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## Influence of Stress and Dietary Natural-Source Vitamin E on Nonspecific Immunocompetence, Tissue Tocopherol Composition, and Postslaughter Fillet Oxidative Stability in Sunshine Bass

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**Abstract.**—We evaluated the effects of stressor exposure and super-requirement levels of *RRR*- $\alpha$ -tocopheryl acetate (natural-source vitamin E [NSVE]) on production performance and nonspecific immunocompetency of juvenile sunshine bass (female white bass *Morone chrysops*  $\times$  male striped bass *M. saxatilis*). Stressor exposure elicited physiological changes consistent with the generalized stress response, and the magnitude of responses generally mirrored stressor severity. Stressor exposure resulted in lower fillet peroxides and greater aldehydes after short-term frozen storage, whereas increasing dietary NSVE was associated with a nonsignificant reduction in aldehydes after long-term storage. Fillet  $\alpha$ -tocopherol content increased linearly with dietary NSVE, exceeding reported tissue levels achieved with synthetic vitamin E (SYNE). Although we observed no significant immunological effects of stress or dietary NSVE content, sampling time and high individual-to-individual variation probably restricted our ability to resolve statistical significance. Similar results of vitamin E supplementation have previously been reported; however, the effective dietary concentrations we observed for NSVE are low in comparison with values reported for SYNE. The potential of *RRR*- $\alpha$ -tocopheryl acetate as a highly active vitamin E source for aquaculture feeds warrants further evaluation of the relative suitability of NSVE in meeting the demands of optimal aquaculture nutrition.

Many necessary culture practices are stressors to aquatic livestock; thus, physiological stress is a regular, if not constant, condition in aquaculture (Barton and Iwama 1991). Chronic stress results in immunosuppression (Tort et al. 2004) culminating in increased vulnerability to pathogens. Increased susceptibility to infection and high animal density make aquaculture production facilities ideal environments for disease outbreak. Furthermore, stressor exposure before slaughter can negatively affect product quality in terms of texture and organoleptic profile (Poli et al. 2005). Stress-related losses are limiting factors in fish culture, and it is clear that if culture activities are to flourish, these problems must be overcome.

Meeting nutritional requirements is essential for health, but some components of the diet may exert influence beyond their purely nutritional value (Gatlin 2002). Immunostimulative properties of certain dietary constituents may improve the overall robustness of innate disease resistance mechanisms (Erickson et al. 2000), and research to this effect is a growing field in animal science, including aquaculture nutrition (Irianto and Austin 2002). Vitamin E has long been associated with proper immune function in vertebrates, including fishes (Blazer and Wolke 1984; Hilton 1989; Fletcher

1997). As a potent lipid-soluble antioxidant (Bowry and Stocker 1993), vitamin E has received considerable attention in aquaculture nutrition as a candidate “nutriceutical” (Raa 2000) and as a means of enhancing postslaughter organoleptic stability of seafood products (Tocher et al. 2002, 2003; Huang and Huang 2004).

Although most of the tocopherols and tocotrienols have similar in vitro antioxidant activity (Zingg and Azzi 2004),  $\alpha$ -tocopherol is acknowledged as being the most biologically active E vitamin (Hosomi et al. 1997). Of the eight possible stereoisomers of  $\alpha$ -tocopherol, *RRR*- $\alpha$ -tocopherol has the greatest potency in vivo (Burton et al. 1998; Brigelius-Flohé and Traber 1999). Despite the greater efficacy of *RRR*- $\alpha$ -tocopherol, animal feed manufacturers typically use synthetic vitamin E (SYNE; all-*rac*- $\alpha$ -tocopheryl acetate) to meet the vitamin E requirements of livestock. Natural-source vitamin E (NSVE) is a purified source of *RRR*- $\alpha$ -tocopherol (as *RRR*- $\alpha$ -tocopheryl acetate) and has been established as a more effective micronutrient source for sunshine bass (female white bass *Morone chrysops*  $\times$  male striped bass *M. saxatilis*; Trushenski and Kohler 2006). Natural-source vitamin E may also be superior to SYNE in enhancing the immunocompetence and performance of aquatic livestock; however, these effects of NSVE in vivo and postslaughter have not previously been addressed. Accordingly, we evaluated the effects of super-requirement dietary levels of NSVE on production performance, stress tolerance, and

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TABLE 1.—Formulation and proximate composition of experimental feeds fed to sunshine bass (based on Brown and Griffin 1993). Natural-source vitamin E (NSVE) was fed at the minimum required level (22 mg/1X) and in multiples of the minimum. The ETH treatment included ethoxyquin. All values are reported as grams per kilogram of dry matter, except where noted.

Variable	Dietary treatment					
	1X	2X	5X	10X	100X	ETH
<b>Ingredient</b>						
Menhaden meal <sup>a</sup>	100	100	100	100	100	100
Casein (vitamin-free)	350	350	350	350	350	350
Menhaden <sup>b</sup> /tocopherol-stripped canola oil (50:50)	70	70	70	70	70	70
Dextrin	330	330	330	330	330	330
Carboxymethyl cellulose	20	20	20	20	20	20
Vitamin premix <sup>b</sup> (vitamin E-free)	5	5	5	5	5	5
Mineral premix <sup>c</sup>	80	80	80	80	80	80
Cellulose	40	40	40	40	40	40
Choline chloride	5	5	5	5	5	5
NSVE <sup>d</sup> (mg)	22	44	110	220	2200	22
Ethoxyquin (mg)	0	0	0	0	0	300
<b>Proximate Composition</b>						
Dry matter	765	825	831	811	846	789
Protein	443	393	436	437	433	443
Lipid	64	69	67	65	70	65
Ash	75	75	76	75	75	76
$\alpha$ -tocopheryl acetate (mg)	31	40	90	243	1698	59

<sup>a</sup> Omega Protein Corporation, Inc., Houston, Texas.

