BIOLOGY OF REPRODUCTION **77,** 614–625 (2007)
Published online before print 16 May 2007.
DOI 10.1095/biolreprod.107.060947

Induction of Three Vitellogenins by 17beta-Estradiol with Concurrent Inhibition of the Growth Hormone-Insulin-Like Growth Factor 1 Axis in a Euryhaline Teleost, the Tilapia (*Oreochromis mossambicus*)[1]

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ABSTRACT

The objective of the present study was to utilize the male Mozambique tilapia (*Oreochromis mossambicus*) as a model for examining the molecular mechanisms that mediate the physiological transition between somatic and gonadal growth in female teleost fish, and in vertebrates in general. Partial cDNAs that encode multiple forms of vitellogenin (Vtg), which is the major precursor of yolk proteins, were cloned from estrogen-treated males and utilized to develop real-time quantitative RT-PCR assays, which were supplemented by an assay for Vtg immunoreactivity in the plasma. Alignment analyses of the amino acid sequences deduced from the *vtg* cDNAs revealed three distinct tilapia Vtgs, which were categorized as Aa-, Ab-, and C-type Vtgs. A single injection of male tilapias with 17beta-estradiol (E_2) at 5 μg/g body weight significantly increased the plasma E_2 and hepatic levels of all three *vtg* transcripts within 1 day. Plasma E_2 levels declined after 3 days, whereas the plasma Vtg immunoreactivity and hepatic levels of the three *vtg* transcripts continued to increase. Hepatic expression of the *estrogen receptor* (*esr*) *1* gene, but not the *esr2* gene, also increased markedly 1 day after E_2 injection and remained elevated for 5 days. While plasma growth hormone (Gh) levels were unaffected, hepatic expression of transcripts that encoded the Gh receptor and insulin-like growth factor 1 (Igf1) was suppressed by E_2, as were the plasma Igf1 levels. These results clearly suggest a distinct negative interplay between the growth and reproductive axes at the molecular level of key hepatic regulatory pathways involved in the control of energy utilization by gonadal and somatic growth processes.

estradiol, estrogen receptor, growth hormone, insulin-like growth factor, vitellogenin

INTRODUCTION

Studies of a number of mammals indicate a close interdependence among the factors that regulate reproduction and growth [1–3]. This interdependence involves interactions between estrogens and multiple growth peptides and with their receptors. While somatic and gonadal growth processes generally compete for energy in mammals and vertebrates, relatively little is known about the regulatory mechanisms that mediate this interaction [3, 4]. The present study utilized estrogen-treated male Mozambique tilapia *Oreochromis mossambicus* (om), which is a teleost fish, as a model for examining the molecular mechanisms that govern the physiological transition between somatic and gonadal growth in females. This euryhaline species is a well-established model for studies of fish reproduction, growth, and development [5]. The physiology of the tilapia growth hormone (Gh)-insulin-like growth factor 1 (Igf1) axis has been studied extensively [6–8], and the female reproductive system has been investigated with respect to estrogen receptors (Esr) [9–11] and vitellogenin (Vtg), which is the major precursor of yolk proteins [12–16].

Vtgs are synthesized and released by the liver in response to endogenous 17β-estradiol (E_2). They are transported to the ovary through the circulatory system and are specifically incorporated into growing oocytes via receptor-mediated endocytosis, before being cleaved into yolk proteins. In teleost fishes, these yolk proteins can include lipovitellin (Lv), phosvitin (Pv), various Lv-Pv complexes, β′ component (β′-c), and another small yolk proteins derived from the C-terminus of Vtg [17]. The existence of multiple forms of Vtg has been reported in several teleost species [17–19].

Recently, Finn and Kristofferson [20] have proposed a new Vtg nomenclature based on phylogenetic analysis of all known vertebrate Vtgs. Vtgs that possess a complete yolk protein domain structure (NH_2-Lv heavy chain [LvH]-Pv-Lv light chain [LvL]-β′c-C-terminal peptide-COOH) are known as "complete" Vtgs. According to Finn and Kristofferson [20], the complete Vtgs of advanced teleosts (*Acanthomorpha*) are derived from the same ancestral form of teleost Vtg (VtgA) and can be divided into two major types, VtgAa and VtgAb (previously VtgA and VtgB [18]). Each type of complete Vtg is selectively proteolyzed into its respective yolk products, which play disparate roles in oocyte hydration and embryonic and larval nutrition [17, 20–22]. The "incomplete" C-type of Vtg (VtgC) lacks the Pv, β′-c, and C-terminal yolk protein domains (NH_2-LvH-LvL-COOH) and is derived from a different ancestral form of vertebrate Vtg [20]. The biochemical features of VtgC include a lower content of phosphorus or serine residues and a considerably lower molecular mass than either VtgAa or VtgAb. The exact physiological functions of VtgC remain to be clarified [17].

[1]Supported in part by an EPA STAR fellowship (FP91642801) to L.K.D., and by grants from the National Science Foundation (IBN 04-17250, IOB 05-17769, OISE 04-36347), US Department of Agriculture (2005-35206-15285), and the Pauley Foundation Summer Program 2004 and 2005 to E.G.G.
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Received: 6 March 2007.
First decision: 2 April 2007.
Accepted: 16 May 2007.

ISSN: 0006-3363. http://www.biolreprod.org

All three types of *vtg* transcript and/or their corresponding protein products have been identified, characterized, and classified in several teleost species [21, 23]. Earlier, two distinct Vtg proteins, which appeared to be complete and incomplete (Pv-less) forms of Vtg, were purified and characterized in tilapia (genus: *Oreochromis*) [12–15]. Nevertheless, in a preliminary study using cloning strategies to detect the expression of three distinct teleost *vtg* genes [21, 23], we obtained evidence for the presence of three different Vtgs (om VtgAa, om VtgAb, and om VtgC) in Mozambique tilapia (Hiramatsu and Davis, unpublished data).

It is well established that teleost Vtgs are induced by E_2 both in vivo and in vitro, and that this induction correlates with increased Esr levels [24–26]. It is apparent that at least two Esr subtypes exist in numerous teleost species [9, 27–29]. In Mozambique tilapia, *esr1* and *esr2* in the brain are differentially regulated by temperature, developmental period, and sex steroids, which suggests that these genes play disparate roles in sexual differentiation and development [10, 11]. Sabo-Attwood et al. [27] have reported that, in largemouth bass, three different *esr* isotypes are regulated differentially among tissues, temporally and in response to E_2. However, little is known about Esr subtype involvement in E_2 stimulation of Vtg production.

The Gh/Igf1 axis is central to the control of growth in vertebrates and is likely to be central to the transition between somatic and gonadal growth. Igf1, which is the primary mitogenic factor in vertebrates, is produced primarily in the liver in response to Gh, which acts through its receptor (Ghr) to initiate a signaling cascade [30]. In addition to promoting somatic growth, Gh and Igf1 play important roles in fish development and reproduction. Several studies have shown that Igf1 is essential for final oocyte maturation [31–33], and in Chinook salmon, growth is the major factor influencing the onset of male sexual maturation [34]. The importance of the Gh/Igf1 axis is further supported by the presence of receptors for these hormones in gonadal tissue [35–38].

In order to gain a better understanding of the interaction between the molecular regulatory mechanisms of reproduction and growth, we first identified and sequenced the partial cDNAs that encode the three different forms of Vtg in Mozambique tilapia. We then examined the effects of E_2 treatment of male fish on both the plasma levels of E_2, Vtg, Gh, and Igf1 and the hepatic expression levels of transcripts for *esr1*, *esr2*, *ghr*, *igf1*, and the three forms of *vtg*. The results presented herein indicate that E_2 stimulates Vtg production by up-regulating the expression of all three *vtg* genes in concert with the activation of *esr1* transcription, while clearly suppressing the Gh/Igf1 axis. These results provide valuable insights into the molecular mechanisms whereby E_2 regulates the transition between somatic and gonadal growth in an important vertebrate model species.

MATERIALS AND METHODS

Fish Sampling and Hormone Treatment

All experiments involving live fish were conducted in accordance to the principles and procedures approved by the Institutional Animal Care and Use Committees of North Carolina State University and the University of Hawaii.

For the cloning of *vtg* cDNAs, adult male Mozambique tilapia (weight 80–100 g) were held in a 900-L freshwater recirculation system maintained at 22–23°C under a constant 14L:10D cycle at the Aquatic Research Laboratory of the North Carolina State University. Fish were administered an i.p. injection of E_2 (5 μg/g body weight [BW]) at weekly intervals for 2 wk. One day after the third injection, liver samples were dissected from the fish, immediately frozen in liquid nitrogen, and stored at −80°C until use.

In order to examine the effects of E_2 treatment on gene and protein expression, male tilapia (60–120 g BW) were maintained in 700-L freshwater flow-through tanks under a natural photoperiod at the Hawaii Institute of Marine Biology, University of Hawaii. The water temperature was maintained at 25 ± 2°C. Prior to the experiment, the fish were fed a maintenance diet of Trout Chow (Silver Cup; Nelson and Sons, Murray, UT) once daily. Fish were not fed during the course of E_2 induction.

