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Induction of vitellogenin production in male tilapia (*Oreochromis mossambicus*) by commercial fish diets

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ABSTRACT

Mozambique tilapia, (*Oreochromis mossambicus*), are a euryhaline teleost and an important biological model species. Captive male tilapia frequently have high levels of the estrogen-induced yolk precursor protein vitellogenin (Vg), a common indicator of exposure to estrogenic compounds. Sex steroids are found in commercial fish diets, but relatively few studies have examined the relationship between commercial diets and Vg production. In a fasting experiment to ascertain a dietary role in male Vg production, plasma Vg was reduced to negligible levels after 2 weeks of fasting, while no change in estrogen receptor (ER) expression was seen. When male tilapia were fed a squid-based diet that replaced the commercial trout diet, plasma Vg was reduced to undetectable levels over 40 days, concomitant with significant reductions in hepatic expression of Vgs A, B, and C, and ERβ, compared with control fish fed commercial trout diet. Female tilapia fed the squid-based for 20 days had no change in these parameters. When male tilapia were fed a defined, soy-based diet, plasma Vg reduced to 20% of levels in fish given either commercial trout diet or a defined, fishmeal-based diet. Overall, results from these studies suggest that estrogens in a commercial trout diet induce vitellogenin production by increasing expression of Vg, but not ER genes in male tilapia.

1. Introduction

Commercial fish feeds utilized in aquaculture often contain fishmeal, which can comprise up to 65% of the diet. Fishmeal is prepared from several sources including whole fish and fish viscera, both of which can contain high levels of sex steroid hormones (Pelissero and Sumpter, 1992). Consequently, these hormones may induce side effects including the production of the yolk precursor protein, vitellogenin (Vg). Vitellogenin is a large phospholipoprotein, present at high levels in the plasma of sexually mature female fish and is produced by the liver in response to estrogens. Under normal circumstances, male fish have little or no circulating Vg; thus, Vg production by male fish has been commonly used as a biomarker for exposure to estrogens or estrogenic compounds (Kaushik et al., 1995; Denslow et al., 1999). While the presence of estrogenic substances in fish feed, including sex steroids and plant-derived phytoestrogens, is well documented, their potential effects on Vg production are often overlooked (Pelissero et al., 1989b; Feist and Schreck, 1990; Matsumoto et al., 2004).

Mozambique tilapia (*Oreochromis mossambicus*) are an important aquaculture species and provide a well-established biological model. Several natural history and physiological characteristics of tilapia make them ideal candidates for endocrine disruptor studies. They are euryhaline, fast growing, resilient and can tolerate a range of temperatures and stresses. Additionally, they are found throughout the tropical and subtropical regions of the world, where agricultural, municipal, and industrial discharges and rainwater run off, exposing them to endocrine disruptors (Okoumassoun et al., 2002; Park et al., 2007). Moreover, many endocrine disruptors mimic estrogens, whose biology is well characterized in the tilapia (Nakamura and Takahashi, 1973; Barry and Grau, 1986; Coward and Bromage, 2000).

The effects of 17β-estradiol (E_2) and many endocrine disruptors are mediated through the estrogen receptor (ER) and the positive relationship between hepatic ER and Vg genes is well-established in many fish species (Pakdel et al., 1991; Flouriot et al., 1996; Bowman et al., 2002; Andreassen et al., 2005). Multiple forms of Vg and at least two ERs exist in teleosts; most common are ERα and ERβ while a second subtype of ERβ (ERβ2/ERγ) exists in some species (Hawkins et al., 2000; Matsubara et al., 2003; Sabo-Attwood et al., 2004; Sawaguchi et al., 2006; Finn and Kristoffersen, 2007). We have identified and sequenced partial cDNAs encoding three different Vgs in tilapia, VgA, VgB and VgC, and identified ERα and ERβ in the tilapia

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liver (Davis et al., 2007). Both female and male tilapia express all three Vgs, with females and E_2-treated males having significantly higher levels of all transcripts than males. In female and E_2-treated male tilapia, hepatic ERα mRNA is expressed predominantly, while ERβ has higher gene expression in male liver (Davis et al., 2008).

Fish species commonly used in endocrine disruptor studies, such as male medaka and killifish, have basal plasma Vg levels that are commonly undetectable. Treatment of these fish with estrogenic compounds increases plasma Vg to mg/mL levels (Chikae et al., 2003; Pait and Nelson, 2003). Male tilapia kept in flow-through tanks at the Hawaii Institute of Marine Biology consistently show basal plasma levels of Vg in the range of 1–5 mg/mL; female levels are approximately 10-fold higher. These relatively high levels of Vg in male tilapia could potentially mask the vitellogenic effects of exposure to exogenous estrogens or endocrine disruptors, reducing efficacy of captive laboratory fish for endocrine disruptor studies. Previously, we have shown that male tilapia injected with o,p'-DDE or heptachlor at 100 μg/g had increased expression of ER and Vg genes, but showed no change in plasma Vg (Davis et al., 2009). Maintenance of fish on commercial diets for aquaculture and biological research is customary, yet the potential implications of diet are often overlooked (Coimbra and Reis-Henriques, 2007; Leanos-Castaneda et al., 2007). Here, through a series of experiments, we aim to determine whether a commercial trout diet may induce Vg production in male fish and to characterize the mechanisms by which this may occur.