<sup>b</sup> Formulated to contain the following per 100 g of premix: 76.1115 g of glucose, 8.8000 g of inositol, 6.0914 g of ascorbic acid, 3.3000 g of nicotinic acid, 2.000 g of calcium pantothenate, 0.9091 g of menadione sodium bisulfate complex, 0.8800 g of thiamine hydrochloride, 0.8000 g of pyridoxine hydrochloride, 0.4400 g of riboflavin, 0.3200 g of vitamin A palmitate, 0.2000 g of folic acid, 0.0800 g of cyanocobalamin, 0.0600 g of cholecalciferol, and 0.0080 g of biotin.

<sup>c</sup> Formulated to contain the following per 100 g of premix: 27.55 g of cellulose, 25.74 g of calcium carbonate, 24.00 g of sodium phosphate, 20.00 g of potassium phosphate, 1.20 g of sodium chloride, 0.50 g of magnesium sulfate, 0.20 g of zinc sulfate, 0.20 g of ferrous sulfate, 0.20 g of manganese sulfate, 0.10 g of aluminum chloride, 0.10 g of potassium fluoride, 0.10 g of cupric chloride, 0.05 g of sodium molybdate, 0.04 g of sodium selenite, 0.01 g of potassium iodide, and 0.01 g of cobalt chloride.

<sup>d</sup> Archer Daniels Midland Company, Animal Health and Nutrition Division, Decatur, Illinois.

nonspecific immunocompetency of juvenile sunshine bass.

### Methods

**Diet preparation and analyses.**—Six isocaloric, isonitrogenous, semipurified diets were prepared based on a formulation previously developed for juvenile sunshine bass (Table 1; Brown and Griffin 1993). The feeds were formulated to vary only in antioxidant composition. One control feed was formulated to meet the minimum dietary requirement of sunshine bass as met by NSVE (22 mg of supplemental NSVE/kg of feed; treatment 1X; Trushenski and Kohler 2006). A second control feed (ETH) was formulated to contain 22 mg NSVE/kg feed and 300 mg ethoxyquin/kg feed. Four test feeds were formulated to contain 44, 110, 220, or 2,200 mg NSVE/kg feed (treatments 2X, 5X, 10X, and 100X, respectively). The feeds were processed into 3-mm sinking pellets, dried at ambient temperature, and stored frozen ( $-20^{\circ}\text{C}$ ) throughout the duration of the study. Proximate analyses of triplicate diet samples were conducted according to standard methods for analysis of animal feeds (AOAC International 1995), confirming diet composition (mean  $\pm$

SE) as  $431 \pm 6$  g crude protein/kg,  $67.0 \pm 0.6$  g crude lipid/kg,  $76.0 \pm 0.2$  g ash/kg, and  $811 \pm 7$  g dry matter/kg.

Total  $\alpha$ -tocopheryl acetate concentrations were confirmed via sample saponification–extraction and quantification by high-performance liquid chromatography (HPLC). Sample saponification and extraction procedures were adapted (performance verified with known samples; lipid matrix; 95% recovery of  $\alpha$ -tocopheryl acetate) from Bourgeois and Ciba (1988). Briefly, 2-g diet samples were ground into a fine powder and were saponified for 20 min with 0.5 mL of iso-octane, 7 mL of ascorbic acid solution (0.25 g of ascorbic acid in 60 mL of 5:1 ethanol : distilled water), and 1.5 mL of concentrated potassium hydroxide solution (50 g of KOH in 50 mL of distilled water) under N at  $120^{\circ}\text{C}$ . Samples were centrifuged at  $2,750 \times$  gravity (g) for 10 min at ambient temperature, and the supernatant was filtered by a diatomaceous earth column. The resulting antioxidant extract was evaporated to dryness under  $\text{N}_2$  and resuspended in iso-octane. The resuspended samples were analyzed with a HPLC system and ultraviolet (UV) detector (Shimadzu Corporation, Kyoto, Japan; models LC-10Ai and SPD-

10AV) equipped with a fused-silica column (4  $\mu\text{m}$ , 250.0  $\times$  4.6 mm; Phenomenex, Inc., Torrance, California; Synergi Hydro-RP) according to the following isocratic elution–detection protocol: 75:25 acetonitrile : methanol mobile phase, 2.0-mL/min flow rate, 20-min run time at ambient temperature, and UV-visible detection at 280-nm wavelength. Discrepancies between formulated and actual tocopherol content (Table 1) were attributed to small batch size and the diet manufacturing issues raised by the viscosity of the NSVE product used.

*Experimental design, feeding trial, and experimental stressor exposure.*—A water recirculation system consisting of twenty-four 150-L fiberglass tanks and associated mechanical and biological filtration units was stocked with 18 age-0 sunshine bass per tank (mean  $\pm$  SE = 23.3  $\pm$  0.5 g; mixed sex; produced in-house from captive broodstock). The six dietary treatments were each randomly assigned to four replicate tanks. Fish were fed their assigned feeds daily for a total of 8 weeks; feeding rates were adjusted weekly according to apparent satiation.

