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Steroidogenic Acute Regulatory Protein Transcription is Regulated by Estrogen Receptor Signaling in Largemouth Bass Ovary

Melinda S. Prucha, Christopher J. Martyniuk, Nicholas J. Doperalski, Kevin J. Kroll, David S. Barber, Nancy D. Denslow

Department of Physiological Sciences and Center for Environmental and Human Toxicology, College of Veterinary Medicine, University of Florida, Gainesville, Florida 32611

Abstract

Estrogenic contaminants in the environment are linked to the occurrence of reproductive abnormalities in many aquatic species, including largemouth bass (*Micropterus salmoides*; LMB). Previous work has shown that many different types of xenoestrogens regulate expression of the Steroidogenic Acute Regulatory protein (StAR), a cholesterol-transporting protein vital to steroid hormone biosynthesis; however, the regulatory mechanisms of StAR are incompletely characterized in fish. To learn more about endogenous expression patterns of StAR in the ovary, LMB were collected from the St. John's River (Florida, USA) over an entire breeding season to investigate StAR expression. Plasma 17 β -estradiol and StAR mRNA levels were positively correlated in females, and StAR mRNA levels displayed ~100-fold increase between primary oocyte growth stages and final maturation. To further study the regulation of StAR, female LMB in the laboratory were fed at ~2% of their weight on a diet laden with 17 α -ethinylestradiol (EE2, 70 or 200 ng EE2 per gram feed). Diets were designed to achieve a physiologically-relevant exposure to EE2, and StAR expression was assessed in vivo. We observed a dose-dependent suppression of StAR mRNA levels, however both diets led to high, pharmacological levels in the blood and do not represent normal physiological ranges of estrogens. In the 200 ng EE2/gm feed group, ovarian StAR mRNA levels were suppressed to approximately 5% of that of the LMB control group. These investigations suggest that LMB StAR increases in expression during oocyte maturation and that it is suppressed by E2 feedback when estrogens levels are high, through the HPG axis. A 2.9 kb segment of the LMB StAR promoter was examined for putative E2 response elements using *in silico* software, and a putative estrogen receptor binding element (ERE/-1745) was predicted in the promoter. The functionality of the ERE was examined using MA-10 mouse Leydig cells transfected with the LMB StAR promoter. ER interaction with ERE/-1745 was evaluated under basal and human chorionic gonadotropin (hCG)-treated conditions in the presence and absence of E2. Chromatin immunoprecipitation (ChIP) experiments revealed that ER1 binding

Corresponding author: Nancy D. Denslow, Department of Physiological Sciences and Center for Environmental and Human Toxicology, University of Florida, PO Box 110885, Gainesville FL 32611, Tel: (352) 294-4642; Fax: (352) 392-4707; ndenslow@ufl.edu.

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to the promoter was enriched under basal conditions and E2 exposure elicited an increase in enrichment (4-fold) above that observed under basal conditions. ER2 was not strongly enriched at the ERE/-1745 site, suggesting that StAR may be preferentially regulated by ER α . Taken together, these different experiments provide evidence that LMB StAR is under the control of estrogens and that ER α binds directly to the LMB StAR promoter in an E2-responsive manner.

Keywords

Steroidogenesis; estrogen receptor; seasonal gene expression; largemouth bass; gene regulation; ChIP

1. Introduction

Steroid hormone biosynthesis is essential for sexual reproduction and a number of autocrine, paracrine, and endocrine factors regulate this process. Estrogens from both endogenous and exogenous sources can regulate steroid biosynthesis, and the effects are mediated via estrogen receptors (ERs), which are ubiquitously expressed members of the nuclear receptor superfamily of transcription factors. Binding of ERs to DNA response elements regulates a wide array of genes, including many involved in regulating steroid hormone biosynthesis and reproduction. There are multiple isoforms of ERs in fish [*esr1*, *era2* (in some fish), *esr2a*, and *esr2b*] (Halm et al., 2004, Hawkins and Thomas, 2004, Hawkins et al., 2000, Menuet et al., 2002, Nagler et al., 2007, Pinto et al., 2006, Sabo-Attwood et al., 2004). These isoforms carry out distinct and independent functions in the regulation of gene expression, suggesting that there is a complex and intricate role for ERs in regulating the transcriptional activity of target genes. The isoforms of ERs in fish resulted from a gene duplication that occurred approximately 300 million years ago, but for most teleost fish the *era2* was lost (Ohno et al., 1968, Tohyama et al., 2016). Rainbow trout, different from other teleosts, retained *era2*, which is expressed in a variety of tissues (Nagler et al., 2007).

The mRNA for steroidogenic acute regulatory (StAR) protein, which is the rate-limiting step in steroid hormone production in all vertebrates (Clark et al., 1994), is a transcript that has been characterized in a number of fish species (Geslin and Auperin, 2004, Goetz et al., 2004, Ings and Van Der Kraak, 2006, Kocerha et al., 2010, Kusakabe et al., 2009, Li et al., 2003, Nunez and Evans, 2007). StAR protein transports cholesterol from the outer mitochondrial membrane to the inner membrane where it is cleaved into pregnenolone and is subsequently converted into other steroid hormones; as such, *StAR* is localized predominantly in steroidogenic tissues (Manna et al., 2009). Studies have also demonstrated that 17 β -estradiol (E2), a natural ligand and activator of the ER signaling cascades, regulates the expression of *StAR* mRNA (Houk et al., 2004, Kortner et al., 2009, Sharpe et al., 2007), but the underlying mechanisms are not completely understood in teleost fish. StAR transcription in mammalian systems is highly regulated through the cAMP/PKA pathway. The promoter is conserved across species with binding elements for steroidogenic factor-1 (SF-1) (Christenson et al., 2001), activator protein 1 (AP-1) (Shea-Eaton et al., 2002), nuclear factor DAX-1, a dominant negative regulator of transcription (Zazopoulos et al., 1997), cAMP response element-binding protein (CREB) (Manna et al., 2003), among others (Manna et al.,

2003) and is controlled by circadian rhythms through ROR α and rev-erba (Kocerha et al., 2010).

