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CLONING AND EXPRESSION OF THE TRANSLOCATOR PROTEIN (18 KDA), VOLTAGE-DEPENDENT ANION CHANNEL, AND DIAZEPAM BINDING INHIBITOR IN THE GONAD OF LARGEMOUTH BASS (*MICROPTERUS SALMOIDES*) ACROSS THE REPRODUCTIVE CYCLE

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Abstract

Cholesterol transport across the mitochondrial membrane is rate-limiting for steroidogenesis in vertebrates. Previous studies in fish have characterized expression of the steroidogenic acute regulatory protein, however the function and regulation of other genes and proteins involved in piscine cholesterol transport have not been evaluated. In the current study, mRNA sequences of the 18 kDa translocator protein (*tspo*; formerly peripheral benzodiazepine receptor), voltage-dependent anion channel (*vdac*), and diazepam binding inhibitor (*dbi*; also acyl-CoA binding protein) were cloned from largemouth bass. Gonadal expression was examined across reproductive stages to determine if expression is correlated with changes in steroid levels and with indicators of reproductive maturation. In testis, transcript abundance of *tspo* and *dbi* increased with reproductive maturation (6- and 23-fold maximal increase, respectively) and expression of *tspo* and *dbi* was positively correlated with reproductive stage, gonadosomatic index (GSI), and circulating levels of testosterone. Testis *vdac* expression was positively correlated with reproductive stage and GSI. In females, gonadal *tspo* and *vdac* expression was negatively correlated with GSI and levels of plasma testosterone and 17 β -estradiol. Ovarian *dbi* expression was not correlated with indicators of reproductive maturation. These studies represent the first investigation of the steroidogenic role of *tspo*, *vdac*, and *dbi* in fish. Findings suggest that cholesterol transport in largemouth bass testis, but not ovary, may be transcriptionally-regulated, however further investigation will be necessary to fully elucidate the role of these genes in largemouth bass steroidogenesis.

Keywords

translocator protein (18 kDa); voltage-dependent anion channel; diazepam binding inhibitor; largemouth bass; endocrine disruption; steroid synthesis

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1. Introduction

In vertebrates, the first and rate-limiting step in steroidogenesis is the transport of cholesterol across the outer mitochondrial membrane (OMM) [51]. Cholesterol is the precursor of steroid hormones and must be made available to the cascade of enzymatic reactions which occur within mitochondria to yield pregnenolone and ultimately estrogens and androgens such as 17 β -estradiol (E2) and testosterone [24]. Limited knowledge exists regarding mitochondrial cholesterol transport (MCT) in piscine steroidogenesis, though previous work in this area has characterized expression of steroidogenic acute regulatory protein (Star) [2, 27, 33, 50]. However, MCT in mammals involves at least three other proteins, the 18 kDa translocator protein (Tspo), voltage-dependent anion channel (Vdac), and the adenine nucleotide transporter (Ant) [44]. The Tspo (formerly peripheral benzodiazepine receptor), which is integral in mammalian MCT, is yet unexplored in fish [31]. The Tspo was initially discovered due to its ability to bind a number of synthetic ligands such as the benzodiazepines [6], but also binds endogenous ligands, including the diazepam binding inhibitor (Dbi; also, acyl-CoA binding protein, Acbp; endozepine) [14, 48]. In mammals, the Tspo acts as a sink to which all cholesterol available for acute transport across the OMM is bound [36, 51]. During the basal, un-stimulated state, the Tspo is thought to interact with the Vdac in the OMM and the Ant within the inner mitochondrial membrane (IMM) (Figure 1). Upon stimulation of steroidogenic cells by gonadotropins, Tspo molecules polymerize and form contact sites between the inner and outer phospholipid layers of the OMM. Release of cholesterol by the Tspo across the OMM is thought to primarily occur upon endogenous ligand binding, leading to an increase in the production of steroid hormones. The Tspo complex also interacts with Star, which purportedly delivers cholesterol to the Tspo within the OMM [51].

The Tspo complex may play a role in the effects of endocrine disrupting compounds in mammals. Prior studies suggest that the Tspo-Vdac-Ant complex binds environmental contaminants including pyrethroid insecticides [9, 10, 49] and hexachlorocyclohexanes (e.g. lindane) [58]. Other studies have reported effects of heptachlor [16], parabens [56], phthalates [35], and pyrethroid exposure [39] on *tspo* expression in rat brain, adrenals, and testis. In mice, permethrin administration resulted in dose-dependent reductions in plasma and testis testosterone levels associated with decreased expression of genes encoding Tspo, Star, and cytochrome P450 side-chain cleavage enzyme [57]. Therefore, there is potential for the Tspo to be a susceptible component of the steroidogenic pathway in other organisms.

The largemouth bass (LMB), an important freshwater fish species in Florida and throughout the United States, is sensitive to endocrine disruptors in the environment [55] and accumulates endocrine disrupting compounds in body tissues [40, 46]. LMB inhabiting sites contaminated with endocrine disrupting compounds such as organochlorine pesticides in Florida have exhibited poor reproductive success and declines in population which have been correlated with reduced levels of circulating sex steroid hormones [11, 22].

LMB exhibit group synchronous oocyte development and spawn multiple times during the reproductive season. During a 2–3 month apex of the reproductive season, LMB maintain elevated plasma E2 and testosterone levels which are several times higher than the remainder of the year. This is accompanied by increases in gonadosomatic index (GSI) and distinct changes in gonadal histology [7, 23]. This seasonal regulation of steroid production offers the opportunity to study the underlying mechanisms of steroidogenesis in LMB gonadal tissue at predictable peak and nadir periods. The goal of this study was to define the normal changes in gonadal expression of *tspo*, *vdac*, and *dbi* at various reproductive stages. Considering the well-established role of MCT proteins in mammalian steroidogenesis, it was

hypothesized that gonadal expression of LMB *tspo*, *vdac*, and *dbi* would mirror seasonal changes in circulating sex steroid hormones and reproductive status.