Temperature, dissolved oxygen (Yellow Springs Instruments, Inc., Yellow Springs, Ohio; Model 55 oxygen meter), and pH (WTW, Weilheim, Germany; Model PH315i handheld pH meter) were measured daily and maintained at 24.0  $\pm$  0.5°C, 7.7  $\pm$  0.2 mg/L, and 7.5  $\pm$  0.3, respectively. Alkalinity, ammonia-N, nitrite-N, and nitrate-N were measured weekly (Hach Company, Loveland, Colorado; Model DR/2010 spectrophotometer). All water quality attributes were maintained within ranges suitable for sunshine bass culture (Kohler 2000). Photoperiod was maintained at 12 h light : 12 h dark. All methods for culture and husbandry, experimental stressor exposure, euthanasia, and sample collection were conducted under the direction and approval of the Southern Illinois University Institutional Animal Care and Use Committee (protocol number 04-016).

After termination of the feeding trial, surviving fish in each tank were divided into three groups according to experimental stressor exposure: (1) acute, (2) subchronic, and (3) control. To simulate an acute stress event, fish assigned to the first group were chased aggressively with a dip net for 1 min. To simulate a more severe or subchronic stress event, fish in the second group were exposed to the acute stress protocol followed by confinement in a submerged dip net for 1 h. Control groups were not exposed to any experimental stress protocol.

*Sample collection and preparation.*—After experimental stressor exposure, fish were anesthetized in a 3-mg/L aqueous solution of Aquacalm (Syndel International, Ltd., Qualicum Beach, British Columbia).

Control fish were anesthetized immediately, whereas acute and subchronic groups were sampled 1 h after stressor initiation. Once fish were sufficiently sedated, individuals were weighed (nearest 0.1 g) and blood samples were collected from the caudal vasculature in lithium heparin-treated blood collection tubes (Becton, Dickinson and Co., Franklin Lakes, New Jersey; Vacutainer). Whole-blood samples were centrifuged (700  $\times$  g for 10 min at 4°C), and resulting plasma samples were pooled according to dietary–stress experimental group within tanks and stored frozen (–80°C) until subsequent analysis. Immediately after blood collection, fish were euthanized by cranial pitting and then were dissected. Muscle and kidney tissue samples were collected from all individuals, and livers and intraperitoneal (IP) fat masses were removed from control fish and weighed to the nearest 0.1 g. Muscle samples were packaged in sterile polyethylene bags (NASCO, Fort Atkinson, Wisconsin; Whirl-Pak) and stored in the same manner as plasma samples. Pooled renal samples were suspended in ice-cold Leibovitz L-15 medium (BioWhittaker, Walkersville, Maryland) supplemented with heparin sodium (10 units/mL; Acros Organics, Geel, Belgium), HyQ penicillin–streptomycin solution (100  $\mu\text{g}$  of penicillin + 100 units of streptomycin/mL; Hyclone, Logan, Utah), and 2% sterile fetal bovine serum (BioWhittaker), and were held refrigerated (4°C) before macrophage collection via the discontinuous Percoll (Amersham Biosciences, Piscataway, New Jersey) separation method of Garduño and Kay (1994).

*Production performance.*—Survival was calculated for each tank, and percentage weight gain, hepatosomatic index (HSI; [liver weight/whole body weight]  $\times$  100), liposomatic index (LSI; [IP fat weight/whole body weight]  $\times$  100), and feed conversion ratio (FCR; dry matter weight of food/wet weight gained) were calculated per individual and then averaged by tank. After tissue lyophilization and pulverization, muscle tocopherols were extracted according to the two-phase extraction method of Huo et al. (1996) and quantified according to the HPLC methodology outlined above for  $\alpha$ -tocopheryl acetate determination of feed samples. To estimate postslaughter fillet oxidative stability, muscle samples were analyzed for the presence of peroxides and aldehydes with Peroxysafe and Aldesafe colorimetric assay kits (Saftest, Inc., Phoenix, Arizona) shortly after termination of the study (1 month; short term) and after 6 months of frozen storage (long term). All peroxide–aldehyde concentrations were standardized for fillet lipid content (Folch et al. 1957).

*Stress physiology.*—Cortisol was isolated from plasma samples with use of an enzyme immunoassay (DRG International, Inc., Mountainside, New Jersey)

and quantified spectrophotometrically (Thermo Fisher Scientific, Inc., Waltham, Massachusetts; Multiskan Plus) according to the manufacturer's instructions. Plasma glucose concentrations were measured with a human diabetic testing kit (Abbott Laboratories, Abbott Park, Illinois; Freestyle glucose monitoring kit). Plasma osmolality was determined with a vapor pressure osmometer (Wescor, Inc., Logan, Utah; Model 5100C).

**Nonspecific immunity.**—Macrophage monolayer cultures were prepared in 96-well microplates by administering 200  $\mu\text{L}$  of the macrophage-enriched suspensions ( $5 \times 10^3$  viable cells/mL) to each well and incubating for 2 h at ambient temperatures to facilitate cell attachment. Macrophage respiratory burst activity was subsequently measured according to the ferricytochrome-c reduction protocol of Secombes (1990). After addition of a ferricytochrome-c-phorbol myristate acetate solution to trigger respiratory burst, absorbance ( $\lambda = 590$  nm) was measured every 30 s for a total of 1 h (Multiskan Plus). Data from duplicate wells were averaged, and the maximum reaction rate ( $V_{\max}$ ) of superoxide generation was reported as milli-optical density units per minute.