Largemouth bass (*Micropterus salmoides*; LMB) is a species of teleost fish that inhabits the greater part of North American freshwater systems and is highly susceptible to the negative effects of a number of endocrine disrupting chemicals (EDCs), including xenoestrogens (Blum et al., 2008, Garcia-Reyero et al., 2006, Hinck et al., 2008, Sabo-Attwood et al., 2007, Sepulveda et al., 2003). LMB are a commercially important game fish and are considered to be top predators in the food chain, making the species prone to bioaccumulation of contaminants in the environment. The reproductive cycles of LMB are semi-synchronous and reproductive progression is predominantly dependent upon water temperature, photoperiod, and steroid hormone levels. E2 tightly controls the expression of genes centrally involved in reproduction via genomic and non-genomic signaling. For example, studies have shown that *ER* and *StAR* mRNA expression levels vary throughout the reproductive cycle in many teleost species (Kocerha et al., 2010, Kusakabe et al., 2006, Nakamura et al., 2005, Rocha et al., 2009, Sabo-Attwood et al., 2004). However, most concerning is that, in various locations throughout the U.S., intersex male smallmouth bass and LMB (bearing both eggs and sperm in testes) have been observed and research has linked this high occurrence of intersex traits with EDCs in the watershed (Blazer et al., 2012, Iwanowicz et al., 2016). These effects can be due, in part, to disruption of steroid biosynthesis and altered ER signaling. Thus, continued research on the regulation of steroidogenesis by estrogens is an important physiological, economic, and ecological topic, in order to ensure healthy fish populations.

The objectives of this study were to determine how *StAR* is regulated during the reproductive cycle in female LMB and more specifically, to determine how it is regulated by estrogens. Analysis of the *StAR* promoter revealed a putative estrogen response element, leading to the hypothesis that *StAR* was directly regulated by estrogens in the LMB ovary. To test the hypothesis, we performed both *in vivo* and *in vitro* experiments to characterize *StAR* expression in LMB and test whether ERs could bind and transactivate expression from the promoter. These studies support the hypothesis that there is a direct role for ERs in regulating *StAR* gene transcription in the female LMB ovary. However, additional studies are necessary to determine if this role is predominant and functional at physiological concentrations of E2.

2. Materials and Methods

2.1. Female Largemouth bass collections from the wild

To determine the expression pattern of *StAR* in LMB ovaries over a reproductive season, wild individuals from the St. John's River in Welaka, FL were collected once per month from the same general location from October 2005 to September 2006. The river at this location has relatively low inputs from agricultural or industrial uses and has been considered by us and by others as a reference site (Martyniuk et al., 2016, Sepulveda et al., 2002). The river's temperature varies from 60 °F in winter to almost 90 °F in summer (Martyniuk et al., 2016, Sepulveda et al., 2002). It is fed by numerous springs that have a relatively constant temperature of 72 °F year-round. The period between sampling events

ranged from 3–5 weeks. Approximately 10 females were collected every month, and body weight, body length (tip of the mouth to tip of the tail), gonad weight, and age were recorded. Gonadosomatic indices (GSIs) were calculated for all individuals used in this study as absolute gonad weight/absolute body weight X 100. Following euthanasia, gonadal tissue and blood were collected from each individual and samples were stored at –80 °C until further processing, as described previously (Doperalski, 2009, Martyniuk et al., 2009).

Reproductive stage of each individual was verified by microscopic examination of ovarian histology samples following staining with hematoxylin/eosin as previously described (Martyniuk et al., 2009). Female LMB were categorized into one of six predominant stages; perinucleolar (PN), cortical alveoli (CA), early vitellogenic (EV), late vitellogenic (LV), maturation (M), and atresia (AT) (Martyniuk et al., 2013). To determine how StAR mRNA levels corresponded to circulating E2, plasma samples were extracted and reconstituted in buffer prior to determination of E2 concentration by enzyme-linked immunosorbent assay (ELISA) as previously described (Doperalski et al., 2011). All animals were treated as per the guidelines outlined by University of Florida Institutional Animal Care and Use Committee under protocol #201202759.

2.2 Experimental feeding studies with 17alpha-ethinylestradiol (EE2)

Female fish were fed a potent estrogen (EE2) in a diet study to determine how estrogens regulate StAR expression *in vivo*. LMB for this study were purchased from American Sport Fish Hatchery (Montgomery, AL, USA). Fish were housed in the Aquatic Toxicology Facility at the Center for Environmental and Human Toxicology at the University of Florida. EE2 was dissolved in 90% ethanol, mixed with menhaden oil, and coated onto a trout diet feed (Silver Cup, Ogden, UT, USA). The high dose of EE2 was approximately 200 ng EE2/g feed and the low dose was approximately 70 ng EE2/g feed. Feed was prepared as per Colli-Dula et al. (Colli-Dula et al., 2014). The feeding study commenced in early March when the LMB were in mid-gametogenesis, and LMB were fed (approximately 2 % body weight/day) for 55 days (~2 months). Natural photoperiod and ambient water temperature were maintained throughout the experiment. At the end of the experiment, fish were anesthetized with MS-222 (200 mg/L buffered with sodium bicarbonate). Blood was collected from the caudal vein and fish were euthanized by spinal dislocation. Ovary samples (along with other tissues) were collected, snap-frozen in liquid nitrogen and stored at –80 °C until processed into RNA.