2. Material and Methods

2.1. Cloning of LMB *tspo*, *dbi*, and *vdac* mRNA sequences

Total RNA was isolated from LMB ovarian tissue by phenol-chloroform extraction (TRIzol Reagent, Life Technologies Corp./Invitrogen, Carlsbad, CA) and resuspended in RNasefree Resuspension Solution (Life Technologies Corp./Ambion, Austin, TX), purified by DNase treatment (DNAfree Kit, Life Technologies Corp./Ambion), and evaluated for nucleic acid concentration and purity by spectrophotometry (NanoDrop ND-1000, Thermo-Fisher Scientific, Inc., Waltham, MA). First-strand cDNA synthesis was performed using 2 µg total RNA in a final reaction volume of 25 µL as follows (all reagents Promega Corp., Madison, WI): total RNA was combined with 2.0 µL of 500 µg/mL random hexamers and brought to 15 µL with nuclease-free water. The RNA/primer solution was heated at 70 °C for 5 minutes, cooled immediately on ice, followed by the addition of 5 µL M-MLV 5X reaction buffer, 5 µL dNTP mix (10 mM), 0.7 µL RNasin (40 U/µL), and 1 µL M-MLV RT (200 U/µL). Reaction tubes were incubated at 42 °C for 1 hour, and then stored at –20 °C. The resultant cDNA was utilized for the cloning efforts described below.

Sequences of the *tspo*, *dbi*, and *vdac* from multiple aquatic species were obtained from the National Center for Biotechnology Information (NCBI) Nucleotide database. Sequences were aligned using the BioEdit Sequence Alignment Editor [25] running the ClustalW multiple alignment algorithm [34] and regions of relative homology among species were identified. In the case of *dbi* and *vdac*, additional LMB sequence homologous to that from other aquatic species was located by using the basic local alignment search tool against an existing LMB normalized cDNA library produced by pyrosequencing [15]. Tentative coding sequence constructs of the LMB *dbi* and *vdac* were gleaned by assembling homologous non-annotated contigs from this cDNA library, which revealed substantial gaps in the putative nucleic acid sequences. Using Primer3 [52], oligonucleotide PCR primers were designed to target homologous sequence regions from the cDNA library constructs or those areas identified as homologous among multiple species for each gene (Table 1). PCR was performed with 100 ng LMB cDNA template, 2.5 µL 10X PCR buffer, 0.75 µL MgCl₂ (50 mM), 0.5 µL dNTPs (10 mM), 0.5 µL forward and reverse primers (10 µM each), and 0.2 µL *Taq* DNA polymerase (1 U/µL) (Life Technologies Corp./Invitrogen). PCR thermocycling parameters were as follows: 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 50–65 °C for 30 s, and 72 °C for 1 min, and final extension at 72 °C for 7 min. Amplification products were visualized on 1–2 % agarose gels stained with 1 µg/mL ethidium bromide. Products of the correct predicted length were ligated into the pGEM-T Easy plasmid vector (Promega Corp.) which was used to transform One Shot TOP10 chemically competent *E. coli* (Life Technologies Corp./Invitrogen). Plasmids were isolated from insert-positive colonies via the PureYield Plasmid Miniprep System (Promega Corp.) and sequencing was performed by the Interdisciplinary Center for Biotechnology Research (University of Florida, Gainesville, FL).

Three-prime rapid amplification of cDNA ends (RACE) was utilized to determine additional nucleic acid sequence of the LMB *tspo* and used exclusively to clone the *vdac* sequence. A commercially-available RACE kit (FirstChoice RLM-RACE, Life Technologies Corp./Ambion) was used per manufacturer guidelines to construct an LMB cDNA library with 3' adapter sequence to enable 3'-RACE as follows: 1 µg total RNA was combined with 2 µL 10X RT Buffer, 3 µL dNTP Mix (2.5 mM), 2 µL 3'-RACE Adapter, 1 µL RNase Inhibitor (10 U/µL), 1 µL M-MLV RT, and 9.25 µL nuclease-free water. First-strand synthesis was carried out at 42 °C for 1 hour. RACE-PCR forward primers (Table 1) were designed based

on cloned LMB sequence (as determined above) or cDNA library contigs and used in conjunction with the provided 3'-RACE Outer (reverse) Primer (10 μ M) to amplify coding sequence as well as the full 3' untranslated regions of *tspo* and *vdac*. Nested PCR using the kit-supplied 3'-RACE Inner Primer (10 μ M) and custom forward primer was required to successfully amplify *vdac* (Table 1). Resulting PCR products were evaluated and sequenced as above.

At least three isoforms of Vdac have been identified in multiple species, encoded by the *vdac1*, *vdac2*, and *vdac3* genes. To determine the isoform of *vdac* cloned from LMB in the current work, phylogenetic analysis was conducted. Twenty-four published *vdac* complete coding sequences from fish, amphibians, mammals, and a copepod were aligned with the novel LMB *vdac* complete coding sequence using ClustalW online (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>) and the resulting alignment file was loaded into the stand-alone ClustalW2 application (v.2.0.12) [34]. A phylogram was constructed using the Neighbour Joining method, without exclusion of gaps or correction for multiple substitution. Bootstrap analysis was performed using 100 trials.