Fifty-six male tilapia were administered an i.p. injection of E_2 dissolved in vegetable oil (5 μg/g BW) or vehicle alone. Before injection, the fish were anesthetized in a bath that contained 0.5 g/L tricaine methanosulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA) neutralized with $NaHCO_3$ (0.5 g/L). Blood was collected on Days 1, 2, 3, and 5 from the caudal vessels using a heparinized syringe (200 U/ml lithium heparin; Sigma). Plasma was isolated by centrifugation of the blood at $10\,000 \times g$ for 10 min and stored at −80°C until analysis of the E_2, Gh, Igf1, and Vtg levels. After blood sampling, the fish ($n = 7$ on each sampling day) were decapitated and liver tissues were placed in TriReagent (Molecular Research Center, Cincinnati, OH) and stored following the protocol provided by the manufacturer until analysis of mRNA transcript levels. Untreated control males ($n = 8$) were sampled similarly for comparison with vehicle-injected and E_2-injected fish.

Molecular Cloning of cDNA Fragments Encoding Tilapia Vitellogenins

Total RNA samples were extracted from the livers of E_2-induced tilapia using TRIzol (Invitrogen, San Diego, CA), and reverse-transcription was conducted using the Superscript First Strand Synthesis System (Invitrogen), to produce template cDNAs for PCR, as described previously [39]. Degenerate PCR primers were designed to target highly conserved regions of the teleost *vtg* sequences available from the National Center for Biotechnology Information (NCBI) database [40]. Only one degenerate primer pair (VtgA/B primer set) was used for the cloning of both the om *vtgaa* and om *vtgab* cDNA fragments: The forward (VtgA/BF) and reverse (VtgA/BR) primers were 5′-CARGT-NYTNGCNCARGAYTG-3′ and 5′-GCAYTCNSWNGCRTCNCKRC-3′, respectively. Two sets of degenerate primer pairs were used for the cloning of the partial om *vtgc* cDNAs (VtgC primer sets). The forward primers (VtgCF1, 5′-GGTRAAYTWTGGRCKKGGSAWRCCAAA-3′; and VtgCF2, 5′-CAR-CACTTCAGTSCYTTCAA-3′) were each used with a single reverse primer (VtgCR, 5′-TACACCAGAGAGCCRTAGGWAAG-3′). PCR was carried out as described previously [39], with the following exceptions. For the VtgA/B primer set, PCR was conducted as follows: denaturation at 94°C for 45 sec, annealing at 55°C for 90 sec, and extension at 72°C for 45 sec for a total of 30 cycles. The PCR thermal parameters for the VtgC primer sets were: denaturation at 94°C for 60 sec, annealing at 55°C for 60 sec, and extension at 68°C for 60 sec for a total of 30 cycles. The resulting PCR products were cloned into the pCRII vector using the TA Cloning Kit Dual Promoter according to the protocol provided by the manufacturer (Version F; Invitrogen). Positive transformants that contained cloned inserts of the expected size (~465 bp for VtgA/B, tentatively termed the *vtga/b* clones; ~610 bp for VtgCF2 and VtgCR, tentatively termed the *vtgc1* clones; and ~1220 bp for VtgCF1 and VtgCR, tentatively termed the *vtgc2* clones) were subcultured, extracted of plasmid DNA as described previously [39], and then unidirectionally sequenced at the University of Chicago Cancer Research Center DNA Sequencing Facility. The resulting sequences were compared with sequences obtained from primary publications or from NCBI using BLAST searches [41] and used to design *vtg* gene-specific primers for use in real-time quantitative RT-PCR (see below). Alignment of the *vtg* sequences returned by BLAST was performed manually using the MacVector software (version 7.0; Oxford Molecular Ltd., Madison, WI) and the CLUSTAL W algorithm [42]. To determine degrees of homology, the following parameters were utilized: the pairwise alignment parameters of Open Gap Penalty = 10.0, Extended Gap Penalty = 0.1, and Similarity Matrix = blosum; and the multiple alignment parameters of Open Gap Penalty = 10.0, Extended Gap Penalty = 0.1, Delay Divergent = 40%, Similarity Matrix = blosum. Similarity discounts conservative substitutions within the following amino acid (aa) groups: basic, acidic, uncharged polar, and nonpolar (no substitutions allowed for cysteine).

Radioimmunoassays

Plasma E_2 levels were estimated using the ImmuChem Double Antibody 17β-E_2 RIA kit (MP Biomedical, Costa Mesa, CA) with the following modifications. The E_2 standards were prepared by dissolving E_2 (Sigma) in 100% ethanol (1 mg/ml) and then diluted to the desired concentrations with assay buffer (0.01 M sodium phosphate, 1% BSA, 0.1% Triton-X, 0.01% sodium azide; all reagents from Sigma). Plasma E_2 was extracted with ether as follows: 2 ml of ethyl ether (Sigma) was added to 50 μl of plasma diluted 1:1 in

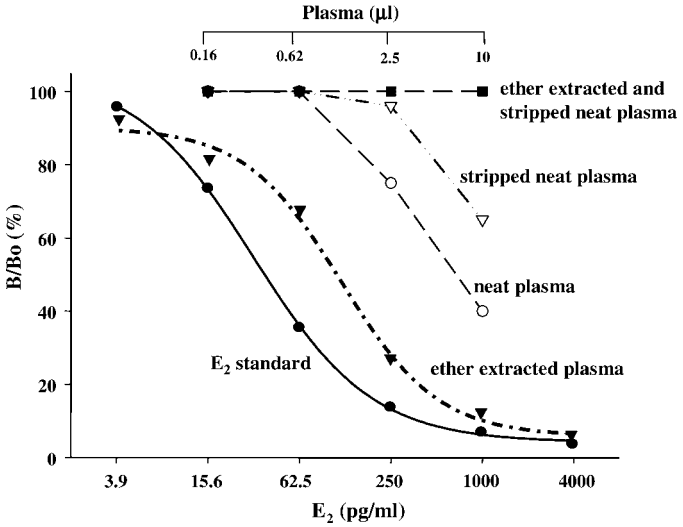

FIG. 1. Validation of E_2 radioimmunoassay for tilapia plasma. Both unextracted (labeled as neat in figure) and ether-extracted plasma samples produced displacement curves that paralleled those of the E_2 standards. When stripped with charcoal, ether-extracted plasma showed no cross-reactivity, whereas charcoal-stripped unextracted plasma retained some cross-reactivity. Each point represents the mean of duplicate determinations.

distilled water, the samples were vortexed, frozen at −80°C for 10 min, and the aqueous organic layer was decanted. The ether extract was evaporated to dryness in a water bath at 40°C for 1 h, and then placed under nitrogen for 5 min to ensure complete evaporation. Extracts were then reconstituted with 50 µl of assay buffer. All tubes received 50 µl of E_2 standards or plasma extracts, 250 µl of ^{125}I-E_2, and 250 µl of anti-E_2 antibody (provided with the kit). The tubes were incubated at 37°C for 90 min. Following incubation, 250 µl of precipitant solution (provided with the kit) was added to each tube and tubes were then centrifuged at 1000 × *g* at 4°C for 20 min. The supernatant was aspirated, and the radioactivity levels of the precipitates were counted using a gamma counter (Cobra II; Packard, Meriden, CT).

The validity of the assay was assessed from the parallel displacement curves obtained with serial dilutions of plasma samples and stripped plasma. An aliquot of pooled plasma from male tilapia was stripped by incubation at room temperature for 15 min with 2% w/v activated charcoal (Sigma), followed by centrifugation at 10 000 × *g* for 5 min, and the supernatant was collected as stripped plasma. As shown in Figure 1, both unextracted and ether-extracted plasma showed parallelism with the standard curve. Unextracted plasma that was charcoal-stripped retained some cross-reactivity, whereas no cross-reaction was seen for plasma that was charcoal-stripped and ether-extracted. The intraassay and interassay coefficients of variation were 10% (n = 5) and 11.8% (n = 5), respectively.

Plasma Gh levels were measured using a homologous radioimmunoassay described previously [43], and Igf1 levels were measured using a commercially available kit (GroPep, Adelaide, Australia), as modified by Shimizu et al. [44].

Enzyme-Linked Immunosorbent Assay (ELISA) for Vitellogenin

Plasma Vtg levels were estimated by ELISA conducted in 96-well plates, as described previously [45]. Briefly, diluted plasma (50 µl) was added to each well and incubated overnight at 4°C. The wells were washed four times with TBST (10 mM Tris-HCl [pH 7.0], 150 mM NaCl, 0.05% Tween-20). Unbound sites were blocked with 200 µl blocking buffer (TBST plus 1% BSA), and incubated for 2 h at room temperature. The wells were then washed four times with TBST and 50 µl of 1C8 monoclonal antibody raised against purified striped bass (*Morone saxatilis*) Vtg [46] diluted 1:1000 in blocking buffer were added to each well. The plates were incubated with the primary antibody for 2 h at room temperature, and then washed with TBST as described above. After washing, 50 µl of mouse alkaline phosphatase-conjugated secondary antibody (Calbiochem, La Jolla, CA) diluted 1:1000 in blocking buffer were added to each well. The plates were incubated for 2 h at room temperature, followed by washing with TBST. One hundred µl of *p*-nitrophenyl phosphate disodium salt (1 mg/ml; Calbiochem) in substrate buffer (30 mM Na_2CO_3, 2 mM $MgCl_2$ [pH 8.3]) were added to each well, and the plates were incubated at room temperature for 15 min. The reaction was stopped by the addition of 100 µl of 3 M NaOH. The intensity of color development was quantified at 405 nm in a SpectraCount plate reader (Packard). Vitellogenin purified from the plasma of E_2-injected tilapia by anion exchange chromatography on POROS 20 HQ (PerSeptive Biosystems, Framingham, MA) [45] was serially diluted and used to create a standard curve.