Concentrations in the plasma of treated animals at the end of the experiment was verified using GC-MS (Colli-Dula et al., 2014) and were 540 ± 0.11 ng/ml and 260 ± 110 ng/ml, respectively (N = 4 to 7 individuals/treatment). Our intent was to achieve an environmental and physiologically-relevant dose of EE2 (pg/ml) in the blood of LMB, and our expectation was that EE2 would be metabolized significantly by the liver over the two-month feeding regime. However, contrary to our expectation, EE2 accumulated in the blood over time. We did not measure EE2 in the ovary, which may be less than that in circulation. Here we point out that EE2 levels in the fish are not physiologically relevant but posit that the experiment offers some useful information about StAR regulation by estrogens, in the context of the other experiments conducted in this study.

2.3 RNA Extraction and Purification

Tissues were homogenized in the phenol-based RNA isolation reagent STAT60™ (Tel-Test, Inc., Friendswood, TX, USA) using a fine-tipped tissue homogenizer (IKA Works, Inc., Wilmington, NC, USA). RNA was extracted according to the manufacturer's protocol (Tel-Test, Inc., Friendswood, TX, USA). Each resulting RNA pellet was washed twice with 75% ethanol, dried, and resuspended in the appropriate amount of RNAsecure™ (Ambion, Inc., Austin, TX, USA) to yield a final concentration of 1 µg/µL. All residual DNA contamination was removed from each sample using a DNAfree™ kit according to the manufacturer's protocol (Ambion, Inc.). Purified RNA samples were stored at -80 °C. Prior to use, RNA concentration and quality were quantified on a NanoDrop Spectrophotometer (Nanodrop Technologies, Wilmington, DE) and all ratios of A260/280 > 1.8.

2.4 cDNA Synthesis and Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Purified RNA samples (500 ng) were reverse transcribed using SuperScript II RT (Invitrogen, Inc.) according to the manufacturer's protocol. Biological replicates for qPCR were measured in duplicate and each reaction contained 1X iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 100 ng first-strand cDNA, corresponding forward and reverse primers, and nuclease-free water in a final reaction volume of 25 µL. Primers used to amplify genes of interest were previously optimized and rigorously tested in our laboratory (Blum et al., 2008, Garcia-Reyero et al., 2006, Martyniuk et al., 2009, Martyniuk et al., 2011, Sabo-Attwood et al., 2007, Sabo-Attwood et al., 2004), and efficiencies were between 90–110% and $R^2 > 0.97$. Primers used in ChIP assays were designed using Primer3 software (Rozen and Skaletsky, 2000) and their efficiencies were between 93–107%. All primer sequences used in this study are listed in Table 1.

All data were generated using an iCycler Thermal Cycler (Bio-Rad Laboratories, Inc.) using PCR parameters recommended by the manufacturer. Standard curves relating initial template copy number to fluorescence and amplification cycle were generated using a pGEM-®T easy vector (Promega Corp., Madison, WI, USA) containing the gene of interest as a template. Expression values of all genes are reported as absolute copy number per µg total RNA.

Seasonal variation in conventional housekeeping genes has been previously reported in ovaries of aquatic species (Doperalski et al., 2011, Ings and Van Der Kraak, 2006, Rocha et al., 2009) and normalization methodologies have been developed to standardize housekeeping gene expression values when such a pattern of variation is found (Billiau et al., 2001, Essex-Fraser et al., 2005). In this study, female 18S values were standardized to an arbitrary “control” group (PN stage) as reported in Doperalski et al. (Doperalski et al., 2011). The standardized 18S values were then used to normalize gene expression data for female LMB as a ratio of target gene expression normalized to standardized 18S values for each individual sample (Martyniuk et al., 2013).

2.5 Gonadal explant cultures.

Explant cultures of control and EE2 treated LMB were tested for E2 production *in vitro* following previously reported methods (Martyniuk et al., 2016). Briefly, ovaries were

obtained immediately upon euthanasia of fish and minced into small pieces of approximately 20 mg and placed into wells of a 24-well plate. The ovarian fragments were cultured in duplicate in 1 ml Leibovitz's L-15 culture media supplemented with 1% antibiotic-antimycotic (ABAM) solution (ThermoFisher). After settling in the well, half of the cultures were treated with 10 U/ml hCG. Culturing continued for 20 h on a rocking platform and at room temperature in the dark.

2.6 Analysis of hormones in plasma and cell culture media.

Estradiol production in the cell culture media was analyzed by ELISA as described (Doperalski, 2009). Briefly, a commercially available ELISA kit (Immuno-Biological Laboratories, Inc., Minneapolis, MN) was utilized. A standard curve for E2 or testosterone (T) was prepared in culture media. Culture media from the explants were tested directly in duplicate on the same plate as the standard curve. To evaluate hormones in plasma samples, 100 μ L of LMB plasma or steroid standards prepared in charcoal-stripped LMB plasma were extracted twice with 1.0 mL of 1-chlorobutane (99%+ purity, Acros Organics). Extracted samples were reconstituted overnight at 4°C with sample buffer (50 mM sodium phosphate, 0.1% gelatin, 0.1% RIA grade Fraction V bovine serum albumin, pH 7.6) and measured by ELISA as above (Doperalski, 2009). Reconstituted samples were spiked with 5 nCi tritiated E2 or T, specific activity of 44 Ci/mmol and 73 Ci/mmol, respectively (Amersham Radiochemicals) to determine matrix effects in the assay.