2.2. LMB sample collection and reproductive stage analysis

LMB were captured once per month from October 2005 to October 2006 via electroshocking from the St. John's River in Welaka, Florida, in conjunction with the Florida Fish and Wildlife Conservation Commission. Female animals were sampled for the months encompassing October 2005 to October 2006, while male samples were taken during the range of October 2005 to September 2006 only. Upon capture, animals were euthanized with a blow to the head and morphological parameters including body weight, body total length, and gonad weight were recorded. Body and gonad weights were utilized to determine GSI by calculating the ratio of gonad weight / body weight and multiplying by 100. Multiple tissue samples from each animal were flash-frozen in liquid nitrogen for RNA analysis, and a separate gonad sample was fixed in formalin for histological examination, as previously described [42]. Blood was collected (3–5 mL) from the caudal vein with a heparinized vacutainer, and plasma was isolated by centrifugation and stored at -80°C prior to sex steroid analysis.

Examination of hematoxylin/eosin-stained gonadal sections to group animals by reproductive stage was performed, as previously described [42]. Male animals were grouped into 4 reproductive stages: 1) immature or non-reproductive, 2) early sperm maturation, 3) mid-sperm maturation (< 50 % of tissue = mature sperm), and 4) late sperm maturation (> 50 % of tissue = sperm). Classification of female animals consisted of five reproductive stages: 1) primary growth perinucleolar, 2) cortical alveoli, 3) vitellogenesis, 4) oocyte maturation, and 5) atresia, and was based on the relative frequencies of ovarian follicles at five classifications of development: A) pre-perinucleolar (oogonia), B) perinucleolar, C) cortical alveoli, D) vitellogenic, and E) oocyte maturation. For purposes of performing correlative analysis, the frequency of each follicle class from six LMB in reproductive stages 1 through 4 was quantified by selecting a representative area of each hematoxylin/eosin-stained tissue section at 2X magnification and counting all follicles within the field of view. The frequency of each follicle class was calculated as the percentage of total follicles counted for each fish, and is reported as the mean percentage for each reproductive stage $\pm$ standard error of the mean (SEM). In addition, the maximum diameter of 10 follicles in each of four follicle classes (perinucleolar, cortical alveoli, vitellogenic, and oocyte maturation) from six female LMB was quantified at 2X magnification. Follicles selected for diameter measurement had been histologically sectioned through the nucleus to minimize shape effects. Follicle diameters are reported as the combined mean of 60 follicles per class (10 from each of six fish) $\pm$ SEM, with the exception of the oocyte maturation class, in which a total of 46 follicles were analyzed. All analyses were conducted using ImageJ software

version 1.43 (National Institutes of Health, available for download at <http://rsbweb.nih.gov/ij/>).

2.3. LMB gonadal gene expression analysis

Gonadal tissue from four LMB of each sex per month was mechanically homogenized (T 10 basic ULTRA-TURRAX homogenizer, IKA Works, Inc., Wilmington, NC), RNA extracted and cDNA synthesized as described previously [42]. Briefly, total RNA was extracted by RNA STAT-60 Reagent (Tel-Test, Inc., Friendswood, TX), resuspended, purified, and quantified as described in section 2.1. First strand synthesis utilizing 1–2 µg total RNA was accomplished via SuperScript II Reverse Transcriptase (Life Technologies Corp./Invitrogen) in a reaction volume of 20 µL. An aliquot of each final cDNA solution was diluted between 15 to 20 times with nuclease-free water for analysis by real-time quantitative PCR (qPCR) and all cDNA samples were stored at –20 °C while RNA was stored at –80 °C.

To quantify expression of *tspo*, *dbi*, and *vdac* in male and female LMB gonadal tissues, qPCR was performed using a Bio-Rad MyiQ Single-Color Real-Time PCR Detection System along with 2X iQ SYBR Green Supermix (Hercules, CA). Serial dilutions of pGEM-T Easy plasmid preparations containing target inserts facilitated quantitation of transcript copy number via the generation of amplification standard curves for all genes. Expression of individual transcripts and 18S ribosomal subunit RNA (*m18s*) in each sample was evaluated by duplicate independent reactions as follows: 5 µl diluted sample cDNA (12.5×10^{-3} to 33.3×10^{-3} µg nucleic acid) or plasmid standard was combined with 0.5 µl forward and reverse qPCR primers (10 mM), 6.5 µl nuclease-free water, and 12.5 µl 2X iQ SYBR Green Supermix in each well of a 96-well reaction plate. QPCR thermocycling parameters included initial denaturation at 95 °C for 3 min, 40 cycles of 10 s at 95 °C followed by 60 s at 58 °C, and final melting curve at 55 to 95 °C. Each qPCR assay included two quality controls in which 1) template was omitted during qPCR reaction creation to ensure absence of contamination in the PCR reagents used (non-template control) and 2) reverse transcriptase was omitted during first strand synthesis to ensure genomic DNA contamination was negligible following DNase treatment (-RT control). Melting curve analysis was used to verify a single PCR product in each assay by plotting the dissociation curve.

The resulting amplification data was analyzed by iQ5 Optical System Software (v. 2.1, Bio-Rad). Signal baseline was assigned to the optimum by the analysis software and the threshold location for the calculation of cycle threshold values was placed within the logarithmic amplification zone for all reactions in a given assay. Copy number of each gene of interest and *m18s* was calculated and expressed as copies per µg of input cDNA. Standard curves maintained R^2 values of at least 0.998 for all assays, and amplification efficiencies ranged from 90 to 96% (Table 1). To compensate for variations in sample preparation and mass, all gene of interest values were normalized to the respective *m18s* value for each sample.