Electrophoresis and Western Blotting

In order to confirm the specificity of the 1C8 monoclonal antibody used for the Vtg ELISA (supplied by Dr. N.D. Denslow, University of Florida at Gainesville), sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blotting analyses were performed using the 1C8 antibody and a polyclonal antiserum raised in rabbits against a preparation of complete tilapia Vtgs (anti-tilapia VtgAa/b). The tilapia VtgAa/b preparation was purified from the blood plasma of estrogen-treated males by anion exchange chromatography on POROS 50 HQ, whereby VtgC was lost in the pass-through fraction, followed by gel filtration on a Superdex 200 (S-200) column (Amersham Pharmacia Biotech, Piscataway, NJ), as described for white perch (*Morone americana*) Vtgs by Hiramatsu et al. [18]. The S-200 chromatography profile exhibited a single sharp and symmetrical peak and, unlike the case with white perch Vtgs, it was not possible to separate further tilapia VtgAa from VtgAb using additional steps of anion exchange on POROS 20 HQ (PerSeptive Biosystems) followed by rechromatography on POROS 20 HQ and gel filtration on S-200 (data not shown). The SDS-PAGE was performed on a 7.5% polyacrylamide gel under reducing conditions. Electrophoresis was carried out at a constant 100 V, and the proteins were subsequently transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) at 35 V for 1 h or stained for the detection of total protein with Coomassie Brilliant Blue R-250 (Sigma). Membranes were blocked in 5% nonfat milk in TBS (10 mM Tris-HCl [pH 7.0], 150 mM NaCl) for 2 h at room temperature, and then probed overnight at 4°C with the 1C8 (1:5000) or anti-tilapia VtgAa/b (1:5000) antibody. Blots were washed twice with TBS that contained 0.05% Tween-20 (TBST), and then incubated with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 2 h at room temperature. Detection of proteins was performed using the Supersignal Chemiluminescent Substrate (Pierce, Rockford, IL). The molecular masses of the immunoreactive bands were estimated using prestained molecular mass marker proteins (Bio-Rad). Chemiluminescent signals were detected using the ChemiDoc XRS system (Bio-Rad). Samples for Western blotting included purified tilapia VtgAa/b [46] and plasma from naturally mature males and females, E_2-injected males, and juvenile fish.

Real-Time Quantitative RT-PCR

Total RNA was extracted from liver samples in TriReagent (Molecular Research Center), following the manufacturer's instructions. The liver om *vtgaa*, om *vtgab*, and om *vtgc* transcripts were measured using real-time quantitative RT-PCR (rtqRT-PCR) as follows. First, 10 ng/µl of RNA were reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad). Liver RNA samples from several mature female fish was pooled, reverse-transcribed, and used as an interassay standard. In addition, samples were taken through the cDNA synthesis reaction without the addition of reverse transcriptase enzyme, to serve as negative controls.

Quantitation of *vtg* transcripts by rtqRT-PCR was performed using iQ Sybrgreen Supermix (Bio-Rad) with gene-specific primers (200 nM) according to the instructions provided by the manufacturer (see Table 1 for primer sequences and probes used in rtqRT-PCR). To ensure gene-specific amplification, melting curve analyses were conducted for all samples and standards. In addition, *vtg* gene-specific primer sets (A, B, and C) were tested for amplification of a nonspecific *vtg* template. None of the primer sets amplified a mismatched template, ensuring specificity of the primers, and the melting curve analyses resulted in a single peak for each reaction, indicating a single product. The primers for the *vtg* rtqRT-PCR assays were purchased from Integrated DNA Technologies (Coralville, IA).

The protocol described by Tsai et al. [11] was adapted for rtqRT-PCR measurement of *esr1* and *esr2* transcripts, with the following modification. Different probes for *esr1* and *esr2*, and different primers for *esr2* were designed for use in the present study, as sequence analysis revealed that the primers published by Tsai et al. [11] were missing several nucleotides necessary for amplification of the specific tilapia mRNAs. In addition, we performed separate reactions for cDNA synthesis and rtqRT-PCR with the iScript cDNA synthesis kit and iQ Supermix, respectively. The *igf1* and *ghr* rtqRT-PCR amplifications were carried out as previously described [47] using the iCycler iQ Real-Time PCR Detection System (Bio-Rad).

The assay for each gene transcript included a standard curve derived from plasmid cDNA diluted to a known copy number, and the cycling parameters for

TABLE 1. Nucleotide sequences for primers and probes used in rtqRT-PCR assays.

Gene	Primer/probe	Sequence
vtgaa	Forward primer	5′-GAATGTGAATGGGCTGGAAATAC-3′
	Reverse primer	5′-TTTGTTTGATCTGGATGTCAGCTT-3′
vtgab	Forward primer	5′-AAGTTGCAGACTGGATGAAAGGA-3′
	Reverse primer	5′-GCGGTACTCGTCTCCGACAT-3′
vtgc	Forward primer	5′-GGACCTTGCAGAACCCAAAG-3′
	Reverse primer	5′-CATCGTTTCTTGCCAGTTCCA-3′
esr1	Forward primer	5′-CATGATGAAAGGAGGTATGCGTAA-3′
	Reverse primer	5′-CATTGCACGCTTCCTTCCAT-3′
	Probe	5′-AAGACCTGCCACACACAAGGGCGTC-3′
esr2	Forward primer	5′-CCAGAGATCTACCTCGTGAAGGA-3′
	Reverse primer	5′-ATCTGCCAGGTTGGTGAGTAACA-3′
	Probe	5′-CGAAAAGGCCGCTGACCGAAGC-3′
arp	Forward primer	5′-TTTGAAAATCATCCAACTTTTGGAT-3′
	Reverse primer	5′-GCAGGGACAGACGGATGGT-3′
	Probe	5′-ACTATCCAAAATGCTTCATCGTGGGCG-3′

all assays were as follows: 2 min at 95°C, 2 min at 50°C, followed by 40 cycles at 95°C for 15 sec, and 60°C for 1 min. The data for all the rtqRT-PCR assays were normalized to the expression level of a reference gene, *acidic ribosomal phosphoprotein P0* (*arp*). The *arp* expression levels did not vary between treatments, which suggests that it is a valid reference gene under our experimental conditions (Pierce, unpublished results). The primers and probes for the *esr1* and *esr2*, *ghr*, and *igf1* expression assays were utilized at 200 nM and were purchased from Biosearch Technologies (Novato, CA).

Statistics

Data on the plasma E_2 and Vtg levels and the hepatic expression of *vtgaa*, *vtgab*, and *vtgc* were analyzed by Kruskal Wallis nonparametric tests followed by Mann-Whitney *U*-tests. Correlations between hepatic expression of *vtg* genes and plasma levels of Vtg were performed using the Pearson product-moment correlation. Plasma Igf1 and Gh levels were analyzed using one-way or two-way analysis of variance (ANOVA), followed by the Fischer least significant difference test. The expression levels of *igf1* and *ghr* were analyzed by one-way ANOVA. Results were considered significant for $P < 0.05$. Calculations were performed using the Statistica program (StatSoft, Tulsa, OK). The data are expressed as mean ± SEM.

RESULTS

Cloning and Sequence Homologies of Tilapia vtgs

The nucleotide sequences obtained from the om *vtgaa* and om *vtgab* partial cDNAs were grouped into two classes in terms of both size (425 or 422 nucleotides, excluding primer sequences) and homology (the results for all sequence analyses described below are summarized in Table 2). The shorter clones (*nh170*, 422 bp) consisted of an uninterrupted open reading frame (ORF) that encoded 140 aa, while the longer clones (*nh172*, 425 bp) encoded 141 aa. Alignment of the aa sequences deduced from the short and long clones using the CLUSTALW algorithm revealed homology scores of 50% identity and 71% similarity. When the deduced aa sequences of clone *nh170* and *nh172* were aligned with the deduced full-length aa sequence for blue tilapia (*O. aureus*) Vtg (*Vtg1*; GenBank accession no. AF017250), both clones shared homology with a region of the blue tilapia *vtg1* sequence that corresponds to the β′c domain (aa positions 1553 to 1693). The NH172 sequence (om*VtgAb*; EF408236) was 99% identical to the blue tilapia *vtg1* sequence, with only one exceptional aa substitution, while the *nh170* clone (om*VtgAa*; EF408235) showed comparatively lower levels of identity and similarity (51% and 72%, respectively). The average identity and similarity of NH170 with the deduced primary sequences of other teleost VtgAas were 65.3% and 80.2%, respectively, while lower values were obtained for comparisons with the VtgAb group (54.7% and 74.8% on average, respectively). Higher sequence homologies (average identity and similarity) for NH172 were obtained with alignment to teleost VtgAb sequences (60.3% and 75.6%, respectively) compared with VtgAa sequences (54.5% and 72.5%, respectively).