2.7 In Silico Transcription Factor Analyses

The 2.9 kb LMB (Acc. No. DQ166819) promoter sequence was analyzed for putative estrogen response/ER binding sites using MatInspector Release Professional (version 8.0.4 August 2010) published by Genomatix, Inc (Cartharius et al., 2005). All elements predicted carried at least 70% to 80% homology to the core vertebrate element sequences.

2.8 Cell Culture

MA-10 mouse Leydig tumor cells were generously provided by Dr. Mario Ascoli (Ascoli, 1981). MA-10 cells were cultured in RPMI-1640 culture medium supplemented with charcoal-stripped horse serum (15% v/v), 20 mM HEPES, pH 7.2, and 50 μ g/mL gentamicin, pH 7.7. Cells were cultured in 75 cm² flasks coated with 0.1% gelatin (dissolved in calcium- and magnesium-free phosphate-buffered saline) prior to seeding into plates or dishes for experimentation. The cells were passaged every 5–6 days and never exceeded 10 passages before a new vial was thawed. Cells were grown and maintained at 37 °C in a humidified 5% CO₂ cell culture incubator.

2.9 Transfection and ChIP Assays in MA-10 cells

MA-10 cells were cultured in 150 mm plates coated with 0.1% gelatin and grown to ~70% confluency; each plate was transfected with the 2.9 kb LMB StAR gene promoter plasmid using Eugene HD (Roche Diagnostics, Indianapolis, IN). Briefly, MA-10 cells were plated in 0.1 % gelatin coated 24-well culture plates (125,000 cells/well) and were cultured for 24 hours prior to transfection. Transfection reactions consisted of a 4:1 ratio of FuGENE HD (Roche Diagnostics, Indianapolis, IN) to plasmid DNA (2 μ l FuGENE HD/0.5 μ g total DNA

per well) suspended in 25 μ L media with no serum or antibiotics. When promoter activity was examined, the StAR promoter construct was cotransfected with a Renilla luciferase construct (400:1 w/w ratio) as a background control. Cells were transfected overnight (18 hours) prior to exposure to chemicals. Cells were treated with 0.2% ethanol (vehicle control) or E2 (50 and 500 nM E2) for 20 hours. E2/vehicle treatments were then refreshed either alone (at the same doses) or supplemented with 10 U/mL human chorionic gonadotropin (hCG) for an additional 4 hours. Following exposures, cells were lysed, and luciferase levels were quantified using the Dual Luciferase Assay (Promega, Inc., Madison, WI) according to the manufacturer's protocol.

ChIP was performed using the ChIP-IT Express Kit (Active Motif, Inc.) according to the manufacturer's protocol. An antibody against mouse IgG was used as a non-specific, background control. Transfected cells were treated with E2 alone or in combination with hCG, a potent stimulator of the steroidogenic pathway. In brief, cells were treated with a fixation solution containing 37% formaldehyde for 10 minutes at room temperature to freeze transcription factors directly on the DNA, followed by washing of the cells in ice cold 1X PBS for 5 seconds. The reaction was stopped using glycine stop-fix solution as per protocol and scraped from the plate in a solution containing 1X PBS and 100 mM phenylmethylsulfonyl fluoride (PMSF).

Following optimization, chromatin was sheared via sonication using a Fisher Sonic Dismembrator (~15 pulses for 15 seconds on ice at a power of 4 with approximately 20 second rest in between pulses). Immunoprecipitation reactions (25 μ g chromatin/2 μ g antibody per reaction) were incubated overnight at 4 °C, beads were washed in 800 μ L ChIP buffer 1 and 2 as per protocol. Following re-pelleting and re-suspension in elution buffer, crosslinks were reversed using 50 μ L Reverse Cross-Linking Buffer provided in the kit. Protein was digested by treating with 2 μ L of 0.5 μ g/U Proteinase K for 1 hour at 37°C and RNA was removed using 1 μ L 10 μ g/U RNase A. Reactions were stopped after 1 hour using the Proteinase K Stop Solution provided in the kit.

DNA was purified using a QIAquick PCR Purification Kit (QIAGEN, Valencia, CA) as per the manufacturer's protocol and qPCR was run on all samples using primers designed against the putative ERE/-1745 binding site in the LMB StAR promoter (Table 1).

Primers were tested and verified using the manufacturer-recommended 2-step PCR protocol with a lowered annealing temperature of 58 °C; products were subjected to a melt curve to verify specificity of the primers for the site. The control antibody (IgG) was purchased from Active Motif, Inc. Polyclonal antibodies specific to mammalian ESR1 and ESR2 used for immunoprecipitation were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Data were normalized to a 1:1000 dilution of the DNA input control within each treatment (due to extremely high transfection efficiency input samples were diluted). Data are reported as % net enrichment (IgG enrichment subtracted as background) normalized to the input for each treatment. Graphed data are representative of one experiment, which was replicated at least three times to independently verify the results.

2.10 Statistical Analyses

Gene expression data were subjected to ANOVA followed by Dunnett's T3 post-hoc test. The assumption of unequal variances was used to evaluate differences in $\log_{10}$ copy number across reproductive stages; however, mean values ($\pm$ SEM) are plotted in all gene expression graphs. All transfection data, ELISA data, and remaining gene expression data were subjected to ANOVA followed by Tukey's post-hoc test. Spearman correlation was used to determine whether or not there were relationships among transcripts, E2, and gonadosomatic index. Statistical tests were conducted using both SPSS Statistics v17.0 (SPSS Inc., Chicago, IL, USA) and SigmaPlot v11.0 (Systat Software Inc., Chicago, IL, USA). P-values < 0.05 were considered statistically significant.

