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[Article]

# Evaluation of Natural-Source Vitamin E, RRR-α-Tocopheryl Acetate, as a Micronutrient in Sunshine Bass Feed

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*Abstract*.—A variety of vitamin E isomers can be used in the manufacture of animal feeds, though the efficacy of these sources varies. Our purpose was to evaluate natural-source vitamin E, RRR-α-tocopheryl acetate (NSVE), as an alternative to synthetic vitamin E, all-rac-α-tocopheryl acetate (SYNE), in the diets of sunshine bass (female white bass *Morone chrysops* × male striped bass *M. saxatilis*). Our specific objectives were to evaluate graded levels of NSVE in terms of meeting the vitamin E requirement of sunshine bass and to address the physiological and economic suitability of NSVE by direct comparison with SYNE. Five isocaloric, isonitrogenous, semipurified diets were prepared and supplemented with NSVE at 0, 10, 20, or 30 mg/kg or SYNE at 30 mg/kg. Each dietary treatment was fed to fish in four replicate tanks, each housing 10 juvenile sunshine bass (mean weight = 25.1 g) in a recirculating system. One-way multiple analysis of variance (MANOVA) indicated that significant differences among treatment groups (Wilks' $\lambda = 0.006$) were most greatly attributable to differential survival, growth, food conversion ratio, and liver aldehyde concentration. Fish fed diets containing NSVE at less than 22 mg /kg (as-fed concentration, or 20 mg/kg supplemental dietary treatment) exhibited significantly lower survival (≤65% versus ≥92%) and weight gain (≤359% versus ≥591%), impaired food conversion ratio (2.9 versus ≤1.9), and evidence of heightened liver tissue oxidation (aldehyde, 0.62 nmol/g of tissue versus ≤0.22 nmol/g). Fish fed diets containing an NSVE as-fed concentration of 22 mg/kg or more exhibited performance equivalent to those fed the SYNE control within all parameters. Accordingly, we conclude that NSVE is as effective as SYNE, but at lower concentrations, and that it may be of greater value as a micronutrient source for aquaculture feeds.

Vitamin E is a collective term for the tocopherols and tocotrienols, the primary lipid-soluble antioxidants in living organisms. The E vitamers trap peroxyl radicals and inhibit their deleterious effects, thereby protecting lipids, vitamins, and other compounds from oxidation (Bowry and Stocker 1993). Vitamin E, because of its antioxidant properties and role as an enzyme cofactor, is essential for vertebrate fitness and vigor, maintaining vascular health and reproductive success, and preventing encephalomacia, exudative diathesis, muscular dystrophy, liver necrosis, sterility, and fetal resorption (Halver 2002). In teleosts, vitamin E deficiency, or hypovitaminosis E, has been linked to reduced survival, poor growth, hemolysis and anemia, erythrocyte malformation, ascites, and muscular dystrophy (Halver 2002); dermal discoloration or melanism, exudative diathesis, ceroid deposition, exopthalmia, edema, and lordosis (Gouillou-Coustans and Guillaume 2001); impaired nonspecific immune function, antibody response, and lymphoproliferation (Verlhac et al. 1991; Montero et al. 1998; Clerton et al. 2001; Pearce et al. 2003); and reduced stress resistance (Montero et al. 2001). Like other fat-soluble vitamins, excess vitamin E is not readily excreted from the body and may also be problematic, leading to vitamin E toxicity or hypervitaminosis E. High tocopherol concentrations can inhibit reactions necessary to form nonreactive products from free radicals. Unstable toco-radical intermediates act as pro-oxidants, stimulating oxidation that vitamin E would otherwise prevent (Bowry and Stocker 1993). Vitamin E toxicity has been demonstrated in both poultry and swine (Hale et al. 1995; Sünder et al. 1999), and though less commonly reported in the literature, vitamin E toxicity has been implicated in cases of poor growth, toxic liver reaction, and mortality in fish (Watanabe et al. 1970).