TABLE 2. Homologies (percentage identity [ID] and percentage similarity [SM]) between deduced amino acid sequences of tilapia vitellogenins (NH170, NH172, *Vtg1*, and om VtgC) and those of selected published teleost vitellogenins.

	NH170[a]		NH172[a]		*Vtg1*[b]		om VtgC	
Group	ID	SM	ID	SM	ID	SM	ID	SM
VtgAa								
Bar *VgA*	69	82	58	74	49	66	25	48
Had *VtgA*	67	80	57	77	48	67	25	50
Med *Vg1*	62	79	48	70	45	65	24	49
Mos *VgA*	64	82	56	73	43	64	24	47
Mum *VgI*	60	76	48	67	43	63	23	47
Pag *VgA*	70	82	60	74	51	69	25	50
Mean	65.3	80.2	54.5	72.5	46.5	65.7	24.3	48.5
VtgAb								
Bar *VgB*	56	75	65	77	58	75	23	47
Had *VgB*	54	76	55	76	53	71	27	50
Med *VgII*	50	73	58	77	56	75	24	50
Mos *VgB*	52	71	58	69	53	72	25	50
Mum *VgII*	56	77	61	77	54	73	25	51
Pag *VgB*	60	77	65	78	61	77	24	48
Mean	54.7	74.8	60.3	75.6	55.8	73.8	24.7	49.3
VtgC								
Gob *Vg-320*	–	–	–	–	18	33	45	64
Mos *PvlVg*	–	–	–	–	18	34	48	66
Pag *VgC*	–	–	–	–	20	37	57	74
Zeb *vg3*	–	–	–	–	19	35	43	65
Mean	–	–	–	–	18.8	34.8	48.2	67.2
Vtg1[c]	51	72	99	99			26	50

[a] A dash (–) indicates that alignment was not possible (see *Materials and Methods* and *Results* for details).
[b] Full-length deduced amino acid sequence of blue tilapia (*Oreochromis aureus*) *Vtg1* (GenBank accession number AAD480) was used in alignment comparisons of full-length sequences listed in matrix.
[c] Partial-length deduced amino acid sequence of blue tilapia (*Oreochromis aureus*) *Vtg1* (GenBank accession number AAD480) was used in alignment comparisons of partial-length sequences listed in matrix.

The *vtgc1* clones (n = 10) were 564 bp in length (ORF encoding 187 aa) while the *vtgc2* clones (n = 5) were 1170 bp in length (ORF encoding 389 aa), excluding the primer sequences. The consensus nucleotide sequences obtained from the *vtgc1* and *vtgc2* clones were 100% identical in the region where they overlapped. Accordingly, the following homology analyses were conducted using the consensus tilapia *vtgc* (om*VtgC*; GenBank accession no. EF408237) sequence (total of 1170 bp/389 aa). The highest homology score by alignment (57% identity and 74% similarity) of the deduced aa sequence of om *vtgc* was with red seabream (*Pagrus major*) VtgC and aligned to a portion of the LvH domain (seabream VtgC aa 47 to 435). Similar homologies were obtained from comparisons of deduced aa sequences between om *vtgc* and other teleost Pv-less Vtg genes, i.e., mosquitofish *pvlvtg* (48% identity and 66% similarity), zebrafish *vtg3* (43% and 65%), and Japanese common goby *vtg-320* (45% and 64%). The aa sequence homologies between om VtgC and complete Vtgs that belong to either the VtgAa or VtgAb group were considerably lower (average 24.5% identity and 48.9% similarity) than the values obtained with Vtgs that belong to the VtgC group (48.2% and 67.2%).

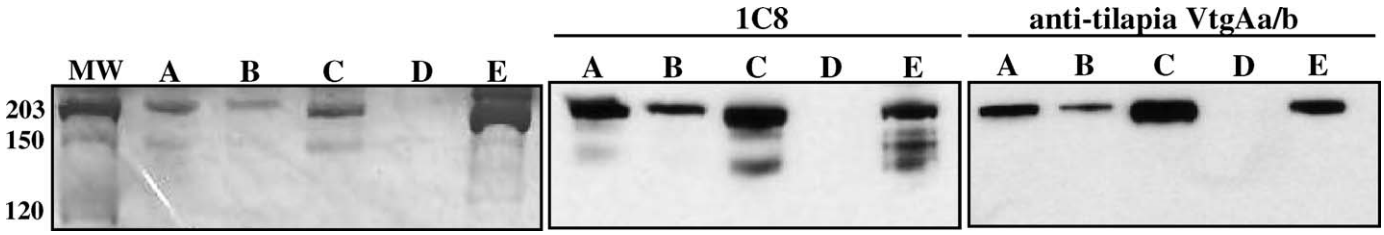

FIG. 2. Western blots of tilapia plasma samples comparing the 1C8 monoclonal antibody raised against purified Vtg from striped bass and anti-tilapia VtgAa/b antibody raised against a purified preparation of complete Vtg (VtgAa and VtgAb) from tilapia. Samples tested included female plasma (lane A), male plasma (lane B), plasma from E_2-injected males (lane C), juvenile plasma (lane D), and purified tilapia Vtg (lane E). Identical plasma samples and molecular mass (MW) standards stained with Coomassie blue are shown in the left panel. Numerals at the left side of the figure indicate the positions of the MW markers.

Immunoreactivities of the 1C8 Antibody and Anti-Tilapia VtgAa/b Antiserum

The SDS-PAGE gels and Western blots (Fig. 2) confirm that the 1C8 monoclonal antibody is strongly immunoreactive with a major band of ~200 kDa, which presumably represents a mixture of intact complete tilapia Vtgs (VtgAa and VtgAb), as this band was exclusively recognized by the anti-tilapia VtgAa/b antiserum. As the 1C8 antibody was the primary antibody used in our ELISAs, the plasma Vtg data reported herein are considered to be mainly representative of circulating complete Vtg (VtgAa/b) levels. Results for reactivity of the 1C8 monoclonal antibody with the smaller, phosvitin-less form of tilapia Vtg (12–15), VtgC (~150 kDa), were equivocal. Due to the well-known tendency of Vtgs to degrade during purification and storage, the tilapia VtgAa/b preparation utilized as the ELISA standard and the plasma samples from mature females and E_2-treated males generated several minor bands that overlapped the 150-kDa position and that immunoreacted with the 1C8 antibody. The anti-tilapia VtgAa/b antiserum did not immunostain these minor bands, perhaps because this antiserum is directed against epitopes that are not present in the complete Vtg degradation products. Both the 1C8 antibody and the anti-tilapia VtgAa/b antiserum detected the ~200 kDa VtgAa/b band in untreated male plasma, which was not unexpected because constitutive expression of Vtg by male tilapia has previously been reported (12). However, both the immunoblots and the stained SDS-PAGE gel show that male plasma contains less Vtg than either female plasma or E_2-injected male plasma, while the plasma from juvenile fish lacks any Vtg that is detectable using these methods.

Effect of Single E_2 Injection on Plasma E_2 and Vtg in Male Tilapia

A single i.p. injection of E_2 at 5 μg/g BW into male tilapia increased the plasma E_2 levels several-fold by Day 1 postinjection (Fig. 3A), and this high level was maintained on Day 2 postinjection. The plasma E_2 levels had declined in the E_2-injected fish by Days 3 and 5 after injection, although they remained elevated relative to the E_2 levels in the plasma of vehicle-injected control fish. In addition, the plasma Vtg levels were elevated several-fold in E_2-injected fish on Day 1, and continued to increase until Day 5 after injection (Fig. 3B). Over the course of the experiment, vehicle-injected control fish retained plasma levels of E_2 at ~0.1 ng/ml and of Vtg at ~1.5 mg/ml.

