REGULATION OF THE STEROIDOGENIC ACUTE REGULATORY PROTEIN BY NUCLEAR RECEPTOR SIGNALING PATHWAYS AND BY ENDOCRINE DISRUPTING CHEMICALS IN LARGEMOUTH BASS (Micropterus salmoides)

By

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To Doris White – your strength to prevail through cancer at such a young age has inspired me more than you could ever fathom. In support of this work, you once stated that you were my “biggest fan;” well I’ll have you know that I am yours.
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Lake Apopka, a federally-appointed Superfund site in Florida, is contaminated with organochlorine pesticides (OCPs) that have been implicated in disrupting sex steroid levels in aquatic wildlife. Largemouth bass (*Micropterus salmoides*; LMB) inhabit Lake Apopka and are subject to exposure to the contaminants. Several of the OCPs in the lake have been reported to disrupt molecules important in steroid hormone biosynthesis, including the steroidogenic acute regulatory (StAR) protein. StAR protein shuttles cholesterol, the precursor of all steroid hormones, to the inner mitochondrial membrane for processing into steroid hormones. The mechanisms through which OCPs disrupt StAR protein are not known. The hypotheses of these studies are that OCPs disrupt gonad-specific *Star* mRNA expression and that the activity of the LMB *Star* gene promoter is regulated by nuclear receptor signaling, which may mediate the disruption by OCPs.

Gene expression changes that encompass the LMB reproductive cycle were examined and gonadal *Star* mRNA levels varied significantly dependent upon reproductive stage. To characterize the LMB *Star* gene, a segment of its promoter was cloned and its activity was
measured using transfections in gonadal and adrenal cell lines. Gonadotropin and cAMP exposure stimulated promoter activity and mutation of several putative transcriptional elements resulted in a loss of response to cAMP, including a site for the orphan nuclear receptors RORα and rev-erbα. RORα and rev-erbα regulate genes involved in mammalian peripheral circadian rhythm. RORα and rev-erbα bind the LMB StAR promoter, implicating the proteins play a role in regulating its activity. It was confirmed that another nuclear receptor, estrogen receptor β, can bind to an element in the StAR promoter. OCPs are known to disrupt nuclear receptor signaling pathways; thus I investigated gonad-specific StAR mRNA expression in response to ex vivo OCP exposure. Several OCPs disrupted StAR mRNA levels, however, transfections with the LMB StAR promoter indicated that the OCPs disrupt StAR mRNA levels at levels either at an upstream site or by indirect mechanisms. Altogether, these studies have provided insight into the regulation of the StAR gene in LMB and have verified that StAR mRNA expression is directly targeted by OCPs in the gonads of LMB.
CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Introduction

As human beings, we produce and expel mass quantities of chemicals into the environment; hence, it is no surprise that many of these chemicals have the capacity to negatively impact wildlife and humans. Endocrine disruption occurs when chemicals in the environment elicit a response in an organism that alters normal function of the endocrine system, which can result in reproductive, developmental, and other abnormalities. Much controversy surrounds the discussion of endocrine disruption in the environment. Recent reports in the United States and in other countries have revealed that several species of male fish, including largemouth bass (LMB, Micropterus salmoides), have been identified in the wild that bear intersex qualities (both eggs and sperm present in the gonad). It is highly speculated that the presence of intersex species is causally linked to exposure to xenoestrogenic compounds in the aquatic environment. However, the exact mechanisms that contaminants found in the waters employ remain unresolved (1, 2).

Reproductive success is vital to the survival of all species and is fundamental to sustaining biodiversity on the planet. Numerous environmental and physiological factors affect the growth, development, and reproductive success of an individual and it is important to understand the factors that control these physiological processes.

Literature Review

Endocrine Disruption

Endocrine disrupting chemicals (EDCs) encompass a very broad class of compounds that disrupt normal endocrine function in a wide range of species. Hormones control reproduction
and development, and disruption of endocrine function can severely impair the development and reproductive capability in an organism.

EDCs can impact reproduction and steroid hormone synthesis by mimicking the actions of endogenous androgens or estrogens or by disrupting the synthesis or metabolism of these key reproductive hormones. There is substantial evidence that links exposure of humans and wildlife to EDCs with changes in steroidogenic capacity, secondary sex characteristics, gonad development, and production and size of eggs and sperm (3-15).

EDCs are ubiquitous in the environment and include a multitude of different compounds, ranging from pesticides and fungicides and their metabolites (16-20) to chemicals found in plastics (21-23), papermill effluent, (24, 25) and sewage wastewaters (26). Hundreds of studies report that a large number of species exhibit reproductive and developmental abnormalities upon exposure to EDCs, including humans, mammals, amphibians, reptiles, birds, invertebrates, and fish (reviewed in (9)).

Many EDCs are quite stable and persist in the environment, and the main sink for many EDCs is in freshwater lakes and rivers; soluble compounds aggregate in the surface waters, whereas less soluble compounds collect in the sediments. It is for this reason that numerous aquatic vertebrates, including multiple species of fish (reviewed in (27)), are at high risk for exposure to EDCs and are susceptible to reproductive abnormalities.

One of the major abnormalities reported in wildlife exposed to EDCs is the presence of intersex fish in the environment; that is, the presence of male fish bearing both immature eggs and sperm in their gonad. Intersex fish species have been identified in freshwater and marine environments on a widespread basis, some of which include largemouth and smallmouth bass in the Potomac River in the United States (2, 28), white perch from the Great Lakes (29), roach fish
from Denmark (30) and the United Kingdom (31), and the three-spined stickleback from Germany (32). The feminization of fish in the wild has been correlated with the presence of xenoestrogens in the environment. Xenoestrogens are EDCs that act as estrogens or as anti-androgens, disrupting normal steroid hormone homeostasis.

The United States Geological Survey reported the presence of intersex male LMB in the Potomac River in the United States (28), yet the exact causes of the reproductive abnormalities observed have not been resolved. Recent studies by the U.S. Fish and Wildlife Service have revealed that many environmental contaminants were found at multiple sites at which intersex LMB were observed in the Potomac River, including many xenoestrogenic agricultural pesticides (1, 2). It is postulated that these chemicals, which are concentrated most near points of release of a local wastewater treatment facility, may be responsible for the reproductive abnormalities observed in the bass, though research efforts are ongoing and mechanisms are unclear (1, 2, 28).

Lake Apopka (Apopka, FL, USA) is the fourth largest lake in Florida and has been appointed by the U.S. Environmental Protection Agency (EPA) as a Superfund cleanup site. Decades ago, the serious spill of the organochlorine pesticides (OCPs) dicofol and dichlorodiphenyltrichloroethane (DDT) by the Tower Chemical Company prompted the EPA’s designation of the lake as a Superfund site. High levels of the contaminants seeped into the surrounding aquifers, contaminating the waters and sediments of Lake Apopka. In addition to the spill, muck farms lined the shores of Lake Apopka years ago where multiple OCPs were used to control pest growth, including the compounds DDT, toxaphene (TOX), dieldrin (DIEL), and methoxychlor (MXC), amongst others. At the Lake Apopka site, p,p’-dichlorodiphenyldichloroethylene (DDE, the persistent and stable breakdown product of DDT),
TOX, DIEL, and MXC have been reported to constitute a predominant part of the OCP load in the sediment, in fish, and in alligators (33-36). Research by a number of investigators has strongly supported the hypothesis that the multitude of OCPs present at this site are to blame for the altered reproductive status and altered circulating steroid hormone levels observed in fish (37-39), turtles (40), and alligators (40, 41).

The continued presence of persistent OCPs and their metabolites in Lake Apopka poses an ongoing potential threat to area wildlife. The OCPs that contaminate Lake Apopka vary substantially, both by mode of action and by chemical structure (Figure 1-1); hence, it is important to understand how these chemicals function and disrupt reproduction and development.

**Largemouth Bass (LMB) as a Model for Endocrine Disruption**

LMB are one of the many species of fish that are susceptible to the deleterious effects of EDCs. LMB, a species of game fish important both commercially and ecologically, are found throughout bodies of freshwater throughout North America, including Lake Apopka in Florida and the Potomac River in Maryland. The effects of many EDCs tend to biomagnify as an organism moves up rank in the food chain, and since LMB are considered a top predator, they are especially susceptible to the effects of high levels of EDCs in the environment and prone to biomagnification of contaminants. Thus, LMB serve as a good model when studying the effects of EDCs in the laboratory.

LMB are a species of teleost fish that reproduce semi-synchronously in the wild, making isolation of various stages of reproduction in male and female LMB generally feasible. It is because of this that LMB make a useful model for the study of EDCs and their mechanisms of action. In order to assess the effects and mechanisms of action that EDCs exert in LMB
reproduction, it is important to gain specific knowledge on the complexities surrounding their reproductive cycles.

The reproductive cycles of female and male teleost fish species have been well characterized previously (42, 43), although a detailed reproductive stage-specific study of gonadal gene expression changes, gonad staging, and other physiological endpoints in wild LMB has not been reported to date. It must be noted that a previous study in pond-reared LMB was conducted that spanned several months of a projected spawning season; plasma steroid hormone levels and various gene expression profiles from different tissues were characterized (44, 45). These previous studies did not examine any reproductive stage-specific gene expression in either sex, and did not characterize the entire reproductive cycle of LMB.

LMB that reside in Florida typically spawn in the wild a few times per year, and the spawning season usually falls between the months of late January and early May (45); however spawning is controlled by several environmental and physiological cues, including photoperiod, temperature, courtship behavior, and endogenous hormonal triggers (46).

It is important to have a firm understanding of the predominant reproductive stages and steroidogenesis in both sexes of LMB in order to understand levels of vulnerability to the disruptive effects of EDCs in the environment.