3. Results

3.1 Plasma E2 and ovarian StAR mRNA levels increase over the female reproductive cycle

LMB were collected monthly from the St. John's River in Welaka, FL, spanning one full reproductive cycle. We have previously described histological assessments for LMB female ovary (Martyniuk et al., 2013). Our focus here was to examine the expression of *StAR* in the ovary in relation to plasma E2 (Doperalski et al., 2011). We re-graphed previously collected E2 data in females (37) and measured *StAR* mRNA levels in the ovary for comparison at each stage of reproductive maturation. Plasma E2 levels significantly varied over reproductive stage and increased more than 10-fold between EV and LV, peaking at > 3000 pg/mL during maturation (M; Figure 1A). Ovarian *StAR* mRNA expression was quantified by qPCR from LMB collected throughout the reproductive season and data were analyzed by reproductive stage (Fig. 1A). Expression levels of *StAR* varied significantly between reproductive stages (d.f. = 5; $F = 29.07$; $p < 0.0001$). *StAR* mRNA levels were constitutively low ($\sim 10^4$ copies/ μ g total RNA) during the early reproductive stages (PN, CA, EV) and levels increased by more than 10-fold and 70-fold during LV and M, respectively. Following a peak at M, *StAR* mRNA levels dropped to levels comparable to that observed in primary growth stages. Plasma E2 and *StAR* mRNA levels were highly correlated and peaked during M, displaying nearly a 100-fold increase in levels between primary growth (PN, CA) and M.

We also examined the expression levels of the estrogen receptors in the ovary at each stage of ovarian maturation. Expression levels of *estrogen receptor alpha* (*esr1*) varied significantly between reproductive stages (d.f. = 5; $p < 0.05$) and were higher in expression levels at M compared to earlier primary growth stages (e.g. cortical alveoli, CA) (Fig. 1B). Interestingly, expression levels of *estrogen receptor beta a* (*esr2a*) showed a decreasing trend in expression in the ovary from early growth stages to M while *estrogen receptor beta b* (*esr2b*) remained relatively consistent in expression over ovarian development (Fig. 1C). For each of the estrogen receptor beta isoforms, there was no significant difference detected between reproductive stages.

We detected a number of pairwise correlations for transcripts in the gonad of female LMB collected from the St John's River (Table 2). Most notably, reproductive stage, GSI, E2, *esr1* mRNA, and *StAR* mRNA were all significantly and positively correlated, and these values

ranged from $r = \sim 0.4\text{--}0.7$. Each of the three ER isoforms also showed a positive correlation in expression with each other. *StAR* expression, however, was only associated with the expression pattern of *esr1* and did not significantly correlate with the expression patterns of the *esr2* isoforms (Table 2).

3.2 Ovarian *StAR* mRNA Levels Are Repressed in Response to Long Term EE2 Treatment

To determine whether exposure to a potent estrogen receptor agonist, EE2, could modulate ovarian *StAR* mRNA expression *in vivo*, a second group of female LMB housed in the laboratory were fed food laced with EE2. The two groups included a low dose feed (70 ng/g feed, 0.07 ppm) or a high dose feed (200 ng/g feed, 0.2 ppm) of EE2 or vehicle ($n = 4\text{--}7$ per treatment) for 55 days. Ovarian *StAR* mRNA levels were quantified by qPCR (Fig. 2A). Exposure to 0.07 ppm EE2 through the diet did not significantly affect *StAR* mRNA levels; however, the highest dose of EE2 elicited a significant reduction in *StAR* mRNA levels, reducing relative abundance to $\sim 4\%$ of that observed in vehicle-treated control animals ($p = 0.018$). We also measured plasma levels of E2 and T (Fig 2B and 2C), and both of these sex steroid hormones were reduced in a dose dependent manner in the fish, although because of fish to fish variation, the differences were not significant. These data indicate that *in vivo* exposure to the potent ER agonist and synthetic estrogen EE2 can suppress ovarian *StAR* mRNA levels in LMB, probably through classic E2 negative feedback through the HPG axis.

Ovaries were removed from control and 0.2 ppm EE2 treated fish and minced into fragments and cultured *in vitro* under basal and hCG treatments. Production of E2 in the culture media was quantified by ELISA (Fig. 2D). There was a significant drop by 27% in the ability of explants from EE2-treated fish to produce E2 compared to controls. These supportive data suggest that gonadal cultures with reduced *StAR* mRNA were not able to make E2.

3.3 In Silico Analysis of the LMB *StAR* Gene Promoter

To determine whether *StAR* is directly regulated by ER signaling at the transcriptional level, computer software designed to analyze DNA sequences for putative transcriptional elements was used to examine a 2.9 kb segment of the LMB *StAR* gene promoter (Kocerha et al., 2010) for putative estrogen-response elements (EREs). There were 9 putative estrogen-response sites predicted in the LMB and all sequences showed greater than 70% homology to consensus vertebrate core element sequences (Fig. 3A). Interestingly, all elements were predicted to be estrogen-related receptor binding elements aside from ERE/−1745 in LMB, which was a putative canonical ER half-site. The sequence shares the GTCA ER half-site to which ERs may directly bind (Fig. 3B).