Expression of Hepatic om vtgaa*, om* vtgab*, and om* vtgc

The expression levels of om *vtgaa*, om *vtgab*, and om *vtgc* transcripts in the liver were significantly elevated on Day 1 after E_2 injection, as compared with the corresponding transcript levels in vehicle-injected control fish (Fig. 4, A–C); the expression levels of all three *vtg* genes remained significantly higher over the course of the experiment. The om *vtgaa* transcripts showed the largest increase after E_2 treatment,

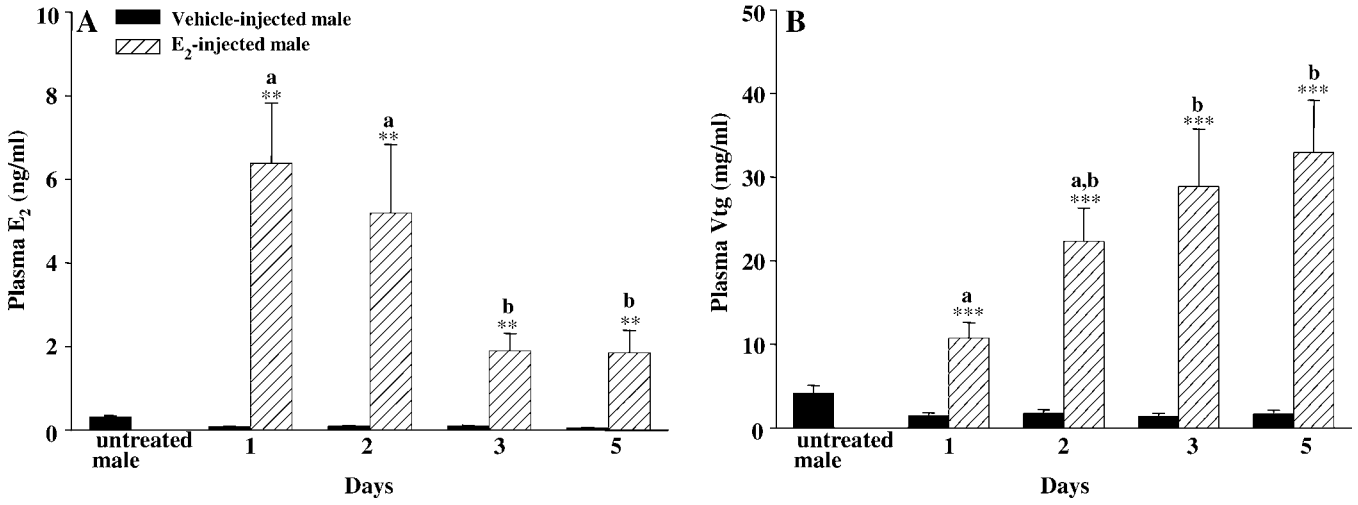

FIG. 3. Effects of a single i.p. injection of E_2 (5 μg/g BW) into male tilapia on the plasma levels of E_2 (**A**) and Vtg (**B**). Vertical bars represent mean ± SEM (n = 7). Asterisks indicate significant differences from vehicle-injected males at each time-point; $^{**}P < 0.01$, and $^{***}P < 0.001$ (Mann-Whitney *U*-test). Different letters indicate significant differences between E_2-injected males over time (one-way ANOVA).

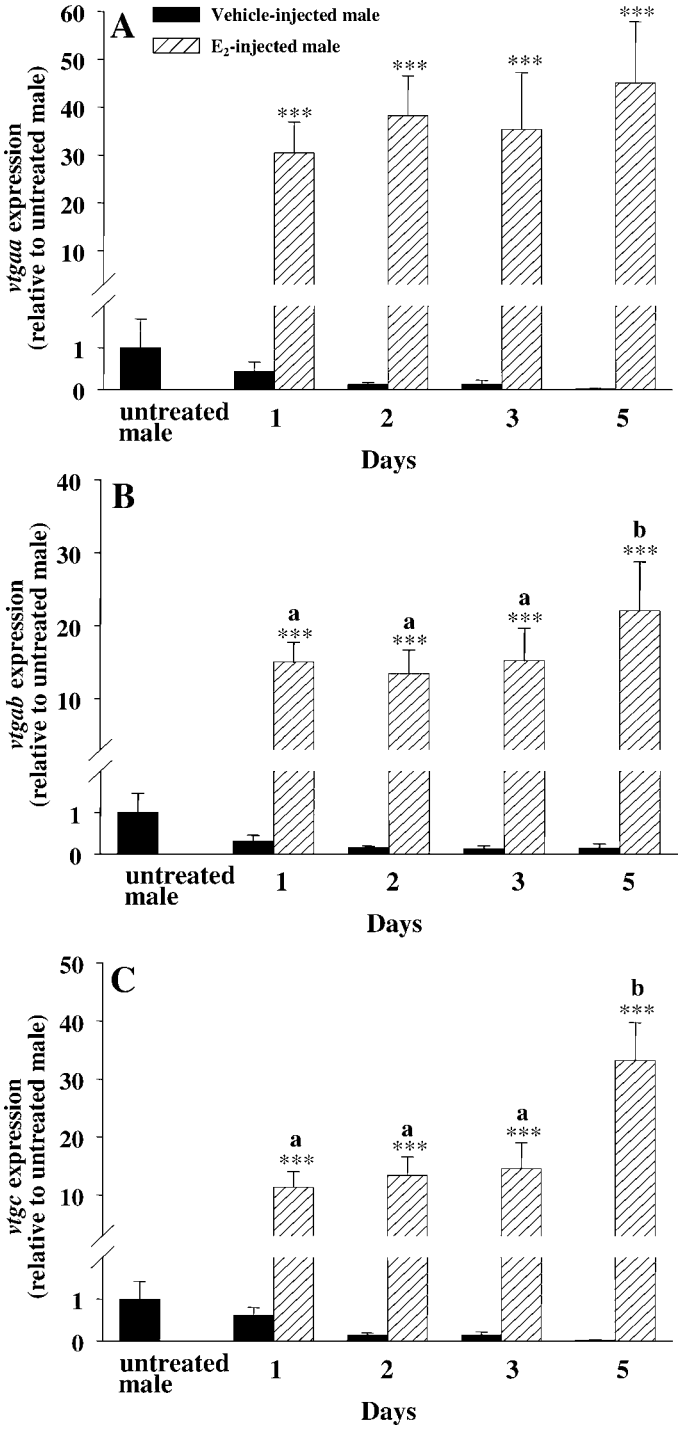

FIG. 4. Effects of a single i.p. injection of E_2 (5 μg/g BW) in male tilapia on hepatic expression of *om vtgAa* (**A**), *vtgAb* (**B**), and *vtgC* (**C**). Data are shown as expression relative to untreated males. Vertical bars represent mean ± SEM (n = 7). Asterisks indicate significant difference from vehicle-injected males at each time-point; ***$P < 0.001$ (Mann-Whitney *U*-test). Different letters indicate significant differences between E_2-injected males over time (one-way ANOVA).

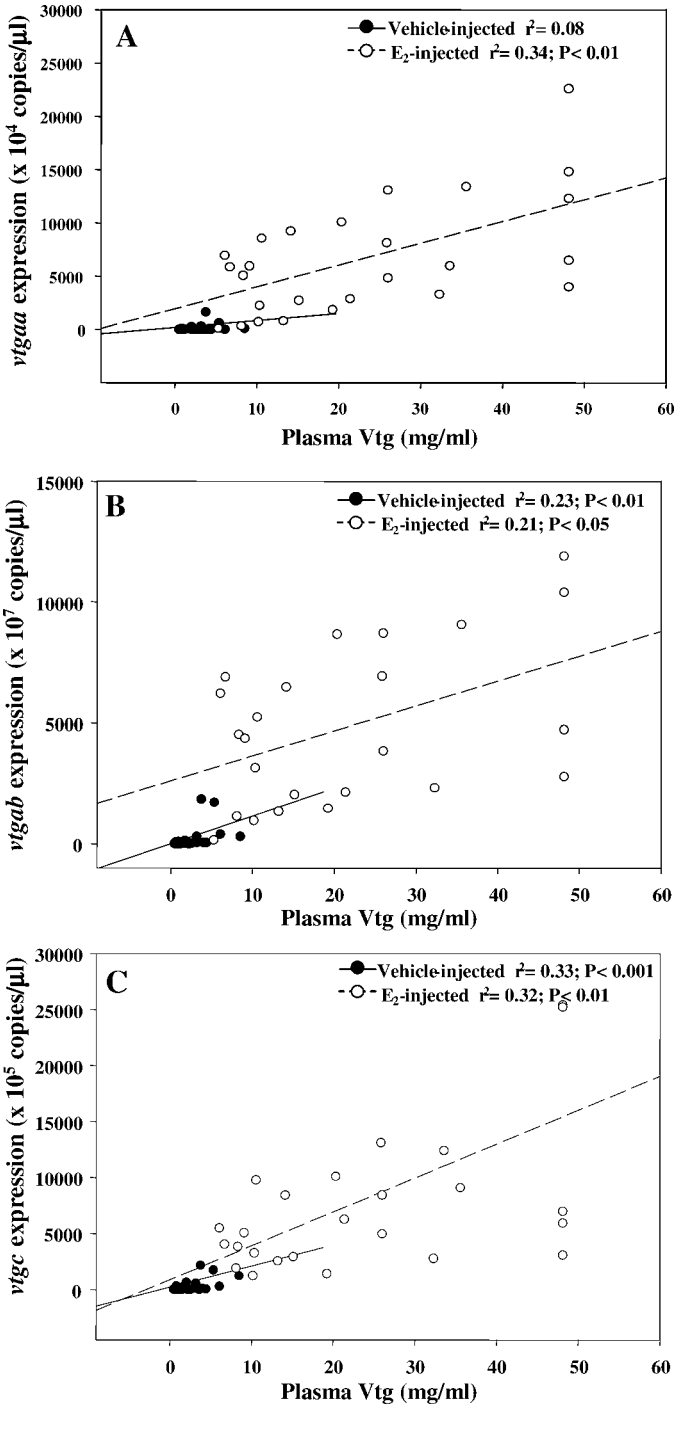

FIG. 5. Correlations between plasma Vtg and hepatic expression of *om vtgAa* (**A**), om *vtgAb* (**B**), and om *vtgC* (**C**) in vehicle-injected Days 1–5 (closed circle) and E_2-injected Days 1–5 (open circle, n = 7). The expression of *vtg* is shown as copy number/μl cDNA.

rising 30–45-fold, as compared to the transcript levels in untreated males, whereas the levels of the om *vtgab* and om *vtgc* transcripts increased 15–30-fold. Among the three gene transcripts, om *vtgab* appeared to be most abundant; in addition to the unquestionably higher copy numbers, cDNA transcript amplification occurred earlier in the rtqRT-PCR cycling for om *vtgab* than for either om *vtgaa* or om *vtgc*, indicating the presence of more templates. On average, the expression levels of all three *vtg* genes significantly correlated with the plasma Vtg levels in E_2-injected fish and in vehicle-injected controls, with the exception of om *vtgaa* expression in the control fish (Fig. 5, A–C).

