.

			Supplementation level					
			Single				Combined	
	Initial fish	C	E0.25%	E0.5%	M0.25%	M0.5%	EM0.25%	EM0.5%
Moisture	77.0 ± 0.28	71.4 ± 0.37	70.0 ± 1.20	70.1 ± 0.53	70.0 ± 0.54	70.9 ± 1.78	70.5 ± 0.82	70.3 ± 1.65
Crude protein	14.1 ± 0.19	14.5 ± 0.39	16.4 ± 0.27	16.5 ± 0.85	16.1 ± 0.23	16.5 ± 0.20	16.1 ± 0.20	16.2 ± 0.00
Crude lipid	4.86 ± 0.26	8.12 ± 0.83	11.2 ± 0.24	9.2 ± 0.54	10.8 ± 0.94	10.3 ± 1.17	8.78 ± 0.92	12.7 ± 2.26
Crude ash	2.19 ± 0.26	1.87 ± 0.01	1.87 ± 0.06	1.73 ± 0.08	1.60 ± 0.23	2.04 ± 0.03	1.99 ± 0.20	1.96 ± 0.20

TABLE 6. Growth, nutrient utilization, and protein apparent digestibility metrics (mean ± SD) of Rainbow Trout fed diets with two inclusion levels (0.25% and 0.5%) of *Ef* and MOS supplemented in a single (E or M) or combined (EM) form; C = control, ADC = apparent digestibility coefficient. Different letters in a row denote significant differences ($P < 0.05$).

		Supplementation level					
		Single				Combined	
Parameter	C	E0.25%	E0.5%	M0.25%	M0.5%	EM0.25%	EM0.5%
Initial body weight (g/fish)	36.4 ± 0.1	36.4 ± 0.7	35.4 ± 0.8	36.3 ± 0.7	36.7 ± 0.5	36.6 ± 0.1	36.0 ± 1.5
Final body weight (g/fish)	104.0 ± 7.7 yx	130.2 ± 8.5 z	98.6 ± 2.0 y	124.2 ± 6.2 zw	118.2 ± 3.8 zxw	109.1 ± 6.3 yxw	133.6 ± 8.4 z
Weight gain (g/fish)	67.6 ± 7.9 yx	93.2 ± 9.3 z	63.2 ± 4.4 y	87.9 ± 5.5 zw	81.5 ± 4.4 zxw	72.4 ± 7.1 yxw	97.4 ± 6.8 z
Specific growth rate (%/d)	1.2 ± 0.1 yx	1.5 ± 0.1 z	1.2 ± 0.0 y	1.4 ± 0.0 zw	1.4 ± 0.1 zxw	1.3 ± 0.1 yxw	1.5 ± 0.0 z
Feed gain ratio	0.9 ± 0.0 y	0.8 ± 0.0 zy	0.9 ± 0.0 zy	0.9 ± 0.1 zy	0.9 ± 0.1 zy	0.9 ± 0.1 zy	0.8 ± 0.0 z
Feed intake (g/fish)	63.1 ± 7.3 zy	77.4 ± 7.0 z	55.7 ± 0.2 y	76.2 ± 0.4 z	74.1 ± 6.0 z	64.3 ± 2.5 zy	75.3 ± 8.9 z
Protein efficiency ratio	2.6 ± 0.0 z	2.9 ± 0.0 zy	2.8 ± 0.0 z	2.8 ± 0.2 z	2.7 ± 0.3 z	2.7 ± 0.1 z	3.2 ± 0.2 y
Protein retention (%)	47.0 ± 0.9	51.2 ± 1.5	49.8 ± 3.6	47.6 ± 3.9	48.1 ± 6.4	46.4 ± 1.2	54.4 ± 2.7
ADC of protein (%)	91.6 ± 2.5	95.2 ± 1.0	93.1 ± 4.5	95.4 ± 2.0	94.3 ± 0.7	92.9 ± 0.9	95.6 ± 0.9

fed a combined higher supplementation of *Ef* and MOS compared with fish that were fed the lower supplementation level diet (Figure 1).

Increased phagocytic activity was observed in experimental fish fed *Ef* and MOS supplemented diets. Phagocytosis remained similar between experimental groups fed two single supplementations of *Ef*. In contrast, a higher single supplementation of MOS resulted in a significant increase in phagocytic activity ($P < 0.05$) compared with that exhibited by fish fed a lower dosage of this prebiotic. A combined 0.5% supplementation of *Ef* and MOS resulted in the highest phagocytic activity ($P < 0.05$) compared with the other experimental groups (Figure 2).

Mucus production was stimulated by *Ef* and MOS supplemented diets. Fish fed either of the two single supplementation levels of *Ef* exhibited similar mucus weights. The same result was shown between experimental fish fed the M0.25% and M0.5% diets. However, a combined higher supplementation of the two additives resulted in increased mucus production ($P < 0.05$) compared with the lower combined dosage (Figure 3).

Challenge Test

The results of the pathogen challenge test can be contrasted with the immunological status of experimental fish.

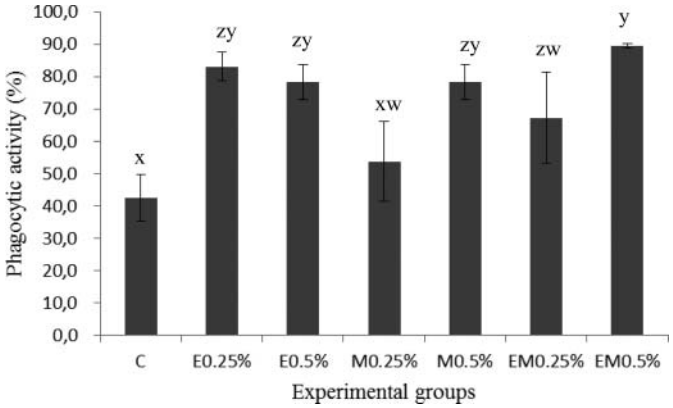

FIGURE 2. Phagocytic activity (mean ± SD, $n = 8$) after a 12-week feeding test of Rainbow Trout fed diets with two inclusion levels (0.25% and 0.5%) of *Ef* and MOS supplemented in a single (E or M) or combined (EM) form. Different letters accompanying bars denote significant differences ($P < 0.05$).

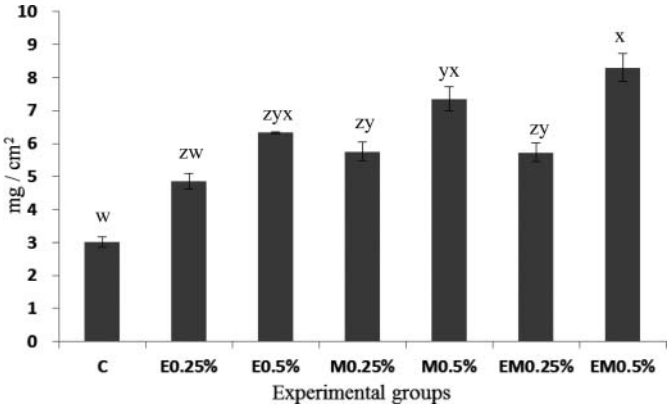

FIGURE 3. Mucus weight (mean ± SD, $n = 8$) after a 12-week feeding test of Rainbow Trout fed diets with two inclusion levels (0.25% and 0.5%) of *Ef* and MOS supplemented in a single (E or M) or combined (EM) form. Different letters accompanying bars denote significant differences ($P < 0.05$).

Supplementation levels of *Ef* and MOS, in either a single or combined form, conferred certain protection against *A. salmonicida.* All experimental fish showed the typical symptoms of furunculosis throughout the experimental period (i.e., lethargy and loss of appetite). From day 3 postinfection (pi), the skin of infected survivors became discolored, and some areas exhibited a necrotic and reddish appearance. In some cases, bloody blotches (erythema) appeared around the fins and mouth. Although no mortality was recorded during days 1–4 pi, massive mortalities (due to the endotoxic acute shock produce by *A. salmonicida* extracellular products) occurred on days 5 and 6 pi. Mortality among individuals fed the C diet remained constant throughout the experimental period; mortality in the experimental groups ceased at day 12 pi. Different patterns of mortality were observed among experimental groups during the 14 d of the challenge test. After the challenge test, surviving fish recovered their appetites and did not exhibit any disease symptoms. Cumulative mortality in group C was significantly higher ($P < 0.05$) than that observed in fish fed experimental diets. A higher dosage (0.5%) of *Ef* recorded a significantly ($P < 0.05$) lower morality compared with a lower (0.25%) *Ef* dosage. A higher dosage of MOS single supplementation significantly reduced ($P < 0.05$) fish deaths compared with that shown for a lower MOS dosage. Mortality registered in fish fed diet EM0.5% was significantly lower ($P < 0.05$) than that observed in EM0.25% (Figure 4). The frequency of *A. salmonicida* in the HK of survivors fed experimental diets was significantly lower ($P < 0.05$) compared with that observed in fish fed the C diet. There was no significant difference in *A. salmonicida* presence in the HK of surviving fish fed the E0.25% and E0.25% diets. However, fish fed M0.25% exhibited a higher frequency of this pathogen in the HK ($P < 0.05$) compared with that observed in the M0.5% group. The EM0.5% experimental group exhibited the lowest presence of *A. salmonicida* ($P < 0.05$) compared with other experimental groups (Figure 5).

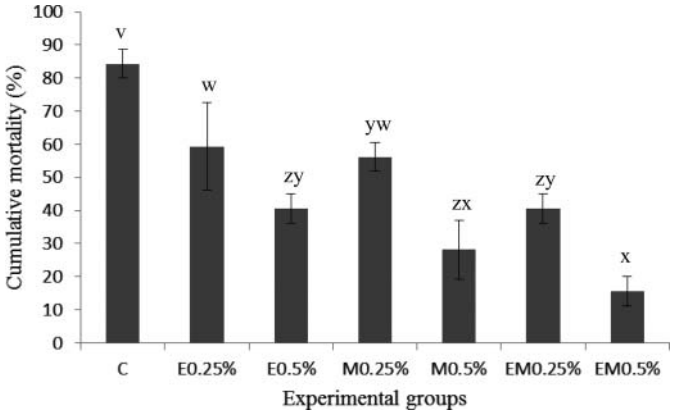

FIGURE 4. Percent cumulative mortality (mean ± SD, $n = 32$) after a 14-d intraperitoneal challenge with *A. salmonicida* of RainbowTrout fed diets with two inclusion levels (0.25% and 0.5%) of *Ef* and MOS supplemented in a single (E or M) or combined (EM) form. Different letters accompanying bars denote significant differences ($P < 0.05$).

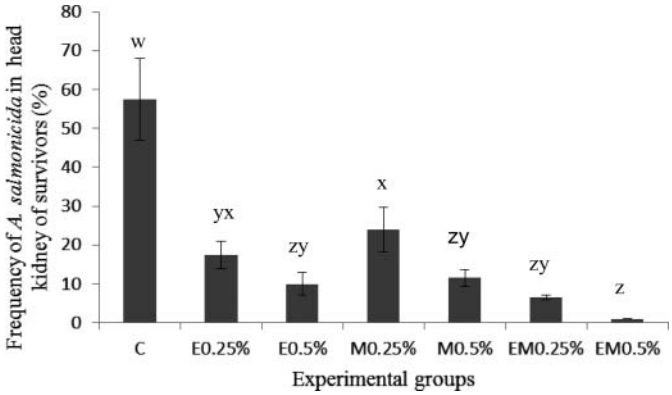

FIGURE 5. Presence (mean % frequency ± SD) of *Aeromonas salmonicida* in the head kidney of survivors after a 14-day intraperitoneal challenge test with *A. salmonicida* of Rainbow Trout fed diets with two inclusion levels (0.25% and 0.5%) of *Ef* and MOS supplemented in a single (E or M) or combined (EM) form. Different letters accompanying bars denote significant differences ($P < 0.05$).

DISCUSSION

Components derived from bacteria and yeast provide health benefits beyond inherent basic nutrition (Guarner and Schaafsma 1998). The present study evaluated the effect of an inactive form of *Ef,* a lactic acid bacteria, and MOS, a yeast cell wall product, on growth performance, nonspecific immunity, and disease resistance in Rainbow Trout, an important species for aquaculture. Although whole-body proximate composition was not significantly affected by *Ef* or MOS supplementation levels in a single or combined form, lipid content in fish with a higher weight gain (E0.25%, M0.25%, and EM0.5% diets) recorded a slightly higher lipid content compared with other experimental groups. Similar results have been shown in previous studies (El-Haroun et al. 2006; Vandenberg and Moccia 1998; Rodriguez-Estrada et al. 2009) where fish with significantly higher growth after experimental treatments also had a slightly higher whole-body lipid content compared with individuals with lower growth. The results of this study indicated that additives of microbial origin exerted a positive effect on growth performance and immune system function in Rainbow Trout. The biological response to *Ef* was previously evaluated by examining immunomodulation and immune-adjuvant effects in animal husbandry (Kotani et al. 2008; Shimada et al. 2009). Likewise, the immuno-nutritional aspects of cultured fish diets supplemented with MOS were evaluated by examining growth performance, immune stimulation, and disease resistance against pathogens (Sang et al. 2009; Dimitroglou et al. 2010; Sang and Fotedar 2010; Gu et al. 2011). A higher growth performance ($P < 0.05$) and a noticeable increase in nutrient utilization of protein was observed in fish fed a lower single *Ef* supplementation diet compared with fish fed a higher single *Ef* supplementation diet.

This result is in accord with previous studies that demonstrated the application (at different levels) of probiotics improves feed conversion and growth rates (Bogut et al. 2000; Taoka et al. 2006a, 2006b; Bagheri et al. 2008; Wang et al. 2008). For example, Wang et al. (2008) observed that Nile Tilapia *Oreochromis niloticus* treated with *E. faecium* exhibited an increased SGR compared with that of control fish. Several authors have suggested that organisms in aquaculture are primarily affected by beneficial bacteria through the enhancement of host nutrition due to the stimulation of digestive enzymes resulting in a higher growth and FCR (Suzer et al. 2008). Furthermore, the presence of beneficial bacterial cells in the intestine improves microbial balance, which in turn improves nutrient absorption and utilization (Gatesoupe 1999, 2007; Lara-Flores 2003). In this study, single supplementation with MOS at two levels did not result in significantly different growth performance. However, a lower supplementation diet resulted in increased growth compared with the control group. The effects of MOS supplementation and dosage levels on growth performance and feed utilization has been reported in previous studies (Dimitroglou et al. 2010; Refstie et al. 2010; Sang and Fotedar 2010; Torrecillas et al. 2011). For instance, Torrecillas et al. (2007) reported that a 0.4% MOS dietary supplementation showed increased growth performance and changes in hepatocyte morphology in European Sea Bass *Dicentrarchus* (*Morone*) *labrax* compared with both fish fed a lower MOS level (0.2%) and controls. In the present study, a slightly higher ADC of protein was detected in groups fed E0.25%, M0.25%, and EM0.5% diets compared with the other experimental groups. Previous studies have demonstrated that growth-promoting additives result in the improved digestibility of nutrients (Ringø and Gatesoupe 1998; Skrede et al. 2002; Sorensen et al. 2011). For example, Grisdale-Helland et al. (2008) found that a 1% supplementation of oligosaccharides significantly increased apparent digestibility in Atlantic Salmon *Salmo salar*.