During analysis of qPCR data, significant variation in *m18s* expression by reproductive stage was noted in ovarian cDNA samples ($p < 0.001$), but not in testis samples ($p = 0.322$). Trends in expression of conventional housekeeping genes by reproductive stage in ovarian tissue from aquatic species has precedent in the literature [27, 50], and normalization methodologies have been developed to standardize housekeeping gene expression values when such a pattern of variation is found [3, 13]. To this end, female *m18s* values were standardized to an arbitrary “control” group (Stage 1) as follows [27]:

$$\frac{\text{Individual values within a group}}{(\text{Mean value within a group}/\text{Mean value of control group})}$$

These standardized *m18s* values were then used to normalize gene of interest data for female animals as a ratio of gene of interest input amount / standardized *m18s* for each individual sample. Since there was no variation in male gonadal *m18s* values across months or reproductive stages, male gene of interest values were normalized via the conventional ratio of gene of interest / raw *m18s* input amount.

2.4. Plasma Sex Steroid Analysis

Circulating levels of E2 and testosterone were determined by commercially-available enzyme-linked immunosorbent assay (ELISA) kits (IBL America, Minneapolis, MN), preceded by liquid-liquid extraction of organic compounds as previously described [41]. Briefly, 25–100 μ L of LMB plasma was added to individual borosilicate glass tubes, brought to a total volume of 100 μ L with assay buffer (50 mM sodium phosphate, 0.1 % gelatin, pH 7.6), and spiked with 100 μ L of tritiated-E2 (50 nCi/mL, specific activity of 44 Ci/mmol; Amersham Radiochemicals, now GE Healthcare Bio-Sciences Corp., Piscataway, NJ) to enable determination of extraction recovery. One mL of 1-chlorobutane (n-butylchloride, 99+ % pure, Acros Organics/Thermo-Fisher Scientific, Inc.) was added to each tube followed by vortex mixing for 30 s. Following centrifugation to separate aqueous and organic phases, the latter was transferred by Pasteur pipet to a clean borosilicate glass tube. The extraction process was repeated once and the final combined extract was evaporated until solvent-free under nitrogen at 37 °C. Samples were reconstituted in BSA buffer (50 mM sodium phosphate, 0.1 % gelatin, 0.1 % RIA grade bovine serum albumin, pH 7.6) at 4 °C overnight with shaking. Concentrations of E2 and testosterone in sample extracts were determined by direct application of reconstituted extract to ELISA plates, using standards which were created in charcoal-stripped pooled LMB plasma and extracted as above. Recovery of extracted steroid was determined by counting an aliquot of each reconstituted sample and standard for 3 minutes in the tritium window and comparing to the original tritiated-E2 spike solution. Final plasma concentration of sex steroids was calculated by correcting the value measured in each sample extract for dilution and extraction recovery and expressed as pg/mL plasma.

2.5. Statistical analysis

Gene expression data was organized by reproductive stage and presented as group mean $\pm$ SEM. Differences in gonadal gene expression were analyzed via 1-way ANOVA with Student-Newman-Keuls *post-hoc* correction for multiple comparisons. Data which were not normally distributed were transformed prior to ANOVA analysis by either logarithmic or reciprocal transformation. Male *vdac* expression data as well as follicle diameter data were not normalized by transformation, thus ANOVA on Ranks (Kruskal-Wallis Method) was performed using Dunn's Method for *post-hoc* correction. Pairwise correlation was performed by Pearson Product Moment. All statistical analysis was performed using SigmaPlot for Windows v. 11.0 (Systat Software Inc., Chicago, IL). P-values < 0.05 were considered significant.

3. Results

3.1 Cloning of LMB *tspo*, *dbi*, and *vdac* mRNA sequences

Analysis of cloned LMB mRNA and deduced amino acid sequences revealed functional domains and similarity to sequences from other species. The partial cloned sequence of the LMB *tspo* (GenBank accession HQ110631) was 746 nucleotides in length covering positions 3–486 of the coding sequence which encodes 161 amino acids (Supplemental Figure 1) and contained the putative tryptophan-rich sensory protein / mitochondrial peripheral benzodiazepine receptor conserved protein domain (TspO_MBR, NCBI pfam03073; E-value = 2.32×10^{-38}). The LMB *tspo* exhibited 92 and 91% homology with

the putative *tspo* nucleotide and amino acid sequences, respectively, from the sablefish, *Anoplopoma fimbria* (BT082208.1). The cloned LMB *dbi* sequence (HQ110633) was 287 nucleotides in length and contained the complete coding sequence, with the open reading frame comprising positions 1–267, encoding a protein 88 amino acids in length (Supplemental Figure 2). The LMB *dbi* deduced protein sequence contained the putative Acbp conserved protein domain (NCBI cd00435; E-value = 1.51×10^{-25}) of the Acbp Superfamily (NCBI cl00221). The coding sequence of the LMB *dbi* was 87 and 81% homologous to the *dbi* nucleotide and amino acid sequence from sablefish (BT082722.1). Lastly, the cloned LMB *vdac* (HQ110632) was 1182 nucleotides long containing the complete coding sequence, with an open reading frame at positions 1–852 encoding a protein 283 amino acids in length (Supplemental Figure 3). The LMB *vdac* deduced amino acid sequence contained the Porin3_Vdac conserved protein domain (NCBI cd07306; E-value = 4.53×10^{-97}) of the Porin3 Superfamily (NCBI cl03224). The LMB *vdac* was 90 and 97% identical to the *vdac* nucleotide and amino acid sequences from the olive flounder, *Paralichthys olivaceus* (DQ821474.1).