Expression of Hepatic esr1 *and* esr2

Expression of *esr1* increased significantly on Day 1 after E_2 injection, and high levels were maintained through Day 5, at

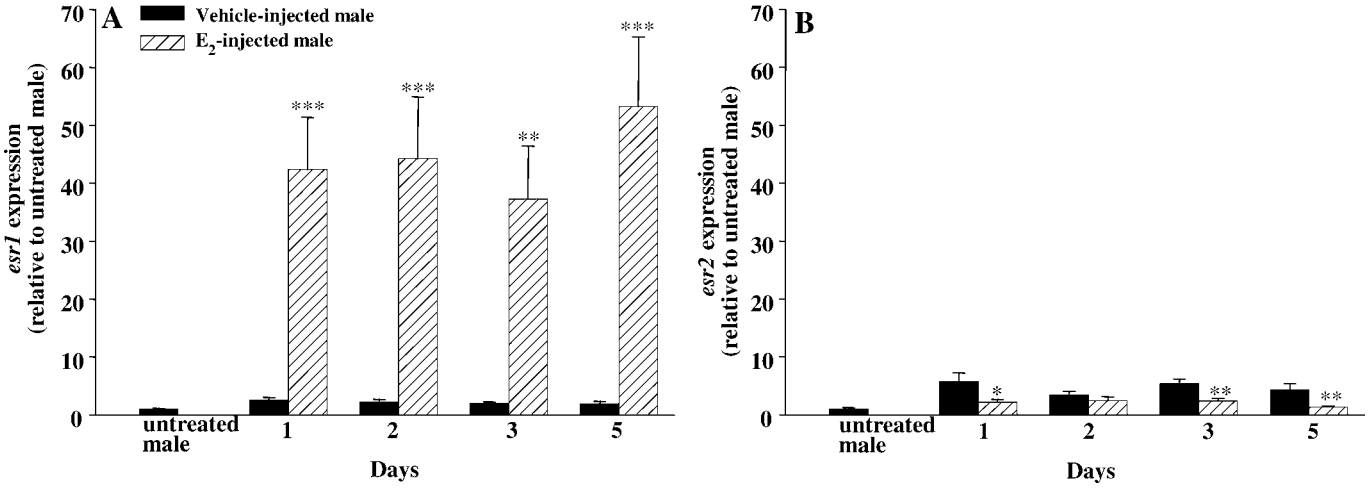

FIG. 6. Effects of a single i.p. injection of E_2 (5 μg/g BW) on hepatic expression of *esr1* (**A**) and *esr2* (**B**) in male tilapia. Data are shown as expression relative to untreated males. Vertical bars represent mean ± SEM (n = 7). Asterisks indicate significant differences from vehicle-injected males at each time point at $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ (Mann-Whitney *U*-test for *esr1*, Student *t*-test for *esr2*).

which time-point the *esr1* transcript levels were about 50-fold higher than in the untreated males (Fig. 6A). In contrast, *esr2* gene expression was suppressed by E_2 treatment at all time-points, as compared with the expression levels in vehicle-injected males (Fig. 6B). Furthermore, *esr2* expression increased in vehicle-injected fish, as compared with untreated males.

Effect of E_2 on the Gh/Igf1 Axis

There was no effect of E_2 treatment on plasma Gh levels, although expression of the *ghr* gene was significantly lower in E_2-treated fish as compared to vehicle-injected fish on Days 1 and 5, and was lower on average on all days after E_2 injection (Fig. 7, A and B). Plasma Igf1 levels were significantly reduced in E_2-treated fish on Days 1 and 2 after injection compared to the levels in vehicle-injected fish (Fig. 8A). The levels of *igf1* transcripts in E_2-treated fish, as compared with control males, were significantly lower on Days 1 and 5 and lower on average on all days after E_2 injection (Fig. 8B).

DISCUSSION

In vertebrates, Gh and Igf1 play pivotal roles in regulating body growth and development. These actions are modulated to a considerable degree by sex steroids. In humans, a close interplay between estrogen and Gh leads to the attainment of gender-specific body composition during puberty [4], and there is clear-cut evidence, particularly in mammalian systems, for cross-talk among estrogen, Igf1, and their receptors [1, 48]. Estrogen also affects Gh and Igf1 at the level of receptor expression and signaling. Recently, estrogen has been shown to oppose the phosphorylation of Janus kinase 2 induced by Ghr binding [49], while increasing the expression of several factors necessary for Igf1 signal transduction [50]. However, the physiological significance of these interactions is not well understood. While species-specific differences exist, the regulation of growth, development, and reproduction is evolutionarily conserved. Mammalian studies are made difficult by in utero development, highly evolved nervous systems, and the presence of additional hormonal targets, such as the uterus and mammary glands. While further studies in

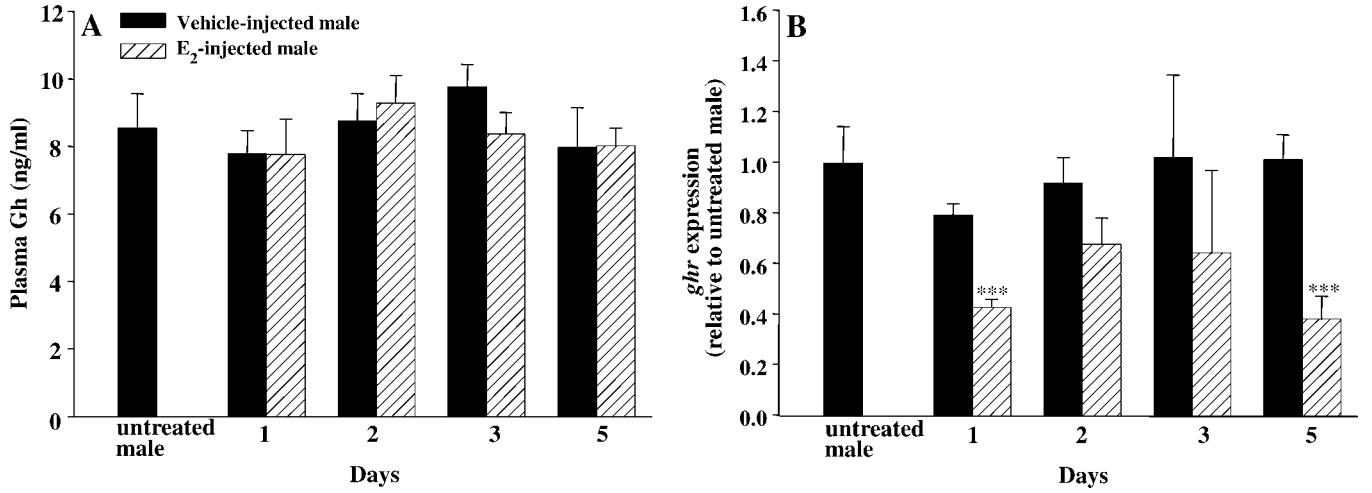

FIG. 7. Effects of a single intraperitoneal injection of E_2 (5 μg/g body weight) on plasma Gh (**A**) and hepatic *ghr* expression (**B**) levels in male tilapia. The expression of *ghr* is shown as expression relative to untreated males. Vertical bars represent the mean ± SEM (n = 7). Asterisks indicates significant differences from vehicle-injected males at each time-point at $^{***}P < 0.001$ (Student *t*-test).