In the present study, supplementation of inactivated *Ef* cells at two supplementation levels enhanced immune parameters, including hematocrit value, phagocytic activity, and skin mucus production. Other inactivated bacteria with beneficial properties have demonstrated a capacity to modify the immune parameters of Gilthead Seabream *Sparus aurata* (Salinas et al. 2006), Nile Tilapia (Taoka et al. 2006b), Ocellate Puffer *Takifugu rubripes*, and Japanese Flounder *Paralichthys olivaceus* (Kotani et al. 2008). The immunomodulating activity of nonviable bacteria may be due to the existence of certain microbial components, such as capsular polysaccharides, peptidoglycans, and lipoteichoic acids, which are potent stimulators of the piscine immune system (Miettinen et al. 1996; Secombes et al. 2001; Nayak 2010). Once ingested, inactivated bacterial cells are transported to the gut lumen where they are recognized and processed by the immune system through three possible routes: (1) bacterial cells may attach to intestinal epithelial cells and modulate function directly; (2) microfold cells localized in the follicle-associated epithelium overlying Peyer's patches may transport particles to the immune cells in the adjacent subepithelial dome region; (3) dendritic cells in the lamina propria may actively extend dendrites to sample microorganisms in the gut lumen, which then triggers a cascade of reactions leading to an immune response stimulation (Shida and Nanno 2008).

Mannan oligosaccharides provide immune stimulation in aquatic organisms (Sang et al. 2009; Zhou et al. 2010). However, dosage is a decisive factor in obtaining the desired results (Merrifield et al. 2010). In our study, a higher single dosage of this oligosaccharide resulted in a more effective stimulant outcome compared with a lower level. The immunomodulation properties of MOS are explained by the presence of mannose receptors, which are involved in phagocytosis (Ofek et al. 1995), that interact with mannose-binding lectin secreted by the liver of animals fed diets supplemented with MOS (Janeway 1993). At the same time, MOS–protein conjugates activate the animal's immune system (Wismar et al. 2010).

In this study the weight of the skin mucus of experimental fish was measured. Mucus weight varied both by *Ef* and MOS dosage and by the formulation used. Epidermal mucus serves as a repository of numerous innate immune factors, such as lysozyme, immunoglobulins, complement, proteins, lectins, C-reactive protein, proteolytic enzymes, and various other antibacterial proteins and peptides (Shephard 1994; Cole et al. 1997). Previous research has indicated the importance of the use of stimulants for enhancing mucus production in fish. For example, the capacity of lactic acid bacteria (Salinas et al. 2008b) and MOS (Sweetman et al. 2010; Torrecillas et al. 2011) to modify the epidermal mucus production in fish has been demonstrated in previous studies. The innate immune system, particularly the external body surface, protects fish from the external environment. Fish have a unique physical barrier composed of skin and epidermal mucus that acts as the first line of defence (Palaksha et al. 2008).

To correlate the stimulated immune status results of fish fed *Ef* and MOS, and to assess the effectiveness of these additives against disease, an intraperitoneal injection challenge test of *A. salmonicida* was conducted. The ability of *A. salmonicida* to produce disease in fish is due to multiple physiological and biochemical mechanisms. These mechanisms include the generation of extracellular products, such as hemolysin (Hirono et al. 1993) and cytokines (Lee and Ellis 1990), surface exposed structures including the A-layer protein (Chu et al. 1991), lipopolysaccharides (Lee and Ellis1990), type IV pili (Masada et al. 2002), and a repertoire of exo-enzymes that digest cellular components such as proteases, amylases, and lipases (Campbell et al. 1990; Whitby et al. 1992). In this study, *Ef* was shown to provide certain protection against *A. salmonicida*. Numerous investigations have reported that lactic acid bacteria provide a protective barrier against disease (Nikoskelainen et al. 2001; Ringø et al. 2007a, 2007b; Salinas et al. 2008b; Balcázar et al. 2009). In addition, the protection conferred against furunculosis through dietary supplementation of different lactic acid bacteria species in salmonids has been widely demonstrated (Gildberg

et al. 1995; Robertson et al. 2000; Nikoskelainen et al. 2001; Balcázar et al. 2007b; Brunt et al. 2007). For example, Gildberg et al. (1995) reported that dietary supplementation of *Lactobacillus plantarum* provided protection against artificially induced furunculosis in Atlantic Salmon. Similarly, Robertson et al. (2000) found that the use of *Carnobacterium* sp. exerted a noticeable protection against disease in Rainbow Trout and Atlantic Salmon. Under experimental conditions, other lactic acid bacteria species, such as *L. rhamnosus* (Nikoskelainen et al. 2001) and *L. sakei* (Balcázar et al. 2007b), also provided protection against *A. salmonicida*. The protection triggered by lactic acid bacteria is due to the reduction of host tissue damage induced by the pathogen, as suggested by Salinas et al. (2008b) who observed that *L. delbrueckii* prevented cellular damage when Atlantic Salmon tissue was artificially exposed to a furunculosis infection. Furthermore, remarkable benefits were obtained when different inactivated microbial cells were administered to Rainbow Trout that were subsequently challenged with *A. salmonicida* (Irianto and Austin 2003). The capacity of MOS to act as a disease-protecting agent has been reported in other studies with Rainbow Trout (Rodriguez-Estrada et al. 2009) and Channel Catfish *Ictalurus punctatus* (Welker et al. 2007; Peterson et al. 2010), as well as with other aquatic organisms (Gu et al. 2011). For example, Gu et al. (2011) reported that dietary supplementation of MOS in sea cucumber *Apostichopus japonicus* diets resulted in reduced mortality after a disease challenge with *Vibrio splendidus*. This conferred protection may be explained by the presence of foreign molecules with pathogen-associated molecular patterns (PAMPs) that can be recognized and bound by pattern-recognition proteins (PRPs) (Ramírez-Gómez et al. 2010). Mannose-specific lectins belong to this group of PAMP molecules. They function not only as adhesins, binding bacteria like *Campylobacter jejuni* (McSweegan and Walker 1986) and *A. hydrophila* (Merino et al. 1996) to epithelial cells, but also as stimulators of phagocytic cell activities (Perry and Ofek 1984; Wright et al. 1989).

The combined use of immunostimulant and growth-promoting additives is a new concept in aquaculture (Daniels et al. 2010). Until now, experiments typically tested the effects of only one additive (Gu et al. 2011). However, a few studies have combined different stimulants to amplify the effects in cultured aquatic animals. The effect of combining immunostimulants results in amplified immune responses and protection against pathogens (Ortuño et al. 2001; Seguin-Devaux et al. 2005; Selvaraj et al. 2006; Zhang et al. 2010). This study demonstrated that, when used in combination at a 0.5% supplementation level, *Ef* and MOS supplementation resulted in enhanced protection against *A. salmonicida*. This result supports the supposition that *Ef* exhibits adjuvant properties when used in combination with other stimulants. The adjuvant effect of inactivated cells of *Ef* has been demonstrated by Kotani et al. (2008), who observed that vaccination effects were improved when *Ef* was supplemented in Japanese Flounder and Ocellate Puffer diets. Furthermore, the effects of mixing lactic acid bacteria and other additives has been demonstrated by Harikrishnan et al. (2011), who observed that a combination of several strains of beneficial bacteria, along with herbal infusions, added to the diets of Olive Flounder *Paralichthys olivaceous* enhanced growth performance, blood constituents, and the nonspecific immune response. Other combinations of natural stimulants have proven to be growth performance inducers and immune stimulators in Japanese Flounder (Kim et al. 1998), Greasy Grouper *Epinephelus tauvina* (Sivaram et al. 2004), Indian white prawn *Penaeus indicus* (Immanuel et al. 2004), and disk abalone *Haliotis discus* (Lee et al. 2001). The improved effect of MOS when combined with other stimulants has been tested in some studies. Daniels et al. (2010) demonstrated that a mixture of this oligosaccharide with *Bacillus* spp. significantly improved weight gain, carapace length, specific growth rate, feed conversion ratio, and postlarval conditions of European lobster *Homarus gammarus* compared with those organisms fed with a single supplementation of either MOS or *Bacillus* spp. In another report, Gu et al. (2011) demonstrated that MOS combined with β-glucan improved growth performance, immunity, and disease resistance in sea cucumber.

The results of this study confirm that dietary supplementation with stimulants may be an effective natural prophylactic alternative for aquaculture. The present study indicated that dosage level and single or combined supplementation of *Ef* and MOS strongly influenced the immune system and growth performance of Rainbow Trout. Combined *Ef* and MOS supplementation at the 0.5% level could further promote growth and immune stimulation. Therefore, combinations of stimulants are worthy of further consideration, as this would be an efficient mode of delivery in aquaculture.

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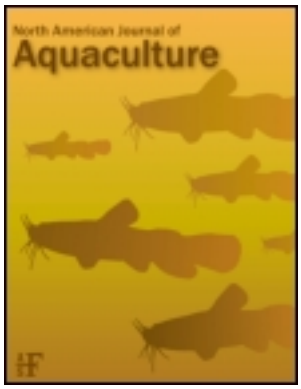

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The Effect of Different Feeding Protocols on Compensatory Growth of Black Sea Trout Salmo trutta labrax

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ARTICLE

The Effect of Different Feeding Protocols on Compensatory Growth of Black Sea Trout *Salmo trutta labrax*

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Abstract

The objective of this study was to determine the effects of different feeding protocols on compensatory growth, feeding rate, and feed conversion efficiency of Black Sea Trout *Salmo trutta labrax*. To our knowledge, no previous feeding experiments with Black Sea Trout have incorporated a fasting period to simulate the handling and acclimation conditions experienced by farmed fish. Fifteen fish per tank (10.69 ± 0.06 cm and 13.22 ± 0.14 g, mean ± SE) were stocked into 40-L fiberglass tanks. Fish were fed with different fasting–feeding regimes for 95 d and were equally allotted to four treatments (T_{cont}: control; $T_{5–10}$: 5 d fasting, 10 days feeding; $T_{10–10}$: 10 d fasting, 10 d feeding; and $T_{15–10}$: 15 d fasting, 10 d feeding) with three replicates per treatment. The results indicated that length ($P = 0.0005$), weight ($P = 0.000$), condition factor ($P = 0.013$), and specific growth rate ($P = 0.014$) were significantly affected by the interaction between feeding and time. All fasting treatments showed partial compensation during refeeding. There was an increase in daily feeding rate and feed conversion efficiency in fasting treatments compared with the control treatment. In contrast, at the end of the experiment specific growth rate, condition factor, and body weight in fasting treatments were significantly lower compared with the control treatment. We concluded that there was partial compensation of growth with regular refeeding after periods of feed deprivation (e.g., 5, 10, or 15 d) over a long term and a shorter fasting period may be preferred in order to achieve compensatory growth.

The Black Sea Trout *Salmo trutta labrax* is one of the nine subspecies of Brown Trout *S. trutta* and is endemic in Turkey (Quillet et al. 1992). The Black Sea Trout is a native finfish of the eastern Black Sea coast and rivers and is a new species for intensive aquaculture (Tabak et al. 2001). The Black Sea Trout is an anadromous subspecies and migrates into the freshwater streams for spawning (Kocabaş 2009).

Food is an important factor for growth, and fish grow more efficiently with a continuous food supply (Skalski et al. 2005). In contrast, many organisms grow faster during recovery from total or partial food deprivation compared with having food available continuously (Wilson and Osbourn 1960; Jobling 1994). Animals experiencing a period of growth depression may achieve the same size and age as those that are

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provided with feed regularly and grow continuously (Ali et al. 2003).

Compensatory growth (CG) is defined as a period of increased growth following food deprivation (Ricker 1969, 1975; Atchley 1984; Riska et al. 1984). Compensatory growth is dependent upon fish species, fish size, dietary nutrient content, duration of feeding trial, feeding regime, social factors, and the physiological–nutritional state of the fish (Wilson and Osbourn 1960; Bilton and Robins 1973; Jobling et al. 1994; Rueda et al. 1998; Jobling and Johansen 1999; Gibson Gaylord and Gatlin 2000, 2001; Hayward et al. 2000; Hornick et al. 2000; Wang et al. 2000; Ali et al. 2003; Oh et al. 2007; Cho and Cho 2009; Cho et al. 2010; Cho 2011).

Although several studies have been performed on compensatory growth of salmonid fish species, for example, Arctic Char *Salvelinus alpinus* (Jobling et al. 1994) and Whitefish *Coregonus lavaretus* (known as Powan in North America [Koskela et al. 1997; Känkänen and Pirhonen 2009]), there is no available information about the possible responses of Black Sea Trout in relation to feed restrictions. A primary objective in aquaculture is to achieve maximum production throughout the year. Determining the optimum feeding protocol is important in terms of achieving maximum growth and low feeding efficiency rate, size distribution, and reducing food wastes (Schnaittacher et al. 2005). Within this framework, the objective of this study was to examine the effects of fasting and subsequent refeeding on growth of Black Sea Trout.

METHODS

Experimental design.—Black Sea Trout ($n = 180$; 13.22 ± 0.14 g in weight and 10.69 ± 0.06 cm in length, mean ± SE) were obtained from Karadeniz Technical University Marine Sciences Faculty Aquaculture Research and Production Center, Trabzon, Turkey. Fish were stocked in equal numbers into 12 tanks, three replicate tanks per treatment, and fed for 95 d. Fish were reared in fiberglass tanks with a rearing volume of 40 L. Each tank contained 15 fish. The water flow was 3 L/min per tank. The dissolved oxygen and pH were measured daily with YSI model 51 oxygen meter (Yellow Springs Instruments). Temperature was measured with a digital thermometer twice a day at 0800–0900 hours and 1600–1700 hours. Temperature of the incoming water was 11.4 ± 2.2°C. The pH and dissolved oxygen levels in the treatments were 7.93 ± 0.18 (range, 7.71–8.19) and 7.83 ± 1.32 mg/L (range, 6.15–10.01 mg/L), respectively. The water for the tanks was supplied from River Çamburnu. The water in the experimental tanks was aerated and 20% of the water was changed daily. The photoperiod was 16 h light : 8 h dark.

Fish in all treatments were hand-fed with a commercial food (2-mm extruded trout feed; 45% crude protein [CP], 18% crude fat [CF], energy [E], 4,100 kcal/kg) and fed the same daily ration twice a day, every day of the week. The proximate composition of the experimental diets is shown in Table 1. The amount fed to each tank was recorded. The experiment lasted for 95 d.

TABLE 1. Proximate composition of the experimental diet used to assess compensatory growth in Black Sea Trout. EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid, BHT = butylated hydroxytoluene.