Macrophage bactericidal activity was measured on stock cultures of *Micrococcus lysodeikthus* according to a protocol adapted from Peddie et al. (2002). Macrophage monolayer microplates prepared as previously described were inoculated with stock *M. lysodeikthus* culture in tryptic soy broth (TSB). After incubation at ambient temperature for 0 (T0) or 5 (T5) h, the plates were washed to remove the bacterial suspension, and bactericidal activity was terminated by the addition of 50  $\mu\text{L}$  of distilled water. Aliquots (100  $\mu\text{L}$ ) of TSB were added to the wells, and surviving bacteria (phagocytosed but not lysed) were allowed to proliferate at ambient temperature overnight. Aliquots of MTT solution (3-[4,5-dimethylthiazol]-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, Inc., St. Louis, Missouri) at 5 mg MTT/mL distilled water were added to the wells, and absorbance ( $\lambda = 590$  nm) was determined. Using averages from duplicate wells, bactericidal activity was calculated as  $(\lambda_{T5}/\lambda_{T0}) \times 100$  and reported as percentage bacterial mortality.

Plasma lysozyme activity was estimated with a *M. lysodeikthus* lysis assay based on the spectrophotometric method of Obach et al. (1993) modified for microplate wells. Aliquots (10  $\mu\text{L}$ ) of whole plasma were added to wells containing 190  $\mu\text{L}$  of *M. lysodeikthus* stock culture. Absorbance ( $\lambda = 450$  nm) was measured immediately and every 15 s thereafter for a total of 2 min (Multiskan Plus). Lysozyme activity was calculated from *M. lysodeikthus* lysis ( $V_{\max}$ ; absorbance units/

min) and reported in enzyme units per milliliter of plasma.

**Statistical analyses.**—Although multiple individual fish were sampled from each tank, replicate tanks served as the experimental units for all statistical analyses ( $N = 4$  replicates/treatment). Production performance and fillet tocopherol data collected before experimental stressor procedures were analyzed by one-way analysis of variance (ANOVA) within the mixed-model framework of the Statistical Analysis System version 9.1 to determine whether differences existed among dietary treatment groups. All remaining data were subjected to two-way ANOVA within the mixed-model framework to determine whether differences existed among treatment groups based on all the response variables and to test for interaction among the stressor and dietary treatments. For variables exhibiting significant differences, CONTRAST statements were used to compare means. In all cases, differences were considered significant at  $P$ -values of 0.05 or less.

## Results

We observed no immediate immunosuppressive effects of stress; however, stressor exposure resulted in increased vulnerability of fillet tissue to oxidative degradation. Oxidation product concentrations were significantly altered by stressor exposure after short-term frozen storage (Table 2); subchronic stress was associated with significantly higher aldehyde and lower peroxide concentrations relative to the control. Providing more NSVE in the diet augmented fillet tocopherol content (Table 3)—linearly, in the case of  $\alpha$ -tocopherol (Figure 1). Survival (mean  $\pm$  SE =  $93.4 \pm 1.7\%$ ), weight gain ( $280 \pm 4\%$ ), FCR ( $1.69 \pm 0.02$ ), HSI ( $1.42 \pm 0.03$ ), and LSI ( $2.48 \pm 0.04$ ) were not significantly affected by dietary or stressor treatments (Table 3). No dietary–stressor treatment interactions were observed for any of the production performance characteristics.

Plasma cortisol, glucose, and osmolality were significantly affected by exposure to the experimental stressors (Table 4). Cortisol concentrations increased significantly with stressor severity. Plasma glucose concentrations were higher in stressor-exposed groups than in control groups; however, acute and subchronic groups were not significantly different from one another. Plasma osmolality was significantly reduced in the subchronic group relative to control and acute stressor groups. Diet and diet  $\times$  stress effects on stress physiology were nonsignificant.

No significant differences in lysozyme activity, macrophage respiratory burst, or bactericidal activity were observed with respect to stressor or dietary treatments (Table 5), and no significant interactions

TABLE 2.—Sunshine bass fillet oxidation byproduct concentrations after short- and long-term frozen storage by dietary and stress treatments. Dietary treatments were natural-source vitamin E (NSVE) at the minimum required level (22 mg/kg; 1X) and in multiples of the minimum. The ETH treatment included 300 mg of ethoxyquin/kg of feed and 22 mg NSVE/kg. Least-squares means ( $\pm$ SE) for each treatment factor combination are shown in normal text; means across stress and dietary treatment factors are shown in italics. The *P*-values for each response variable and their interaction are also provided. Means with different letters are significantly different ( $P < 0.05$ ).