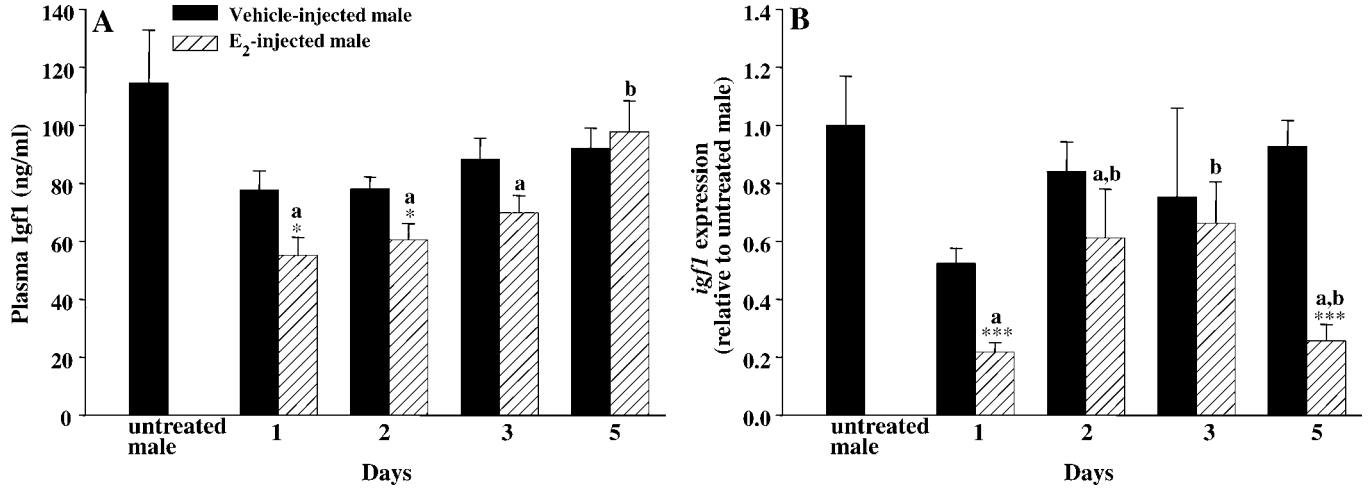

FIG. 8. Effects of a single i.p. injection of E_2 (5 μg/g BW) on plasma Igf1 (**A**) and hepatic *igf1* expression (**B**) levels in male tilapia. The expression of *igf1* is shown as expression relative to untreated males. Vertical bars represent the mean ± SEM (n = 7). Asterisks indicate significant differences from vehicle-injected males at each time-point at $*P < 0.05$ and $***P < 0.001$ (Student *t*-test). Different letters indicate significant differences between E_2-injected males over time (one-way ANOVA).

females are warranted to verify that male tilapia provide a good model for understanding the interaction between growth and reproduction in general, we believe that the relatively simple physiology of male teleost fish makes them good models for understanding the mechanisms that underlie somatic growth and ovarian growth (vitellogenesis) in female vertebrates.

Vitellogenin, which is the major precursor of egg yolk proteins in oviparous vertebrates, is a definitive marker of the onset of puberty and the progression of maturation in female teleosts, and is the major protein contributing to the massive growth of the ovary in these species [17]. Normally only strongly activated in females, vitellogenesis can be induced in male fish under experimental conditions by exposure to estrogen or through contact with estrogenic pollutants in the environment [18]. In the present study, the induction of Vtg production by E_2 in male fish was used to simulate the transition from somatic to gonadal growth in naturally maturing females, so that the endocrine mechanisms that underlie this transition could be investigated. Initially, we cloned and sequenced the cDNAs that encode portions of three different Vtgs in Mozambique tilapia and examined the effects of E_2 treatment in male fish on the plasma profiles of E_2, Vtg, Gh, and Igf1 and on the hepatic expression of gene transcripts for the three forms of Vtg and two Esr subtypes, as well as *ghr* and *igf1*. The results clearly indicate that E_2 stimulates vitellogenesis by up-regulating the expression of all three *vtg* genes in concert with increased *esr1* transcripts, while suppressing the activity of the Gh/Igf1 axis. This is the first study to document sustained, up-regulated expression of multiple *vtg* genes by E_2, together with simultaneous suppression of the Gh/Igf1 axis. Our data also support the recent report by Filby et al. [51] on the down-regulation of liver *igf1* and *ghr* mRNA transcripts in fathead minnow (*Pimephales promelas*) by the synthetic estrogen 17α-ethinylestradiol.

The discovery of three *vtg* gene transcripts (*vtgaa*, *vtgab*, and *vtgc*) in the Mozambique tilapia adds to the growing number of teleosts known to express multiple *vtg*s. Nevertheless, despite gene and protein characterizations, the differential regulation of these distinct forms of *vtg* has received relatively little attention. We have shown that expression of all three tilapia *vtg* genes is strongly induced by E_2 and is significantly correlated with Vtg immunoreactivity in the blood plasma. The VtgAa/b primer set used in the present study to amplify two distinct tilapia *vtg* transcripts is the same degenerate primer pair used in a previous study by Bowman et al. [40] for the cloning of partial cDNAs that encode Vtgs in the sheepshead minnow (*Cyprinodon variegatus*). In the sheepshead minnow, this primer set successfully amplified two distinct *vtg* cDNAs (*vtg1* and *vtg2*) from hepatic transcripts present in E_2-treated fish. Alignment analyses confirmed that sheepshead *vtg1* and *vtg2* are homologues of mummichog *vtgI* (*vtgaa*) and *vtgII* (*vtgab*), respectively. Our comparisons of the deduced aa sequences of multiple Vtgs from several teleost species with peptide sequences encoded by the Mozambique tilapia *vtg* cDNAs, *nh170* and *nh172*, support the grouping of the two tilapia sequences with the complete Aa- and Ab-types of Vtg, respectively. Although our alignment analyses were based on only partial cDNA sequences, this classification is almost certainly correct for the Vtg polypeptide encoded by the *nh172* cDNA because it was nearly 100% identical to blue tilapia *vtg1* within the aligned regions; the full-length aa sequence encoded by tilapia *vtg1* is clearly a *vtgab* homologue [52].

Alignments of the Vtg aa sequences deduced from *nh170* and *nh172* with those of C-type Vtgs are not possible, since the region amplified by the VtgAa/b primer set encodes a portion of the cysteine-rich C-terminal yolk protein domain (β′c) that is typically absent in C-type Vtgs [19]. However, this also serves as evidence that neither the NH170 nor NH172 sequences belong to the VtgC group. The partial om VtgC aa sequence aligned to a portion of the LvH domain present in all three classes of teleost Vtgs (Aa-, Ab-, and C-type Vtgs), and the alignment analyses clearly classified this tilapia sequence as belonging to a C-type Vtg, based on homology scores (Table 2).

As previously mentioned, only two distinct Vtg proteins have been detected previously in the circulation systems of *O. mossambicus, O. niloticus*, and *O. aureus* [12–15]. However, the present study reveals the expression of at least three functional *vtg* genes in *O. mossambicus*. We are confident that the partial om *vtgc* cDNA obtained in the present study encodes the minor (small) Mozambique tilapia Vtg (VTG-140 or tVTG130), which has been purified and characterized in previous studies [13, 14], as this minor tilapia Vtg appears to be missing a Pv domain based on its low phosphorus content. On the other hand, it is apparent that clones *nh170* and *nh172* encode a C-terminal cysteine-rich region of Mozambique

tilapia Vtgs, which is characteristic of complete Vtgs (VtgAa and VtgAb, respectively). The mass of the major Vtg monomer was estimated on SDS-PAGE to be 210 kDa [14] or 200 kDa [13] in Mozambique tilapia, which is very close to the mass predicted for the deduced aa (193.5 kDa, excluding the signal peptide) of blue tilapia VtgI (VtgAb), which suggests that VtgAb is a component of the major tilapia Vtg polypeptide band evident in the SDS-PAGE. Thus, it is highly likely that the major tilapia Vtg described in previous studies is actually a mixture of om VtgAa and om VtgAb, as has been reported for the white perch [39], mosquitofish [53], and red seabream [21].

To date, the simultaneous regulation of three distinct *vtg* genes has not been characterized in any species. In the present study, after a single injection of 5 μg/g E_2 into male tilapia, the om *vtgaa*, om *vtgab*, and om *vtgc* mRNA levels rose significantly by 24 h postinjection, and the peak transcript levels, which were 22–45-fold higher than in untreated males, were reached 5 days postinjection. Plasma Vtg immunoreactivity had also increased significantly at 24 h and continued to rise over the course of the experiment, even after a decline in the plasma E_2 levels. The maintenance of high transcript levels in the face of declining E_2 in the circulation may be due to the increased stability of mRNA in association with the characteristic "memory effect" following estrogen induction. This effect, whereby a second E_2 treatment induces Vtg expression more quickly and often more robustly than the first treatment, has been described in numerous species, including rainbow trout [54], blue tilapia [55], and sheepshead minnow [40]. The detection of circulating Vtg proteins and hepatic *vtg* transcripts in our untreated male fish suggests that E_2, either exogenous or endogenous, stimulated their livers to produce Vtg prior to the start of the experiment, possibly sensitizing the liver for the experimental injection. The constitutive expression of *vtg* by male tilapia at levels lower than those seen in mature females or estrogen-treated males has been reported previously (12).