3.4 ERs Directly and Differentially Bind to the LMB *StAR* Promoter

To examine whether ERs functionally bind to the predicted ER binding site ERE/−1745 in the LMB *StAR* promoter sequence, ChIP assays were performed using MA-10 mouse Leydig cells transfected with the LMB *StAR* promoter construct with antibodies designed against mammalian ESR1 and ESR2. The LMB *StAR* promoter displayed a unique pattern of enrichment of ESR1 and ESR2 (Fig. 4). ESR1 was enriched by 15% under basal conditions (in the absence of added E2) and increased 4-fold (up to 60% enrichment of input) upon E2 exposure. ESR1 enrichment was minimal under hCG-stimulated conditions

(in the presence or absence of added E2). ESR2 was detectable and minimally enriched (~6 – 8%) under hCG-induced conditions only, with no ESR2 enriched when cells were treated with E2 under basal or induced conditions. Noteworthy, under basal conditions, ESR1 was more highly enriched than ESR2, whereas under hCG-induced conditions, ESR2 appeared more enriched than ESR1 (Fig. 4).

3.5 Gonadotropin-Induced Activation of the LMB *StAR* Promoter is decreased in Response to E2

To investigate whether estrogen regulates the transcriptional activation of LMB *StAR* at the promoter level, transient transfections were carried out in MA-10 mouse Leydig tumor cells using a luciferase reporter construct containing a 2.9 kb segment of the LMB *StAR* promoter. Transfected cells were exposed to vehicle or E2 (50 or 500 nM) for 20 h followed by a replenished exposure to E2/vehicle for an additional 4 hours. Cells were lysed and luciferase levels were quantified. Changes in activation of the promoter in response to the treatments are reported as fold-change from vehicle control. E2 treatment had no significant effect on the basal activity of the LMB *StAR* promoter (Fig. 5A). To examine whether E2 might affect gonadotropin-induced activation of the LMB *StAR* promoter, the same exposures were conducted as in the E2 study, except after 20 hours when the E2/vehicle was replenished, 10 U/mL hCG was also added for the additional 4 hours. The activity of the LMB *StAR* promoter was stimulated greater than 4-fold upon exposure to hCG, whereas the combination of either dose of E2 and hCG elicited an approximately 25% decrease in activation of the promoter as compared to the intact response to hCG alone ($p < 0.001$) (Fig. 5B). Therefore, E2 significantly reduced the response of the LMB *StAR* promoter to gonadotropin stimulation. All graphs represent data collected from at least two independent experiments conducted in triplicate.

4.0 Discussion

4.1 *StAR* is differentially expressed in the LMB ovary during sexual maturation

This is the first study investigating the molecular mechanisms underlying E2-driven regulation of the *StAR* gene in LMB. This study demonstrated that ovarian *StAR* mRNA levels were significantly and positively correlated with plasma E2 levels throughout the reproductive cycle. *StAR* mRNA abundance in the LMB ovary peaked markedly during late reproductive stages and expression levels increased nearly 3fold during EV and nearly 100-fold during M from levels observed during earlier reproductive stages in the ovaries of LMB. Gonadotropins drive gonadal steroid hormone biosynthesis in LMB and other teleost species. A study of gonadotropin subunit expression in the pituitary of LMB by reproductive stage utilizing the same individuals studied in our study revealed that gonadotropin subunit expression peaked during LV and M in female LMB pituitary (Martyniuk et al., 2009), paralleling expression patterns observed in *StAR* mRNA in female ovary observed here. Gonadal *StAR* expression has been characterized throughout the reproductive stages of multiple species of fish including rainbow trout and European sea bass (Campbell et al., 2006, Kusakabe et al., 2006, Rocha et al., 2009), and all studies report an increase in *StAR* expression during late reproductive stages, as observed here in LMB. A transcriptomics study in LMB also confirms that there is an increase in *StAR* mRNA in the ovary throughout

oocyte maturation, as this transcript was increased approximately 5-fold in late maturation phases compared to early vitellogenic stages (Martyniuk et al., 2009, Martyniuk et al., 2013). Both the aforementioned microarray study and the present study demonstrate that, if the oocytes are reabsorbed and the ovary becomes atretic, relative *StAR* mRNA dramatically decreases 10–30-fold. Thus, *StAR* protein, which is required for transporting precursor cholesterol across the plasma membrane of mitochondria during the first step of steroid hormone biosynthesis, is likely increased in order to accommodate steroid production for reproduction; furthermore, *StAR* mRNA expression closely parallels LMB plasma E2 levels throughout the stages.