Fillet storage duration	Variable	Stress treatment <sup>a</sup>	Dietary treatment					ETH
			1X	2X	5X	10X	100X	
1 month	Peroxides (mEq/kg)	Control	0.50 $\pm$ 0.14	0.47 $\pm$ 0.14	0.28 $\pm$ 0.14	0.51 $\pm$ 0.14	0.54 $\pm$ 0.14	0.52 $\pm$ 0.14
		Subchronic	0.00 $\pm$ 0.14	0.00 $\pm$ 0.14	0.00 $\pm$ 0.14	0.00 $\pm$ 0.14	0.00 $\pm$ 0.14	0.00 $\pm$ 0.14
		Mean	<i>0.25</i>	<i>0.23</i>	<i>0.14</i>	<i>0.25</i>	<i>0.27</i>	<i>0.26</i>
	Aldehydes (mg/kg)	Control	0.00 $\pm$ 0.12	0.06 $\pm$ 0.12	0.04 $\pm$ 0.12	0.00 $\pm$ 0.12	0.00 $\pm$ 0.12	0.00 $\pm$ 0.12
		Subchronic	0.20 $\pm$ 0.12	0.27 $\pm$ 0.12	0.34 $\pm$ 0.12	0.28 $\pm$ 0.12	0.25 $\pm$ 0.12	0.63 $\pm$ 0.12
		Mean	<i>0.10</i>	<i>0.16</i>	<i>0.19</i>	<i>0.14</i>	<i>0.12</i>	<i>0.32</i>
6 months	Peroxides (mEq/kg)	Control	0.00 $\pm$ 0.28	0.00 $\pm$ 0.28	0.00 $\pm$ 0.28	0.00 $\pm$ 0.28	0.00 $\pm$ 0.28	0.00 $\pm$ 0.28
		Subchronic	0.00 $\pm$ 0.28	0.91 $\pm$ 0.28	0.00 $\pm$ 0.28	0.00 $\pm$ 0.28	0.40 $\pm$ 0.28	0.09 $\pm$ 0.28
		Mean	<i>0.00</i>	<i>0.46</i>	<i>0.00</i>	<i>0.00</i>	<i>0.20</i>	<i>0.04</i>
	Aldehydes (mg/kg)	Control	3.09 $\pm$ 0.89	1.39 $\pm$ 0.89	2.48 $\pm$ 0.89	0.96 $\pm$ 0.89	1.58 $\pm$ 0.89	2.03 $\pm$ 0.89
		Subchronic	2.71 $\pm$ 0.89	1.63 $\pm$ 0.89	0.20 $\pm$ 0.89	2.24 $\pm$ 0.89	0.50 $\pm$ 0.89	2.14 $\pm$ 0.89
		Mean	<i>2.90</i>	<i>1.51</i>	<i>1.34</i>	<i>1.60</i>	<i>1.04</i>	<i>2.08</i>

<sup>a</sup> Subchronic stress event involved confinement in a submerged dip net for 1 h.

among the treatments were observed for these variables.

### Discussion

In this study, sunshine bass fillet tocopherol composition tended to reflect dietary NSVE content; significantly increased levels of deposition were associated with the 100X treatment level. Fillet  $\gamma$ -tocopherol concentration was also increased at the highest level of NSVE supplementation, probably because minute amounts of this compound occurred as an impurity in the NSVE product used. Linear regressions of mean fillet  $\alpha$ -tocopherol concentrations (standardized to fillet dry matter) versus as-fed dietary vitamin E concentration revealed diet-to-fillet transfer greater than reported for SYNE in other aquatic species (Stéphan et al. 1995; Ruff et al. 2002; Chaiyapechara et al. 2003). The increased potency of NSVE is associated with superior binding of *RRR*- $\alpha$ -tocopherol to hepatic  $\alpha$ -tocopherol transport protein ( $\alpha$ -TTP) and therefore greater retention of *RRR*- $\alpha$ -tocopherol compared with its racemates or other tocopherol isomers (Stone and Papas 2003). The  $\alpha$ -TTP facilitates selective uptake of *RRR*- $\alpha$ -tocopherol from hepatic circulation and may enhance systemic trafficking and tissue deposition of *RRR*- $\alpha$ -tocopherol (Blatt et al. 2001). Although  $\alpha$ -TTP has not been isolated from a teleost model, apparent patterns of discriminatory tocopherol trafficking in sunshine bass and Atlantic salmon *Salmo salar* (Hamre and Lie 1997) are similar to those observed in mammalian models. It is reasonable to assume the increased biological activity (Trushenski and Kohler 2006) and the high level of tissue retention we

observed for NSVE is related to the affinity of  $\alpha$ -TTP for *RRR*- $\alpha$ -tocopherol.

As the secondary byproducts of oxidation, measurable levels of aldehydes indicate fillet oxidation has surpassed the initial phase of peroxide formation. Thus, elevated aldehyde fillet content among subchronic stressor-exposed fish suggests an advanced state of oxidative degradation after short-term storage. Stressor exposure, particularly preslaughter, has long been associated with reduced flesh quality. Severely stressful harvesting procedures result in mechanical damage (i.e., scaling and bruising), reduced fillet pH and energy content, and rapid onset of rigor (Poli et al. 2005). Our results suggest even comparatively benign procedures can negatively affect oxidative stability of sunshine bass fillets during short-term storage. Tocopherols prevent free radical oxidation of lipids and fatty acids in vivo (Bell et al. 2000); postslaughter, they can reduce rates of oxidation and lengthen shelf life of fresh or frozen fillets (Gatta et al. 2000; Ruff et al. 2002; Hamre et al. 2004). Tissue  $\alpha$ -tocopherol content and oxidative stability have been enhanced via dietary supplementation with SYNE in sunshine bass (Kocabas and Gatlin 1999), rainbow trout *Oncorhynchus mykiss* (Chaiyapechara et al. 2003), European seabass *Dicentrarchus labrax* (Gatta et al. 2000), Atlantic salmon (Hamre et al. 1998, 2004; Scaife et al. 2000), Atlantic halibut *Hippoglossus hippoglossus* (Ruff et al. 2002, 2004), and turbot *Scophthalmus maximus* (Ruff et al. 2003, 2004). Although no significant differences were noted after long-term storage, fillet aldehydes numerically decreased with increasing dietary NSVE but not with ethoxyquin. All fillets were kept frozen through-

TABLE 2.—Extended.