Takemura and Kim [14] have observed that E_2 treatment of Mozambique tilapia stimulates dose-dependent production of two different Vtgs, VTG210 and VTG140, both in vivo and in vitro. Although production of the two Vtgs followed similar time courses, i.e., increasing on Day 1 postinjection and declining after Day 3, VTG210 was present at 5–8 times the level of VTG140. In hepatocyte cultures, the production of VTG210 was induced more rapidly than that of VTG140. Lim et al. [55] have suggested that the difference in the relative proportions of these two forms of Vtg in blue tilapia (*O. aureus*) may be due to unequal activation of their genes. In the present study, om *vtgaa*, om *vtgab*, and om *vtgc* were all strongly induced by E_2 at 24 h. However, for Days 1–3, om *vtgc* showed a smaller increase (14-fold) than om *vtgaa* (35-fold), thus lending support to the hypothesis proposed by Lim et al. [55]. Despite the fact that absolute transcript copy numbers may vary due to the efficiency of cDNA synthesis, we approximate the om *vtgab* transcript levels as approximately10-fold higher than those of either om *vtgaa* or om *vtgc*. Thus, om VtgAb may be the predominant monomer in the ~200-kDa band observed in the present study (Fig. 2), the VTG-200 band seen by Kishida and Specker [13], and the VTG210 band seen by Takemura and Kim [14] in SDS-PAGE and Western blots of blood plasma from vitellogenic Mozambique tilapia. Other studies of tilapia have shown that the lower molecular mass form of Vtg (categorized here as VtgC) is less abundant than the complete form(s) of Vtg [13, 14]. As noted, the plasma levels of Vtg protein (Vtg immunoreactivity) in the present study are mainly indicative of the total circulating levels of the complete form(s) of Vtg, and it is uncertain whether the Vtg ELISA detects VtgC in Mozambique tilapia. We are currently working to establish an immunoassay for the specific measurement of VtgC protein levels in this species. This type of assay would allow us to characterize better the relationship between the regulation of the om *vtgc* gene and its protein products.

Concomitant with the increase in gene expression for om *vtgaa*, vtg*ab*, and vtg*c*, E_2 treatment resulted in the elevated expression of *esr1* transcripts. Hepatic expression of *esr*s, which are categorized as type 1, is positively regulated by E_2 in zebrafish and fathead minnows, while the expression of *esr2* isoforms is unchanged or reduced by E_2 [27, 56]. Although samples were not taken until Day 1 after E_2 injection, we speculate that augmentation of *esr1* expression occurs in less than 24 h and precedes the increases in transcript levels for om *vtgaa*, *vtgab*, and *vtgc* in estrogen-treated fish. An up-regulation of liver *esr* expression prior to *vtg* expression has been observed in some species, supporting the role of Esr in the mediation of vitellogenesis [26, 57, 58]. Receptor-mediated estrogen induction of *vtg* expression has been described quite thoroughly in other species of fish, and there is no reason to suppose that a separate mechanism is occurring in this particular species. However, further studies are necessary to define the relationship between *esr1* and vitellogenesis in tilapia as more than correlative. In contrast to our present results for Mozambique tilapia, *erβ-1* is increased by E_2 and appears to modulate vitellogenesis in goldfish, although the effects of E_2 on goldfish *erα* and *erβ-2* remain to be reported [59, 60]. In the present study, the effects of E_2 on hepatic *esr2* gene expression were not apparent. However, vehicle-injected control males had *esr2* transcript levels that were 3.4–5.7-fold higher than those measured in untreated fish at each time-point. While it is evident that in Mozambique tilapia, *esr2* is unlikely to be the primary mediator of E_2 actions on the liver associated with the induction of vitellogenesis, the increase in *esr2* transcript levels in vehicle-injected fish is puzzling. This may be a consequence of injection that is negated by exposure to E_2, although the physiological relevance of altered hepatic expression of the *esr2* gene in these fish remains to be elucidated.

The inhibitory effects of E_2 on the Gh/Igf1 axis were also evident in the present study. Although no such effect was seen on plasma Gh levels, the plasma Igf1 levels and hepatic expression levels of the *ghr* and *igf1* genes were all significantly reduced by E_2 treatment. Recently, Filby et al. [56] have demonstrated that male fathead minnows exposed to E_2 for 14 days show no change in liver *ghr* expression but have reduced levels of *igf1* mRNA. In the present study, E_2 treatment of male tilapia clearly reduced hepatic expression of *ghr* transcripts without affecting the plasma Gh levels, which suggests that in tilapia, E_2 down-regulates the Gh/Igf1 axis by desensitizing the liver to circulating Gh. In contrast to the present study, others have shown that E_2 treatment increases the Gh levels in goldfish [61], rainbow trout [62], and tilapia [63]. These discrepancies may be due to species variation, the time of treatment, sampling or the nutritional and/or maturation state of the animal. The relationships between body growth, Igf1, and reproductive maturation in some teleosts suggest that responsiveness to sex steroids and growth factors may vary with maturity and size, making direct comparisons difficult [31–34].

We have reported previously that E_2 treatment of male tilapia alters the Gh/Igf1 profile to one that resembles that found in female fish, including increased plasma Gh levels combined with reductions in plasma Igf1 levels and in hepatic expression of *igf1* [63]. This suggests that E_2 treatment of the male fish may shift energy utilization away from somatic

growth (Gh/Igf1 axis) and towards vitellogenesis (egg production). Although plasma Gh levels were not significantly affected by E_2 treatment in the present study, the reduction of *ghr* expression in E_2-treated fish is in agreement with our previous results and suggests that down-regulation of *ghr* may provide an additional mechanism through which E_2 suppresses somatic growth. In mammals, regulatory interactions between E_2 and the Gh/Igf1 axis have been thoroughly described, including roles in postnatal growth, puberty, sexual dimorphism, bone growth, metabolism, neurological regeneration and function, and tumorigenesis [1, 4]. While a great deal remains undefined regarding the relationship between reproduction and the Gh/Igf1 axis in teleosts, a wide range of evidence suggests an important regulatory interplay between the two systems on multiple levels. This includes the characterization of changes in ovarian Ghr binding throughout gametogenesis in rainbow trout [36], the presence of Igf1 receptors and *igf1* mRNA in the testes of trout [37], and sex- and age-specific differences in the expression of genes important for somatic growth in several tissues of fathead minnows [64]. In differential display and gene arrays for E_2-treated fish, the down-regulation of several genes has been noted, although their identities are mostly unknown [65, 66]. As genomic and proteomic techniques evolve, currently unknown transcripts that are down-regulated by E_2 may be identified as genes that are important in the regulation of somatic growth.

In the current study, Vtg was detected in the untreated male plasma along with significant expression of all three *vtg* genes in the liver. Although Vtg is not usually assumed to occur naturally in males, there are several reports indicating the presence of Vtg proteins in the plasma or *vtg* mRNA transcripts in the livers of male fish [19, 45]. The ability and extent to which a male can synthesize Vtg could be influenced by several factors, including diet, temperature, season, and profile of exposure to estrogens. Most, if not all, diets used in fish culture and in experiments on captive fish contain measurable amounts of sex steroids and steroid-like substances, and these substances can be estrogenic and induce vitellogenesis [67]. Even in the wild, omnivorous and piscivorous species are routinely exposed to estrogens in their diet when feeding on sexually maturing or mature fish prey, and all types of fish can be impacted by the presence in the environment of estrogenic pollutants known as endocrine disrupters [19, 46]. In addition, season and age, as well as the number and length of treatments have all been shown to alter the vitellogenic response to estrogen in several species [40, 54, 55, 58]. Most female teleosts produce significant quantities of testosterone, which is largely converted to estradiol during vitellogenesis [68]. However, some studies have shown that androgens also are capable of inducing or potentiating vitellogenesis in females. In tilapia hepatocyte cultures, 5α-dihyrotestosterone (DHT) increases Vtg production, while testosterone and methyltestosterone (MT) potentiate the effect of E_2, suggesting that androgens play a role in vitellogenesis [69, 70]. In contrast, MT decreases plasma Vtg in female eelpout [71], while DHT has similar effects on plasma Vtg in female tilapia in addition to suppressing *vtg* expression [72]. These varied results may reflect hormone dosage, method of treatment or the maturational status of the fish; further studies are warranted to characterize further the effects of androgens in females. To describe better the disparate regulation of the different types of Vtg (Aa, Ab, and C) in male and female Mozambique tilapia in future studies, all of the above factors should be considered.

In conclusion, we have obtained evidence for Vtg multiplicity in tilapia by molecular cloning of partial cDNAs that encode three distinct forms of tilapia Vtg. The resulting sequence analyses categorized these forms of tilapia Vtg as homologues of teleost VtgAa, VtgAb, and VtgC. This is the first study to describe the physiological effects of E_2 on the expression of three different *vtg*s, two *esr*s, and several aspects of the Gh/Igf1 axis. The three forms of tilapia *vtg* are estrogen-sensitive, and significant increases in their expression occur within 24 h of E_2 treatment in male tilapia, lasting at least 5 days. Esr1 may play a role in modulating the induction of vitellogenesis by E_2, as suggested by the large induction of *esr1* in correlation with all three *vtgs*. The results of the present study also clearly indicate that, simultaneous with Vtg induction, E_2 suppresses the Gh/Igf1 axis. These results fully support our hypothesis that E_2 represses somatic growth while promoting vitellogenesis during gonadal recrudescence in females. Such interactions between regulatory mechanisms in reproductive and growth physiology are often overlooked, especially in piscine studies, and the implications of the results presented here are far-reaching in several areas of basic and applied biology, including biomedical research using fish model systems and research on aquaculture and endocrine disruption.

ACKNOWLEDGMENT

We thank Dr. N. Denslow (University of Florida at Gainesville) for kindly providing the 1C8 monoclonal antibody for the Vtg ELISA.

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