Diet component	Percent of dry weight (or total amount)
Crude protein (%)	45.0
Crude fat (%)	18.0
Ash (%)	12.0
Crude cellulose (%)	3.0
Moisture (%)	12.0
Metabolic energy (kcal/kg)	4,100
Calcium and phosphorus (%)	2.0
Phosphorus (%)	1.5
EPA and DHA (%)	2.5
Lysine (%)	1.8
Methionine and cysteine (minimum) (%)	0.5
BHT (%)	1.7

Fish in the control treatment were fed to satiation throughout the 95-d period. Fish in the other three treatment groups were deprived of feed for 5-, 10-, or 15-dintervals during the feed restriction period, and then fed to satiation for 10 d during the refeeding periods. Food deprivation was timed so that the end of deprivation period occurred in all treatments at 95 d. Fish were fed according to the following four fasting–feeding treatment (T) schedules as described by Heide et al. (2006): (1) T_{cont}: feeding for 95 d as control; (2) $T_{5–10}$: 5 d fasting, 10 d feeding; (3) $T_{10–10}$: 10 d fasting, 10 d feeding; (4) $T_{15–10}$: 15 d fasting, 10 d feeding. The $T_{15–10}$ and $T_{10–10}$ treatments only had 5 d of feeding at the end of the 95-d study (Figure 1).

Growth measurements.—Each fish was anesthetized in a 30-mg/L solution of benzocaine, and body weight (BW; to 0.01 g) and FL (to 0.1 cm) were measured and recorded. Specific growth rate (SGR) was calculated as $[(\log_e W_2 - \log_e W_1) / (t_2 - t_1) \times 100]$, where W_1 and W_2 represent BW at times t_1 and t_2, respectively. Condition factor (CF) was calculated as [BW (g)/FL^3 (cm)] × 100. Feed conversion efficiency (FCE) was calculated as biomass (B) gain per weight unit of consumed feed (C): i.e., FCE = $(B_2 - B_1)/C$ (Heide et al. 2006). Uneaten pellets were flushed out of the tanks within 15 min and filtered from the outlet water using a manual collection system. Uneaten pellets were removed from the filters and immediately put into a drying chamber for 24 h (70°C). The amount of food consumed was calculated as the difference between dry weight of the feed presented and dry weight of the uneaten pellets (Handeland et al. 2008). Food consumption (%FC) as a percent of BW per day (% BW/d) was estimated based on the mean weight of fish in each tank between two consecutive measurements and the total amount of food offered per tank. No mortality occurred during the entire experimental period.

Statistic analysis.—Statistical analyses were performed using SPSS (version 14.0). Possible differences in fish weight, length, and CF were tested using repeated measures ANOVA,

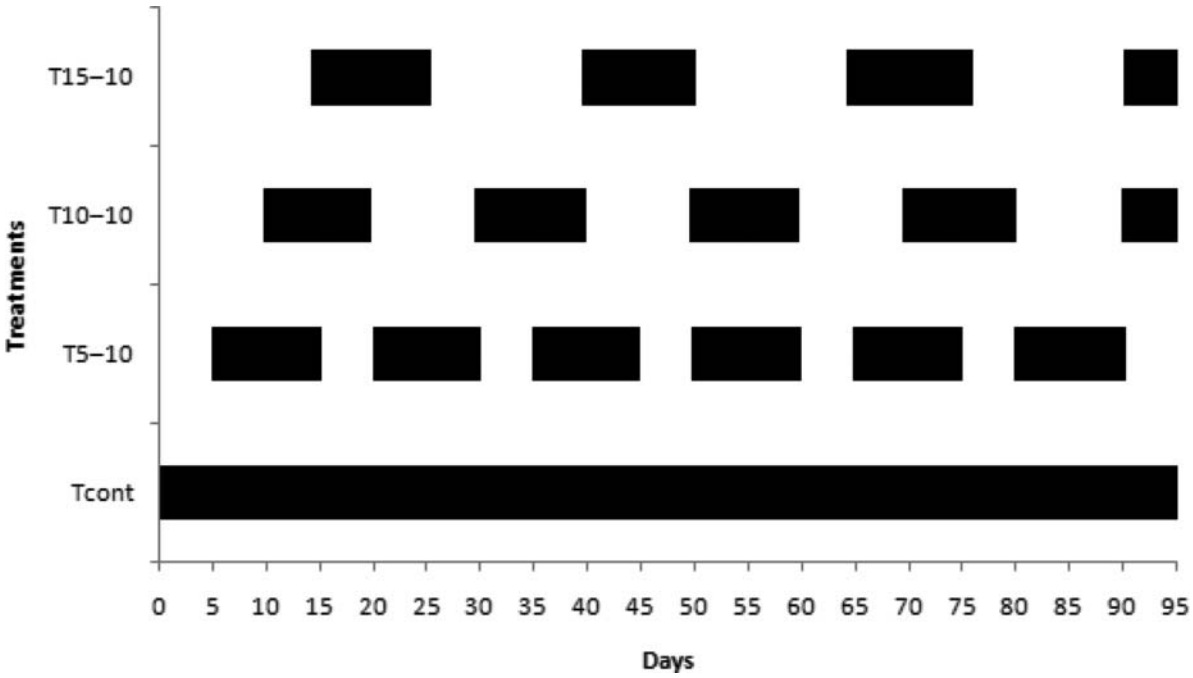

FIGURE 1. Schematic overview of the experimental fasting–feeding treatments used to assess compensatory growth in Black Sea Trout (black fields indicate feeding days; periods in between represent days of fasting). See text for full description of fasting–feeding treatments.

and other parameters (SGR, %FC, FCE) were tested with one-way ANOVA using the tank mean value as the observational unit. Post hoc comparisons between sample means were tested by Tukey's test and 5% was taken as the level of significance (Känkänen and Pirhonen 2009).

RESULTS

The initial mean weight and length of Black Sea Trout were 13.22 ± 0.15 g (range, 10.18–16.66 g) and 10.73 ± 0.05 cm (range, 9.62–11.91 cm), respectively. Mean final lengths and weights (Figure 2) of Black Sea Trout in T_{cont}, $T_{5–10}$, $T_{10–10}$, and $T_{15–10}$ were 15.44 ± 0.08 cm and 51.44 ± 1.35 g, 14.10 ± 0.29 cm and 39.51 ± 2.24 g, 12.73 ± 0.13 cm and 67.61 ± 14.63 g, and 12.49 ± 0.09 cm and 20.20 ± 0.16 g, respectively. Weight was significantly affected by the interaction between feeding ($P = 0.0005$) and time ($P < 0.05$). At the end of the experiment, fish weights and lengths from fasting treatments were significantly lower than those from the control treatment.

At the beginning of the study, the mean final CF values in T_{cont}, $T_{5–10}$, $T_{10–10}$, and $T_{15–10}$ were 1.09 ± 0.002, 1.08 ± 0.004, 1.08 ± 0.034, and 1.07 ± 0.008, respectively. At the termination of study, CF values of Black Sea Trout in the treatments were 1.35 ± 0.018, 1.08 ± 0.004, 1.08 ± 0.034, and 1.11 ± 0.005, respectively (Figure 3). There were differences among the treatments ($P = 0.014$); CF was higher in T_{cont} compared with the other treatment groups.

Specific growth rate of Black Sea Trout was calculated as 1.51 ± 0.03 in T_{cont}, 1.29 ± 0.04 in $T_{5–10}$, 0.84 ± 0.03 in $T_{10–10}$, and 0.71 ± 0.18 in $T_{15–10}$. Changes in SGR at different feeding regimes are presented in Figure 4. Specific growth rate was significantly affected by the interaction between feeding and time ($P = 0.014$). At the end of the experiment, SGR of fish from fasting treatments was significantly lower compared with the control treatment.

Food consumption (%FC) of Black Sea Trout was calculated as 1.11 ± 0.01 in T_{cont}, 0.86 ± 0.37 in $T_{5–10}$, 0.89 ± 0.02 in $T_{10–10}$, and 0.77 ± 0.09 in $T_{15–10}$ (Table 2). There were

TABLE 2. Final food consumption (%FC) and food conversion efficiency (FCE) of Black Sea Trout cultured with one of four fasting–feeding regimes over 95 d. See text for full description of fasting–feeding treatments. Values followed by different lowercase letters are significantly different ($P < 0.05$).

	Fasting–feeding treatment			
	T_{cont}	$T_{5–10}$	$T_{10–10}$	$T_{15–10}$
Final %FC	1.06 ± 0.010 z	1.07 ± 0.160 z	2.01 ± 0.120 y	2.32 ± 0.190 x
Final FCE	1.11 ± 0.05 z	0.86 ± 0.37 y	0.89 ± 0.02 y	0.77 ± 0.09 y

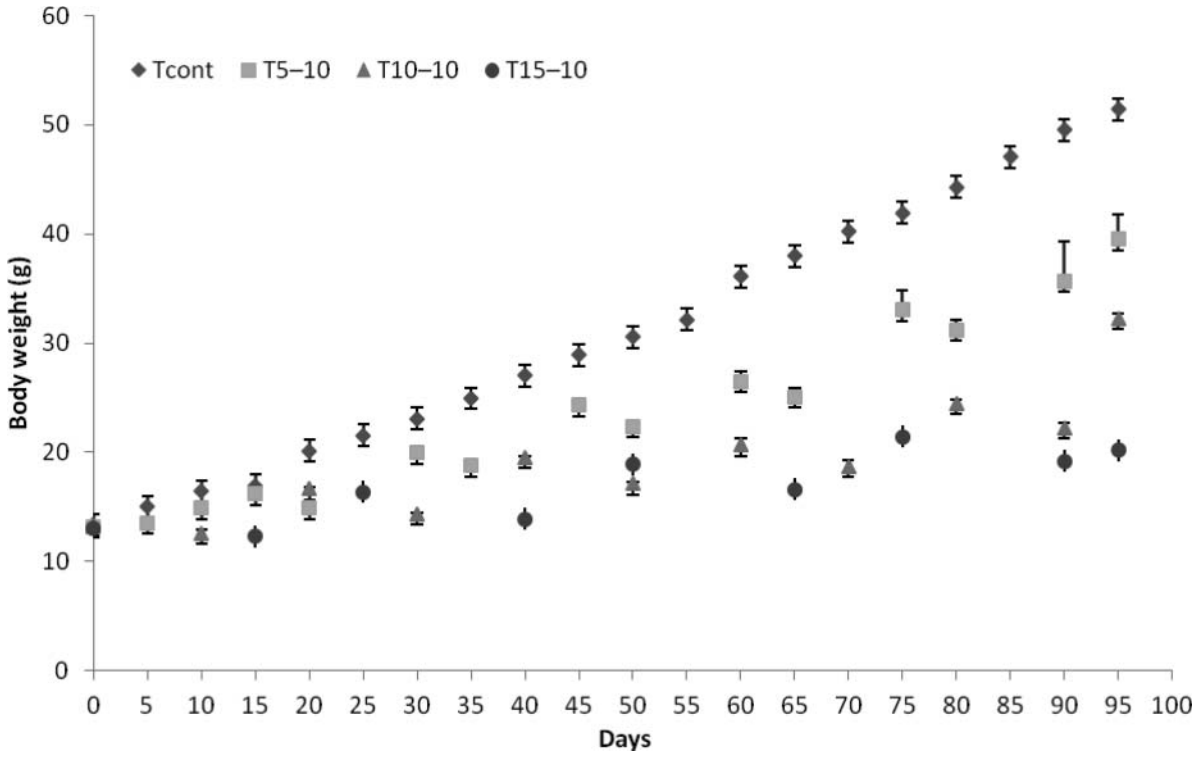

FIGURE 2. Changes in mean body weight of Black Sea Trout cultured with one of four fasting–feeding regimes over 95 d. See text for full description of fasting–feeding treatments.

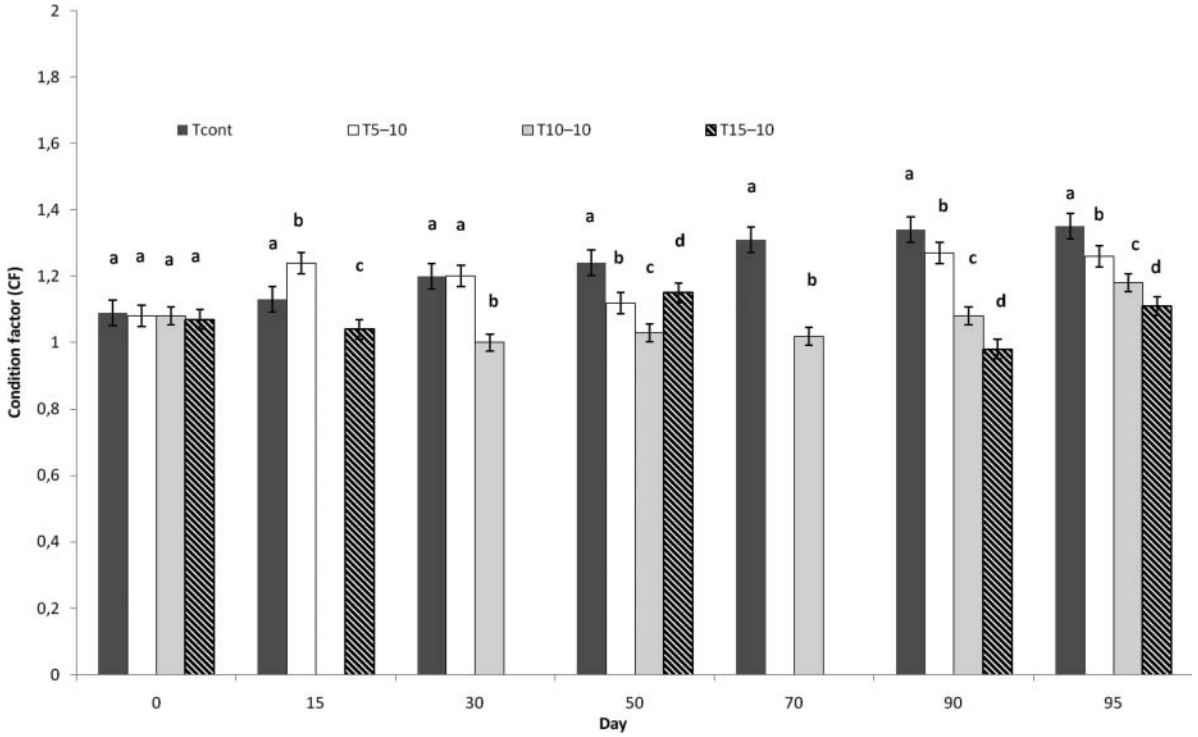

FIGURE 3. Condition factor of Black Sea Trout between each fasting and feeding period and overall (95 d) in Black Sea Trout reared under one of four fasting–feeding regimes. See text for full description of fasting–feeding treatments. Vertical lines accompanying bars indicate SE. Different letters above bars indicate statistical differences (Tukey's test, $P < 0.05$).