Fillet storage duration	Means	Two-way ANOVA <i>P</i>		
		Diet	Stress	Diet × stress
1 month	0.47 z	0.94	<0.01	0.94
	0.00 y			
	0.02 y	0.54	<0.01	0.50
	0.33 z			
6 months	0.00	0.53	0.17	0.53
	0.23			
	1.92	0.39	0.50	0.48
	1.57			

out the storage period, minimizing overall oxidation and potentially masking the influence of fillet antioxidant content. Had the fillets been exposed to warmer temperatures, freeze-thaw cycles, or both, the magnitude of oxidation and perhaps the protective effect of increased fillet tocopherol would have been more pronounced.

We were unable to resolve any significant immunological effects of stress; however, nonsignificant reductions in bactericidal and lysozyme activities were noted in association with stressor exposure. These reductions in activity agree with the well-established relationship between stress and immunosuppression (Barton and Iwama 1991; Bly et al. 1997; Magnadóttir 2006); the lack of significance we observed is probably an artifact of our sampling protocols. Collection of tissue samples 1 h after stressor exposure allowed for development of the generalized adaptation syndrome, as indicated by the elevated plasma cortisol and glucose concentrations and depressed osmolality (Bar-

ton and Iwama 1991; Mommsen et al. 1999) measured in the experimentally stressed animals. Stress-related immunosuppression may take longer to develop; thus, our sampling window may have been too narrow to capture any immunological perturbations caused by stressors employed.

Super-requirement levels of vitamin E have elicited positive immunological effects in a variety of aquatic species, though supplemental vitamin E has been reported to have little-to-no effect on immune response or disease resistance of sunshine bass (Sealey and Gatlin 2002a, 2002b, 2002c). Elevated levels of vitamin E were accompanied by an increase in phagocytic activity in rainbow trout (Clerton et al. 2001) and gilthead seabream *Sparus aurata* (Mulero et al. 1998; Ortuño et al. 2000). Enhanced lysozyme activity has been reported for malabar grouper *Epinephelus malabaricus* (Lin and Shiau 2005), rainbow trout (Kiron et al. 2004), and Indian major carp *Labeo rohita* (Sahoo and Mukherjee 2002) fed increasing levels of vitamin E. Increased oxidative burst has been reported in association with vitamin E supplementation, but these studies typically observed enhanced responses in conjunction with increased levels of other micro- or macronutrients, such as vitamin C (Mulero et al. 1998; Wahli et al. 1998; Sealey and Gatlin 2002b) or lipid (Lin and Shiau 2005). Statistically significant effects were not reported in all studies, and the variability of the data was often cited as a limitation. We also noted high individual-to-individual variation within our immunological data, which may have obscured patterns of dietary effect. The reported potency of NSVE relative to SYNE suggests that it may possess greater immunomodulatory activity, but experimentation directed at reducing the confounding effects of individual variability is needed to fully address this hypothesis.

Advances in stereochemistry and the analytical capacity to separate and define specific vitamin E isomers and stereoisomers have vastly improved our

TABLE 3.—Production performance and fillet tocopherol content of sunshine bass fed natural-source vitamin E (NSVE) at the minimum required level (22 mg/kg of feed; 1X) and in multiples of the minimum. The ETH treatment included 300 mg of ethoxyquin/kg of feed and 22 mg NSVE/kg. Within a row, means with common letters are not significantly different ( $P > 0.05$ ).

Variable	SE	Dietary treatment					
		1X	2X	5X	10X	100X	ETH
Survival (%)	3.9	90.3	98.6	100.0	87.4	90.0	94.4
Weight gain (%)	9.3	292.2	290.2	284.5	258.0	281.2	272.5
Feed conversion ratio	0.04	1.32	1.35	1.38	1.35	1.45	1.32
Hepatosomatic index	0.07	1.36	1.35	1.48	1.41	1.42	1.49
Liposomatic index	0.21	2.29	2.42	2.57	2.13	3.10	2.34
Fillet $\alpha$ -tocopherol concentration ( $\mu\text{g/g}$ , dry tissue)	70.4	35.8 y	81.7 y	105.0 y	213.0 y	1,148.4 z	126.0 y
Fillet $\gamma$ -tocopherol concentration ( $\mu\text{g/g}$ , dry tissue)	1.1	1.4 y	1.7 y	1.5 y	2.1 y	8.4 z	2.1 y
Fillet $\delta$ -tocopherol concentration ( $\mu\text{g/g}$ , dry tissue)	0.5	0.4	0.5	0.8	0.8	2.5	0.6