To determine the isoform of *vdac* which was cloned from LMB, phylogenetic analysis was performed utilizing mRNA coding sequences representing *vdac* isoforms from fish, amphibians, and mammals, while a copepod *vdac* was used to root the phylogram. The analysis clearly separated *vdac* subtypes and placed the cloned LMB *vdac* with *vdac2* sequences from all other included species (Figure 2). Based on these findings, we have designated the newly cloned LMB mRNA sequence as encoding the *vdac2* gene of the LMB.

3.2 Female reproductive stage analysis

Female LMB reproductive stage was determined by analysis of the relative frequencies of each of five follicle developmental classes within histological sections of the gonad. In addition to evaluating morphological characteristics, the mean diameter of follicles in the perinucleolar, cortical alveoli, vitellogenic, and oocyte maturation developmental classes was quantified. Significant differences were found between all classes (overall $p < 0.001$), with mean diameters of 215 ± 8 , 291 ± 17 , 575 ± 36 , and 1275 ± 37 μm for follicles in the perinucleolar, cortical alveoli, vitellogenic, and oocyte maturation classes, respectively.

Distinct changes in gonad follicle composition occurred across the reproductive stages (Figure 3). The relative abundance of pre-perinucleolar and perinucleolar follicles decreased as reproductive stage advanced and was negatively correlated with the assigned reproductive stage ($R^2 = -0.628$ and -0.652 , respectively; $p < 0.001$). The frequency of pre-perinucleolar follicles across reproductive stages was also negatively correlated with GSI and levels of circulating E2 and testosterone ($R^2 \leq -0.638$; $p < 0.01$). Cortical alveoli follicles first appeared in ovaries during the cortical alveoli reproductive stage, but their frequency did not correlate with indicators of reproductive maturation. The appearance of vitellogenic follicles was a hallmark of the vitellogenesis reproductive stage, and the abundance of vitellogenic follicles was positively correlated with reproductive stage, GSI, and circulating levels of E2 and testosterone ($R^2 \geq 0.557$; $p < 0.01$). Lastly, follicles in the oocyte maturation class were observed exclusively during the oocyte maturation reproductive stage.

3.3 Relationship between reproductive stage, plasma steroid levels, GSI, and gonadal gene expression

Teleost reproductive status is dependent on a number of environmental factors including water temperature and quality, photoperiod, and availability of food [28], the timing of which may change from year to year. In addition, animals at different stages of reproductive maturation may coexist in a specific habitat simultaneously. Therefore, gene expression data

were organized by histologically-determined reproductive maturation stage for each animal to examine stage-specific patterns of expression and to allow comparison with two indicators of reproductive maturation: circulating levels of sex steroid hormones and GSI. GSI values from animals included in the current work have been reported in detail previously [42]; these data were utilized in the current study for correlation analysis only.

Testis expression of *tspo* varied significantly with reproductive stage, being low during the immature (1) and early maturation (2) stages and consistently high during mid- (3) and late-sperm maturation (4), with a maximal fold change of 5.9 (stage 1 versus 3; overall $df = 3$, $F = 13.8$, $p < 0.001$; Figure 4). Moreover, significant correlation was detected between testis *tspo* expression and plasma testosterone concentration as well as GSI ($R^2 = 0.563$ and 0.800 , respectively; $p < 0.01$; Table 2). Male gonadal *dbi* expression changed dramatically from $1.0 \times 10^{-6} \pm 5.5 \times 10^{-7}$ to $2.4 \times 10^{-5} \pm 4.7 \times 10^{-6}$ copies normalized to *m18s* in stages 1 and 4, respectively, constituting a 23-fold increase ($df = 3$, $F = 14.5$, $p < 0.001$; Figure 4). *Dbi* expression was also strongly correlated with the concentration of circulating testosterone and GSI across reproductive stages ($R^2 = 0.903$ and 0.887 , respectively, $p < 0.001$). Lastly, while expression of *vdac* was found to vary significantly by reproductive stage in male gonad ($df = 3$, $H = 9.7$, $p = 0.021$), the stage-dependent increase in transcript abundance was much less pronounced than that of *tspo* or *dbi*, and was not found to correlate significantly with the level of plasma testosterone.

In female LMB, gonadal expression of *tspo* varied significantly by stage ($df = 4$, $F = 4.2$, $p < 0.01$; Figure 5), with stage 4 (oocyte maturation) exhibiting lower transcript levels than stages 1, 2, and 3 ($p < 0.04$). Ovarian *tspo* expression was negatively correlated with circulating levels of E2 and testosterone, and with GSI ($R^2 \leq -0.376$; $p < 0.05$). The expression profile of ovarian *vdac* was similar to that of *tspo*, and was also negatively correlated with plasma levels of E2 and testosterone, as well as GSI ($R^2 \leq -0.344$; $p < 0.05$). Ovarian *dbi* transcript abundance was not correlated with indicators of reproductive maturation, and was lowest during stage 1 (perinucleolar) versus stages 2, 3, and 4 ($p < 0.01$).

3.4 Influence of sex on gonadal expression of MCT genes

Comparison of ovarian and testis transcript levels across reproductive maturation stages revealed several distinctions. The maximal percent increase in normalized gonadal *tspo* transcripts across reproductive stages was 588% in males and 128% in females, and the abundance of *tspo* mRNA remained at least 12-fold higher in ovary versus testis across all stages. With regard to *dbi*, normalized mRNA abundance in the gonad increased a maximal 2300% from the lowest value in males, and 131% in females. The range of expression of *dbi* overlapped between the sexes, with males exhibiting approximately 9-fold higher maximum expression than females. Of the three transcripts studied in males, gonadal *vdac* levels changed the least (59% increase from the lowest value) while in females, ovarian expression of *vdac* exhibited a maximal increase of 149% and maintained levels approximately 2 orders of magnitude higher than males across reproductive stages.