Clearly, proper dietary concentration of vitamin E is essential to animal health, particularly in the case of intensive aquaculture production, where prepared feeds are the sole source of nutrition. The source of vitamin E used in diet manufacture may be equally important; although all E vitamers have similar antioxidant properties, their efficiency in vivo varies. The most biologically active vitamer is α-tocopherol (Hosomi et al. 1997), and of the α-stereoisomers RRR-α-tocopherol, hereafter referred to as natural-source vitamin E (NSVE), possesses the greatest potency (Burton et al. 1998; Brigelius-Flohé and Traber 1999). Animal feed manufacturers typically use all-rac-α-tocopherol (SYNE), a synthetically prepared racemic mixture of

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all possible α-tocopherol stereoisomers. In the case of both NSVE and SYNE, the compounds are usually esterified, forming stable tocopheryl acetates that are more resilient to diet processing and storage. This process is reversed in vivo during gastric hydrolysis, allowing normal uptake and antioxidant function.

The enhanced biological activity of NSVE suggests it is a superior alternative to SYNE; however, this possibility has not previously been addressed in aquaculture nutrition. Therefore, the purpose of this study was to investigate NSVE as an alternative micronutrient source in diets for a cultured teleost, sunshine bass (female white bass *Morone chrysops* × male striped bass *M. saxatilis*). Our specific objectives were to evaluate graded levels of NSVE in terms of meeting the vitamin E requirement of sunshine bass and to evaluate the suitability of NSVE in this context by comparison with synthetic vitamin E.

## Methods

*Diet preparation and analyses*.—Five isocaloric, isonitrogenous, semipurified diets (Table 1) were prepared based on a formulation previously developed for juvenile sunshine bass (Brown et al. 1993). Proximate analyses of duplicate diet samples were conducted according to standard methods for analysis of animal feeds (AOAC 1995), confirming diet composition as 35 ± 0.5% crude protein (mean ± SD), 9.2 ± 0.9% lipid, and 86.7 ± 3.9% dry matter. The four test diets were formulated to contain 0 (vitamin E free), 10, 20, or 30 mg of supplemental RRR-α-tocopheryl acetate per kilogram of diet (treatments N/S0, N10, N20, and N30, respectively). Based on the previously determined requirement for SYNE, the fifth (control) diet was formulated to contain all-rac-α-tocopheryl acetate, 30 mg/kg diet (treatment S30). The diets were processed into 3-mm sinking pellets, dried at ambient temperature, and stored frozen (−20°C) before use.

TABLE 1.—Composition of basal diet prior to vitamin E supplementation (based on Brown et al. 1993).

| Ingredient | Quantity (g/kg dry weight) |
|---|---|
| Menhaden fishmeal | 100 |
| Casein | 350 |
| Tocopherol-stripped soybean oil | 70 |
| Dextrin | 330 |
| Carboxymethyl cellulose | 20 |
| Vitamin premix[a] (vitamin E free) | 5 |
| Mineral premix[b] | 80 |
| Cellulose | 35 |
| Choline chloride (50%) | 10 |

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

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

Total α-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 α-tocopheryl acetate) from Bourgeois and Ciba (1988). Briefly, 2.0-g diet samples were ground into a fine powder; saponified for 20 min with 0.5 mL of iso-octane, 7.0 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 nitrogen at 120°C; and centrifuged at 2,750 × gravity (*g*) for 10 min at ambient temperature. The supernatant obtained was filtered through a diatomaceous earth column. The resulting antioxidant extract was evaporated to dryness under nitrogen and resuspended in 1.0 mL of iso-octane. The resuspended samples were analyzed by HPLC with a UV–visible detector (LC-10Ai, SPD-10AV; Shimadzu Corporation, Kyoto, Japan) equipped with a fused silica column (Develosil-5 μm, 250 × 4.6 mm; Phenomenex, Torrance, California) according to the following elution/detection protocol: 95:5 methanol : distilled water mobile phase, 1.0 mL/min flow rate, 10 min run time at ambient temperature, and UV–visible detection at 284 nm wavelength.