We also measured the expression of estrogen receptors in the ovary and observed that *esr1* mRNA increased in the ovary when the LMB were becoming sexually mature. Conversely, *esr2a* expression decreased in the ovary as the females matured and *esr2b* remained relatively constant in expression over ovarian maturation. These patterns are consistent with data from other fishes; *esr1* is expressed at higher levels in the ovary at advanced maturity stages (IV and V) in Asian swamp eel (*Monopterus albus*) compared to earlier stages (Ding et al., 2016) and *esr1* expression in the orange-spotted grouper (*Epinephelus coioides*) (Chen et al., 2011) is lower in primary growth stages within the ovary, reaching a peak in mRNA levels at maturation. In the study by Chen et al. (Chen et al., 2011), it was also observed that *esr2b* (ER β b) is primarily expressed in immature ovaries and that *esr2a* (ER β a) showed higher variation in expression during ovarian development. These data correspond to the expression patterns observed here in LMB and suggests that the estrogen receptor beta subunits are more abundant in early stages of oocyte maturation compared to later stages. In addition, Sabo-Attwood et al. (Sabo-Attwood et al., 2004) demonstrated that over the months of November to March, as the female ovary tissue grows, there was a marked decrease in the expression of the *esr2a* and *esr2b* in the ovary of laboratory reared female LMB, a trend noted here in the wild-caught LMB. However, the study by Sabo-Attwood reported a decrease in *esr1* mRNA in the ovary with maturation, thus the discrepancy between the present study and that previously observed in LMB may be related to how the females were grouped prior to the study analysis (i.e. by month rather than reproductive stage). Ideally, because LMB ovary contains oocytes at different stages of maturation at any given time, future studies should segregate individual oocytes for a more accurate quantitation of estrogen receptor expression at different stages of oocyte maturation. The expression pattern of *StAR* was significantly and positively associated with both *esr1* and E2, suggesting a direct (or indirect) relationship between the enzymes and circulating plasma E2. It is perhaps not surprising that E2 is correlated with *StAR* expression, as it is one of the products of steroidogenesis. These studies demonstrate that LMB *StAR* is regulated in expression during oocyte maturation and is responsive to E2 feedback in the ovary.

4.2 ChIP analysis of the LMB *StAR* promoter

To determine whether E2 directly regulates *StAR* at the transcriptional level, a 2.9 kb segment of the LMB *StAR* promoter was examined for putative E2 response elements. A putative ER binding site (ERE/–1745) carrying ~80% homology to a perfect consensus for an ER binding site was predicted in the distal segment of the LMB *StAR* promoter sequence.

To test the hypothesis that estrogens directly regulate StAR expression, we used the established MA10 cell line derived from a mouse Leydig tumor (Ascoli, 1981). Characterization of these cells showed that they had functional LH/hCG receptors and could respond to signals from exogenously added LH or hCG and were able to produce steroid hormones. Thus, they have all the machinery required for hormone production, in addition to StAR. In fact, this cell line was used in the first studies to characterize mammalian StAR protein (Clark et al., 1994). The ChIP analysis was used to determine how the StAR promoter is regulated within cells that have all the available machinery.

ER interaction with the LMB ERE/-1745 was evaluated under basal and hCG treated conditions. The results indicated that, under basal conditions, E2 stimulates the binding of ESR1 to the LMB promoter by a factor of about 4–5-fold. ESR2, on the other hand, was not enriched at this site by the addition of exogenous E2. Treatment of the cells with hCG effectively antagonized the binding of ESR1 to the promoter, suggesting that during ovarian maturation influenced by pituitary gonadotropins, estrogen receptors are inhibited from binding to this site on the promoter. This result suggests that StAR is preferentially regulated by ESR1

Interestingly, when cells were treated with hCG in the absence of E2, there was a slight increase in the binding of ESR2 to the LMB site, but neither ESR1 nor ESR2 was enriched at this site in the presence of additional exogenous E2. The significance of this slight increase is not apparent at this time.

4.3 Response of LMB to exogenous estrogens in vivo

To test the responsiveness of *StAR* mRNA to estrogens, *in vivo* exposure of female LMB to the potent ER agonist EE2 elicited a marked decrease in ovarian *StAR* mRNA levels. In the high EE2 group, ovarian *StAR* mRNA levels were suppressed to ~5% of that of the LMB control group. At lower EE2 concentration, the suppression of *StAR* mRNA was not as notable, suggesting that the primary control of *StAR* is at the level of the pituitary or hypothalamus, through a classic negative feedback loop. Other studies have demonstrated that E2 treatments can elicit a decrease in *StAR* gene expression in goldfish and mouse (Houk et al., 2004, Kortner et al., 2009, Sharpe et al., 2007) and rainbow trout (Nakamura et al., 2009); however, the mechanisms that regulate *StAR* are not fully elucidated and many other transcriptional co-factors are needed for expression (Christenson et al., 2001, Manna et al., 2009, Manna et al., 2003, Shea-Eaton et al., 2002, Zazopoulos et al., 1997). Additional experiments may be warranted to determine the physiological significance of control of the promoter by estrogen receptors compared to other known regulatory factors. It is possible that E2 exerts control only when its concentrations in the plasma are high enough to also shut down the system through the pituitary.

We propose that in early growth stages of the ovary, the estrogen receptor beta isoforms show high relative expression and act to regulate initial growth of the oocytes, along with *esr1* (Fig. 6). During this period, gonadotropins are increasingly expressed in the LMB pituitary (Martyniuk et al., 2009) to promote the production of sex steroid hormones. As the ovary matures, the expression of estrogen receptor beta isoforms gradually declines. As the expression of *esr1* is auto-regulated by E2, further binding and activation of StAR is

promoted with the increase in E2. At later stages of maturation, there is a down-regulation of ESR1, which acts to reduce StAR expression and decreases circulating E2.

Important for the control of StAR transcription is the stimulation of steroidogenic cells by LH/FSH or ACTH, resulting in cAMP production, which in turn activates PKA (Hatano et al., 2016, Manna et al., 2009). But other activation pathways, such as those through PKC, have also been described. Stimulation through these pathways activates several transcription factors including SF1, CREB, GATA4 and Ap1 that bind to proximal elements in the StAR promoter, and with the additional action of coactivators, act to increase transcription. Specifically PKA phosphorylates CREB and GATA4 and is involved in de-sumoylation of SF1 (Jefcoate and Lee, 2018, Manna et al., 2009, Martyniuk et al., 2011, Yang et al., 2009). The exact mechanisms involved in regulating StAR expression are complicated and involve chromatin remodeling (Jefcoate and Lee, 2018), histone deacetylation (Hiroi et al., 2004), and both positive and negative effectors binding to the proximal promoter. Here we demonstrate that estrogen regulates the binding of ERs to the proximal promoter of StAR under basal conditions, which may directly affect the expression of StAR in the ovary of LMB and provide a new angle for understanding the mechanism of its regulation in the LMB ovary.