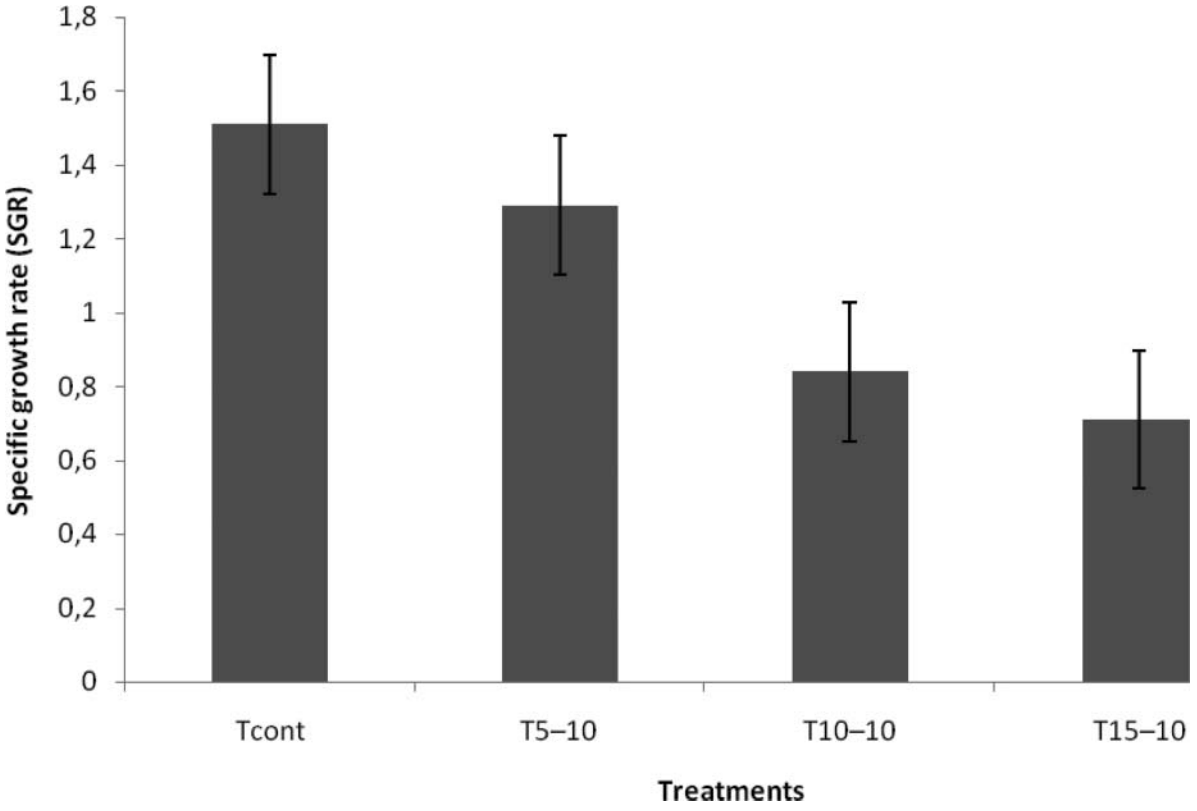

FIGURE 4. Specific growth rates overall (95 d) in Black Sea Trout reared under four fasting–feeding regimes. See text for full description of fasting–feeding treatments. Vertical lines accompanying bars indicate SE.

differences among the treatments ($P = 0.013$), and food consumption was higher in the $T_{15–10}$ treatment compared with the other groups.

Food conversion efficiency of Black Sea Trout was calculated as 1.06 ± 0.01 in T_{cont}, 1.07 ± 0.16 in $T_{5–10}$, 2.01 ± 0.12 in $T_{10–10}$, and 2.32 ± 0.19 in $T_{15–10}$ (Table 2). Food conversion efficiency was significantly affected by the interaction between feeding and time ($P = 0.006$). At the end of the experiment, FCE in fish from the control treatment was significantly lower compared with the fasting treatments.

DISCUSSION

By the end of this experiment, food-restricted fish appeared to show partial compensation. In partial compensation, the food-deprived animal does not achieve the same size and age as nonrestricted contemporaries. However, an increase in growth rates can occur in some cases (Heide et al. 2006). In previous studies, partial CG was resulted after fasting in Alaska Yellowfin Sole *Pleuronectes asper* (Paul et al. 1995), Arctic Char (Jobling et al. 1993), Atlantic Halibut *Hippoglossus hippoglossus* (Heide et al. 2006), and Black Rockfish *Sebastes schlegeli* (Oh et al. 2007). In contrast, some studies have reported that a full growth recovery occurred in Atlantic Cod *Gadus morhua* (Jobling et al. 1994) and Turbot *Scophthalmus maximus* (Sæther and Jobling 1999) after a period of feed restriction.

Specific growth rate and CF can be influenced by restricted rations (Stefansson et al. 2009). Nicieza and Metcalfe (1997) stated that positive growth and weight loss in food-restricted fish are general phenomena and may be adaptive in fish experiencing fluctuating (seasonal) changes in food abundance. Stefansson et al. (2009) determined that growth rate increased in fish after refeeding. Heide et al. (2006) reported that in the final sampling period, SGR was higher in fish from fasting treatments compared with those from control treatments. This may be attributed to lower energy expenditures during the fasting-induced resting periods (Heide et al. 2006). The present results agree with these reports. In addition, the present results showed that treatment $T_{5–10}$ displayed the highest overall SGR of the fasting treatments. For aquaculture purposes, one initial short period of fasting is preferred in order to achieve a clear compensatory effect. We also observed that weight loss occurred in fasting treatments. Loss of body weight may be due to redirection of energy reserves to meet the demands of a fish's metabolic rate (Beamish 1964; Love 1970, 1980; Blaxter and Ehrlich 1974; Jobling 1980; Johnston 1981; Du Preez et al. 1986a, 1986b; Du Preez 1987; Wieser et al. 1992; Ali et al. 2003).

The results from the present study indicated that FCE and%FC in fish were significantly higher in fasting treatments

compared with the control treatment. Feed conversion efficiency and food consumption changed depending on duration of the feed deprivation period (Eroldoğan et al. 2006b) compared with the control treatment (Eroldoğan et al. 2006a). Fish have responded to different feed-restriction refeeding protocols. Some studies have reported that feed efficiency increased in fasting treatments (Qian et al. 2000; Gibson Gaylord and Gatlin 2001; Li et al. 2005; Känkänen and Pirhonen 2009). In contrast, other studies have determined that there was no difference in feed efficiency between the fasting treatments and the control treatment (Miglavs and Jobling 1989a; Kim and Lovell 1995; Hayward et al. 1997; Wang et al. 2000, 2005; Xie et al. 2001; Nikki et al. 2004). In some studies, researchers have stated that the improvement of feed conversion efficiency was only temporary (Miglavs and Jobling 1989b; Hayward et al. 1997). The present results may be due to hyperphagia being the main mechanism involved in the compensatory growth response. This is because in a hyperphagic phase food consumption rates are high in fish when continually provided a ration ad libitum, and there is an increase in FCE (Ali et al. 2003; Heide et al. 2006).

The results obtained from the present study indicated that there was partial compensation of growth in Black Sea Trout. Fish had experienced CG upon the termination of the experiment. For commercial aquaculture, the expenses associated with feed are important and a shorter fasting period may be preferred in order to achieve compensatory growth. To further assess the potential growout performance of this subspecies, additional research is needed to investigate cost-effective feeding protocols.

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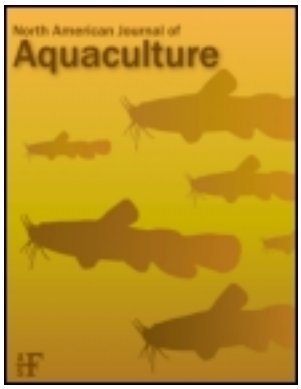

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Growth and Survival of Juvenile Gulf Killifish Fundulus grandis in Recirculating Aquaculture Systems

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COMMUNICATION

Growth and Survival of Juvenile Gulf Killifish *Fundulus grandis* in Recirculating Aquaculture Systems

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Abstract

Gulf Killifish *Fundulus grandis* is one of the largest killifish species. It is a euryhaline teleost and a popular baitfish occurring along the coastal Gulf of Mexico and southern Atlantic states. There is currently limited information on the grow-out characteristics of killifish in indoor recirculating aquaculture systems (RAS). This preliminary study aims at determining the effects of stocking density on the growth and survival of juvenile Gulf Killifish in indoor RAS. Individuals of 0.45 ± 0.01 g (mean ± SE) weight were stocked at initial densities of 2 and 5 fish/L in 60-L tanks in RAS with four replicates each. After 82 d of culture, difference in growth at these densities was not significant ($P > 0.629$). Mean survival and gross and net yields differed by stocking density. Survival (94.6%) in the lowest density was significantly high (ANOVA: $P < 0.01$) compared with survival (83.9%) in the highest density. Gross and net yields were significantly high (ANOVA: $P < 0.01$) at the 5 fish/L stocking densities. Compared with the traditional pond system, the use of RAS in this preliminary study enabled stocking of killifish at very high densities. However, cultured fish grew relatively slow in RAS compared with pond culture and did not reach market size (≈3.3 g).

The Gulf Killifish *Fundulus grandis* is one of the largest killifish species with a blunt head and short snout; it is a popular baitfish and occurs along the coastal Gulf of Mexico and southern Atlantic (Tatum et al. 1979). It is a popular baitfish in estuarine and marsh environments, and it is referred to regionally by anglers as Cocahoe Minnow, Bull Minnow, or Mud Minnow (Waas et al. 1983; Oesterling et al. 2004). This species was the first among candidate species, along with its sister species the Mummichog *F. heteroclitus*, to demonstrate the greatest potential for successful development as a live bait for marine and estuarine anglers (Oesterling et al. 2004). The Gulf Killifish is characterized as a hardy species that has the ability to withstand a wide range of temperatures and salinities that could potentially lend them to perform well on the hook for anglers (Umminger 1971; Perschbacher et al. 1990). The majority of Gulf Killifish sales to anglers are harvested from the wild, resulting in an inconsistent supply due to seasonal variation and irregular harvest sizes (Hanson et al. 2004; Oesterling et al. 2004). Aquaculture production of this species and its related sister species could provide year-round availability, something which should be well received based on the reputation of Gulf Killifish and Mummichog as hardy and effective bait.

Adams and Lazur (2001) estimated a net return of US$3,714/ha for a 4-ha Gulf Killifish production facility, indicating possible profitability in killifish culture. Gulf Killifish are adaptable to aquaculture (Tatum et al. 1982; Perschbachner and Strawn 1985) but are not commonly cultured as bait (Phelps et al. 2010). Previous work has demonstrated that a market-size Gulf Killifish (6.3 cm) can be obtained in less than 115 d in ponds by modifying stocking densities and feed inputs (Tatum et al. 1982; Perschbacher and Strawn 1985). Production trials in static pools stocked at rate of 25, 50, and 100 fry/m^2 produced mean

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harvest weights of 5.1, 2.8, and 2.2 g, respectively, for a production period of 104 d (Phelps et al. 2010). Previous pond studies have reported obtaining 2.8–3.4-g individuals within 70 d at a stocking density of 200,000 fish/ha when initially stocking larger 0.5-g individuals (Waas and Strawn 1982). It is clear that most growth studies have been performed in ponds; however, there is a dearth of information on the use of recirculation aquaculture systems and grow-out phase research in these systems for the Gulf Killifish as well as the Mummichog. Compared with the traditional systems, the ability to culture juvenile Gulf Killifish at high densities with a control over the culture environment in recirculatory systems will enable aquaculturists to raise and market more fishes per unit volume of water. Juvenile rearing utilizing recirculation capabilities could further increase the number of market-sized individuals within a production system and hence the numbers of adults and broodstock.

Growth is a result of the accretion of body components. Exogenous inputs from dietary and environmental sources are therefore needed in the buildup of these components (Bureau et al. 2000). Energy accumulated for growth is the difference between quantity of food eaten and the amount required for catabolism; hence, large differences in growth can result from slight differences in the energy budget. Growth is, therefore, extremely variable in fishes (Weatherley and Gill 1987; Diana 2004). Supply of diet in the right quantity and quality, and proper timing is very essential to the realization of growth potential in fish. Fish culture practices such as varying stocking density and feeding rate take advantage of response of fish to manipulations of stocking density and nutrition that influences these factors, thereby impacting individual fish growth rates. This preliminary study aims at determining the growth and survival of juvenile Gulf Killifish in an indoor recirculating aquaculture system (RAS).

METHODS

Culture system.—This study was conducted at the Aquaculture Research Station (Louisiana State University Agricultural Center, Baton Rouge, Louisiana) in an indoor recirculating system. The Institutional Animal Care and Use Committee of the Louisiana State AgCenter approved in advance all procedures used in this study under protocol AE2010–14. Water used in the culture system originated from a dechlorinated municipal source and was maintained at a salinity of 12‰ using Crystal Sea Marinemix (Marine Enterprises International, Baltimore, Maryland). Replicate circular tanks each contained 60 L of recirculating water with continuous aeration of atmospheric air in each tank from a regenerative blower. The system had two sumps containing about 30 L of water each and a flow rate of 50 mL/s. Photoperiod was set to light cycle of 14 h light and 10 h darkness. Water was added biweekly to account for losses through evaporation and maintained at ambient temperature. The recirculating system was serviced by a 170-L bubble-washed bead filter and a 40W ultraviolet sterilizer.

Stocking and feeding.—Prior to stocking, juvenile Gulf Killifish of the same cohort were withheld feed for 24 h and then graded. A sample of 240 individuals was sampled from the graded cohort to measure their weight (g) and TL (cm). Juveniles with a weight of 0.45 $\pm$ 0.01 g (mean $\pm$ SE) were then randomly assigned to treatments at a density of 2 or 5 fish/L, with four replicates for each density treatment. The Gulf Killifish were then allowed a period of 72 h to recover from stress while being observed for signs of infections, abnormal swimming behavior, or both. Fish were fed a commercially available extruded feed (50% crude protein, 14% crude fat, 0.8-mm diameter; Burris Mill and Feed, Franklinton, Louisiana) and fed daily at 9% body weight divided into six feeding times, between 0800 hours and 1800 hours at 2 h-intervals using automatic feeders (Sweeney Enterprises, Boerne, Texas). Quantity of feed given was adjusted every 2 weeks according to mean body weight after weighing and assessing the number of fish per tank for each density treatment.

Growth and survival parameters.—Growth parameters measured included weight gain, specific growth rate (SGR), condition factor (CF), coefficient of variation (CV), feed conversion ratio (FCR), gross yield (GY), and net yield (NY). We calculated specific growth rate as

$$\mathrm{SGR} = 100 \times [(\log_e W_f - \log_e W_i)]t^{-1},$$

coefficient of variation as

$$\mathrm{CV} = [(\mathrm{SD}/W_n)] \times 100\%,$$

feed conversion ratio as

$$\mathrm{FCR} = F/(W_f - W_i),$$

and condition factor as CF using the Fulton formula (Fulton 1904), where $\log_e$ is natural log, W_f is the final mean wet weight (g), W_i is the initial mean wet weight, t is the time in days, W_n is the mean fish weight, F is the quantity of feed given fish, and SD is the standard deviation of the Gulf Killifish weight. Survival rate (SR) was determined biweekly and on conclusion of the study. We determined GY by multiplying the mean total weight of Gulf Killifish by the total survival, and NY as the difference in biomass between the biomass harvested and biomass stocked (expressed as g/60 L).

Water quality.—Temperature, salinity, dissolved oxygen (DO), pH, total alkalinity, total hardness, total ammonia nitrogen (TAN), and nitrite were measured prior to stocking and weekly thereafter. Total alkalinity and hardness were determined with standard titration techniques, while TAN (salicylate method) and nitrite (diazotization method) were determined with a Hach DR 4000 spectrophotometer (Hach, Loveland, Colorado). Dissolved oxygen was measured with a YSI Model 55 DO meter (YSI, Yellow Springs, Ohio), salinity and temperature were measured with a YSI Model 30 salinity–conductivity-temperature meter,

and pH was determined with an Orion Model 330 pH meter (Thermo Fisher Scientific, Waltham, Massachusetts).