**Folliculogenesis and oogenesis in female LMB**

The reproductive cycles of female teleost fishes are quite complex; there are multiple stages and sub-stages that occur throughout the season and these vary greatly across species. Female LMB typically have two ovaries that are suspended in the dorsal part of the abdomen that are linked by a gonoduct to the genital pore. In female LMB, as plasma sex steroid hormones and vitellogenin (VTG; egg yolk precursor protein) profiles increase, the onset of reproduction occurs. In general, increasing plasma concentrations of VTG, 17β-estradiol (E2), 17α,20β-
dihydroxy-4-pregnen-3-one (P4), and testosterone (T) are highly involved in the growth and development of the ovaries (45, 47).

In a study conducted throughout the projected spawning season in pond-reared LMB, plasma sex steroid hormone and VTG levels were quantified and analyzed. The study revealed that E2 and T levels peaked in February and March and plasma VTG peaked during December-February. VTG, E2, and T gradually decreased concomitantly through May and remained constitutively low after spawning (45).

As defined by Grier et al. (42), the female teleost reproductive cycle is encompassed by six distinct stages, including: proliferation of oogonia (during adolescence), chromatin nucleolus (CN), primary growth (PG), secondary growth (vitellogenesis; SG), oocyte maturation (OM), and ovulation (OV). Details of the stages and descriptions from Grier’s system are outlined in Table 1-1 below (42). In LMB inhabiting Florida, following ovulation and when water temperatures rise to > 75 °F, follicles are resorbed and the ovaries undergo atresia. Although there are multiple sub-stages assigned to each of the reproductive stages, it is often quite difficult to isolate specific steps in the reproductive cycle of wild LMB due to the fact that the species isn’t completely synchronous in its reproductive capacity. The reproductive cycle of female LMB is extremely complex and highly controlled by physiological and environmental parameters.

**Spermatogenesis in male LMB**

Slightly less complex than the reproductive cycle of female LMB, but still interspersed with multiple stages and tightly controlled physiological changes, is the reproductive cycle of male teleost fishes. Male LMB typically bear two testes, both attached to the dorsal wall in the abdomen of the fish; the testes converge into a central efferent duct system which is linked to the exterior of the body at the urogenital pore. In males, increases in plasma T and 11-
Ketotestosterone (11-KT) are associated with the growth and development of the testes throughout the reproductive cycle (47). 11-KT has been identified as the predominant male hormone, and consistently peaks in several teleost species during final reproduction and spawning (45, 48). In the study of pond-reared LMB conducted by Gross et al. in 2002, plasma levels of 11-KT and T in male LMB peaked in February and March, respectively, and circulating levels of both hormones diminished as water temperatures exceeded 75 °F in the summer months (45).

In all male teleost species, the testes form spermatozoa during spermatogenesis, deliver them to the efferent ducts during spermiation, and secrete male sex hormones (49). Similar to the reproductive cycles of female LMB, male LMB undergo a number of physiological changes throughout spermatogenesis. As described by Grier and Aranzábal, there are distinct stages that the testes undergo throughout spermatogenesis, including: early germinal epithelium (GE) development, mid GE development, and late GE development. Early GE development is characterized by the presence of spermatogonia and spermatocytes; mid GE development is characterized by the presence of spermatids, as well as spermatogonia and spermatocytes. During late GE, spermatozoa are produced in pockets in the testes in preparation for spermiation during spawning (43).

Altogether, the reproductive cycles in both female and male LMB are tightly controlled by physiological and hormonal cues, and each distinct stage of reproductive development is characterized by the presence and variable abundance of several different types of cells. Sex steroid hormones tightly control reproduction in both female and male LMB. With much variation in cell type population and in plasma sex steroid hormone levels throughout LMB
reproductive cycling, it is likely that susceptibility to the disruptive effects of xenoestrogenic OCPs in the environment varies as well.

**Sex Steroid Hormone Biosynthesis in LMB**

In all vertebrates, development, homeostatic regulation, and reproduction are essential and complex physiological processes that are all tightly regulated by steroid hormones. Steroid hormones are small, cholesterol-derived molecules, which are produced by specialized cells in different steroidogenic tissues. The two major organs in fish that are responsible for steroid hormone biosynthesis are the gonads and the head kidney. In mammals, the gonads and the adrenal cortex produce steroid hormones (reviewed in (50)).

There are five major types of steroid hormones, including glucocorticoids and mineralocorticoids (involved in stress response and homeostatic maintenance), progestagens, androgens, and estrogens (involved in sexual development and reproduction). All five classes of steroid hormones play complex and integral roles in the survival of an organism. However, because many OCPs, including those found at the Lake Apopka site, have been reported to alter plasma sex steroid hormone levels and disrupt other reproductive parameters in LMB and in other species, it is important to have a solid understanding of sex steroid hormone biosynthesis in LMB.

Reproductive success in all vertebrates is dependent upon an intricate balance of circulating sex steroid hormones. Sex steroid hormone biosynthesis is acutely regulated by tropic hormones released by the anterior pituitary, including luteinizing hormone (LH) and follicle stimulating hormone (FSH). Sex steroid hormones control the expression of genes involved in a number of physiological processes, including reproduction. In general, sex steroid hormones bind to and activate specific nuclear receptor proteins, resulting in receptor dimerization and translocation into the nucleus of a cell where the receptor complex and other
transcription factors control the expression of target genes (51). Nuclear receptors control the expression of genes centrally involved in reproduction, and these important and complex pathways and genes will be discussed in further detail in the next section.

Relative to reproduction in adult vertebrates (including LMB), there are three major classes of sex steroid hormones, including estrogens, androgens, and progestogens. Sex steroid hormones are produced by specialized cells found in the gonad; theca and granulosa cells (in the female ovary) and Sertoli and Leydig cells (in the male testis) work in a concerted manner to generate sex steroid hormones from cholesterol (reviewed in (50)). These specialized cells express tightly regulated steroidogenic enzymes that are involved in the processing and modulation of cholesterol, the precursor molecule of all steroid hormones, into progestogens, androgens, and estrogens, which in turn regulate progression and cycling of LMB reproduction. A generalized diagram depicting the major steps and enzymes involved in teleost steroid hormone biosynthesis can be found in Figure 1-2.

It has been well characterized in all vertebrates that the production of different classes of steroid hormones hinges on the delivery of cholesterol to the inner mitochondrial membrane, where P450 side chain-cleavage (P450scc) enzyme metabolizes cholesterol into pregnenolone (the basal steroid that is metabolized into other steroid hormones). In mammals, the sterol transfer protein required to deliver cholesterol to the inner mitochondrial membrane was first purified and sequenced in steroidogenic cells in 1994 by Clark et al (52); this protein was termed the steroidogenic acute regulatory (StAR) protein and it controls the rate-limiting step in steroid hormone biosynthesis. StAR protein is acutely regulated protein responsible for the translocation of cholesterol across the outer mitochondrial membrane prior to enzymatic cleavage into steroid hormones. Other proteins work together with StAR protein at the
mitochondrial membrane, including an essential complex of protein kinases (53), voltage-dependent anion channel 1, the peripheral benzodiazepine receptor, hormone-sensitive lipase, among others (reviewed in (54)). StAR protein activity is highly regulated by the Protein Kinase A (PKA) pathway; in general, binding of tropic hormones (produced by the pituitary) to membrane-bound tropic hormone receptors in steroidogenic cells activates the PKA pathway. PKA, with help from Protein Kinase C (PKC) and other pathways, phosphorylates StAR protein, resulting in its activation (55-59). Because StAR protein plays such an integral and essential role in steroid hormone biosynthesis, further discussion regarding its regulation at the transcriptional and translational levels will be addressed in more detail later in this chapter.

Altogether, the biosynthesis of sex steroid hormones is a complex process and involves multiple signaling pathways and numerous different signaling molecules and enzymes. Because sex steroid hormone biosynthesis is so complex and so tightly regulated, it is likely that OCPs known to alter the levels of circulating sex steroid hormones in LMB disrupt the biosynthetic process at multiple steps.

**Nuclear Receptor Signaling in Reproduction**

Nuclear receptor signaling pathways play integral roles in controlling the expression of genes involved in a number of physiological processes, including those central to reproduction and development (51). Nuclear receptors have been extensively researched and reviewed in the literature, and the information discussed here is a conglomeration of a huge subset of data most recently reviewed on the superfamily (60, 61).

Nuclear receptors have the capacity to bind directly to DNA sequences as monomers, homodimers, and heterodimers, and are considered to be one of the most diverse and important families of transcription factors involved in controlling gene expression. In general, nuclear receptors are composed of six functional domains (A-F, from the N-terminal to the C-terminal
end), and each domain is responsible for a specific part of receptor function. The poorly defined and highly variable A/B domains contain an activation function (AF-1) domain. The highly conserved C domain contains regions that are responsible for receptor dimerization and for DNA binding. The poorly conserved D domain is the hinge region which contains a localization signal. The functionally complex E domain is the region that binds ligands and also plays an integral role in receptor dimerization and the F domain is thought to contain a modulatory function that mediates how ligands affect the transcriptional activity of nuclear receptors in cells (reviewed in (60, 62-64)).

Currently, there are roughly 50 different types (each type having multiple isoforms) of functional nuclear receptors identified in vertebrates. This family includes two general types of receptors, including ligand-dependent receptors and orphan nuclear receptors (for which no ligand is known or may not exist).

Bearing in mind that nuclear receptor signaling controls the expression of numerous genes and is integral to multiple physiological processes; this review will focus on a few key players of the nuclear receptor superfamily, including both ligand-mediated and orphan receptors. These receptors have been implicated in the control of genes that are fundamentally involved in reproduction. When trying to assess how xenoestrogenic EDCs in the environment disrupt reproduction, it is vital to have a solid understanding of the endogenous signaling pathways involved in regulating genes that control reproductive functions.

**Estrogen receptors (ERs) and androgen receptors (ARs)**

ERs and ARs, belonging to the ligand-dependent subset of the nuclear receptor superfamily, are key players in mediating the transcriptional control of genes in response to hormonal cues central to reproduction in all vertebrates. However, the two sex steroid hormone
receptors vary substantially with regards to isoform expression, ligand binding, and functional specificity.