*Experimental design and feeding trial*.—A water recirculation system consisting of 150-L fiberglass tanks and associated mechanical and biological filtration units was stocked with juvenile sunshine bass (weight, 25 ± 7 g; total length, 131 ± 10 mm [mean ± SD]) at a rate of 10 fish per tank. After a 2-week acclimation and feed training period, the five dietary treatments were assigned to four replicate tanks per treatment. The fish were fed the experimental diets twice daily for a total of 16 weeks. Feeding rates were adjusted weekly according to apparent satiation.

Temperature, dissolved oxygen (YSI Model 55 oxygen meter; Yellow Springs Instruments, Yellow Springs, Ohio), and pH (WTW Model PH315i handheld pH meter; WTW, Weilheim, Germany) were measured daily and maintained at 24.0 ± 0.5°C, 7.7 ± 0.2 mg/L, and 7.5 ± 0.3 (mean ± SD), respectively. Ammonia-, nitrite-, and nitrate-nitrogen as well as

alkalinity were measured weekly (Hach DR/2010 spectrophotometer; Hach Company, Loveland, Colorado). All water-quality parameters were maintained within ranges suitable for sunshine bass culture (Kohler 2000). Photoperiod was maintained at a 12 h light : 12 h dark cycle.

*Sample collection and analyses*.—After 16 weeks, fish were anesthetized in a 3.0-mg/L solution of Aquacalm (Syndel International, Inc., Vancouver, Canada), and their total length (nearest mm), weight (nearest 0.1 g), and survival were recorded. Blood samples were collected from the caudal vasculature in EDTA-treated evacuated blood-collection tubes. A small volume of each blood sample was immediately used to determine hematocrit by the method of Houston (1990). The remaining whole-blood samples were centrifuged (700 × *g* for 5 min at 4°C) and the plasma was collected and stored frozen (−80°C) until subsequent analysis. Immediately after blood collection, fish were euthanatized by single pithing, and the livers and intraperitoneal (IP) fat masses were removed, weighed to the nearest 0.1 g, and stored in the same manner as plasma samples.

Plasma osmolality was determined with a vapor pressure osmometer (Model 5100C; Wescor, Inc., Logan, Utah). Whole liver tissue was analyzed for the presence of aldehydes by using the Aldesafe colorimetric assay kit (Saftest, Inc., Tempe, Arizona). Percent weight gain, hepatosomatic index (HSI; 100 · liver weight/whole body weight), percent IP fat (100 · IP fat weight/whole body weight), and food conversion ratio (FCR; weight of food fed/weight gained) were also calculated.

*Statistical analyses*.—All data were subjected to one-way multiple ANOVA (MANOVA) within the general linear model framework of the Statistical Analysis System, version 8.1 (SAS Institute 2004) to determine whether overall differences existed among treatment groups based on all the response parameters and to assess the relative contributions of the individual response parameters to those differences. According to the statistical interpretation outlined by Scheiner (1993), response parameters that contributed most to statistical separation of treatment groups (hereafter referred to as "key parameters") were subjected to individual ANOVA (ANOVA). In all cases, differences were considered significant at critical values (Wilks' $\lambda$ for MANOVA, $P$ for ANOVA) less than 0.05.

During the course of the feeding trial, two replicates in treatments N/S0 and N10 exhibited heavy mortality, presumably as a result of chronic vitamin E deficiency. Although it was possible to collect data for some parameters for these treatments at 12 weeks, these data are not necessarily representative of fish maintained on these diets for a full 16 weeks. Moreover, MANOVA is not robust to missing values, and any experimental units lacking complete data sets must be excluded. Accordingly, the two replicates suffering excessive mortality were eliminated from both treatment N/S0 and N10, reducing the total number of experimental units to 16.