Data analysis.—Data obtained during the study were analyzed using XLSTAT 2012 computer software. There was an initial exploration of data for normality using the Shapiro–Wilk and Anderson–Darling tests. Data were reported as mean ± SE, and data expressed as percentages were arcsine-transformed prior to analysis. In order to determine differences in density treatments, one-way ANOVA was conducted on data. This was done after data passed the normality test. Ryan–Einot–Gabriel–Welsch (REGWQ) multiple-range test was conducted if statistically significant differences ($P \leq 0.05$) were detected among the density treatments.

RESULTS

Growth Performance and Survival

The initial mean weight of juvenile Gulf Killifish stocked at 2 and 5 fish/L was not significantly different (ANOVA: $F = 0.259$, df = 1, $P = 0.629$). After 8 weeks of culture, Gulf Killifish had doubled their initial weight in each of the stocking densities. At the conclusion of the study, juveniles initially stocked at the 2 and 5 fish/L had tripled their mean weight. Weight of Gulf Killifish was not significantly different (ANOVA: $F = 0.012$, df = 1, $P = 0.918$; Table 1) at the stocking densities.

After 82 d of culture, final weight of fish were 1.45 ± 0.14 g (mean ± SE) and 1.43 ± 0.05 g for juvenile Gulf Killifish stocked at 2 and 5 fish/L, respectively. Survival rate of 94.6 ± 0.8% in the lowest density was significantly high (ANOVA: $F = 15.022$, df = 1, $P = 0.008$) compared with the 83.9 ± 2.3% in the highest density. Weight gain, SGR, CF, and CV did not differ significantly between the stocking densities (Table 2). Gross and net yields were highest (ANOVA: $F = 96.655$, df = 1, $P < 0.0001$; ANOVA: $F = 34.810$, df = 1, $P = 0.001$, respectively) at the 5 fish/L stocking density.

TABLE 1. Weight (g; mean ± SE) of Gulf Killifish during culture period at initial stocking densities of 2 and 5 fish/L for 82 d in a 60-L recirculating system. Different lowercase letters within a row denote significant differences between density treatments during a given culture period (REGWQ multiple-range test: $P \leq 0.05$).

	Stocking density (fish/L)	
Culture period	2	5
At stocking (week 0)	0.47 ± 0.05 z	0.44 ± 0.02 z
Week 2	0.54 ± 0.05 z	0.50 ± 0.02 z
Week 4	0.66 ± 0.06 z	0.60 ± 0.03 z
Week 6	0.80 ± 0.06 z	0.76 ± 0.03 z
Week 8	1.01 ± 0.09 z	0.97 ± 0.04 z
Week 10	1.17 ± 0.11 z	1.15 ± 0.05 z
Week 12	1.45 ± 0.14 z	1.43 ± 0.05 z

TABLE 2. Summary of growth and survival parameters (mean ± SE) for juvenile Gulf Killifish cultured for 82 d in a recirculating system at 2 and 5 fish/L initial stocking density. Different lowercase letters within a row denote significant differences between density treatments for a given parameter (REGWQ multiple-range test: $P \leq 0.05$).

	Stocking density (fish/L)	
Parameter	2	5
Initial weight (g)	0.47 ± 0.05 z	0.44 ± 0.02 z
Final weight (g)	1.45 ± 0.14 z	1.43 ± 0.05 z
Weight gain (g)	0.98 ± 0.07 z	0.99 ± 0.06 z
SGR (%)	1.38 ± 0.12 z	1.44 ± 0.06 z
CF (g/cm^3) × 10^{-2}	1.37 ± 0.04 z	1.46 ± 0.04 z
CV (%)	41.8 ± 7.1 z	57.4 ± 4.1 z
Feed consumption (g)	601.6 ± 12.7 z	1,277.7 ± 27.4 y
FCR	5.5 ± 0.7 z	4.3 ± 0.2 z
GY (g/60 L)	163.8 ± 14.7 z	358.2 ± 8.7 y
NY (g/60 L)	107.5 ± 12.8 z	226.6 ± 11.9 y
Survival (%)	94.6 ± 0.8 z	83.9 ± 2.3 y

Water Quality

Results of the water quality parameters can be seen in Table 3. Results show an optimum in the condition of the water in which fish were cultured.

DISCUSSION

This study is the first, to our knowledge, to investigate the grow-out phase of the Gulf Killifish within a recirculation system. Traditionally, Gulf Killifish has been cultured in earthen ponds (Trimble et al. 1981; Tatum et al. 1982; Waas and Strawn 1982; Perschbacher and Strawn 1985, 1991), and recently in static pools (Phelps et al. 2010) relatively near the coast where there is ready access to saline water which will likely impact the environment negatively. The cost of such a facility near the coast is very expensive and continues to rise. Recirculating aquaculture systems, being the most sustainable type of aquaculture production (Martins et al. 2009), is an alternative to

TABLE 3. Water quality parameters for juvenile Gulf Killifish cultured for 82 d in a recirculating system at 2 and 5 fish/L initial stocking density.

Parameter	Mean ± SE	Range
Temperature (°C)	24.9 ± 0.1	24.2–25.2
DO (mg/L)	7.78 ± 0.05	7.39–8.12
Salinity (‰)	11.8 ± 0.08	11.4–12.4
pH	8.71 ± 0.01	8.60–8.80
TAN (mg/L)	0.002 ± 0.001	0.000–0.010
Nitrite (mg/L)	0.025 ± 0.006	0.0001–0.0500
Alkalinity (mg/L)	495.2 ± 32.6	440–533
Hardness (mg/L)	2,312 ± 21.6	2,210–2,500

opening the culture of Gulf Killifish in areas farther away from the coast, where the cost is relatively low. However, juvenile Gulf Killifish cultured in such an indoor system showed a relatively slower growth compared with that of pond culture as fish had poor FCR (Table 2). Due to this, fish at neither densities reached market size (2.5 in, about 3.3 g; Adams and Lazur 2001) within 82 d of culture, although fish were fed a high protein diet (50% crude protein) at 9% body weight daily in multiple times a day. This can be attributed in part to the high stocking density used in this study. The 2 fish/L density (the lowest of the stocking densities used in this study) was about 45–150-fold higher than the densities used in earthern ponds and static pools (Trimble et al. 1981; Tatum et al. 1982; Waas and Strawn 1982; Perschbacher and Strawn 1991; Phelps et al. 2010). Lack of natural production in the indoor RAS could be another reason for fish not attaining market size within the culture period. Natural production has been seen to be very important in the diet of killifish in culture (Tatum and Helton 1977; Ogle and Solangi 1982; Perschbacher and Strawn 1991). Studies by Perschbacher and Strawn (1985) and Phelps et al. (2010) conclude that a positive relationship exists between killifish growth and natural production.

Previous studies have found killifish growth to decrease with increasing stocking density (Trimble et al. 1981; Waas and Strawn 1982; Perschbacher and Strawn 1985; Phelps et al. 2010). Tatum et al. (1982) therefore proposed a manipulation in the stocking density to meet the demands of the prevailing market. In the current study, growth parameters (final weight, weight gain, SGR, and CF) did not differ at the two stocking densities. This study was carried out in the same RAS; hence, fish at both densities shared the same water. Due to this, the presence of any species-specific metabolites that could have built up and inhibited growth to a greater degree at the higher fish density would have been shared between both densities, possibly reducing any difference in the relative growth of fish at the two densities. The presence of such growth inhibitors might have contributed to the overall slower growth in the RAS compared with ponds and static pools.

Survival rates achieved in the study were similar to the rates achieved by Trimble et al. (1981) and Tatum et al. (1979) but was 20–25% higher than that reported by Phelps et al. (2010). This might be due to the relatively large (0.44-g) juveniles used in the study and the differences between static pools and recirculation systems. In the current study juvenile Gulf Killifish culture was performed in the same RAS; hence, the same water quality parameters existed in all tanks. The values from these parameters were the conditions for optimum growth, survival, and condition for Gulf Killifish as seen in Patterson et al. (2012), Phelps et al. (2010), and Green et al. (2010). It can therefore be said that water quality did not affect growth of Gulf Killifish negatively. Unlike outdoor culture systems, the use of indoor RAS in the current study eliminated predation by birds, reptiles, amphibians, and mammals aside the possibility of infesting cultured fish with diseases.

Results from this preliminary study showed that growth of Gulf Killifish in an indoor system is slow even when fed multiple times a day at 9% body weight daily with an expensive high-protein diet. Gulf Killifish cultured in a system without natural productivity show reduced growth. The study showed survival rate of juvenile Gulf Killifish was highest at the lowest density. It could also be seen that growth did not differ at the 2 and 5 fish/L stocking densities. Juvenile Gulf Killifish can be cultured in indoor RAS to a density of 5 fish/L without deteriorating water quality. Results obtained from the growth parameters (Table 2) were similar between the 2 and 5 fish/L in this preliminary study, and indicate that greater densities such as 8, 10, 12, and perhaps 15 individuals/L would be needed to determine a relationship between stocking density and growth of Gulf Killifish in recirculating systems. Further, it might be possible to incorporate these results not only in this species but also other sister species such as Mummichog and Seminole Killifish *F. seminolis*, both species being used as important baitfish along the East Coast and in Florida.

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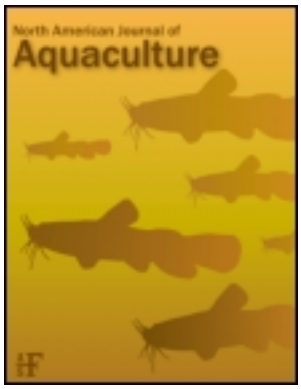

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Studies on the Salinity Tolerance of the Juvenile Dark Sleeper

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COMMUNICATION

Studies on the Salinity Tolerance of the Juvenile Dark Sleeper

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Abstract

Acute and chronic experiments were conducted to evaluate the effect of salinity on survival and growth of the juvenile Dark Sleeper *Odontobutis potamophila*. For the acute experiment, half-lethal concentrations (LC50) of salinity for the juvenile Dark Sleeper after exposures for 12, 24, 48, 72 and 96 h were determined. Mean survival time (MST) and half-survival time (ST50) at salinity of 32‰ were also recorded. For the chronic experiment, a 22-day trial focused on comparing the effects of different salinity levels (0, 2, 4, 6, 8, and 10‰) on survival rate and growth of the juvenile Dark Sleeper. Results revealed that the 96-h LC50 was 13.79‰ salinity. MST and ST50 at 32‰ were 35.4 min and 37.0 min, respectively. In the 22-d trial, survival rate and specific growth rate (SGR) decreased with salinity. Survival rate of groups in 8‰ and 10‰ salinity were significantly lower than that of other groups. The SGR for body length and body weight of the 10‰ group was significantly lower than that of the other groups. This study indicates that the optimal salinity level for satisfactory survival and growth of juvenile Dark Sleeper in cultures should be below 6‰.

Dark Sleeper *Odontobutis potamophila* is a popular and important commercial fish species in China, due to its high value and delicious taste (Sun and Guo 1996). There is a growing interest in developing aquaculture techniques for Dark Sleeper to meet its market demand. A first step in expanding and enhancing the local Dark Sleeper industry is to examine their tolerance of salinity and their environmental needs to provide information for the selection of appropriate sites for Dark Sleeper culture.

Among the environmental factors, salinity is one of the most conspicuous physiological challenges that might influence survival and growth of aquatic organisms (Secor et al. 2000; Castro-Mejía et al. 2011). Rearing fish at optimum salinity reduces the fish's standard metabolic rate, which should provide more energy for growth (Neill and Bryan 1991). Freshwater fish showed higher growth rates at salinity levels higher than 0‰. Kibria et al. (1999) recorded that Silver Perch had maximum growth rates in salinities between 4‰ and 8‰. Martínez-Palacios et al. (2008) indicated that Blacknose Silversides survived a salinity change from 0 to 5‰ without an acclimation period; after acclimation, however, they can survive salinity up to 15‰. Hence, salinity might have a significantly positive effect on growth for many freshwater species (Boeuf and Payan 2001).

Moreover, we can utilize certain salinity of water in prevention and cure of aquatic animal diseases, including saprolegniasis, parasitosis, and bacterial infectious disease. In addition, this study offers scientific support for expanding aquaculture from inland freshwater areas to coastal areas.

The present study aimed to determine the effects of salinity on growth rate and survival of the juvenile Dark Sleeper. Two toxicity experiments were conducted: one acute and one chronic tests of salinity on survival and growth of the juveniles.

METHODS

Juvenile Dark Sleeper source and experimental conditions.— The juvenile Dark Sleeper were obtained by artificial breeding in Shanghai Fisheries Research Institute (Shanghai, China) in April 2011. Prior to the experiment, juveniles were carefully acclimated to rearing conditions for 1 week in an indoor tank (diameter: 80 cm, water volume: 300 L), equipped with a water-recirculating system consisting of two aquarium pumps and a filter tank. The filter tank was filled with biorings and active carbon. Local aquaculture water (salinity, 0.7‰) was recirculated through the filter at a rate of 4 L/h. During acclimatization, fish were fed an equal mixture of brine shrimp nauplii, cladoceransk, and cyclops twice a day (0800 and 1500 hours). At the beginning of the experiments, small and large fish were removed, and 2,000 fish of similar size were selected (body length 17.23 ± 2.06 mm, body weight 106 ± 36 mg, mean ± SD).

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Both the acute and chronic experiments were conducted in fiberglass tanks (diameter: 20 cm, water volume: 25 L). Each tank was aerated continuously with water containing dissolved oxygen maintained at 5.5 mg/L. Water temperature was maintained at 27°C by a water bath throughout the experiment. The photoperiod was 10 h:14 h (dark:light), the light period lasting from 0730 to 1930 hours. Both water sources, local aquaculture water and seawater, were filtered through a 200-μm sieve to remove bivalve larvae and other small organisms possibly present in the water. Filtered local aquaculture water was used as dilution water and for the control. Filtered seawater (salinity, 32‰) was diluted with the filtered freshwater to the various test salinities. Each treatment in both the acute and the chronic experiments had three replicates.

Acute salinity exposure.—The acute experiment, which included two tests, used no food addition. Before the acute experiment, we did a previous trial to determine a plausible range of water salinity. In this previous trial, there was no death of the juvenile Dark Sleeper when the salinity was below 10‰, but nearly all the juveniles died when the salinity was above 15‰. For the first acute test, based on the results of the previous trial, we designed eight salinity levels according to arithmetic series (9, 10, 11, 12, 13, 14, 15, and 16‰). The 9‰ salinity group was used as a control. Groups of 20 juvenile Dark Sleeper were placed in 25-L tanks, each containing 15 L of water at one of the eight salinity levels for 96 h. Trying to avoid stressing the fish, we transported the fish in a small bowl containing water. The salinity was measured by instrument (YSI-30 m; Yellow Springs Instruments, Yellow Springs, Ohio, USA) twice a day. Thus, increasing salinity attributable to evaporation could be detected and adjusted to the value being tested. Death is defined as loss of opercular movement and of reaction to physical stimuli. Any dead fish were removed from the tank in a timely way to keep the water clean. The number of dead fish after 12, 24, 48, 72, and 96 h were recorded. The water in each tank was changed by 90% once a day. Both the salinity and water temperature were kept constant during the water-exchange process. In the second acute test, three groups of 30 juvenile Dark Sleeper were removed to three 25-L tanks containing 15 L of filtered seawater (salinity, 32‰; Wang et al. 2004). Individual survival time was recorded for each group. Mean survival time (MST) and half survival time (ST50) were also calculated.