ERs have been extensively investigated and well-characterized in many species, including many species of fish (44, 65-67). In mammals, there are two known isoforms of ERs, including ERα and ERβ. Each of the two ERs is transcribed from a unique gene and multiple isoforms are expressed in a tissue-specific manner throughout the body. Similar to mammals, fish also express two main classes of ERs, each class encompassing multiple isoforms of each receptor.

It is now well understood that many teleost species, including LMB, express three unique isoforms of ERs that are products of different genes, and these include ERα, ERβa, and ERβb (44, 66, 68-72). Work by Sabo-Attwood et al. showed that the three ERs in LMB are expressed in the liver and in the gonad; however ERβa and ERβb were more highly expressed in the gonad, whereas ERα was more highly expressed in the liver (44).

Equally as important in controlling signal transduction, ARs have been well characterized. In mammals, two isoforms of ARs have been described, including AR-A and AR-B; AR-A is an N-terminal truncated version of the full length AR-B isoform, and both isoforms have been reported to function in a similar manner (73-75). Similarly, two distinct AR isoforms have been characterized in several species of fish (76-81), including ARα and ARβ (synonymous with AR1 and AR2); however, it has been projected that, due to the lack of sequence homology between the two isoforms, each isoform is likely transcribed from its own gene rather than a single gene as observed in mammals. In addition, 11-KT is an additional functional androgen in fish and it has been reported that a variant of ARβ binds preferentially to this important signaling steroid (82). It is likely that LMB also express two isoforms, though the fragment that has been
sequenced in our laboratory is located in a very highly conserved region (homologous in all isoforms of AR in fish).

In fish and mammals, ERs and ARs mediate the expression of reproductive genes in response to sex steroid hormonal cues throughout the reproductive cycle. Regulation of genes via classical genomic signaling entails the binding of a dimerized, sex steroid hormone-bound receptor complex to a hormone response element (HRE) found in the promoter of a target gene, inducing activation/repression of expression (reviewed in (60)); ERs bind to estrogen response elements (EREs) and ARs bind to androgen response elements (AREs). However, numerous studies have shown that both ERs and ARs can influence gene expression in multiple other non-classical ways by interacting with and influencing the actions of other transcription factors that differentially alter the expression of target genes (reviewed in (83, 84)). Signaling mechanisms surrounding ER- and AR-mediated gene expression are extremely complex and have yet to be perfectly understood.

ERs and ARs are integrally involved in the control of genes involved in reproduction, and it is vital to have an understanding of those genes and the pathways that regulate them, especially when considering the effects of EDCs on reproduction in the environment.

**Orphan nuclear receptors: retinoic acid-related receptors (RORs) and rev-erb receptors**

Orphan nuclear receptors are extensively similar in structure to all members of the nuclear receptor family; however, orphan receptors are receptors that were identified without any prior knowledge of their association with a ligand (reviewed in (61)). In addition to sex steroid hormone receptor signaling, less thoroughly understood orphan nuclear receptor signaling has also been implicated in the regulation of expression of many genes that are critical to reproduction in mammals.
Circadian control of gene expression at the cellular level is important in regulating steroid hormone production in vertebrates. It is known that the orphan receptors RORα and rev-erbα are two signaling proteins that play integral roles in controlling genes central to the circadian cascade (85-87). RORα and rev-erbα both bind to similar core sequences (ROR elements – ROREs), however they induce opposing effects on the transcription of target genes (61). Interestingly, the recently completed crystal structure of human RORα revealed cholesterol in its binding pocket (88). Cholesterol is the backbone to all steroid hormones, and cholesterol as a putative ligand for RORα implicates that the molecule may play an interesting role in regulating steroid hormone biosynthesis.

**Steroidogenic Acute Regulatory (StAR) Protein**

StAR protein, a rapidly synthesized 37 kDa mitochondrial phosphoprotein, is responsible for the translocation of cholesterol, the precursor of all steroid hormones, from the outer mitochondrial membrane to the inner membrane where it is converted into pregnenolone by P450scc (52, 56). Importantly, StAR protein, in concert with other co-factors, controls the first and rate-limiting step in steroidogenesis in all vertebrates. Based on previous studies in our laboratory, it is likely that StAR protein plays the same integral role in LMB (89, 90). Numerous studies have shown that the StAR protein plays an integral role in the biosynthesis of hormones essential to survival (adrenal hormones) and reproduction (gonadal hormones) in vertebrates (52, 91, 92).

The critical role that StAR protein plays in proper development and steroid hormone production is evident upon examination of patients that suffer from lipoid congenital dysplasia (lipoid CAH). Lipoid CAH is an autosomal recessive disorder which is characterized by severely impaired adrenal and gonadal steroid hormone productive capacities (58, 93). In studies
where the expression of the \textit{StAR} gene was disrupted in the mouse, the same phenotype was observed in the experimental model that is observed in patients that suffer from lipoid CAH (94, 95). Proper StAR protein function is essential for steroid hormone production in vertebrates and steroid hormones control both reproduction and survival in an organism.

In mammals, StAR protein expression is primarily associated with steroidogenic tissues, including the adrenal cortex, gonad, placenta, and brain (reviewed in (54)); however, it has been shown that StAR protein is also minimally expressed in the liver (96). It is only in the last several years that researchers have begun to characterize StAR protein and \textit{StAR} gene expression in different fish species (90, 97-107). In the limited studies that have examined the tissue distribution of \textit{StAR} mRNA in teleost species, \textit{StAR} mRNA expression was, as observed in mammals, limited to the ovary, testis, head kidney (analogous to the adrenal cortex in mammals), and, to a lesser extent, the liver (100, 107). Previous work in our laboratory has confirmed expression of \textit{StAR} mRNA in female LMB ovaries (90), and it is likely that the tissue distribution of \textit{StAR} mRNA in LMB is isolated to steroidogenic tissues in a manner similar to that observed in other fish and mammalian studies.

In steroidogenic tissues, acute steroidogenesis is mediated by multiple complex mechanisms (including pathways stimulated by gonadotropin signaling) that alter the transcription, translation, or the functionality of StAR protein (55, 108, 109). However, in mammalian systems, it has been shown that inhibition of the StAR protein at any level, transcriptional or translational, substantially decreases the capacity for steroid biosynthesis and approximately only 10% of steroid hormone production can occur via StAR protein-independent mechanisms (58, 110, 111).
It has been shown in multiple studies that StAR protein is a target of various EDCs in many different species (reviewed in (112)). For example, in vivo exposure of male and female LMB to the OCPs DDE and DIEL caused altered steady-state StAR mRNA levels in the testis and ovary, respectively (113), although the tissue-specific responses have not yet been elucidated. In a study conducted in goldfish, in vivo exposure to the phytosterol β-sitosterol yielded changes in gonadal StAR transcript levels in male fish (114). It is understood that a wide array of compounds have been shown to disrupt StAR gene expression at the transcriptional level, including the pesticides Roundup (19), dimethoate (115), and lindane (20), though the mechanisms through which these contaminants act to disrupt StAR mRNA expression and gene transcription are unresolved. Thus, it is possible that EDCs target StAR protein expression at a multitude of different levels.

It is evident that the StAR protein is very important in steroid hormone production in vertebrates, and further discussion of its regulation at the transcriptional, translational, and post-translational levels is necessary in order to understand how various EDCs may impact the expression of the vital protein.

**Transcriptional regulation of the StAR gene**

Transcriptional regulation of the StAR gene is a key control mechanism in vertebrate reproduction, and it is mediated by a large family of cyclic adenosine monophosphate (cAMP)-responsive nuclear factors that act in response to the stimulation of the adenylate cyclase signaling pathway. It has been well established that StAR gene transcription and activation of its promoter are highly stimulated by cAMP signaling; StAR gene transcription is also highly responsive to other agents that stimulate the adenylate cyclase pathway via G-protein-coupled receptor-mediated signaling, including LH, FSH, adrenocorticotropic hormone (ACTH), human
chorionic gonadotropin (hCG), retinoic acid, amongst many others (reviewed in (116)).

However, the exact mechanisms that are involved in the cAMP-mediated activation of the StAR promoter are not completely understood. Although the activation of transcription by cAMP signaling is classically mediated through the interaction of the cAMP response element (CRE)-binding protein (CREBP) with a conserved CRE sequence located in the promoters of cAMP-responsive genes, mammalian StAR promoters lack perfect CRE sequences (52, 117, 118). It is apparent that other signaling components must be responsible for the activation of the StAR promoter. The promoter for the StAR gene has been extensively characterized in mammalian systems, and research has implicated that there are numerous pathways involved in mediating the cAMP-induced activation of the very complex promoter (reviewed in (54)).

Previous work in our laboratory investigating the activity of a 2.9 kb portion of the StAR gene promoter cloned from LMB revealed that, as observed with mammalian StAR gene promoters, the LMB StAR promoter is inducible by dibutyryl cAMP (dbcAMP), a stable homolog of cAMP (90). The complete cDNA coding region of the StAR gene from LMB and a 2.9 kb fragment of its promoter were previously cloned and sequenced in our laboratory. Sequence alignment of the cDNA with the sequences of other species revealed high homology, thus, it is possible that the signal transduction pathways that control the promoters for the StAR gene are similar and evolutionarily conserved.

Multiple signaling pathways are involved in the regulation of cAMP-induced StAR promoter activation in mammalian models. Initially, the interaction of tropic hormones (LH/ACTH (91, 119)) with specific membrane-bound gonadotropin receptors initiates the activation of G protein coupled receptor signaling pathways, which turns on the membrane-associated enzyme adenylyl cyclase that generates the second messenger cAMP.
activates the PKA pathway, which stimulates the phosphorylation and consequent activation of numerous transcription factors that mediate the activation and activity of the StAR promoter (reviewed in (54)).