## Results

We found that NVEV at 22 mg/kg diet (as fed) was sufficient to meet the vitamin E requirement of sunshine bass under the present dietary regime and that it was biologically equivalent to a higher inclusion rate of SYNE. Multiple ANOVA indicated significant differences among the treatment groups ($\lambda = 0.006$) based on percent survival, percent weight gain, FCR, hematocrit, plasma osmolality, percent IP fat, and liver aldehyde concentration (Table 2). Further, statistical differences among treatment groups were most greatly attributed to differences in percent survival, percent weight gain, FCR, and liver aldehyde concentrations (absolute value of standardized canonical coefficients $\geq 6$ versus $\leq 4$ for other parameters).

Survival ($\leq 65\%$) and percent weight gain ($\leq 359\%$) were significantly lower in the N/S0 and N10 treatments. For all remaining treatment groups, these parameters were to those of the SYNE control (survival $\geq$ 92%, weight gain $\geq$ 591%). Liver aldehyde concentration and FCR were significantly higher in treatment N/S0 (aldehyde 0.62 nmol/g tissue; FCR = 2.9), with no significant differences among remaining treatments (aldehyde $\leq$ 0.22 nmol/g tissue; FCR $\leq$ 1.9). Fish in treatment N/S0 had a significantly lower percentage of IP fat than those in treatments N20, N30, and S30; however, the difference between N/S0 and N10 was not significant. Treatment S30 had significantly higher percent IP fat than treatments N/S0 and N10 but did not differ significantly from treatments N20 and N30. Hematocrit, plasma osmolality, and HSI did not differ significantly among treatments.

## Discussion

The response parameters used in the present study were selected as indicators of hypo- and hypervitaminosis E to identify a dietary concentration that prevents both conditions in sunshine bass. Four parameters emerged as primary indicators of vitamin E deficiency: reduced survival, reduced weight gain, increased FCR, and elevated liver aldehyde concentration. On the basis of differential performances within these key parameters, we concluded that NSVE at 22 mg/kg diet (as fed) was sufficient to prevent vitamin E deficiency in sunshine bass.

TABLE 2.—Percent survival, % weight gain, food conversion ratio (FCR; weight of food fed/weight gain), liver aldehyde concentration, hepatosomatic index (HSI; 100 · liver weight/whole body weight), hematocrit, % intraperitoneal (IP) fat (100 · IP fat weight/whole body weight), and plasma osmolality of sunshine bass fed different levels and sources of vitamin E (mean ± SD). As-fed tocopheryl acetate concentrations are also reported for the dietary treatments. Values within columns with common letters are not significantly different ($P > 0.05$).

| | | Key variables[d] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Treatment[a] | Vitamin E[b] (mg/kg) | % Survival | % Weight gain | FCR | Aldehydes (nmol/g) | HSI | Hematocrit (% packed volume) | % IP Fat | Plasma osmolality (mosmols/kg) |
| N/S0 | [c] | 50 ± 0 y | 252 ± 121 y | 2.9 ± 2.1 z | 0.62 ± 0.57 z | 2.2 ± 0.2 z | 33 ± 11 z | 3.2 ± 1.5 x | 463 ± 37 z |
| N10 | 18 | 65 ± 0 y | 359 ± 56 y | 1.9 ± 0.2 y | 0.22 ± 0.06 y | 2.9 ± 0.9 z | 37 ± 6 z | 4.4 ± 1.8 xy | 396 ± 73 z |
| N20 | 22 | 98 ± 0 z | 591 ± 94 z | 1.7 ± 0.1 y | 0.17 ± 0.12 y | 2.4 ± 0.1 z | 41 ± 5 z | 5.5 ± 0.2 yz | 438 ± 23 z |
| N30 | 28 | 92 ± 0 z | 617 ± 71 z | 1.6 ± 0.1 y | 0.11 ± 0.05 y | 2.6 ± 0.3 z | 39 ± 4 z | 5.1 ± 0.8 yz | 397 ± 8 z |
| S30 | 29 | 100 ± 0 z | 647 ± 160 z | 1.5 ± 0.0 y | 0.33 ± 0.24 y | 2.6 ± 0.2 z | 41 ± 5 z | 5.9 ± 0.5 z | 421 ± 32 z |

[a]N = natural-source vitamin E (RRR-α-tocopheryl acetate); S = synthetic vitamin E (all-rac-α-tocopheryl acetate); number = mg of vitamin E/kg of diet.
[b]As fed.
[c]Below detection limits.