Chronic salinity exposure.—Groups of 50 juvenile Dark Sleeper were placed in 25-L fiberglass tanks containing 15 L of water at six salinity levels (0, 2, 4, 6, 8, and 10‰). The chronic experiment lasted for 22 d. Each day, 50% of the water in each tank was replaced by water of similar salinity. Fish were fed an equal mixture of brine shrimp nauplii, cladocerans, and cyclops twice a day (0800 and 1500 hours). At each feeding, an excess amount of feed (100 live feed organisms /L) was fed to fish and any uneaten feed was removed 1 h after feeding. Prey densities were standard across all treatments. Considering that uneaten feed might lead to deterioration of water quality, the water was changed 1 h after the 800 hours feeding to keep the fish from adverse effects. Survival and growth of the juvenile Dark Sleeper were recorded. Survival rate was calculated as the percentage of fish surviving to the end of the experiment at each salinity treatment. Growth was described as the daily specific growth rate (SGR) for body length (SGRL) and body weight (SGRL).

Calculation and statistical analysis.—Specific growth rates for body length and body weight were calculated as follows:

$$\mathrm{SGRL} = \{[\ln(L_t) - \ln(L_0)]/t\} \cdot 100$$
$$\mathrm{SGRW} = \{[\ln(W_t) - \ln(W_0)]/t\} \cdot 100$$

where L_t is the body length of the juvenile Dark Sleeper at time t, L_0 is the initial body length, W_t is the wet body weight of the juvenile Dark Sleeper at time t, W_0 is the initial wet body weight, and t is the period of the experiment.

Body length was measured with a vernier caliper (0.01 mm, CR2032, Guanglu, Guilin, China); wet body weight was measured with an electronic balance (0.1 mg, AL204-IC, Mettler-Toledo, Shanghai, China).

Probit analysis (Finney 1977) was used to calculate the 12-, 24-, 48-, 72-, and 96-h LC50 values for the acute test and the 22-d LC50 for the chronic test on the juvenile Dark Sleeper at the studied salinities. Results were presented as mean ± SD of three replicates. One-way analysis of variance (ANOVA) was performed to examine the effects of salinity. If a significant effect was found, Tukey's honestly significant difference post hoc multiple range test was used for multiple comparisons. The significance level for all statistical tests was set at $P < 0.05$. All the statistical analyses were undertaken using SPSS 16.0 (SAS).

RESULTS

Survival rates of the juvenile Dark Sleeper in the 96-h acute experiment are shown in Table 1. The 12-, 24-, 48-, 72-, and

TABLE 1. Survival rate (%) of the juvenile Dark Sleeper exposed to different salinities for various times up to 96 h.

	Hours of exposure				
Salinity (‰)	12	24	48	72	96
9	100	100	100	100	100
10	100	100	100	100	100
11	100	100	100	100	100
12	100	100	100	100	95
13	100	100	100	100	87.5
14	100	97.5	95	77.5	47.5
15	75	60	27.5	10	5
16	37.5	5	0	0	0

TABLE 2. Survival rate (%) and specific growth rates for length (SGRL) and weight (SGRW) of the juvenile Dark Sleeper at different salinities after 22 d of exposure. Values with different lowercase letters in the same column show statistical difference between treatments ($P < 0.05$).

Salinity (‰)	SR (%)	SGRL (%)	SGRW (%)
0	97.33 ± 0.02 z	1.05 ± 0.12 z	3.74 ± 0.37 z
2	95.33 ± 0.03 zy	1.04 ± 0.13 z	3.62 ± 0.35 z
4	90.7 ± 0.08 zy	0.92 ± 0.15 z	3.23 ± 0.28 zy
6	84.67 ± 0.10 y	1.03 ± 0.04 z	3.17 ± 0.14 zy
8	66.7 ± 0.01 x	0.93 ± 0.15 z	3.00 ± 0.49 y
10	35.3 ± 0.11 w	0.68 ± 0.07 y	2.00 ± 0.15 x

96-h LC50 salinity values were 15.84, 15.01, 14.66, 14.34, and 13.79‰, respectively.

The MST and ST50 of juvenile Dark Sleeper at 32‰ salinity were 35.4 min and 37 min, respectively.

Survival and growth performance of the juvenile Dark Sleeper in the 22-d chronic experiment are provided in Table 2. The 22-d LC50 salinity value was 9.73‰. The highest survival rate and best growth (SGRL and SGRW) were found in 0‰ salinity group. Survival rate, SGRL, and SGRW decreased with increasing salinity. No significant difference of survival rate was observed between the 0, 2, and 4‰ salinity groups, and the survival rate of the 6‰ salinity group was not significantly different from the 2‰ and 4‰ groups ($P > 0.05$). The lowest survival rate (35.3%) was found in the 10‰ salinity group. The SGRL of the 10‰ group (0.68%) was significantly lower than that for other salinity groups ($P < 0.05$), but none of the SGRLs of the other groups differed significantly from each other s ($P > 0.05$). No significant difference of SGRW was observed between the 0, 2, 4, and 6‰ groups ($P > 0.05$). As with SGRL, the SGRW of the 10‰ salinity group (2.00%) was significantly lower than that for other salinity groups.

DISCUSSION

In the present study, the results for 96-h LC50 of salinity for the juvenile Dark Sleeper and the MST and ST50 at 32‰ salinity were 35.4 min and 37 min, respectively (Table 1) consistent with previous observations of Grass Carp *Ctenopharyngodon idella* (Li et al. 2007), Darkbarbel Catfish *Pseudobagrus vachelli* (Wang et al. 2004), Snakehead *Channa argus*, and Channel Catfish *Ictalurus punctatus* (Liu 2007), and indicated that several freshwater species were able to tolerate rapid salinity changes.

Lasker and Theilacker (1962) proposed that the effect of salinity on aquatic animals might be a result of several factors, such as the effects of the total osmotic concentration, the incidence and concentration of particular ions, and the availability of oxygen due to the inverse correlation between salinity and oxygen. Hence, salinity can be used to decrease transportation mortality of freshwater fish by reducing osmoregulatory demands (Hattingh et al. 1975). Salinity concentrations between 3‰ and 7‰ are widely used for transportation of freshwater fish, but species-specific requirements should also be taken into account (Hattingh et al. 1975; Urbinati and Carneiro, 2006). The present study showed that the juvenile Dark Sleeper should be transported to stocking sites at 6‰ salinity. The juvenile Dark Sleeper can tolerate a lethal salinity level (32‰) without an acclimation period for up to 35.4 min before mortality occurs. Culturists may be able to use high-salinity baths for a short period (10–30 min) to combat fungal, copepod, or trematode outbreaks, which could make production and restocking programs more efficient and reduce disease (Piper et al. 1982). Although some individuals were able to tolerate full saltwater for up to 35 min, their recovery and the potential long-term effects are unknown, especially if kept in a bath for up to 30 min. This warrants further study.

Morgan and Iwama (1991) reported that energetic costs were lower in an iso-osmotic environment than in hyper- and hypo-osmotic environments and that the energy saving was sufficient to permit increased growth. Therefore, for some aquatic animal species, optimal salinity can minimize osmoregulatory demands and thus increase the amount of energy available for growth (Iwama 1996). However, optimal salinity for growth and survival might differ between species, life stages, and seasons (Lambert et al. 1994). By rearing juvenile fish species at the optimal salinity level, the growth, SGR, and feed conversion rate levels can be maximized, possibly increasing production efficiency and the efficiency of stocking efforts (Altinok and Grizzle 2001).

Boeuf and Payan (2001) demonstrated that most fish species have faster growth rates in salinity between 5‰ and 18‰ than in freshwater. Kojima et al. (1993) also found that salinity improved food conversion and increased growth rate of some freshwater fish species, such as Common Carp *Cyprinus carpio*, Grass Carp, and juvenile Russian Sturgeon *Acipenser gueldenstaedtii*. However, in the present study, survival and growth of the juvenile Dark Sleeper decreased in a high-salinity environment, and the highest survival rate and SGR were observed in freshwater groups. Optimal salinity for the juvenile Dark Sleeper is below 6‰ based on the chronic experiment results. Species-specific differences may explain differences between our results and previous studies. The present study is useful to fish culturists and biologists by providing species-specific salinity ranges for the juvenile Dark Sleeper that increase production and stocking efficiency by decreasing mortality level.

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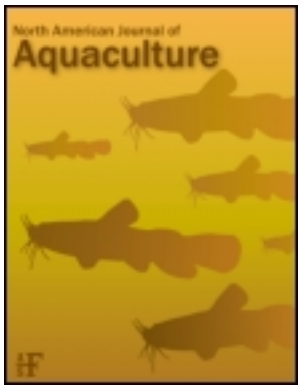

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Antimicrobial Activity of Honey Bee Venom against Select Infectious Fish Pathogens

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COMMUNICATION

Antimicrobial Activity of Honey Bee Venom against Select Infectious Fish Pathogens

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Abstract

In this study, bee venom (BV) isolated from honey bees *Apis mellifera* was assessed for its potential use as an antimicrobial agent against fish pathogenic bacteria. We used three bacterial isolates (*Edwardsiella tarda*, *Vibrio ichthyoenteri*, and *Streptococcus iniae*) that were originally obtained from the gastrointestinal tracts of moribund Olive Flounder *Paralichthys olivaceus*. Bee venom exhibited antibacterial activity against all three infectious fish pathogens. The minimum inhibitory concentration and minimum bactericidal concentration (mean ± SE) of BV were 17.6 ± 2.6 and 34.9 ± 3.4 μg/mL, respectively, against *E. tarda*; 1.76 ± 0.3 and 6.8 ± 2.6 μg/mL against *V. ichthyoenteri*; and 3.49 ± 0.9 and 11 ± 1.6 μg/mL against *S. iniae*. The postantibiotic effect of BV was 5 ± 0.6 h for *E. tarda*, 6 ± 0.3 h for *V. ichthyoenteri*, and 7 ± 0.6 h for *S. iniae*. In addition, the antimicrobial activity of BV was not pH dependent, as inhibition zones were identical at pH levels ranging from 2 to 11. These results indicate that BV inhibits the growth and survival of bacterial strains and that BV may be a useful complementary antimicrobial agent against fish pathogenic bacteria.

Compared with other animal production sectors, aquaculture is highly dynamic and characterized by enormous diversity in both the range of farmed species and the nature of production systems (Walker and Winton 2010). Driven by human population growth coupled with a high demand for seafood, the aquaculture industry has expanded rapidly; hence, this industry is an important component of global food production and contributes significantly to the economic base of many countries around the world (Kim et al. 2011). The emergence and spread of an increasing array of pathogens from anthropogenic management in fish farming were inevitable. From a practical perspective, the search for alternatives to the use of antibiotics, which can result in resistant strains of pathogens in aquaculture, is an important task. Therefore, emphasis has been placed on establishing high standards for aquaculture systems to reduce the potential negative impact of pathogens on fish production. In this context, attention has been focused on identifying antimicrobial agents, including vaccines, as preventive measures against diseases. One such antimicrobial agent is whole bee venom (BV).

Bee venom has been used as a complementary drug for treating various inflammation-associated medical conditions. The anti-inflammatory property of BV has been demonstrated either by (1) inhibiting inducible nitric oxide synthase and tumor necrosis factor-α expression (Han et al. 2006) or (2) the regulation of nitric oxide generation that is dependent on nuclear factor kappa B and activator protein 1 through downregulation of protein kinase C-α-related MEK/ERK (mitogen-activated protein kinases/extracellular signal-regulated kinases) signaling pathways (Lee et al. 2009). Pure venom of honey bees *Apis mellifera* is generally obtained by using a BV collector to electrically stun the honey bees without causing them harm. Impurities are then removed from the collected BV, and the final product is lyophilized. The resultant BV comprises a number of bioactive

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substances, such as melittin, apamin, adolapin, and mast cell degranulating peptide (Son et al. 2007). In addition, it contains biologically active amines (histamine and epinephrine) and a few nonpeptide components, including lipids, carbohydrates, and free amino acids (Lariviere and Melzack 1996). Bee venom is also known to be involved in antibacterial action (Fennell et al. 1968; Perumal Samy et al. 2007; Han et al. 2010).

The antimicrobial activity of BV against fish pathogenic bacteria is not yet known. Therefore, we conducted trials to evaluate BV as an alternative to antimicrobial agents in the fish production industry. This paper is the first to present results of trials assessing the potential use of BV as an antimicrobial agent against fish pathogens.

METHODS

Bacterial species.—Three bacterial isolates—*Edwardsiella tarda*, *Streptococcus iniae*, and *Vibrio ichthyoenteri*—were purchased from the National Fisheries Research and Development Institute in Busan, Korea. Each isolate was originally obtained from the gastrointestinal tracts of moribund Olive Flounder *Paralichthys olivaceus* with bacterial enteritis. The isolates were grown for 18 h on a rotary shaker (100 rotations/min), inoculated into brain–heart infusion (BHI) broth containing 1% sodium chloride (weight/volume), and incubated at 25°C. Brain-heart infusion broth is a rich medium and closely resembles natural foods. The inoculum of each pathogen was prepared from a broth that had been incubated for 4–6 h.

Antimicrobial susceptibility testing.—Testing was performed by using the disk diffusion method on BHI agar as recommended by the Clinical and Laboratory Standards Institute (CLSI 2008). The three species of bacteria were screened for susceptibility to BV diffused on a paper disk (diameter = 8 mm; Advantec, Tokyo, Japan), with a concentration of 50, 25, 10, 2.5, or 1.0 μg of BV per disk. The density of bacterial suspension was adjusted by adding the suspension to a sterile saline tube to match the density at the desired McFarland turbidity of 0.5. Each bacterial isolate was uniformly and aseptically inoculated onto BHI agar plates by the spread plate method using sterile cotton wool. Each BV disk was aseptically placed onto an inoculated agar plate by using sterile forceps. The plates were then incubated at 25°C for 24 h. After incubation, the zones of inhibition (mm) of organisms around the disks were measured and documented. For comparative purposes, bacteria were also inoculated onto plates and then incubated with ampicillin and oxytetracycline (BBL; BD Biosciences, San Jose, California).

Determination of minimum inhibitory and minimum bactericidal concentrations.—Bacterial isolates that were used for determining minimum inhibitory concentrations (MICs) were inoculated overnight. The top of each bacterial colony was touched with a sterile loop, and the growth was transferred to a tube containing sterile saline. The broth culture was incubated until it achieved a McFarland turbidity of 0.5, which resulted in a suspension containing approximately 2×10^6 CFU/mL. Each culture was then spotted with a multipoint inoculator on agar containing serial dilutions of BV. After incubation at 25°C for 24 h, the MIC was identified as the lowest BV concentration that inhibited visible growth of bacteria. For determination of minimum bactericidal concentrations (MBCs), serial dilutions of BV on BHI agar were transferred to plates containing approximately 2×10^6 CFU/mL of each bacterium. The plates were incubated at 25°C for 24 h. After inoculation of a 100-μL suspension into BHI agar and further incubation, the MBC was determined as the lowest concentration of BV that prevented visible growth of bacteria.