In addition to the PKA/PKC induced activation of the StAR gene promoter, the arachidonic acid signaling pathway is integrally involved in StAR gene expression; studies have demonstrated that tropic hormone stimulation causes both the formation of cAMP and the release of arachidonic acid from phospholipids and that both molecules are necessary for proper activation of StAR gene expression (120-123). Other studies have shown that steroid hormones, including E2 and T, can stimulate and/or repress StAR gene expression (124, 125); however, the mechanisms by which steroid hormones impact the expression of the StAR gene are not currently understood.

Extensive studies indicate that numerous transcription factors (TFs) and signal transduction pathways mediate the regulation of the StAR gene promoter. Several TFs have been identified that function to activate the mammalian StAR promoter, including CREBP and steroidogenic factor 1 (SF-1) (108, 126, 127), activator protein 1 (AP-1) and Sp1 (128, 129), GATA-4 and CCAAT/enhancer-binding protein (C/EBP) (130, 131), Clock/Bmal1 (132), aryl hydrocarbon receptor (AhR) (133), and sterol regulatory element-binding protein (SREBP) (134). Although less extensively studied, some negative regulators of the StAR gene promoter have been identified, including yin yang 1 (YY1) (135), DAX-1 (136), and AP-1/c-fos (137). Interestingly, SF-1, AhR, and DAX-1 are considered orphan nuclear receptors. Based upon the promiscuous activities surrounding nuclear receptor signaling, it is likely that other nuclear receptor pathways function in the complex regulation of the StAR gene promoter. Altogether, it
is well recognized that the \textit{StAR} promoter is regulated in a very complex manner involving multiple signal transduction pathways.

\textbf{Post-transcriptional regulation of StAR protein}

It is apparent that transcriptional regulation of the \textit{StAR} gene is critical in controlling the tissue-specific expression of the protein. However, recent research has implicated that a number of post-transcriptional processes function to regulate StAR protein-mediated steroidogenesis, including factors that alter the stability of \textit{StAR} mRNA and post-translational modifications of StAR protein (reviewed in (54)).

In mammals, there is only one isoform of StAR protein, yet northern blots for human \textit{StAR} mRNA commonly show the presence of at least two predominant species of mRNA; the predominant 1.7 kb transcript is present in both the adrenal and gonads and the longer 2.4 and 4.4 kb transcripts present in the adrenal and gonads, respectively (117, 127). The absence of the poly-A 3’ tail found in the longer \textit{StAR} mRNA transcripts has been shown to be highly correlated with increased StAR protein expression (138). Since the stability and localization of many mRNAs are influenced by sequences found in their 3’UTR and because the longer 3’UTR in \textit{StAR} mRNA results in reduced protein expression, it is likely that \textit{StAR} mRNA stability is tightly controlled by a number of mechanisms (reviewed in (54)). Due to the high evolutionary conservation of StAR protein function, it is likely that \textit{StAR} mRNA is regulated in a similar manner in LMB.

A great deal of research has shown that, following translation from mRNA to protein, StAR protein interacts with a number of different factors that enhance the delivery of cholesterol to the mitochondrial membrane. Some of the proteins that have been identified to act in concert with StAR protein in mammals include the peripheral benzodiazepine receptor (139) and hormone sensitive lipase, amongst others (140, 141). In addition, it is well known that, in order
to support cholesterol transport at its fully capacity, StAR protein must be phosphorylated by PKA pathways (142).

Although StAR protein has been studied at the transcriptional, translational, and post-translational levels, the exact mechanisms of regulation of the protein that is so critically involved in steroid hormone biosynthesis remain unresolved. Seemingly, StAR protein is a target of various EDCs and further characterization of the regulation of the critical protein is essential in order to understand how it is that EDCs disrupt its expression.

Mechanisms of OCP Toxicity in LMB

Many laboratory studies have attempted to identify the mechanisms which OCPs present in Lake Apopka employ to adversely affect sex steroid hormone levels in wildlife, but to this day, mechanisms of action are not well defined. The OCPs present at the Lake Apopka site are likely not restricted to a single mode of action. There is significant evidence that implicates the OCPs DDE, MXC, DIEL, and TOX as xenoestrogenic and/or anti-androgenic by nature, and that they can exert numerous effects acting on both ER signaling pathways and/or on AR signaling pathways (4, 11, 65, 143-151). Previous work that examined gene expression profiles of pond-reared LMB throughout a portion of the reproductive cycle in female LMB revealed that $ER$ mRNA expression varied significantly over time (44); thus it is likely that $ER$ mRNA expression varies by reproductive stage. Intuitively, if xenoestrogens, such as the OCPs found in Lake Apopka and the Potomac River, disrupt ER signaling, then susceptibility to the effects of the EDCs will likely vary throughout the reproductive cycle in LMB.

Classically, research in the field of endocrine disruption has focused on how EDCs target ER- and AR-signaling pathways. However, more recent research has shown that the expression of key steroidogenic enzymes, including those involved in the metabolism and production of steroid hormones can be significantly impacted by multiple EDCs (3, 13, 19, 25, 112-115, 152-
Thus, it is likely that OCPs, such as those found at the Superfund site in Lake Apopka, could impact the expression of such enzymes in addition to the classical ER and AR-signaling pathways.

Although it is well understood that OCPs can significantly alter steroid hormone production and reproduction in LMB, no individual study has been able to replicate the reproductive abnormalities exhibited by LMB that inhabit OCP-contaminated waters, implicating that pathways in steroidogenesis (other than those already investigated) are likely to be involved in further mediating the effects of EDCs in the species.

**Research Objectives**

The objectives of the research discussed in this dissertation are: 1) to characterize the natural reproductive cycle of a wild subset of LMB, analyzing gonadal changes both morphologically and at the level of critically regulated gene expression centrally involved in reproduction, 2) to characterize the *StAR* gene in LMB at the mRNA and promoter levels to gain a better understanding of how this protein, vital to steroid hormone production in vertebrates, is acutely regulated in LMB, and 3) to examine the potential for several xenoestrogenic OCPs to impact the *StAR* gene and other classical steroid hormone receptor signaling pathways in the gonads of LMB.
<table>
<thead>
<tr>
<th>Stage</th>
<th>Sub-Stages/Steps</th>
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<tr>
<td>Oogonia Proliferate (OP)</td>
<td>Frequently form cell nests</td>
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<tr>
<td>Chromatin Nucleolus (CN)</td>
<td>Leptotene</td>
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<td>Early diplofene</td>
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<td>Primary Growth (PG)</td>
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<td>Multiple nucleoli</td>
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<td>Perinucleolar</td>
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<td>Circumnuclear oil droplets</td>
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<td>Cortical alveoli</td>
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<td>Secondary Growth: Vitellogenesis (SG)</td>
<td>Early secondary growth</td>
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<td>Late secondary growth</td>
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<td>Full-grown oocyte</td>
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<td>Oocyte Maturation (OM)</td>
<td>Eccentric germinal vesicle (oil drops coalesce)</td>
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<td>Germinal vesicle migration to animal pole</td>
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<td></td>
<td>Germinal vesicle breakdown (oocyte hydrated)</td>
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<td></td>
<td>Meiosis resumes, 2\textsuperscript{nd} arrest</td>
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<tr>
<td>Ovulation (OV)</td>
<td>Oocyte emerges from follicle becoming an egg</td>
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Known Modes of Action in Disrupting Reproduction

**DDE**  
ER Agonist  
AR Antagonist  
Disrupts ERK1/2 Signaling  
Membrane ER Agonist

**MXC**  
ER Agonist  
AR Antagonist  
Disrupts Gonadotropin Signaling

**DIEL**  
Associated with Neurological Disorders  
Disrupts ERK1/2 Signaling

**TOX**  
Unknown

Figure 1-1: Chemical structures and reproductive targets of select OCPs.
Figure 1-2: Reproductive steroid hormone biosynthesis in teleost fishes.
CHAPTER 2
MATERIALS AND METHODS

The overall aims of this project were collectively to characterize the regulation and expression of \textit{StAR} mRNA levels in the gonads of a healthy subset of wild LMB, and using a multitude of molecular techniques, to characterize novel regulatory mechanisms involved in the activation of the LMB \textit{StAR} gene promoter. The potential for OCPs to disrupt \textit{StAR} expression at the mRNA and gene promoter levels (and steroidogenesis) was also investigated using a variety of molecular biological techniques.

\textbf{Animals}

\textbf{Wild LMB Seasonal Study}

Male and female LMB were collected from the St. John’s River in Welaka, FL once per month, ranging from October 2005 to September 2006. The period between sampling events ranged from 3-5 weeks. Additional details of the study site can also be found in (159).

Approximately 10 males and 10 females were collected each month. Body weight, body length (tip of the mouth to tip of the tail), gonad weight and age were recorded. Gonadosomatic indices (GSI) were calculated for all individuals used in this study as [absolute gonad weight/absolute body weight] X 100. Approximately 3-5 mL of blood was drawn from the caudal vein using a heparinized vacutainer and stored for later studies. Fish were euthanized with a blow to the head and gonad samples (along with other tissues) were collected, rapidly dissected, and snap-frozen in liquid N\textsubscript{2}. All samples were stored at -80°C until processed.

A gonad sample was collected for histological examination and placed in buffered formalin (Protocol®, Fisher Scientific, Waltham, MA, USA) to determine the reproductive stage of the animal. Gonads were plastic embedded and cut to 5-10 micron sections. Staining of the gonad sample was done with standard hematoxylin (basophilic dye) and eosin (acidophilic dye) staining.
protocols. Vitellogenic oocytes are acidic and stain more red with eosin. Female histological samples were examined under a microscope and each individual used in this study were categorized into one of six predominant stages; perinuclear (PN), cortical alveoli (CA), early vitellogenic (EV), late vitellogenic (LV), maturation (M), and atresia (AT). Male testes histological samples were also examined under a microscope and were categorized into one of four predominant reproductive stages; spermatogonia (SG), spermatocytes (SC), < 50% spermatozoa (SZ), and >50% SZ. Figures 3-2 (female) and 3-3 (male) contain representative images of each reproductive stage classification, including labels of characteristic cell types indicative of each distinct stage throughout the reproductive cycle.