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Highlights

1. Largemouth bass steroidogenic acute regulatory protein is regulated by estradiol
2. Estrogen receptors bind to the promoter of steroidogenic acute regulatory protein
3. Chromatin immunoprecipitation assays suggest the ERE binding sites are functional

Fig. 1:

(A) Plasma 17 β -estradiol (E2) and *StAR* mRNA levels by female LMB reproductive stage. Plasma E2 levels and gonadal *StAR* mRNA levels were quantified by ELISA and qPCR, respectively, from blood and ovary samples collected throughout the reproductive season. E2 data (left axis) have been published previously (Doperalski et al., 2011). The normalized copy numbers of *StAR* closely follow the levels of plasma E2. (B) The normalized copy numbers of *esr1* and (C) *esr2a* and *esr2b* are quantified at each stage of ovary development. Perinucleolar (PN), cortical alveoli (CA), early vitellogenic (EV), late vitellogenic (LV), maturation (M), and atresia (AT). Different letters indicate statistical differences among individual stages ($P < 0.05$).

Fig. 2:

Dietary exposure of LMB to 0.07 ppm and 0.2 ppm EE2. (A) *StAR* mRNA levels in the ovary following a 2-month *in vivo* dietary exposure to 0.07 and 0.20 ppm EE2. (B) Plasma levels of E2 in treated fish. (C) Plasma levels of T in treated fish. (D) 17 β -estradiol production in media of ovarian explants from control and 0.2 ppm EE2-treated fish cultured under basal and hCG stimulation. Different letters indicate statistical differences among samples.

Fig. 3.

Putative ER binding sites in LMB *StAR* promoter. (A) Following *in silico* analysis of the LMB *StAR* promoter sequence, one putative ER binding sites was predicted amongst several ERR sites, and sites are graphically represented. All elements plotted carried >80% homology with core vertebrate sequences. (B) Sequence information for the putative ER binding site (ERE/-1745) is listed along with a perfect canonical mammalian ER binding element. Capital letters indicate a match to the perfect consensus ERE. Lower case letters denote the variable nucleotides separating the canonical sequences.

Fig. 4:

ChIP analysis of MA-10 cells transiently transfected with the LMB *StAR* promoter construct. Examination of ER binding to the LMB *StAR* promoter was done by ChIP enrichment and the ERE/-1745 was analyzed by qPCR using the purified DNA samples. ChIP data were normalized to dilutions of chromatin input and are plotted as net % enrichment above background controls (with IgG-background subtracted).

Fig. 5:

Transactivation of the LMB StAR promoter using the StAR promoter/luciferase construct.

(A) Effects of E2 on StAR promoter construct luciferase expression under basal conditions.

(B) Effects of E2 on hCG-induced response of StAR promoter construct luciferase expression. All data were plotted as fold-change above vehicle only control; Different letters indicate a significant difference amongst treatments within the experiment ($P < 0.05$).

Fig. 6.

Model of estrogen regulation of StAR in the largemouth bass ovary. Female stages are classified as follows: a) perinucleolar (PN); b) cortical alveoli (CA); c) early vitellogenic (EV); d) late vitellogenic (LV); e) maturation (M); and f) atresia (AT). Scale bars correspond to 200 μ m. Abbreviations are as follows: germinal vesicle (GV); nucleolus (N); primary growth follicle (PGF); ovarian lumen (OL); oil droplet (OD); cortical alveoli (CA); germinal epithelium (GE); zona pellucida (ZP); and yolk globule (YG).

Table 1:

qPCR primer sequences for gene expression and ChIP assays. All sequences are listed 5' → 3' exactly as they were ordered (Forward – sense strand, Reverse primer – anti-sense strand).

	Forward Strand	Reverse Strand
qPCR Primers		
ER α	5'-CGA CGT GCT GGA ACC AAT GAC AGA G-3'	5'-TCC GGT CAC GA TGA TTT TCC TCC TCC A-3'
ER β a	5'-GTG ACC CGT CTG TCC ACA-CA-3'	5'-TCT GGG GTC AGT GCA GGA GA-3'
ER β b	5'-CCG ACA CCG CCG TGG TGG ACT C-3'	5'-AGC GGG GCA AGG GGA GCC TCA A-3'
StAR	5'-GGG CTC CACCTG CTT CTT G-3'	5'- ACC CCT CTG CTC AGG CAT TT -3'
ChIP Primers		
LMB ERE/-1745	5'-CCC TCC ATC CAG CCA GAG-3'	5'-GCA AAA TCC AAA CTA TCT AAC AGC AA-3'

Table 2.

Spearman correlation for expression patterns of transcripts in relation to largemouth bass gonadosomatic index

	GSI	E2	StAR	ERa	ERba	ERbb
Stage	0.640 ***	0.622 ***	0.744 ***	0.401 **	-0.332 *	0.111
GSI		0.682 ***	0.852 ***	0.383 *	-0.313 *	-0.075
E2			0.721 ***	0.458 **	-0.195	0.055
StAR				0.516 ***	-0.199	0.109
ERa					0.471 **	0.687 ***
ERba						0.705 ***

*
p < 0.05**
p < 0.01***
p < 0.001