Determination of postantibiotic effect.—Postantibiotic effect (PAE) refers to the continued suppression of bacterial growth after the organisms received limited exposure to BV. The bacterial inoculum was prepared by suspending growth in broth. The suspension was diluted to approximately 1×10^8 CFU/mL. For PAE experiments, tubes with broth and a BV concentration equal to 2× the MIC were inoculated. The tubes were then vortexed, and the contents were plated for viability count determinations. Growth controls containing inoculum but no BV were included with each experiment. The inoculated test tubes were placed in a shaking water bath at 25°C for 2 h, after which the BV was removed. The PAE was calculated using the following formula: $\text{PAE} = T - C$ (where T = time required for viability counts at 100× and 400× magnification of each BV-exposed culture to increase by one $\log_{10}$ above the counts observed immediately after dilution; C = the corresponding time for the growth control). For each experiment, viability counts expressed as $\log_{10}$(CFU/mL) were plotted against time. Results were expressed as the means of two separate assays for quality control.

Effect of pH on antimicrobial activity of bee venom.—The effect of pH on the antimicrobial activity of BV was evaluated by incubating BV with different pH buffers for 24 h at room temperature and then neutralizing the mixture to pH 7.0. The pH buffers used were sodium citrate (pH 2.0–5.0), sodium phosphate (pH 6.0–8.0), and glycine–NaOH (pH 9.0–11.0). Determination of BV stability as a function of pH was carried out by measuring the antimicrobial activity.

Statistical analysis.—All data are expressed as mean ± SE from 10 replicates. No further statistical analysis was done.

RESULTS

Table 1 shows the antibacterial activity of BV against the bacterial strains. Bee venom exhibited antibacterial activity with inhibition zones in the range of 10.2 ± 0.9 to 22.6 ± 0.5 mm against *E. tarda*; 10.1 ± 0.3 to 32.5 ± 1.0 mm against *V. ichthyoenteri*; and 9.5 ± 0.3 to 31.3 ± 1.5 mm against *S. iniae*. Ampicillin and oxytetracycline showed activity against *V. ichthyoenteri* with inhibition zones of 14 and 13 mm, respectively.

The MIC and MBC results are shown in Table 2; the MICs indicate the inhibitory potential of BV, while the MBCs show the bactericidal potential of BV against these fish pathogens.

TABLE 1. Antibacterial activity of bee venom (BV), measured as inhibition zones (mm; mean ± SE) in the agar diffusion assay (— = no inhibition zone was found). For comparison, results for ampicillin (AM; 10 μg/disk) and oxytetracycline (OT; 10 μg/disk) are also shown.

	BV concentration (μg/disk)						
Bacterial strain	50	25	10	2.5	1.0	AM	OT
Edwardsiella tarda	22.6 ± 0.5	21.2 ± 1.3	19.6 ± 2.4	10.2 ± 0.9	—	—	15
Vibrio ichthyoenteri	32.5 ± 1.0	29.6 ± 0.9	24.8 ± 1.3	14.5 ± 2.1	10.1 ± 0.3	14	13
Streptococcus iniae	31.3 ± 1.5	27.8 ± 1.6	22.8 ± 0.9	13.1 ± 1.3	9.5 ± 0.3	16	—

The MICs of BV were 17.6 ± 2.6 μg/mL for *E. tarda*, 1.76 ± 0.3 μg/mL for *V. ichthyoenteri*, and 3.49 ± 0.9 μg/mL for *S. iniae*. As expected, the MBCs for BV in our study were higher than the MICs. The MBCs of BV were 34.9 ± 3.4 μg/mL against *E. tarda*, 6.8 ± 2.6 μg/mL against *V. ichthyoenteri*, and 11 ± 1.6 μg/mL against *S. iniae*.

A PAE was demonstrated for each bacterium after a 2-h exposure to BV at a concentration of 2 × the MIC (Table 3). The PAEs were 5 ± 0.6 h for *E. tarda*, 6 ± 0.3 h for *V. ichthyoenteri*, and 7 ± 0.6 h for *S. iniae*.

Table 4 shows the results for the antimicrobial activity of BV in relation to pH. The diameters of inhibition zones in the acidic, neutral, and alkaline pH states were identical. Therefore, the antimicrobial activity of BV was not pH dependent.

DISCUSSION

Although it has been reported that BV has an antibacterial effect on a variety of bacteria (Fennell et al. 1968; Perumal Samy et al. 2007; Han et al. 2010), information about the effects of BV on infectious fish pathogens was previously absent from the published literature. In this study, we demonstrated that BV inhibited the growth of three fish pathogenic bacteria: *E. tarda*, *V. ichthyoenteri*, and *S. iniae*.

The Olive Flounder is an important fish species for both fisheries and aquaculture in Korea. Bacterial enteritis and subsequent abdominal swelling are regarded as serious since bacterial infections cause problems in the early rearing stage of Olive Flounder. *Vibrio ichthyoenteri* is the most common bacterium found in the gastrointestinal flora of marine fish and can cause intestinal necrosis in Olive Flounder larvae; this disease can be devastating, with a high mortality rate (Kim et al. 2004). Several antibiotics have been demonstrated as effective in controlling mortality caused by a variety of bacterial diseases (Walker and Winton 2010). However, the use of antibiotics in aquaculture should be limited due to several practical issues. First, injection of individual fish is time consuming and costly, in addition to producing inevitable handling stress in fish. Second, in the case of bath treatments, antibiotics can be inactivated due to a chemical reaction with cations in seawater. Third, pathogenic organisms are becoming resistant to an ever-increasing number of drugs. In fact, the three fish pathogenic bacteria used in our current study have exhibited multidrug resistance against more than two antibiotics (Kim et al. 2010). Recently, probiotics with live microorganisms have attracted significant interest in aquaculture research (Hjelm et al. 2004; Planas et al. 2006). Based on the results from our study, BV offers a health benefit for fish, and it is considered to have strong antimicrobial effects against Gram-positive and Gram-negative bacteria. Compared with the narrow antimicrobial activity observed for ampicillin and oxytetracycline in this study, the inhibition zones generated by BV were substantially greater.

According to Jung and Kim (2000), amoxicillin and ampicillin had MICs of over 100 μg/mL for *E. tarda*, *V. ichthyoenteri*, and *S. iniae*. Furthermore, two synthetic, broad-spectrum quinolone compounds, flumequine and pefloxacin, showed MICs greater than 50 and 100 μg/mL, respectively, against *E. tarda* and *S. iniae*. In addition, two macrolides-lincosamides-streptogramin B antibiotics, erythromycin and spiramycin, demonstrated MICs of over 100 μg/mL against *E. tarda* and *V. ichthyoenteri*. These results support the assertion that BV possesses better antimicrobial potency than conventional antibiotics. However, it must be noted that measured MIC values can change depending on the medium used in the assay, as the physiology of bacteria and the components of the medium may have further chemical interactions with the antimicrobials (Lunestad and Samuelsen 2001).

TABLE 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC; mean ± SE) of bee venom against fish pathogenic bacteria.

Bacterial strain	MIC (μg/mL)	MBC (μg/mL)
Edwardsiella tarda	17.6 ± 2.6	34.9 ± 3.4
Vibrio ichthyoenteri	1.76 ± 0.3	6.8 ± 2.6
Streptococcus iniae	3.49 ± 0.9	11 ± 1.6

TABLE 3. Postantibiotic effect (PAE; mean ± SE) of bee venom (at 2× the minimum inhibitory concentration [MIC]) against three fish pathogenic bacteria.

Bacterial strain	PAE (h) at 2 × MIC
Edwardsiella tarda	5 ± 0.6
Vibrio ichthyoenteri	6 ± 0.3
Streptococcus iniae	7 ± 0.6

TABLE 4. Effect of pH level on the antimicrobial activity of bee venom (BV; diameter of inhibition zones: + = 10–15 mm; ++ = 16–20 mm; +++ = 21–25 mm). The concentration of BV loaded onto each disk was 10 μg.

	pH				
Bacterial strain	2	4	7	9	11
Edwardsiella tarda	+++	+++	+++	+++	+++
Vibrio ichthyoenteri	+++	+++	+++	+++	+++
Streptococcus iniae	+++	+++	+++	+++	+++

Postantibiotic effect appears to be a property of the antimicrobial agents; the mechanism underlying PAE is not known at this time, but we speculate that DNA synthesis in the three fish pathogenic bacteria might have been suppressed for up to 7 h after exposure to BV. The fish bacteria tested during this period of growth suppression might have shown low metabolic activity (Odenholt et al. 1989) or sequential inhibition of nucleic acids and protein syntheses (Barmada et al. 1993).

The antimicrobial activity of a compound depends on in vitro environmental factors, including pH. For example, caffeic acid (a polyphenol found in fruits) was reported to have different antimicrobial activities at different pH values, which were further related to differing MIC values (Almajano et al. 2007). In contrast to the findings by Almajano et al. (2007), our results demonstrate that the resistance of the tested pathogenic bacteria to BV did not differ under the different pH levels. This is largely due to the fact that the degree of sensitivity of pathogenic bacteria to antimicrobial compounds depends on the composition of the cell wall membrane.

We conclude that BV inhibits the growth of the three tested bacterial strains, suggesting that BV may be a useful antimicrobial agent against fish pathogenic bacteria. A greater understanding of BV as a probiotic is essential if current data are to be used optimally in the aquaculture industry. Follow-up studies will need to evaluate whether BV can be incorporated into fish feed, whether fish will eat the treated feed, whether BV will pass the gut wall and be available to kill systemic bacterial infections, and whether BV helps to control fish mortality. Furthermore, there is considerable interest in combining natural products with other means of controlling mortality, such as vaccines and antibiotics, to assess whether there is an additive effect.

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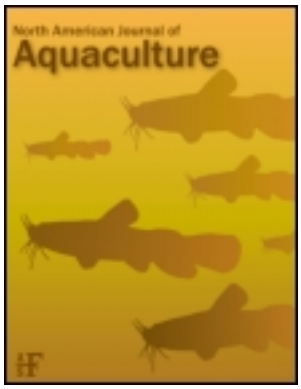

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Physiological Stress Response of Yellow Perch Subjected to Repeated Handlings and Salt Treatments at Different Temperatures

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COMMUNICATON

Physiological Stress Response of Yellow Perch Subjected to Repeated Handlings and Salt Treatments at Different Temperatures

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Abstract

Yellow Perch *Perca flavescens* were subjected to handling stress and salt treatments at different temperatures to determine their physiological changes. Yellow Perch, held at 8–10°C, were divided into three groups with four replicates and subjected to water temperatures of 14, 20, and 26°C to acclimate. Then they were subjected to acute handling twice within separate intervals, in addition to a salt treatment at a salinity of 5‰ for each of the fish groups. Plasma cortisol was used as a stress indicator, and blood samples were taken periodically for plasma cortisol concentration analysis. An increase in plasma cortisol after handling occurred in all groups, but the lowest level of plasma cortisol was in fish subjected to 20°C. We concluded that the optimum water temperature for handling Yellow Perch that results in minimal stress may be 20°C. Salt treatment after handling further stimulated the stress response and increased plasma cortisol levels. Research is needed to identify the optimal salinity to use for Yellow Perch and other fish species when handling fish during common aquaculture practices.

Fish exposed to stressors can exhibit physiological and behavioral changes; these alterations are often referred to as the stress response. The stressors are environmental factors, such as temperature extremes, salinity, water-borne pollutants, social interactions, or aquaculture practices such as handling and sorting (Stratholt et al. 1997). A fish's response to stress represents the perception of an altered state and is characterized by many responses; the primary response includes the release of catecholamines (Reid et al. 1998) and stimulation of the hypothalamic–pituitary–interrenal (HPI) axis to release the corticosteroid hormones into the circulation (Wendelaar Bonga 1997; Barton 2002; Lowe and Davison 2005; Hight et al. 2007; Hosoya et al. 2007; Pankhurst 2011), and secondary stress responses include changes in metabolism, respiration, acid–base status, hydromineral balance, immune function, and cellular responses (Mommsen et al. 1999). When the stressor is acute and short term, the response pattern is stimulatory, and the fish immune response shows an activating phase that specially enhances innate responses; but, if it is chronic, the immune response shows suppressive effects, and therefore the chance of infection may be enhanced (Tort 2011).

The predominant corticosteroid in teleost fishes is cortisol, which has long been used to quantify the stress response (Romero 2002). Under stressful conditions, cortisol and catecholamines are important for several reasons, including central nervous system stimulation and blood glucose elevation; cortisol mobilizes energy to provide metabolic substrates to adjust physiology and behavior aimed at restoring the organism to homeostasis (Rottmann et al. 1992; Vijayan et al. 1997; Mommsen et al. 1999; Barton 2002; Skomal and Mandelman 2012).

Fish immersion in salt water has been theorized to prevent loss of blood ions due to acute stress events (Wedemeyer 1972; Carmichael et al. 1984). While changes in salinity can induce a stress response in fish (Fiol and Kültz 2007), some studies have reported that fish immersion in isotonic saline water after a stressful event can help to reduce the stress and decreasing recovery time (Barton and Peter 1982; Barton and Zitzow 1995; Reubush and Heath 1997). Measurement of the corticosteroid

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stress response after salt treatment has shown conflicting results; some studies recorded an increase in the plasma cortisol level of fish treated with salt (Harrell 1992; Barton and Zitzow 1995), while other studies noted a decrease in cortisol response (Mazik et al. 1991; Carneiro and Urbinati 2001).

The response of fish to typical stressors in aquaculture practices has been extensively examined in the literature (Barton 2002; Acerete et al. 2004; Bertotto et al. 2011). Most of these studies have been carried out on some major aquaculture species, but other species such as Yellow Perch *Perca flavescens* have received less attention. The Yellow Perch is an important potential candidate species for aquaculture in North America and is highly prized as a recreational and food fish. Its culture is limited by its high sensitivity to handling and disturbances in intensive culture conditions (Head and Malison 2000). In order to overcome this limitation, more information on the cortisol stress response in perch is required.

The objective of this study was to develop a greater understanding of the stress response in Yellow Perch through the assessment of the effect of handling stress under different temperatures on the plasma cortisol level in Yellow Perch and to characterize the physiological changes associated with environmental exposures to varying temperatures and salinities.

METHODS

Experimental Fish

Yellow Perch (48 ± 10 g, mean ± SD) were obtained from the Aquaculture Research Center, The Ohio State University South Centers, Piketon, Ohio. Fish were held at a water temperature of 8–10°C in 800-L experimental tanks before transfer. Fish were fed twice with a commercial diet at a rate of 2.5% of average body weight daily. Two weeks prior to experimentation, fish were transferred to twelve 400-L experimental tanks (80 fish/tank) to acclimate them to the experimental system and the target temperatures of 14, 20, and 26°C. Fish were acclimated to the final temperatures by increasing the temperature in each tank gradually until the target temperature was reached for each group, and then the final temperature was maintained.