**Captive LMB**

LMB used in all *ex vivo* testis and ovary culture experiments were purchased from American Sport Fish Hatchery (Montgomery, AL). All fish were housed in the Aquatic Toxicology Facilities in the Center for Environmental and Human Toxicology at the University of Florida in accordance with the National Institute for Health (NIH) Guide for the Care and Use of Laboratory Animals.

**Methods**

**Ribonucleic Acid (RNA) Purification**

For each experiment, tissues (stored at -80 °C until RNA was processed) were homogenized in a 2 mL microcentrifuge tube 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 by adding chloroform, vortexing briefly, and incubating at room temperature for 5 minutes. Each sample was then centrifuged for 15 minutes at 14,000 rpm at 4 °C in a refrigerated microcentrifuge. The organic layer (containing proteins) was discarded and the aqueous layer that contained the RNA was then re-
extracted by repeating the same procedure. Following the second extraction, RNA was precipitated out of the aqueous solution by adding isopropanol followed by storage overnight at -20 °C. The next day, samples were spun at 14,000 rpm at 4 °C for 45 minutes to pellet the RNA. Each 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 deoxyribonucleic acid (DNA) contamination was removed from each sample using the DNAfree™ kit according to the manufacturer’s protocol (Ambion, Inc., Austin, TX, USA). Purified RNA samples were stored at -80 °C. Prior to use, RNA concentrations were quantified by spectrophotometer analysis and RNA integrity was verified by 260/280 Absorbance ratios and by agarose/ethidium bromide electrophoresis. A representative image of a typical denaturing agarose/ethidium bromide gel can be found in Appendix A, Figure A-1.

**Quantitative Reverse Transcriptase Real-Time Polymerase Chain Reaction (qPCR)**

Primers used to amplify all genes of interest in this study have been previously published in (44, 90, 113, 144) and are listed in Table 2-1. Standard curves relating initial template copy number to fluorescence and amplification cycle were generated using pGEM-®T easy vector (Promega Corp., Madison, WI, USA) containing the gene of interest as a template. The equation used to calculate copy number is as follows:

\[
\{6 \times 10^{23} \text{ (copies/mol)} \times [\text{plasmid}] \text{ (g/µl)} \}/\text{molecular weight of the plasmid (g/mol)} = \text{copies/µl}
\]

Standard curves ranged from 1 x 10⁹ to 1 x 10² copies. Standard curves ranged between 95-105% efficiency and were linear at R²>0.99.

qPCR analysis of gene expression was conducted in duplicate from each sample, each reaction containing 1X iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 100 ng first-strand cDNA derived from DNase-treated RNA samples, corresponding
forward and reverse primers (sequences and final concentrations located below in Table 2-1), and nuclease-free water in a final reaction volume of 25 µL per reaction. The two step thermal cycling parameters were as follows; initial 1 cycle Taq activation at 95 °C for 3 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60°C for 1 minute. After 40 cycles, a dissociation curve was produced starting at 55 °C (+1 °C/30 seconds) to 95 °C. qPCR expression was assayed on an iCycler Thermal Cycler (Bio-Rad, Inc.). All gene expression values are reported as absolute copy number per µg total RNA, and all values were normalized to ribosomal 18S.

**Cloning of the 5.6 kb LMB StAR Gene Promoter**

2.9 kb of the LMB StAR gene promoter was cloned and methods were described previously in (90). The cloning of the remaining distal 2.6 kb of the promoter was done using high quality genomic DNA isolated from 50 mg of LMB ovarian tissue using the Wizard Genomic DNA Isolation Kit according to the manufacturer’s protocol (Promega Corp., Madison, WI, USA). The genomic DNA was purified twice by phenol/chloroform extraction to ensure all residual protein was removed. The genomic DNA pellet was resuspended in TE buffer (10 mM Tris-HCl, pH 7.3; 1 mM EDTA). Using the GenomeWalker Kit (Clontech, Mountain View, CA, USA), three digested genomic DNA libraries were generated using the restriction enzymes FspI, HpaI, and NaeI, and ligated to adaptor primers for polymerase chain reaction (PCR) amplification according to the manufacturer’s protocol. PCR amplification of the LMB StAR gene promoter from the restriction libraries was done using a combination of the adaptor primers included in the kit and gene specific primers. Two different gene specific primers were used; one primer started closer to the 5’ end of the 2.9 kb promoter piece than the other (primary PCR), which yielded overlapping sequences and provided for further sequence verification. Nested
gene specific primers were used to reduce non-specific amplification (secondary PCR). All gene specific primer sequences for cloning both the 2.9 kb proximal and the 2.6 kb distal promoter segments are listed in Table 2-2. All secondary PCR reactions using the nested primers were performed using a 1:50 dilution of the primary PCR product in a final volume of 50 µl. Thermocycler conditions for the amplification are listed in Table 2-3 below. PCR products were cloned into the pGEM-T Easy Vector (Promega Corp., Madison, WI, USA). The 5.6 kb promoter for the LMB StAR gene has been deposited in the GenBank database under GenBank Accession Number DQ166819.

**In silico Transcription Factor Analyses**

Putative transcription factor binding elements were identified using MatInspector Release Professional (version 7.4.8.2, July 2007) published by Genomatix, Inc. The 2.9 kb or 5.6 kb LMB StAR promoter consensus sequences were input into the software program and multiple putative elements were identified. All elements reported carried ≥ 80% homology to each core vertebrate consensus sequence.

**Culturing of MA-10 Mouse Leydig Tumor Cells**

MA-10 mouse Leydig tumor cells were generously provided by Dr. Mario Ascoli (160). MA-10 cells were cultured in RPMI-1640 culture medium supplemented with 15% horse serum, 20 mM HEPES pH 7.2, and 50 µg/mL gentamicin, pH 7.7. Cells were grown in 100 mm culture plates (Corning) coated with 0.1% gelatin. 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 ºC in a humidified 5% CO₂ cell culture incubator.

**Culturing of Y-1 Mouse Adrenocortical Cells**

Y-1 mouse adrenocortical cells purchased from ATCC (Manassas, VA, USA) were cultured in Ham’s F12K culture medium containing 2 mM L-glutamine, supplemented with 1.5
g/L sodium bicarbonate, 15 % horse serum, 2.5 % FBS; and 1 % penicillin-streptomycin mix. The cells were passaged every 4-5 days and never exceeded 20 passages before a new vial was thawed. The cells were grown at 37 °C in a humidified 5 % CO₂ cell culture incubator.

**Isolation of Nuclear Extracts**

MA-10 mouse Leydig cells were plated on 100 mm cell culture plates coated with 0.1% gelatin (dissolved in 1X calcium- and magnesium-free phosphobuffered saline (44 g/L KH₂PO₄, 9 g/L NaCl, 0.795 g/L Na₂HPO₄, pH 7.4; PBS), allowed to grow to ~80% confluence, and either untreated or treated with 500 nM E₂ in growth medium for 20 hours. Y-1 mouse adrenocortical cells were plated on 100 mm cell culture plates, allowed to grow to ~80% confluence, and either untreated or treated with 1 mM dbcAMP in growth medium for 20 hours. Nuclear fractions were prepared from basal and treated cells using a Nuclear Extract Isolation Kit (Panomics, Inc., Fremont, CA, USA) according to the manufacturer’s protocol. Specifically, following preparation of Buffers A and B (addition of protease and phosphatase inhibitors and dithiothreitrol to provided buffers), the cell culture medium was aspirated from each plate and each plate was washed twice with ice cold 1X PBS. 1 mL of ice cold prepared Buffer A was added to each 100 mm plate followed by incubation on ice on a rocking platform at 200 rpm for 10 minutes. Following incubation, cells were scraped with a sterile disposable cell scraper. The cells were pipetted up and down to remove clumps and transferred to a 1.5 mL microcentrifuge tube. Samples were spun at 14,000 x g for 3 minutes at 4 °C in a refrigerated microcentrifuge. The supernatants were removed and discarded and the pellets were kept on ice. 150 µL of prepared Buffer B was added to each tube and vortexed for 10 seconds on the highest setting. The tubes were placed on ice for 60 minutes; each tube was gently agitated by hand every 20 minutes. Following incubation, all samples were spun at 14,000 x g for 5 minutes at 4 °C in a
refrigerated microcentrifuge. The supernatants (containing the nuclear extracts) were then aliquotted and stored immediately at -80 °C. The concentrations of all nuclear extracts were quantified by Bradford assay using Coomassie Plus Reagent (Pierce Inc., Rockford, IL, USA) and UV spectrophotometry.

**Oligonucleotide Annealing Reactions for Electromobility Shift Assays**

Sense and anti-sense oligonucleotides designed against the ERE/-2678 element and the ROR/-1969 element were generated based on bioinformatic analysis of the LMB *StAR* promoter. Several control probes were used, including one designed against a perfect human RORE (161) and one designed against a perfect canonical mouse ERE (provided by Panomics, Inc., Fremont, CA, USA). All oligonucleotide sequences are listed in Table 2-4 below. Probes were prepared and obtained commercially (Eurofins MWG Operon, Huntsville, AL, USA). The probes were biotinylated only at the 5' end of each sense strand; “cold” probes lacked biotinylation. Sense and anti-sense oligos were annealed by adding equimolar amounts of each probe to a reaction containing 10 mM Tris, pH 8.0, 1 mM EDTA, 20 mM NaCl, 10 mM MgCl₂, and 5 mM DTT. The reactions were boiled for 10 minutes at 95 °C, allowed to gradually return to room temperature, and stored at 4 °C for use within 48 hours. A polyacrylamide gel stained with SYBR green was run on the annealed oligonucleotides to confirm annealing (Appendix A, Figure A-2).