Survival and weight gain were approximately two times lower among fish fed the vitamin E–free and 10-mg/kg dietary treatments with NSVE, whereas FCR was two times higher in the vitamin E–free dietary treatment than in the other treatments,. Feeding vitamin E–deficient diets caused similar mortality in Atlantic salmon *Salmo salar* (Poston et al. 1976), turbot *Scophthalmus maximus*, gilthead bream *Sparus auratus* (Stephan 1991), and blue tilapia *Oreochromis aureus* (Roem et al. 1990). Reduced weight gain, decreased food conversion efficiency, or both has also been noted with vitamin E deficiency in turbot (Stephan 1991), Korean rockfish *Sebastes schlegeli* (Bai and Lee 1998), blue tilapia (Roem et al. 1990), and hybrid tilapia *O. niloticus* × *O. aureus* (Shiau and Shiau 2001). The decreased survival, growth, and food conversion efficiency observed in the present study agrees well with observations of vitamin E deficiency in other teleost species.

Overt liver necrosis was not observed, but aldehydes—by-products and indicators of lipid peroxidation—were present in liver tissue at substantially higher concentrations in fish fed the vitamin E–free dietary treatment. Cowey et al. (1981) found liver tissue peroxidation to be a good indicator of vitamin E status in fish, and similar observations of hepatic oxidative degradation have been made in vitamin E–deficient channel catfish (Wilson et al. 1984), Atlantic salmon (Poston et al. 1976), European bass *Dicentrarchus labrax*, turbot, gilthead bream (Stephan 1991), rainbow trout *Oncorhynchus mykiss* (Cowey et al. 1983), African sharptooth catfish *Clarias gariepinus* (Baker and Davies 1997), and hybrid tilapia (Shiau and Shiau 2001).

Intraperitoneal fat stores were smaller in fish fed the vitamin E–free and the diets containing10 mg/kg supplemental NSVE. Arguably, this is a positive attribute, because IP fat storage is inversely related to dressout. However, the reduced fat storage we observed is most likely an indirect effect of vitamin E deficiency.

Significant differences in the four key response parameters indicate a clear pattern: fish fed the diet containing no supplemental vitamin E and, in some cases, those fed the supplemental NSVE diet at 10 mg / kg, exhibited inferior performance relative to the those in the other NSVE treatments and the SYNE control. At the end of the trial, remaining fish within the 0 and 10 mg /kg supplemental NSVE treatments exhibited all known clinical signs of hypovitaminosis E in sunshine bass (Kocabas and Gatlin 1999), including emaciation, hyperpigmentation, and greatly reduced consumption and growth rates. Deficiency signs were not observed in any other dietary treatment groups. It is likely that the absolute basal requirement lies between the as-fed concentrations in the diets supplemented with NSVE at 10 and 20 mg/kg (18 and 22 mg/kg, respectively). Regression between 18 mg/kg and 22 mg/kg might identify a theoretical basal requirement; however, interpolation without supporting data are an unnecessary conjecture, considering the narrow range of diets assessed.

Results from all response parameters indicate that NSVE, provided in the diet at 22 mg/kg (as fed), is equivalent to SYNE at 29 mg/kg (as fed). The SYNE requirement reported for sunshine bass is 28–60 mg/kg (Kocabas and Gatlin 1999), whereas commercial feeds used in sunshine bass production typically contain SYNE of at least 90 mg/kg (Nelson and Sons, Inc. 2004). Dietary demand for vitamin E is altered by the lipid content and composition of the diets and body; therefore, precise inclusion rates for animal feeds

should reflect the total potential for lipid oxidation in the feed and in vivo. Given the greater antioxidant potential of NSVE observed in the present and other studies (Burton et al. 1998; Brigelius-Flohé and Traber 1999), we conclude that NSVE is a suitable micronutrient source for aquaculture feeds. Further research to evaluate its biochemical and physiological roles in vivo is warranted.

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