2. Materials and methods

2.1. Animals

Mozambique tilapia (*O. mossambicus*), weighing 50–120 g, were tagged with passive integrative transponder tags (PIT tags, Biomark, Inc., Boise, ID, USA) and maintained in 700-L freshwater flow-through tanks under natural photoperiod at the Hawaii Institute of Marine Biology, University of Hawaii. Water temperature was maintained at 26±2 °C. Prior to the experiments, fish were maintained on a commercial trout diet (Silver Cup, Nelson and Sons, Murray, UT) at approximately 2% body weight per day. Experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawaii.

Experiment 1. To determine if diet was the primary factor underlying male Vg production, an experiment was undertaken to assess the effect of fasting on Vg production in male tilapia. Sexually mature males were either fed commercial trout diet at 4% body weight daily (control) or fasted for 4 weeks; plasma and liver were sampled from 6 fish per treatment at weeks 0, 1, 2, and 4. Fish were anesthetized with tricaine methanosulfonate (MS-222, Argent Chemical Laboratories, Redmond, WA, 0.5 g/L) neutralized with NaHCO3 (0.5 g/L), and blood was collected from the caudal vessels using a heparinized syringe (200 U/mL, lithium heparin, Sigma). Plasma was collected by centrifugation at 10,000*g* for 10 min and stored at −80 °C. After bleeding, fish were decapitated and a sample of liver placed in Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) and stored at −80 °C until RNA isolation for analysis of gene expression by rtqRT-PCR, as described below. Plasma Vg levels were estimated by an enzyme-linked immunosorbent assay (ELISA) based on Denslow et al. (1999) and modified by Davis et al. (2007).

Experiment 2. In order to determine how commercial trout diet affects Vg production in male tilapia, a second experiment was done. Male fish were randomly divided into 4 tanks (10 fish/tank) and acclimated for 2 weeks prior to the experiment. They received the following treatments: ethanol-sprayed commercial trout diet plus vehicle injection (control), E_2-sprayed commercial trout diet (E_2-fed), untreated commercial trout diet plus four injections of E_2 at 10 day intervals (E_2-injected), and a diet composed of squid and mixed vegetables (squid-based diet). The control diet and E_2-sprayed diet consisted of commercial trout diet sprayed with ethanol or E_2 (Sigma-Aldrich, St. Louis, MO, USA) dissolved in ethanol (20 mg/kg), respectively. The squid-based diet consisted of equal parts by weight squid and mixed vegetables, including carrots, peas, green beans, lima beans, and corn; the mixture was frozen in gelatin. Fish were fed to apparent satiation once daily.

On days 0, 10, 20, 30, and 40, fish were anesthetized and blood was collected. After blood withdrawal, fish in the E_2-injected group received an intraperitoneal injection of E_2 (5 μg/g, except for day 40) and fish in the control group received a vehicle (corn oil) injection. On day 40, fish were bled, decapitated, and a sample of liver collected for analysis of gene expression, as above.

Experiment 3. A third experiment was carried out to rule out possible inhibitory effects of squid/vegetable mix on Vg production. Sexually mature female tilapia were fed commercial trout diet (control) or squid-based diet daily. Blood samples were taken at days 0, 10 and 20 for plasma Vg measurement; fish were sacrificed on day 20 for measurement of hepatic expression of Vgs A–C and ERs α and β.

Experiment 4. A fourth experiment was conducted using custom-made, isonitrogenous, isocaloric diets to compare Vg production in male tilapia fed a fish meal-based diet versus that in fish fed a soybean meal-based diet. The ingredients and estimated nutrients of the defined feeds are shown in Table 1. Fish were kept in separate tanks and fed commercial trout diet, a custom-designed fish meal-based diet, or a custom-designed fish meal-free diet (soy-based diet), containing soybean meal as the primary protein source (10 fish/treatment). They were fed to apparent satiation once daily. Blood was sampled after 20 days, as described above.

2.2. Real-time quantitative RT-PCR (rtqRT-PCR)

Liver tissue was homogenized in Tri-Reagent and total RNA was isolated using the manufacturer's instructions. Transcripts for Vgs A, B,

Table 1
Composition of defined tilapia diets with and without fishmeal.

Ingredients	Percent in diet	
	With fishmeal	Without fishmeal
Fishmeal (menhaden)	25.0	–
Soybean meal	21.0	46.4
Cottonseed meal	–	12.0
Wheat middlings	16.0	16.0
Corn meal	32.5	16.0
Carboxy-methyl cellulose	3.0	3.0
Dicalcium phosphate	–	1.50
Soybean oil	1.5	3.90
DL-methionine	–	0.20
Vitamin mix[a]	0.5	0.50
Mineral mix[b]	0.5	0.5
Estimated nutrients		
Crude protein (% as is)	32.0	32.0
Crude fat (% as is)	6.0	6.0
Digestible energy (kcal/diet)	2930	2910

[a] Vitamin mix, diluted in cellulose, provides the following in mg/kg diet: vitamin A (500,000 IU/g), 12; vitamin D_3 (1,000,000 IU/g), 2; vitamin K (51%), 20; vitamin E (50%), 200; thiamin, 15; riboflavin, 30; pyridoxine, 20; pantothenate, 200; nicotinic acid, 150; folic acid 2; vitamin B_{12} (0.1%), 2; biotin, 1; inositol, 200; choline chloride, 1000; Stay C (50%, Na-ascorbyl-phosphate) 300.

[b] Mineral mix, diluted in cellulose, provides the following in mg/kg diet: zinc (as ZnSO4·7HO), 150; iron (as $FeSO_4 \cdot 7H_2O$), 40; manganese (as $MnSO_4 \cdot 7H_2O$), 25; copper (as $CuCl_2$), 3; iodine (as Kl), 5; cobalt (as $CoCl_2 \cdot 6H_2O$), selenium (as $NaSeO_3$), 0.1.