**Electromobility Shift Assays (EMSA)**

Fluorescence-based EMSAs were conducted using a modification of the commercially available EMSA Gel Shift Kit protocol (Panomics, Inc., Fremont, CA, USA) with double-stranded oligonucleotides (described above) and freshly prepared nuclear fractions from either Y-1 mouse adrenocortical or MA-10 mouse Leydig tumor cells. In EMSA/supershift experiments conducted with MA-10 nuclear extracts, 10 µg of nuclear extract and 1 µg of poly
d(I-C) were utilized in each binding reaction. In the instances where antibody was added, 1 µg of ERβ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) antibody was used per reaction. In experiments conducted with Y-1 nuclear extracts, 5 µg of nuclear extract and 1 µg of poly d(I-C) were utilized in each binding reaction. When recombinant RORα protein was used in place of nuclear extracts, 1 µg of protein was used in place of the nuclear extracts. In the instances where an antibody was used, 1 µg of RORα antibody was added to the reaction; Lechtken et al. have previously described the recombinant protein and antibody used in these experiments (161). For all experiments, prior to the addition of double-stranded probes encompassing each site the binding reactions were incubated overnight at 4 °C. The next day, the concentration of each of the double-stranded probes was quantified using a spectrophotometer; biotinylated probes were diluted to 10 ng/µl and 10 ng of probe was added to each reaction. If a competition assay was performed, the cold probes were diluted to 1000 ng/µl prior to adding 1000 ng of cold probe to the specified reaction. The binding reaction conditions are outlined in detail in the manufacturer’s protocol. Once the probes were added, the binding reactions were incubated at 17.5 °C for 30 minutes. A 6% non-denaturing Tris-borate EDTA (TBE) polyacrylamide gel was then prepared and pre-run in pre-chilled 0.5X TBE for 10 minutes at 120 V at 4 °C. Once loaded, gels were electrophoresed for 20 minutes at 60 V at 4°C, followed by 80 minutes at 100 V at 4 °C. The EMSA Gel-Shift Kit protocol and reagents from the manufacturer were used for the remainder of the EMSA/Supershift. All blots were visualized using a gel and blot photo docking system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Ex vivo Testis and Ovarian Tissue Cultures**

Ovarian and testis tissue cultures from LMB were cultured to detect tissue-specific responses to hCG in combination with E2, ICI 182,780, and a number of OCPs. Following
humane euthanasia by exposure to MS-222, gonads from male and female LMB were minced into ~20 mg pieces and carefully cultured in 1 mL L-15 culture medium (supplemented with 1 % antibiotic-antimycotic solution) in 24 well plates. Tissues were immediately pre-exposed for 2 hours to 100 µM doses of each OCP (DDE, DIEN, MXC, TOX) or 10 µM ICI 182,780 and 100 nM E2, followed by exposure to either medium alone or medium supplemented with 1 or 10 U/mL hCG for either 6 or 20 hours (in addition to the same chemical and dose during pre-exposure). All exposures were conducted at ambient temperature and in the dark on a rocking platform, ensuring proper aeration and mixing. Each treatment was conducted in duplicate from four individual LMB per sex. Tissues were snap-frozen in liquid N2 and stored at -80 °C until RNA was processed, while culture medium was aliquotted into 2 mL low adhesion microcentrifuge tubes and stored at -20 °C for future T analysis.

**Chromatin Immunoprecipitation (ChIP) Assays**

Y-1 cells were plated on 2 x 100 mm cell culture plates and allowed to grow to ~70% confluency. Each plate was transfected with the 2.9 kb LMB StAR gene promoter plasmid using Fugene HD (Roche Diagnostics, Indianapolis, IN). For ChIP assays, transfection efficiency must be high, and transfections using GFP in the Y-1 cells confirmed high transfection efficiency (Appendix A, Figure A-3). After transfecting overnight, medium was changed and 1 of the transfected plates was treated with 1 mM dbcAMP for 20 hours. Samples were processed for ChIP analysis using a modified version of the ChIP-IT Kit protocol (Active Motif, Carlsbad, CA). Following two washes with 10 mL ice cold 1X PBS, each plate was fixed for 10 minutes on a rocking platform at room temperature. The fixation solution consisted of formaldehyde diluted in serum-free and antibiotic-free culture medium ([formaldehyde Final] = 0.01%). Following fixation, the used fixation solution was aspirated and each plate was washed with 10
mL ice cold 1X PBS and the wash was discarded. To stop the fixation reaction, 10 mL of Glycine Stop-Fix Solution were added to each plate and each plate was incubated on a rocking platform for 5 minutes at room temperature. The solutions were aspirated following the incubation and each plate was washed with ice cold 1X PBS one final time. 2 mL of Cell Scraping Solution (1X PBS containing PMSF) were added to each plate and cells were scraped using a sterile disposable cell scraper. Cells from each treatment (1 plate basal and 1 plate stimulated) were pooled into multiple 2 mL low adhesion microcentrifuge tubes and centrifuged at 2,500 rpm at 4 °C to gently pellet the cells. The supernatant from each tube was discarded. The pellets from each individual treatment were pooled into one tube by resuspending all individual pellets in a total of 333 µL of ice cold Cell Lysis Buffer (supplemented with PMSF and protease inhibitor cocktail) and placed on ice for 30 minutes. Each cell suspension was then transferred to a pre-chilled 2 mL dounce homogenizer and dounced on ice with 10 strokes to help aid in the release of the nuclei from the cells. The lysed cells were then transferred to 1.5 mL low adhesion microcentrifuge tubes and centrifuged at 5,000 rpm for 10 minutes at 4 °C. The supernatant was carefully removed and each nuclei pellet was resuspended in 333 µL Shearing Buffer (supplemented with protease inhibitor cocktail). Chromatin was sheared using a Fisher Sonic Dismembrator (25% power; 20 second pulse, 30 second rest in ethanol ice bath, repeated 5X). Shearing efficiency was checked on an agarose/ethidium bromide gel (Appendix A; Figure A-4). Crosslinked sheared chromatin obtained from both treatments was pre-cleared with Protein G agarose beads for 2 hours at 4 °C on a rotating mixer. Samples were spun briefly to collect beads and the remaining supernatant (pre-cleared chromatin) from both treatments was split equally into 4 individual low adhesion 0.6 mL tubes. 10 µL of the pre-cleared chromatin from each treatment was frozen at -20 °C for use as input control (used to normalize and control
for the amount of DNA input into each immunoprecipitation reaction under different conditions) later in the protocol. All samples were immunoprecipitated at 4°C overnight on a rotating wheel, each containing 2 µg of antibody (against either IgG, RORα (Santa Cruz Biotechnology, Santa Cruz, CA), or rev-erbα (Cell Signaling Technology, Beverly, MA), or with no antibody (background control). The next day, Protein G agarose beads were added to each sample and samples were incubated on a rotating wheel at 4 °C for 1.5 hours. Beads were pelleted by brief centrifugation and the supernatants were discarded. Collected bead-protein-DNA complexes were washed (2 minutes per wash on rotating mixer at room temperature) with ChIP IP Buffer 1X, Wash Buffer 1 4X, Wash Buffer 2 1X, and Wash Buffer 3 2X. Bound protein-DNA complexes were eluted from the beads by adding ChIP Elution Buffer (containing NaHCO3 and SDS) to each sample and rotating on a rotating mixer at room temperature for 15 minutes. Elution was repeated to maximize yield. Crosslinks were then reversed and protein and RNA were digested from each sample. DNA was column purified and qPCR was run on each sample as described above. Data was normalized to a 1:10 dilution of the DNA input control (pre-cleared chromatin collected from each treatment immediately following agarose bead preclearing). The primers used in qPCR to detect ChIP enriched DNA are listed in Table 2-5. The mouse primers were designed against a putative RORE (mROR/-634) identified in the mouse StAR gene promoter using the Genomatix MatInspector software as described in the in silico analysis section above. The putative site was located between bp -619 and -641 is relative to the transcriptional start site of the gene. Each assay was repeated at least twice to verify results observed.
Transient Transfection Assays in MA-10 Cells

100,000 cells/well were plated in 24-well culture plates coated with 0.1 % gelatin (dissolved in 1X PBS) and allowed to grow for 24 hours prior to treatment with chemicals. Transfection reactions consisted of a 4:1 ratio of FuGENE HD (Roche Diagnostics, Indianapolis, IN) to plasmid DNA (2 µl FuGENE HD/ 0.5 µg total DNA per well in a 24 well plate) suspended in 25 µl media with no serum or antibiotics. All transfections were carried out for 24 hours and normalized to Renilla luciferase (ratio of the mass of LMB StAR gene promoter construct: Renilla luciferase construct used was 0.49875 µg: 0.00125 µg, or approximately 400:1). 24 hours post-transfection, cells were treated for 20 hours with either DMSO (vehicle), or with 1 or 10 µM of each OCP, followed by a 4 hour exposure to growth medium alone or growth medium supplemented with 10 U/mL hCG. Firefly and Renilla luciferase were quantified following transfections and exposures. Note: the set of experiments presented in Chapter 5 were only replicated one time due to the lack of significant response by the promoter observed in response to OCP exposure.

Luciferase Measurements

In MA-10 cells transfected and treated with various chemicals, the Dual Luciferase Kit (Promega Corp., Madison, WI, USA) was used to quantify Renilla and Firefly luciferase values. Immediately following exposure, each well was washed twice with 200 µL 1X PBS and 200 µL of 1X passive lysis buffer (Promega Corp., Madison, WI, USA) was added. Plates were placed on a shaking platform (~50 rpm) for 20-30 minutes at room temperature. Using sterile pipette tips, each well’s layer of cells was agitated and scraped to ensure all cells were lysed and detached. Following lysis, the lysates were transferred to low adhesion 1.5 mL microcentrifuge
tubes. To remove any residual gelatin or cell debris contamination, all lysates were centrifuged at 3,000 x g for 3 minutes at 4 °C. 120 µL of each lysate was transferred to new tubes.