Experimental Design

First handling stressor at different temperatures.—Three experimental groups (four replicates in each) represented the fish acclimated to the different temperatures: 14, 20, and 26°C (Figure 1). Weighing fish from all groups tank by tank using standard practices was used as the handling stressor. Before weighing the fish, half of the water from the tank was siphoned into an empty tank for holding the weighed fish. After the fish were weighed, they were returned to the original tank by netting them from the second tank and transferring them by means of a net, and then the water was returned to the tank. The same method and timing of the handling stressor were applied to all tanks. Handling time was monitored and recorded for each tank. This procedure was carried out at 1000 hours and the timing for one feeding was adjusted to take place 1 h after handling. During this study, fish were sampled before handling (time = 0, prehandling samples), immediately after handling (i.e., 10 min after handling was completed), and 24 h posthandling (Figure 1).

Salt treatment study.—One hour after the samples were collected for the handling stress test, the remaining fish in experimental groups (two tanks from each experimental group and two other tanks as control) were subjected to a daily salt treatment at a salinity of 5‰ for 6 d. The salt treatment procedure was performed daily by adding 5‰ to treat fish in tanks for 2 h: a preweighed amount of salt for each tank was dissolved in water of the same temperature in a large bucket; water flow in each tank was stopped just before the salt water was added. Half of the salt water was added to each tank at the beginning of the trial, and then the other half was added an hour later. Water flow was restored at the end of 2 h at the rate of 1 L/min. Fish were sampled at 72 and 144 h after the last salt treatment.

Second handling stressor.—After the last samples were collected from the salt treatment and control groups, the fish groups were subjected to handling stress using the same protocol as in the first handling stress trial. Samples were collected immediately after handling and again 24 h later. Salt treatment was performed daily in the same manner as before and the last samples were obtained 144 h after the second handling.

Data Collection and Analysis

Blood sampling.—Fish were quickly captured and immediately placed in a bucket containing water and a lethal dose (400 mg/L) of tricaine methanesulfonate (Syndel Laboratories, Vancouver, British Columbia). Blood samples, collected from the caudal vein using a 5-mL heparinized syringe, were obtained within 2 min of the fish being captured. The blood was then stored on ice, and then plasma was separated by centrifugation (1,000 × *g* for 10 min) at 4°C, removed, and stored in 1.5-mL microcentrifuge tubes at −80°C for subsequent analysis.

Determination of plasma cortisol.—Total plasma cortisol levels were determined using an enzyme-linked immunosorbent assay (ELISA kit; NEOGEN, Lexington, Kentucky) according to the manufacturer's instructions, and plates were read with a BioTek microplate reader (BioTek Instruments, Winooski, Vermont) at an absorbance of 650 nm.

Statistical analysis.—An ANOVA was performed with the SPSS Statistical Package using GLM (SPSS 2004). The Duncan's multiple range test was used for testing mean differences and a *t*-test was used for determining statistical differences between groups at a significance level of $P < 0.05$.

RESULTS

Plasma Cortisol Levels after the First Handling Stressor

The total plasma cortisol concentrations in the prehandling samples from Yellow Perch subjected and acclimated to 20°C and 26°C were approximately two- to threefold higher than in those subjected to 14°C (Table 1).

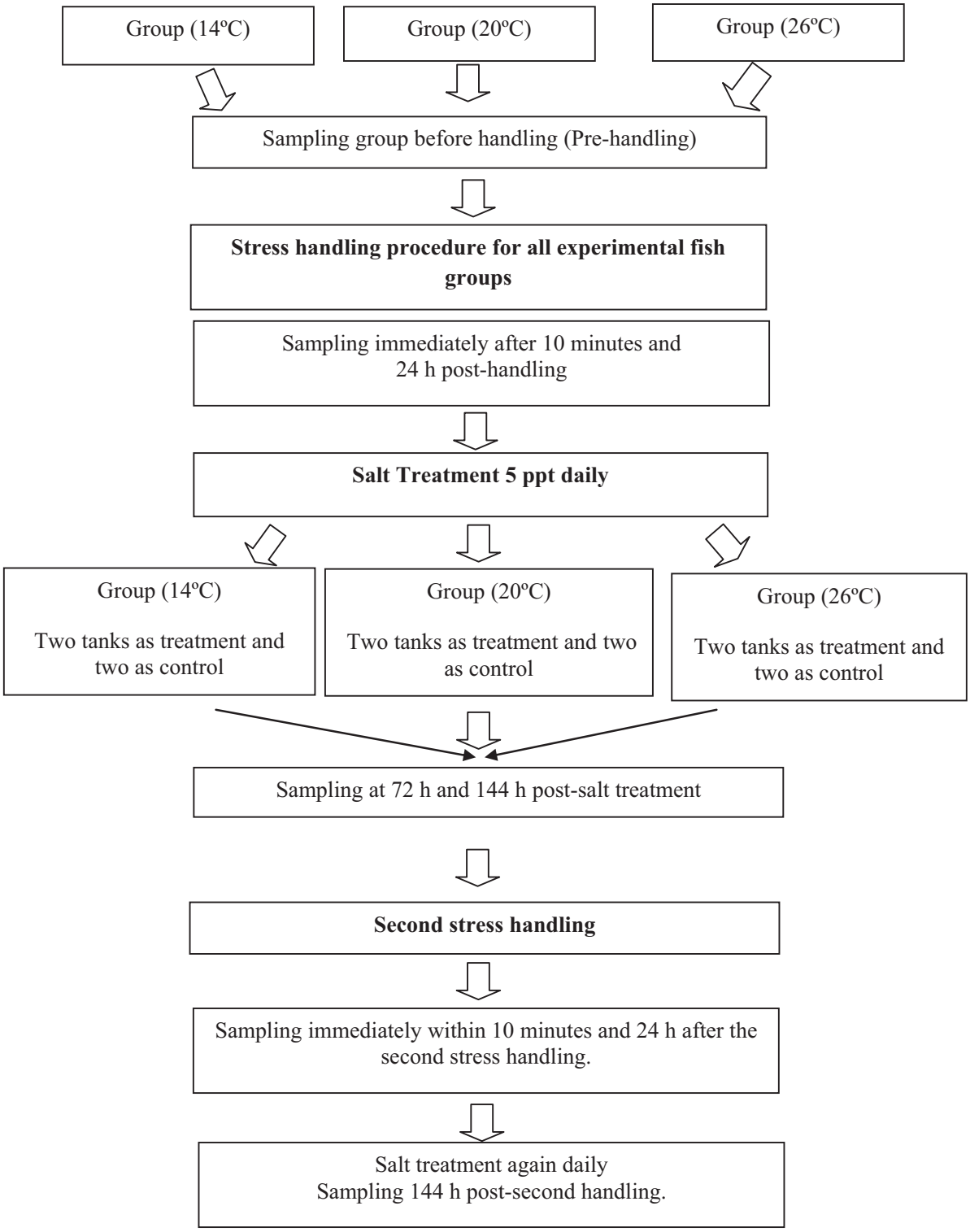

FIGURE 1. Experimental design and sampling schedule used to assess handling stress responses in Yellow Perch. Bold text indicates the times at which the stressors were applied.

TABLE 1. Plasma cortisol concentrations (ng/mL) of plasma in Yellow Perch subjected to handling and salt treatment (ST; 5‰) at different temperatures; values are mean ± SD. Values followed by the same letter are not significantly different from each other ($P < 0.05$).

	Water temperature		
Handling time	14°C	20°C	26°C
Prehandling	33.25 ± 18.6 y	92.0 ± 8.4 z	110.0 ± 51.0 z
Immediately after first handling	169.75 ± 12.5 z	107.75 ± 53.5 y	157.50 ± 13.6 z
24 h after first handling	72.50 ± 45.9	85.70 ± 54.4	78.48 ± 43.6
72 h after first handling with ST	119.75 ± 29.9 zy	79.75 ± 51.9 y	165.50 ± 27.0 z
72 h after first handling with ST: controls	111.75 ± 19.5 y	103.50 ± 45.9 y	184.25 ± 10.2 z
144 h after first handling with ST	128.25 ± 19.1 y	126.00 ± 7.7 y	166.75 ± 21.1 z
144 h after first handling with ST: controls	106.0 ± 37.7	147.25 ± 31.4	107.25 ± 85.9
Immediately after second handling	185.50 ± 7.0	187.25 ± 1.7	189.75 ± 7.9
24 h after second handling with ST	142.50 ± 24.2 zy	116.75 ± 34.7 y	166.00 ± 10.1 z
24 h after second handling with ST: controls	163.50 ± 31.4	168.00 ± 23.1	156.50 ± 24.3
144 h after second handling with ST	110.75 ± 57.9	118.75 ± 47.1	126.50 ± 26.9
144 h after second handling with ST: controls	131.00 ± 40.1	86.75 ± 71.6	92.25 ± 43.7

Plasma cortisol levels increased by approximately 0.5- to 5.0-fold immediately after handling in all fish groups, but increased levels were more prominent in Yellow Perch groups held at 14°C and 26°C than in the group exposed to 20°C (Table 1). No significant differences between the three groups in cortisol levels were detected 24 h after handling. However, cortisol levels were decreased by approximately 50% 24 h after handling in all groups compared with cortisol levels measured immediately (10 min) after handling.

Plasma cortisol levels in the control groups at 72 and 144 h in the daily salt treatment experiment had increased significantly compared with those measured in the groups without salt treatment at 24 h after handling. Also, there was an increase in all treatment groups, except for the plasma cortisol level of the group exposed to 20°C (Table 1).

Plasma Cortisol Levels after the Second Handling Stressor

The highest plasma cortisol levels for all Yellow Perch groups occurred immediately after the second handling, and there was no significant difference between groups. All three groups had nearly similar cortisol levels comparised with all previous cortisol levels. Plasma cortisol levels showed a slight decrease 24 and 144 h after the second handling in all groups, and a significant decrease was recorded in fish exposed to 20°C (Table 1).

General ANOVA of plasma cortisol concentrations for all groups showed high significance with time ($P = 0.000$), temperature ($P = 0.006$), and time versus temperature ($P = 0.020$) (Table 2). The *t*-test values were highly significant ($P = 0.000$) for the comparisons between time and salt, temperature and salt, and temperature and time (Table 3). The general mean determined for temperatures over all times showed that plasma cortisol in the group subjected to 26°C was significantly different from the two other groups, and cortisol levels in fish subjected to 14°C and 20°C were not statistically different (Table 1).No fish mortalities were observed during acclimation or throughout either of the experiments.

TABLE 2. General ANOVA results of plasma cortisol concentrations (ng/mL) in Yellow Perch subjected to three different temperatures and two handling stressors (significance: $P < 0.05$).

Source of variation	df	Mean square	*F*-value	*P*-value
Time	11	12,070.27	8.72	0.000
Temperature	2	7,406.88	5.35	0.006
Time × temperature	22	2,567.32	1.86	0.020
Error	108	1,384.37		
Total	144			

DISCUSSION

Cultured fish in intensive rearing facilities are continuously exposed to management practices, such as handling or transportation, which elicit stress responses (Davis et al. 2002). Repeated or prolonged exposure of fish to common stressors activates the HPI axis leading to increased plasma cortisol and an increased risk of disease (Wendelaar Bonga 1997; Mommsen et al. 1999).

TABLE 3. Analysis (*t*-test) of plasma cortisol concentrations (ng/mL) in Yellow Perch subjected to three different temperatures and two handling stressors (significance: $P < 0.05$).

Pairs compared	df	*t*-value	*P*-value
Time–salt	143	18.80	0.00
Temperature–salt	143	8.46	0.00
Temperature–time	143	15.17	0.00

In the present study, Yellow Perch had relatively high cortisol levels at different water temperatures before handling. The prehandling plasma cortisol levels in the groups held at 20°C and 26°C were two- to threefold higher than in the Yellow Perch exposed to 14°C. After handling, all groups exhibited a one- to fivefold increase in plasma cortisol, which then decreased within 24 h after handling. Other studies have also illustrated that Yellow Perch responded with initial increases in plasma cortisol right after handling, and then decreased gradually (Haukenes and Barton 2004; Hosoya et al. 2007; Haukenes et al. 2008). Acerete et al. (2004) also observed that cortisol levels in Eurasian Perch *P. fluviatilis* increased more than threefold after transportation. Transportation procedures induced rapid elevations in plasma cortisol from 100 to 160 ng/mL within 15–30 min of capture and loading in cultured juvenile Red Drum *Sciaenops ocellatus* (Robertson et al. 1988). Striped Bass *Morone saxatilis* after handling showed a 3.5-fold mean increase in cortisol level up to 400 ng/mL (Cech et al. 1996). In Golden Perch *Macquaria ambigua*, plasma cortisol increased to 240 ng/mL after 30 min of netting and confinement stress (Carragher and Rees 1994).

In this study, Yellow Perch held at 20°C showed the lowest increase in cortisol when first handled, and those held at 26°C and 14°C exhibited significantly higher cortisol levels when handled. This result suggests that Yellow Perch are minimally stressed at a temperature of 20°C. A temperature of 22°C appeared best for growth of Yellow Perch in tank culture systems regardless of the fish stocks (Brown et al. 2002), whereas a temperature of 28°C appeared sufficiently high to represent chronic stress conditions in Yellow Perch (Tidwell et al. 1999). Scott and Crossman (1973) and Brown et al. (2009) reported optimum temperature ranges from 21°C to 24°C for Yellow Perch with an upper lethal limit of 26.5°C for growth. The significantly higher cortisol level in Yellow Perch subjected to handling at14°C may be attributed to the cold water, which could be considered as an additional stress factor other than handling, since the preferred summer temperature of Yellow Perch ranges from 17.6°C to 25°C (Ferguson 1958; Krieger et al. 1983).

There was a significant increase in plasma cortisol from the first handling in Yellow Perch after salt treatment. This is attributed to the increase in water salinity, which is considered to be a stress factor due to alteration of the normal environment. Salinity stimulated the stress response and increased the cortisol level, which may help promote salinity acclimation (Fiol and Kültz 2007; Kammerer et al. 2010). Using saline water (0.5% NaCl) as recovery medium did not attenuate the corticosteroid responses of Walleye *Sander vitreus* to handling, but salt may have allowed the fish to recover more quickly (Barton and Zitzow 1995; Forsberg et al. 2001). Some studies reported that fish immersion in isotonic saline water after a stress event can help to reduce the stress and decrease recovery time (Barton and Peter 1982; Reubush and Heath 1997). In aquaculture practice, aquaculturists usually treat fish with salt after handling to eliminate bacteria and disease. Based on current results, this practice may increase the level of stress at the same time, as indicated by increased plasma cortisol. The salinity of 5‰ may be high for treating Yellow Perch after handling, and research is needed to identify the optimal dosage to treat Yellow Perch and other fish species after handling for common aquaculture practices. Also, lower cortisol levels occurred in groups that were subjected to 20°C during the period of salt treatment, and similar results were observed after the second handling stressor. From this study, we concluded that a water temperature of around 20°C may be desirable for handling Yellow Perch, but salt treatment after handling further stimulates the stress response and increases the circulating cortisol level. Thus, research is needed to identify the optimal salinity to treat Yellow Perch and other fish species after handling during common aquaculture practices.

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