4. Discussion

4.1 Cloning of LMB *tspo*, *dbi*, and *vdac* mRNA sequences

MCT is a fundamental process required for steroidogenesis in vertebrates and therefore conservation of the proteins involved is expected. As predicted, novel LMB mRNA sequences representing the *tspo*, *dbi*, and *vdac* exhibited high homology to those from varied phylogenies. Based on these findings, the newly identified LMB genes are expected to be

involved in MCT as in mammals, however mechanistic studies would be necessary to confirm the precise roles of each protein in teleost cholesterol transport.

Although the function of individual Vdac isoforms in steroidogenesis is unknown, data in this study suggest a role for Vdac2 in LMB gonadal steroidogenesis. Upon examination of expression of *vdac2* across tissue types in LMB, *rn18s*-normalized transcript abundance in testis was higher than in brain, gill, head kidney, or liver. In ovary, *vdac2* expression was higher than all other tissues studied by at least 8-fold (Supplemental Figure 4). These data are similar to studies in mice in which the greatest expression of *vdac2* was detected in testis, followed by kidney, skeletal muscle, heart, and brain [54]. However, Vdac isoforms are expressed in mammalian germ-line cells and are necessary for sperm viability, thus data from whole-tissue studies may not accurately describe expression in steroidogenic cells [37]

Most research investigating the interaction of Vdac with the Tspo has focused on a single subtype: Vdac1 [20, 38]. While studies investigating tissue distribution of Vdac isoforms in mammalian gonadal tissue are few, findings indicate that Vdac1 may not be expressed in the steroidogenic cells of the testis [26, 54], while Vdac3 protein appears abundant in all murine testis cell types including Leydig cells [53]. However, tissue distribution results do not appear consistent across species. Furthermore, the seminal work of McEnery and colleagues [44] identified six Vdac-immunoreactive proteins which interacted with the Tspo in rat kidney mitochondria, all exhibiting an apparent MW of ~32 kDa. Though post-translational modification may account for differences in molecular weight, the interaction of the Tspo with multiple Vdac subtypes is also plausible. Collectively these data suggest that Vdac2 may be an important component of gonadal steroid biosynthesis, while not excluding a potential role for other subtypes.

4.2 Expression of LMB testis *tspo*, *dbi*, and *vdac* across reproductive maturation stages

Cloned mRNA sequences were utilized to evaluate steady-state gonadal expression of transcripts involved in cholesterol transport across reproductive maturation stages. *Tspo* mRNA abundance in testis was lowest during stage 1, and increased during stages 2 and 3 before stabilizing. Differences in testis *vdac* expression were not detected among stages, however both *vdac* and *tspo* transcript levels were positively correlated with reproductive maturation stage and GSI, and *tspo* expression was associated with increased circulating testosterone concentration. Expression of *dbi* increased dramatically in the testis with reproductive maturation and was associated with elevated circulating testosterone levels, GSI, and reproductive maturation stage. Association of the expression of LMB testis *tspo* and *vdac* with reproductive maturation may indicate an increase in mitochondrial contact sites in steroidogenic cells of the testis, resulting in greater potential for steroid production. Furthermore, Dbi and its cleavage products are potent agonists of the Tspo and ultimately steroid biosynthesis in mammalian Leydig cells [12, 18, 19] and *dbi* expression is required by Leydig cells for gonadotropin-stimulated steroidogenesis [5]. In LMB, previous work has demonstrated an increase in pituitary expression of gonadotropin subunit mRNA during reproductive maturation in the male, which peaked during the mid-sperm maturation stage [42]. These gonadotropin subunit transcript levels were also positively correlated with the expression of pituitary estrogen receptor mRNA and coincide with the elevated plasma steroid levels and increased abundance of gonadal transcripts found in the current work, suggesting a possible influence of gonadotropins on expression of MCT genes in male LMB gonad.

The current findings indicate that there is an increase in expression of genes involved in MCT in the testis during maturation. However, the relative expression of MCT genes in various cell types and changes in the number of steroidogenic cells relative to non-steroidogenic cells may complicate interpretation of gene expression data from whole tissue.

Germline cells (spermatocytes, spermatids and spermatozoa) comprise an increasing percentage of total testis cells with maturation. It is unknown to what degree genes involved in MCT are expressed in teleost germline cells. In mammals, Kolmer and colleagues [30] found a high abundance of *dbi* transcripts in developing spermatids, followed by non-detection of *dbi* during the later stages of spermatid differentiation. *Vdac2* is expressed and translated in mammalian spermatocytes and spermatids, and the protein can be found in mature spermatozoa [37]. Expression of *tspo* in germline cells of the mammalian testis has not been described in detail, however studies suggest that Tspo protein is primarily found in Leydig cells [1, 8, 19].

The observed increases in MCT gene expression may also be due to proliferation of Leydig cells during reproductive maturation rather than increases in transcript abundance on a per-cell basis. The ratio of Leydig cells to non-steroidogenic cells in the LMB testis has not been systematically studied in relation to reproductive maturation. However, Kusakabe and colleagues [32] found that the number of Leydig cells on the surface of rainbow trout testis is greater during reproductive immaturity and decreases with maturation. Assuming similarity to rainbow trout and considering the tissue volume occupied by germline cells during advanced reproductive maturation, LMB testis tissue likely contains far fewer steroidogenic cells on a mass basis as maturation progresses. This suggests that changes in MCT gene expression in Leydig cells are diluted during whole-tissue analysis. Additional studies that focus on transcript or protein levels in individual LMB testis cells will be required to determine if changes in gene expression detected in the current study are indicative of regulated expression within individual Leydig cells or changes in the cytological composition of the testis.