Luciferase measurements were made using reagents from the Dual Luciferase Kit (Promega Corp., Madison, WI, USA) and a 96 well plate-reading luminometer (LMax II³⁸⁴, Molecular Devices, Sunnyvale, CA). 20 µl of each cell lysate (from each well of a 24 well plate transfected and treated) was added per well in a 96 well plate. First, Firefly luciferase was measured; 50 µL of Firefly Luciferase Reagent was injected into an individual well. Following a 10 second delay, Firefly Luciferase activity was quantified (corresponding to LMB StAR gene promoter activity), followed by the immediate addition of 50 µL of Stop & Glo Substrate, which contains reagents that quench the Firefly reaction and contains the substrate to quantify the Renilla luciferase (transfection control). The ratio of Firefly luciferase/Renilla luciferase was used to generate graphs reported in Relative Luciferase Units (RLUs).

**Green Fluorescent Protein (GFP) Imaging**

Optimization of transfections in MA-10 cells and in Y-1 cells was conducted using a green fluorescent protein construct (pEGFP, Clontech, Mountain View, CA, USA). Briefly, for MA-10 cells, Thermanox (NUNC) coverslips were placed in 24-well plates and coated with 0.1 % gelatin. Cells were transfected as described earlier and GFP was visualized using fluorescent microscopy for verification of high transfection efficiency. Representative images of these optimization experiments for Y-1 cells (Figure A-3) and for MA-10 cells (Figure A-5) can be found in Appendix A.

**Statistical Testing**

For statistical testing of all data generated in the wild LMB seasonal studies, a one-way analysis of variance (ANOVA) followed by a Tukey’s HSD post hoc pairwise multiple
comparison was performed on log-transformed expression data (JMP v7, SAS, Cary, NC, USA) to determine statistical differences between individual stages and months. All results are plotted as mean copy number/\( \mu g \) total RNA ± standard error mean (SEM) of untransformed data (n = 3-4/month per sex). LMB were also grouped according to gonad stage and results are plotted as mean copy number/\( \mu g \) total RNA ± SEM of untransformed data. Body weight, length, gonad weight, GSI, age, and expression data for each transcript throughout the year were determined to be normally distributed and Pearson multivariate correlations were performed for all variables followed by a p-value correction for multiple tests. All differences were considered significant with a p-value < 0.05.

For all qPCR experiments on LMB ex vivo gonad cultures, a one-way ANOVA followed by post hoc Dunnett’s pairwise multiple comparison were performed on log-transformed gene expression data. All analyses were performed using JMP®Genomics (SAS, Cary, NC, USA).
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Strand (5’ – 3’)</th>
<th>Reverse Strand (5’ – 3’)</th>
<th>[Final] in Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMB</td>
<td>CACCACAGAGAATGTGCCTGA</td>
<td>AATGCCGCGTGAGTGGAC</td>
<td>400 nM each</td>
</tr>
<tr>
<td>LMB AR</td>
<td>CGACGTGCTGGAACCAATGACAGA</td>
<td>ACCTCCTCTTTTAGTAGTCACCTGCCT</td>
<td>400 nM each</td>
</tr>
<tr>
<td>LMB ERα</td>
<td>GTGACCGTCTGTCCTCCACA</td>
<td>AGAGGACGTGACTGGGTCT</td>
<td>200 nM each</td>
</tr>
<tr>
<td>LMB ERβα</td>
<td>CCGACACCGCCGTGGTGGACTC</td>
<td>AACTCCGAGGGGAACGGGGCGA</td>
<td>200 nM each</td>
</tr>
<tr>
<td>LMB ERβb</td>
<td>ACCCCTCTGCTCAGGCATT T</td>
<td>GTTCTTCGTCCACCTCGGG</td>
<td>400 nM each</td>
</tr>
<tr>
<td>Segment</td>
<td>Reaction</td>
<td>Sequence (5’ – 3’)</td>
<td>Additional Comments</td>
</tr>
<tr>
<td>---------------</td>
<td>----------------</td>
<td>-------------------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>2.9 kb (proximal)</td>
<td>Primary PCR</td>
<td>CAGGCAACATCTTTACTCAGGACTTTGTC</td>
<td>See reference (90) for further info.</td>
</tr>
<tr>
<td>2.9 kb (proximal)</td>
<td>Secondary PCR</td>
<td>TCACCTTGCTTCACATAAGACATCTCT</td>
<td>See reference (90) for further info.</td>
</tr>
<tr>
<td>2.9 kb (proximal)</td>
<td>Primary PCR</td>
<td>TTCCACTCCCCCATTTTGCTCCATATT</td>
<td>See reference (90) for further info.</td>
</tr>
<tr>
<td>2.9 kb (proximal)</td>
<td>Secondary PCR</td>
<td>CAGGCAACATCTTACTCAGGACTTTGTCC</td>
<td>See reference (90) for further info.</td>
</tr>
<tr>
<td>2.6 kb (distal)</td>
<td>Primary PCR</td>
<td>TGGCGTTTATGGACCTTTGTGAAACACA</td>
<td></td>
</tr>
<tr>
<td>2.6 kb (distal)</td>
<td>Secondary PCR</td>
<td>GGGGGGAAAAACATCAGTCCTCACTCTGT</td>
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Table 2-3. List of PCR thermal cycler parameters used to clone the proximal 2.9 kb and distal 2.6 kb (total 5.6 kb) StAR gene promoter.

<table>
<thead>
<tr>
<th>Primary PCR Conditions</th>
<th>Secondary PCR Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 cycles: 94 °C/2 seconds, 72 °C/3 minutes</td>
<td>5 cycles: 94 °C/2 seconds, 72 °C/3 minutes</td>
</tr>
<tr>
<td>37 cycles: 94 °C/2 seconds, 67 °C/3 minutes</td>
<td>24 cycles: 94 °C/2 seconds, 67 °C/3 minutes</td>
</tr>
<tr>
<td>Hold: 67 °C/4 minutes</td>
<td>Hold: 67 °C/4 minutes</td>
</tr>
</tbody>
</table>
### Table 2-4. List of oligonucleotide sequences used in EMSA experiments.

<table>
<thead>
<tr>
<th>Site Encompassed</th>
<th>Species</th>
<th>Forward Strand (5’ – 3’)</th>
<th>Complementary Strand (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perfect ERE</td>
<td>Mouse</td>
<td>GTCCAAAGTCAGGTCACAGTGACCTGATCAAAGTT</td>
<td>AACTTTGATCAGGTCACTGTGACCTGACTTTGGAC</td>
</tr>
<tr>
<td>ERE/-2678</td>
<td>LMB</td>
<td>AGCGCCTTTCTAGTCTTTTTGACCACTCAAAGCGC</td>
<td>GCGCTTTGAGTGGTCAAAAAGACTAGAAAGGCGCT</td>
</tr>
<tr>
<td>Perfect RORE</td>
<td>Human</td>
<td>TCGAGTCGTATAACTAGGTCAAGCGCTGGAC</td>
<td>GTCCAGCGCTTGACCTAGTTATACGACTCGA</td>
</tr>
<tr>
<td>ROR/-1969</td>
<td>LMB</td>
<td>AATAGGCATATGACCTACTTTGGCTC</td>
<td>GAGCCAAAGTAGGTGATATGCTGCTATT</td>
</tr>
<tr>
<td>Scrambled RORE</td>
<td>LMB</td>
<td>CCTCTATAACGGGTCCGATACTATTA</td>
<td>TAATAGTATCCGACCACGTATAGAGG</td>
</tr>
<tr>
<td>Site Encompassed</td>
<td>Species</td>
<td>Forward Primer (5’ – 3’)</td>
<td>Reverse Primer (5’ – 3’)</td>
</tr>
<tr>
<td>-----------------</td>
<td>---------</td>
<td>---------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>mROR/-634</td>
<td>Mouse</td>
<td>AGAGTGGAGTTGGTATCAATGGGT</td>
<td>TGGCTCAAAGACAAACAGGATG</td>
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<tr>
<td>ROR/-1969</td>
<td>LMB</td>
<td>AGGTTTCACACCCCTTGCTAGAAA</td>
<td>TCTCTTCTGCGTAAAAGCTCTTTT</td>
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</table>
CHAPTER 3
SEASONAL EXPRESSION OF THE STEROIDOGENIC ACUTE REGULATORY PROTEIN AND SEX STEROID HORMONE RECEPTORS IN THE GONADS OF WILD LARGEMOUTH BASS¹

Introduction

Normal steroid hormone synthesis is a critical component of reproductive success in all vertebrates and for many years the signaling pathways involved in steroid hormone production have been extensively studied and well characterized in vertebrates, including multiple species of fish. The ER and AR are two of many steroid hormone receptor proteins that play critical roles in the regulation of a wide array of genes involved in steroid hormone synthesis and reproduction. Current understanding in the literature is that there are multiple isoforms of ERs (α, βa, and βb) (44, 66, 68-72) and ARs (A and B in mammals; α and β or 1 and 2 in teleosts, respectively synonymous (75, 77-82)) in fish, implicating that signaling pathways are intricate and quite complex. The StAR protein, a more recently identified protein (52), is the protein that facilitates the rate-limiting step in steroid hormone production in all vertebrates, and has only recently been characterized in teleost fishes (89, 100, 104, 106, 162-164). StAR protein transports cholesterol across the outer mitochondrial membrane where it is cleaved into pregnenolone and subsequently into other steroid hormones, and is found predominantly in reproductive tissues (116).