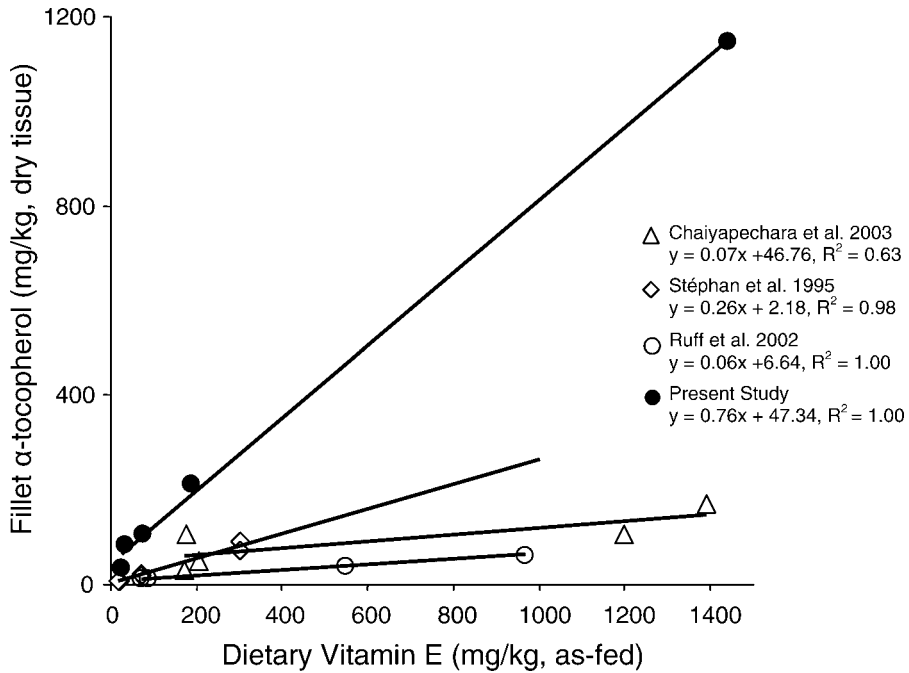


FIGURE 1.—Sunshine bass fillet incorporation of  $\alpha$ -tocopherol according to dietary supplementation with vitamin E. Closed circles represent mean fillet  $\alpha$ -tocopherol concentrations resulting from dietary supplementation with natural-source vitamin E in this study. Open symbols represent mean fillet  $\alpha$ -tocopherol as reported in Stéphan et al. (1995), Ruff et al. (2002), and Chaiyapechara et al. (2003) as a result of synthetic vitamin E supplementation. Lines represent best-fit linear regressions through the respective means.

understanding of the specific roles of these compounds, though many questions remain to be answered. Regardless, the importance of stereoisomeric composition in determining the efficacy of vitamin E

supplements for human and animal nutrition is becoming increasingly clear, and *RRR*- $\alpha$ -tocopherol has emerged as a highly potent micronutrient source. Our results suggest that stress exposure reduces

TABLE 4.—Physiological stress response variables measured in sunshine bass subjected to different dietary and stress treatments. Dietary treatments were vitamin E (NSVE) at the minimum required level (22 mg/kg of feed; 1X) and in multiples of the minimum. The ETH treatment included 300 mg of ethoxyquin/kg feed and 22 mg NSVE/kg. Least-squares means ( $\pm$ SE) for each treatment factor combination are shown in normal text; means across stress and dietary treatment factors are shown in italics. The *P*-values for each response variable and their interaction are also provided. Means with different letters are not significantly different (*P* < 0.05).

Variable	Stress treatment <sup>a</sup>	Dietary treatment						Two-way ANOVA <i>P</i>			
		1X	2X	5X	10X	100X	ETH	Mean	Diet	Stress	Diet $\times$ stress
Plasma cortisol (ng/mL)	Control	36 $\pm$ 31	53 $\pm$ 31	38 $\pm$ 31	70 $\pm$ 31	36 $\pm$ 31	50 $\pm$ 31	47 x	0.74	<0.01	0.51
	Acute	141 $\pm$ 31	161 $\pm$ 31	225 $\pm$ 31	88 $\pm$ 31	170 $\pm$ 31	147 $\pm$ 31	155 y			
	Subchronic	240 $\pm$ 31	222 $\pm$ 31	249 $\pm$ 31	240 $\pm$ 31	218 $\pm$ 31	216 $\pm$ 31	231 z			
	Mean	<i>139</i>	<i>145</i>	<i>171</i>	<i>132</i>	<i>141</i>	<i>138</i>				
Plasma glucose (mg/dL)	Control	128 $\pm$ 28	141 $\pm$ 28	145 $\pm$ 28	192 $\pm$ 28	133 $\pm$ 28	158 $\pm$ 28	149 y	0.76	<0.01	0.63
	Acute	291 $\pm$ 28	342 $\pm$ 28	341 $\pm$ 28	301 $\pm$ 32	364 $\pm$ 28	366 $\pm$ 28	334 z			
	Subchronic	364 $\pm$ 28	369 $\pm$ 28	386 $\pm$ 28	356 $\pm$ 28	345 $\pm$ 28	360 $\pm$ 28	363 z			
	Mean	<i>261</i>	<i>284</i>	<i>290</i>	<i>283</i>	<i>280</i>	<i>294</i>				
Plasma osmolality (mOsm/kg)	Control	390 $\pm$ 21	439 $\pm$ 21	427 $\pm$ 21	426 $\pm$ 21	376 $\pm$ 21	388 $\pm$ 21	408 z	0.39	0.02	0.64
	Acute	390 $\pm$ 21	417 $\pm$ 21	428 $\pm$ 21	382 $\pm$ 24	428 $\pm$ 21	407 $\pm$ 21	408 z			
	Subchronic	371 $\pm$ 21	382 $\pm$ 21	386 $\pm$ 21	358 $\pm$ 21	395 $\pm$ 21	372 $\pm$ 21	377 y			
	Mean	<i>384</i>	<i>412</i>	<i>414</i>	<i>389</i>	<i>400</i>	<i>389</i>				

<sup>a</sup> Acute stress even involved aggressive chasing with a dip net for 1 min. Subchronic stress event involved confinement in a submerged dip net for 1 h.