4.3 Expression of LMB ovarian *tspo*, *dbi*, and *vdac* across reproductive maturation stages

In female LMB, ovarian expression of MCT genes across reproductive maturation stages was not as anticipated. Normalized *tspo* and *vdac* mRNA levels were lowest during stage 4 (oocyte maturation) and were negatively associated with GSI as well as circulating E2 and testosterone levels. *Dbi* transcript abundance was lowest during stage 1 and positively correlated with reproductive maturation stage, but was not associated with morphometric parameters or plasma sex steroid levels. Expression of genes encoding pituitary gonadotropin subunits in female LMB increases several-fold during reproductive maturation, peaking during late secondary growth and oocyte maturation, which coincides with elevated levels of circulating sex steroid hormones found in the current study. In addition, gonadotropin subunit expression has been positively correlated with levels of estrogen receptor subunit mRNA in the pituitary [42]. Kocerha and colleagues [29] studied female LMB on a monthly basis throughout the reproductive season, noting apparent coordinated increases in ovarian *star* mRNA and plasma E2 levels. The current data, however, indicate that expression of genes involved in MCT in LMB ovary may not be regulated by gonadotropins or sex steroid input.

The cholesterol transport functionality of the Tspo complex in LMB ovary may not be transcriptionally regulated. This hypothesis is reinforced by studies which utilized chemical suppression of the ovary in the rat, demonstrating that reductions in ovarian Tspo binding capacity were not associated with alterations of steady-state levels of *tspo* mRNA nor Tspo or Vdac protein concentrations, but appeared to be due to changes in the number of Tspo subunits which interacted with each Vdac [20]. In another study, a drop in uterine Tspo binding density of up to 67% was noted following ovariectomy in the rat, an effect which was not associated with changes in mRNA levels, again demonstrating the potential existence of regulatory means not directly linked to transcription [43]. Evaluation of Tspo binding capacity in the LMB ovary may provide additional clues regarding how the Tspo is functionally regulated throughout the reproductive cycle in teleosts.

Expression of the endogenous ligand Dbi may also represent an important regulatory mechanism for the MCT process. One report suggests that large mammalian luteal cells may utilize the Dbi cleavage product, endozepine, to maintain basal production of large amounts of progesterone relative to small luteal cells. While both cell types express similar amounts of Star, large luteal cells maintain ~3.5-times the level of endozepine protein [47]. Since the *dbi* gene product is processed into various active peptides [12], care must be taken when extrapolating activity from gene expression data. Nevertheless, transcript abundance of *dbi* in LMB ovary exhibited a significant profile across maturation stages and was found to be lowest during the perinucleolar stage of reproductive maturity.

As with testis, care must be taken with interpretation of gene expression data from whole ovary samples. Steroid synthesis in the teleost ovary occurs within a thin layer of thecal and granulosa cells which surrounds the follicle. Changes in follicle composition (Figure 3), expansion of the ooplasm as follicle diameter increases during development, and the deposition of a high level of ribonucleic acid within oocyte cytoplasm [21, 45] all complicate interpretation of whole tissue data. The data in this study demonstrated that the lowest transcript abundance of *tspo* and *vdac* was detected during the peak of reproductive maturation, which was opposite of the expected result. To confirm that these observations are actually due to changes in MCT gene expression in steroidogenic cells, studies that dissect individual follicles and/or remove the ooplasm (deyolking) will be required [17].

The present work represents the first investigation of the potential involvement of the *tspo*, *vdac*, and *dbi* gene products in gonadal steroid biosynthesis in fish. The gene expression profiles exhibited by testis and ovary suggest possible alternate strategies of MCT regulation between the sexes, such as modulation of protein subunit interaction. Furthermore, large differences in reproductive physiology exist between mammals and piscine species, raising the possibility of distinct regulation of expression or precise function of these gene products in steroidogenic tissues in teleosts. Therefore, future work is necessary to solidify the link between the Tspo, Vdac, and Dbi proteins and steroidogenesis in fish. Nevertheless, these studies begin to establish baseline patterns of expression of genes likely involved in piscine steroidogenesis. MCT proteins may represent important targets of endocrine disrupting compounds in teleosts, and therefore understanding the mechanism(s) by which they contribute to steroid biosynthesis is crucial.

Research Highlights

- Largemouth bass (*Micropterus salmoides*) express *tspo*, *vdac*, and *dbi* in gonad.
- Testis expression of *tspo*, *vdac*, and *dbi* is correlated with reproductive stage.
- Changes in ovarian transcript expression are less pronounced.
- Data suggest a role for *tspo*, *vdac*, and *dbi* in teleost gonadal steroid synthesis.
- Differences may exist in transcriptional regulation of these genes between sexes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

OMM	outer mitochondrial membrane
E2	17 β -estradiol

MCT	mitochondrial cholesterol transport
Star	steroidogenic acute regulatory protein
<i>star</i>	gene encoding the steroidogenic acute regulatory protein
Tspo	18 kDa translocator protein
<i>tspo</i>	gene encoding the 18 kDa translocator protein
Vdac	voltage-dependent anion channel
<i>vdac</i>	gene encoding the voltage-dependent anion channel
Ant	adenine nucleotide transporter
Dbi	diazepam binding inhibitor
<i>dbi</i>	gene encoding the diazepam binding inhibitor
Acbp	acyl-CoA binding protein
IMM	inner mitochondrial membrane
LMB	largemouth bass
GSI	gonadosomatic index
NCBI	National Center for Biotechnology Information
RACE	rapid amplification of cDNA ends
SEM	standard error of the mean
qPCR	real-time quantitative PCR
<i>rn18s</i>	gene encoding the 18S ribosomal subunit RNA
ELISA	enzyme-linked immunosorbent assay

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Figure 1.

Graphical representation of the Tspo protein complex that exists in mitochondria under basal conditions, in which Tspo and Vdac within the OMM interact with the Ant in the IMM. Upon acute stimulation of steroidogenesis, multiple events lead to additional protein interaction and activation, ultimately resulting in cholesterol translocation across the OMM to Cyp11a1 in the IMM [adapted from 51].

**Paralichthys* (DQ821474) sequence is an unnamed isoform of VDAC.

Figure 2.

Phylogram demonstrating the ancestral relationship between the cloned largemouth bass *vdac* mRNA coding sequence (underlined) and coding sequences of three isoforms of *vdac* from other species. Node labels represent the positive bootstrap results of 100 trials.

Figure 3.

Relative frequencies of follicle classes across reproductive stages, as percent. Reproductive stage 1, perinucleolar; 2, cortical alveoli; 3, vitellogenesis; 4, oocyte maturation. Follicle class p-pn, pre-perinucleolar; pn, perinucleolar; ca, cortical alveoli; vtg, vitellogenic; om, oocyte maturation. n = 6 animals for all stages.

Figure 4.

Normalized testis expression of *tspo*, *dbi*, and *vdac* (open, hashed, and cross-hatched boxes, respectively) by reproductive stage (1=immature or non-reproductive, 2=early sperm maturation, 3=mid sperm maturation, 4=late sperm maturation). For *tspo* expression, different letters indicate significant differences between stages. †, significantly lower expression of *dbi* vs. stages 3 and 4 ($p < 0.05$). Line plots illustrate plasma sex hormone levels for each stage (filled, testosterone; open, 17β-estradiol). All values are reported as mean $\pm$ SEM. Sample size for each group is indicated below the x-axis.

Figure 5. Normalized ovarian expression of *tspo*, *dbi*, and *vdac* (open, hashed, and cross-hashed boxes, respectively) by reproductive stage (1=perinucleolar, 2=cortical alveoli, 3=vitellogenesis, 4=oocyte maturation, 5=atresia). a, †, and ‡; significantly lower expression of *tspo*, *dbi*, and *vdac*, respectively, versus all other stages ($p < 0.05$). Line plots illustrate plasma sex hormone levels for each stage (filled, testosterone; open, 17β-estradiol). All values are reported as mean $\pm$ SEM. Sample size for each group is indicated below the x-axis.

Table 1

Oligonucleotide primers utilized for cloning efforts and qPCR, along with amplicon size for each primer pair. All primers were synthesized by Integrated DNA Technologies (Coralville, IA) with the exception of 3'-RACE reverse primers, which were provided in the commercial 3'-RACE kit (FirstChoice RLM-RACE, Life Technologies Corp./Ambion). The qPCR primer set for LMB *m18s* was developed by Blum and colleagues [4].

	Forward 5'-3'	Reverse 5'-3'	Amplicon	Length; PCR efficiency ^a
Cloning <i>tspo</i>	CCTACCTGGTGTGGAAGGAG	GGTTGTCTCTCCATATGCAGTAGTT	285	bp
<i>dbi</i>	ATGTCTAATCGGGCAGAGTTT	ACAGGCCATATTTGCTGATG	265	bp
3'-RACE <i>tspo</i>	CGGTTACATCACACGCAAAG	GCGAGCACAGAATTAATACGACT	725	bp
<i>vdac</i>	AAGGCTATGGATTGGCTTG	CGCGGATCCGAATTAATACGACTCACTATAGG	1156	bp
qPCR <i>tspo</i>	ACCCAACCCTACAGAAACCA	GAGTCCAGGCCAGTTAAGA	189	bp; 0.95
<i>dbi</i>	CCAGGAACGCTGGACTTT	GGCCATATTTGCTGATGATT	127	bp; 0.90
<i>vdac</i>	GCTATGGATTTGGCTTGGTG	GCCGTATTCTGACCTCTTGATTT	143	bp; 0.94
<i>m18s</i>	CGGCTACCACATCCAAGGAA	CCTGTATTGTTATTTTCGTCACTACCT	86	bp; 0.96

^aValues represent the average efficiency of 4 individual assays.

Table 2

Pairwise correlation of gonadal *tspo*, *dbi*, and *vdac* expression normalized to *m18s* against circulating sex steroid concentration and morphometric parameters for male and female LMB. T, testosterone.

	Stage	GSI	Circ. T	Circ. E2	<i>tspo</i>	<i>dbi</i>	<i>vdac</i>
<i>Females</i>							
Stage	1						
GSI	0.692	*** 1					
Circ. T	0.639	*** 0.704	1				
Circ. E2	0.623	*** 0.781	*** 0.878	1			
<i>tspo</i>	-0.275	*** -0.394	** -0.387	* -0.376	1		
<i>dbi</i>	0.427	** 0.266	0.172	0.175	0.399	** 1	
<i>vdac</i>	-0.199	* -0.344	* -0.369	* -0.358	0.920	*** 0.606	*** 1
<i>Males</i>							
Stage	1						
GSI	0.557	*** 1					
Circ. T	0.443	* 0.759	*** 1				
Circ. E2	0.148	0.160	0.264	1			
<i>tspo</i>	0.492	*** 0.800	*** 0.563	0.326	1		
<i>dbi</i>	0.505	*** 0.887	*** 0.903	0.158	0.759	*** 1	
<i>vdac</i>	0.385	** 0.431	** 0.050	0.104	0.385	** 0.501	*** 1

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