TABLE 5.—Nonspecific immune variables in sunshine bass subjected to different dietary and stress treatments. Dietary treatments were vitamin E (NSVE) at the minimum required level (22 mg/kg of feed) and in multiples of the minimum. The ETH treatment included 300 mg of ethoxyquin/kg of feed and 22 mg NSVE/kg. Least-squares means ( $\pm$ SE) for each treatment factor combination are shown in normal text; means across stress and dietary treatment factors are shown in italics. The *P*-values for each response variable and their interaction are provided; no difference was noted for any variable.

Variable	Stress treatment <sup>a</sup>	Dietary treatment						Two-way ANOVA <i>P</i>			
		1X	2X	5X	10X	100X	ETH	Mean	Diet	Stress	Diet $\times$ stress
Respiratory burst activity ( $V_{\max}$ milli-optical density units/min)	Control	3.4 $\pm$ 0.9	2.5 $\pm$ 0.8	2.5 $\pm$ 0.8	2.0 $\pm$ 0.8	3.5 $\pm$ 0.8	2.0 $\pm$ 0.8	2.6	0.75	0.98	0.50
	Acute	2.8 $\pm$ 0.8	2.5 $\pm$ 0.8	2.2 $\pm$ 0.8	4.2 $\pm$ 0.8	2.0 $\pm$ 0.8	2.5 $\pm$ 0.8	2.7			
	Subchronic	4.0 $\pm$ 0.8	3.0 $\pm$ 0.8	2.2 $\pm$ 0.8	2.3 $\pm$ 1.1	2.5 $\pm$ 0.8	2.2 $\pm$ 0.8	2.7			
	Mean	<i>3.4</i>	<i>2.7</i>	<i>2.3</i>	<i>2.8</i>	<i>2.7</i>	<i>2.3</i>				
Bactericidal activity (% mortality)	Control	22.0 $\pm$ 6.7	26.5 $\pm$ 5.8	4.8 $\pm$ 5.8	0.0 $\pm$ 5.8	10.2 $\pm$ 5.8	5.2 $\pm$ 5.8	<i>11.4</i>	0.09	0.26	0.25
	Acute	7.3 $\pm$ 6.7	14.5 $\pm$ 5.8	0.5 $\pm$ 5.8	4.8 $\pm$ 5.8	5.5 $\pm$ 5.8	6.5 $\pm$ 5.8	<i>6.5</i>			
	Subchronic	3.0 $\pm$ 5.8	4.8 $\pm$ 5.8	0.0 $\pm$ 5.8	11.0 $\pm$ 8.2	18.0 $\pm$ 5.8	1.5 $\pm$ 5.8	<i>6.4</i>			
	Mean	<i>10.8</i>	<i>15.3</i>	<i>1.8</i>	<i>5.2</i>	<i>11.2</i>	<i>4.4</i>				
Plasma lysozyme activity (enzyme units/mL)	Control	2.8 $\pm$ 2.6	3.0 $\pm$ 2.6	3.2 $\pm$ 2.6	2.8 $\pm$ 2.6	4.0 $\pm$ 2.6	13.7 $\pm$ 2.6	<i>4.9</i>	0.51	0.66	0.42
	Acute	3.5 $\pm$ 2.6	4.0 $\pm$ 2.6	4.8 $\pm$ 2.6	3.6 $\pm$ 2.6	3.7 $\pm$ 2.6	3.6 $\pm$ 2.6	<i>3.8</i>			
	Subchronic	2.6 $\pm$ 2.6	3.2 $\pm$ 2.6	5.7 $\pm$ 2.6	3.7 $\pm$ 2.6	3.5 $\pm$ 2.6	3.2 $\pm$ 2.6	<i>3.6</i>			
	Mean	<i>3.0</i>	<i>3.4</i>	<i>4.5</i>	<i>3.4</i>	<i>3.7</i>	<i>6.8</i>				

<sup>a</sup> Acute stress event involved aggressive chasing with a dip net for 1 min. Subchronic stress event involved confinement in a submerged dip net for 1 h.

postslaughter fillet oxidative stability; however, providing *RRR*- $\alpha$ -tocopherol as NSVE in excess of the dietary requirement may offer some protection against reductions in shelf life of aquaculture products. The particular roles of the vitamin E isomers and stereoisomers, specifically the unique functionality of *RRR*- $\alpha$ -tocopherol, may warrant a reevaluation of vitamin E as a micronutrient and the relative suitability of NSVE and SYNE preparations as dietary supplements. As an antioxidant and mediator of various physiological processes, *RRR*- $\alpha$ -tocopherol merits additional investigation to further elucidate its role in modulating critical biological functions in aquatic livestock.

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