ERs and ARs are essential transcriptional factors that control the expression of genes involved in steroid hormone production and reproduction; StAR protein is a critical player in steroid hormone biosynthesis. ERs, ARs, and StAR protein have all been identified as targets of a number of EDCs in the environment (112, 165). EDCs are chemicals that disrupt normal

endocrine function through a number of mechanisms, including altering steroid hormone levels by disrupting the expression of different genes and proteins involved in steroidogenesis. LMB inhabit the greater part of North American freshwater systems and is highly susceptible to the negative effects of a number of EDCs (14, 67, 113, 144, 166). LMB are a commercially important game fish and are considered top predators in the food chain, making the species highly prone to bioaccumulation of contaminants and toxicants in the environment. In the Potomac River in Maryland (USA), intersex male smallmouth bass and LMB (bearing both eggs and sperm in testes) have been reported and research has linked the high occurrence of this abnormality with EDCs in the watershed (28).

The reproductive cycles of female and male teleost species have been well characterized previously (42, 43). Egg and sperm production in teleost fish is tightly controlled by physiological and environmental cues, and each distinct stage of reproductive development is characterized by the presence and variable abundance of several different types of cells in both sexes. The reproductive cycles of LMB are semi-synchronous and reproductive progression is predominantly dependent upon water temperature, photoperiod, and steroid hormone synthesis. Plasma sex steroid hormones, including 11-KT and E2, tightly control the expression of genes centrally involved in reproduction via genomic (AR- and ER-mediated) and non-genomic signaling.

Studies have shown that ER, AR, and StAR mRNA expression levels fluctuate throughout the reproductive cycles of teleost fish (44, 89, 105, 106, 167). Our laboratory has previously cloned the entire gene coding region for LMB ERα, ERβα, and ERβb (44) and StAR (90), as well as a partial region of the AR (identical between isoforms) coding region. However, reproductive stage-specific expression profiles of these genes are not known for LMB.
Interestingly, studies have shown that estrogens and androgens regulate the expression of the StAR gene (102, 114, 124), but the mechanisms underlying this regulation are currently undefined. Our laboratory has previously characterized the LMB StAR gene promoter (89) and its regulation is extremely complex. It was hypothesized that a direct interaction between an ERβ isoform with a putative element in the very complex LMB StAR gene promoter is possible and that ERs may play a role in controlling StAR gene expression. This is the first study to characterize ER, AR, and StAR mRNA levels in the gonads of a wild subset of male and female LMB. In addition, this study examines changes in StAR mRNA expression in LMB testis cultures in response to E2 and ICI 182,780 (ICI, a potent ER inhibitor) under basal and hCG-stimulated conditions to identify potential ER involvement in StAR gene transcription. A 5.6 kb portion of the LMB StAR promoter was cloned and, using computer software, multiple putative ER/AR elements were identified. In addition, in vitro binding of ERβ to a putative ER-binding element in the LMB StAR gene promoter is confirmed.

**Results**

**Gonadosomatic Indices and Water Temperature**

Both female (Figure 3-1A) and male (Figure 3-1B) LMB GSI reached maximum levels when the water temperature was between 66-71 °F in the St. John’s River in Welaka, FL. Female GSI peaked in February and March (~ 3.4-4.0% ± 0.5%); however, male GSI distinctly peaked in February (~ 0.5% ± 0.05%). During the summer months, when water temperature exceeded 80-85° F, both male and female GSI’s plummeted, indicating regression of the gonads throughout the summer season, as expected. The GSI throughout the year ranged from 0.22-3.96% for females and 0.02-0.52% for males. Data graphed in this figure included values from
all individuals whose gonads were utilized for gene expression data analysis in this study (approximately 4 individuals per month per sex).

**Staging of Gonads from Male and Female LMB Throughout the Reproductive Season**

Representative samples from gonads of fishes exhibiting each major stage of reproduction were collected for histological screening. Micrographs from female (Figure 3-2) and male (Figure 3-3) gonads exhibit distinct cellular and morphological characteristics that vary greatly throughout various reproductive stages, and the staging of all individuals in these studies was done in a similar manner described by Grier et al. (42, 43).

For females (Figure 3-2), stages are categorized as follows: perinucleolar (PN; panel A), cortical alveoli (CA; panel B), early vitellogenesis (EV; panel C), late vitellogenesis (LV; panel D), maturation (M; panel E), and atresia (AT; panel F). The PN and CA stages are characterized by the presence of primary ~100-200 μM growth follicles (PGFs), however presence of oil droplets and CA mark the CA stage. In EV, the follicles start to grow bigger, numerous oil droplets (ODs) begin to accumulate, and the germinal vesicle (GV) stains darkly. In LV, the follicle has grown to its maximum size and yolk globules (YGs) populate the follicle, where you can begin to see the development of the zona pellucida (ZP) and germinal epithelium (GE). During M, there are many different sub-stages, however, for the purpose of this study, females were classified into one group. M is characterized by the coalescence of all oil droplets into an oil globule (OG), GV migration, and ovulation. AT is the stage at which follicles of all stages are resorbed and the cycle restarts.

For males (Figure 3-3), reproductive stages were classified into four categories, including the α stage (marked by the predominance of spermatagonia (SG; panel A)), the β stage (at which spermatocytes (SC; panel B) become present), and the γε and γλ stages, at which spermatozoa
(SZ) and spermatids (ST) are present (panels C and D). In the γε stage, SZ composed < 50% of the area view, whereas in the γλ stage, SZ composed > 50% of the area.

In both sexes of LMB, different stages are represented by a number of different cell types that are differentially involved in the reproductive cycle.

**StAR, AR, and ER mRNA Expression in LMB Ovary and Testes by Month**

In the LMB ovary (Figure 3-4), StAR (3-4A); d.f. = 11; F = 8.31; p < 0.0001, ERβa (3-4D); d.f. = 11; F = 2.50; p < 0.05, and ERβb (3-4E); d.f. = 11; F = 2.63; p < 0.05) transcripts varied significantly by month, whereas AR (3-4B) and ERα (3-4C) did not vary significantly throughout the year. In the LMB testes (Figure 3-5), StAR (3-5A); d.f. = 11; F = 2.67; p < 0.05, AR (3-5B); d.f. = 11; F = 5.12; p < 0.0001, ERα (3-5C); d.f. = 11; F = 2.80; p < 0.05, ERβa (3-5D); d.f. = 11; F = 5.71; p < 0.0001, and ERβb (3-5E); d.f. = 11; F = 6.67; p < 0.0001) all varied significantly throughout the year. Notably, StAR transcript levels were lower in the females than in the males, and there was, on average, 10-fold less copies of StAR mRNA in both sexes when compared to the other genes quantified. StAR mRNA levels peaked significantly in February in the females, whereas StAR didn’t peak during any month in the males, although a trend for higher expression occurred January through March. Throughout the entire year, StAR mRNA levels in the testes were at least 2-fold more abundant than levels observed in the ovary. Interestingly, AR mRNA was more abundant in the ovary than in the testes. ERα mRNA didn’t vary significantly throughout the year in the ovary; however, in the testes ERα peaked in August and decreased in September. ERβα mRNA levels in the ovary peaked in October and diminished throughout the rest of the months until gradually increasing in August-September. In the males, ERβα transcript levels were consistently much lower than the levels observed in the females. In the females, ERβb mRNA peaked in October and decreased throughout the year until gradually
increasing in August through September. \( ER\beta b \) mRNA levels in the males remained fairly consistent from October-March, increased significantly in August, and returned to the levels observed in earlier months until transcript levels diminished in September. \( ER\beta b \) transcript levels peaked at higher levels throughout the year in the testes than the values observed in the ovary. Altogether, several of the genes quantified varied significantly throughout the year in both sexes.

**StAR, AR, and ER mRNA Expression in LMB Ovary and Testes by Reproductive Stage**

Based upon reproductive stage in the females (Figure 3-6), \( StAR \) ((3-6A); d.f. = 5; \( F = 18.65; p < 0.0001 \)), \( ER\alpha a \) ((3-6D); d.f. = 5; \( F = 7.39; p < 0.0001 \)), and \( ER\beta b \) ((3-6E); d.f. = 5; \( F = 4.54; p < 0.01 \)) mRNA levels varied significantly throughout the different stages, while \( AR \) (3-6B) and \( ER\alpha (3-6C) \) mRNA levels did not vary significantly. There was a trend in all \( ER \) isoforms to be highest expressed during earlier stages and less expressed during vitellogenesis and maturation. In the ovary, \( StAR \) mRNA was very minimally expressed through the perinucleolar, cortical alveoli, and early vitellogenic stages. \( StAR \) mRNA increased gradually through the late vitellogenic stage and peaked at maturation. As expected, \( StAR \) mRNA decreased when follicles were undergoing atresia and the spawning season was over. During the perinucleolar and cortical alveoli stages, \( ER\alpha a \) mRNA levels were approximately 3-fold greater than \( ER\beta b \) mRNA levels. During maturation and atresia, the levels of both \( ER\beta \) transcripts were relatively even.

In the testes of the males (Figure 3-7), \( StAR \) ((3-7A); d.f. = 3; \( F = 9.84; p < 0.0001 \)), \( AR \) ((3-7B); d.f. = 3; \( F = 14.97; p < 0.0001 \)), \( ER\alpha a \) ((3-7D); d.f. = 3; \( F = 5.21; p < 0.01 \)), and \( ER\beta b \) ((3-7E); d.f. = 3; \( F = 6.30; p < 0.01 \)) varied significantly through different reproductive stages, while \( ER\alpha (3-7C) \) did not significantly vary. Again, there was a trend for all \( ER \) isoforms to be
expressed during earlier reproductive stages and to taper off during the later stages of spermatogenesis and reproduction. *StAR* transcript levels did not change during the α and β stages, whereas there was an approximately 3-fold increase in the γε and γλ stages. *AR, ERβα*, and *ERββ* mRNA levels in the males were significantly decreased during the β stage when compared to the rest of the stages.

**Ex Vivo Exposure of LMB Testis to E2 and ICI 182,780 Alters StAR mRNA Expression**

To investigate the potential for ER involvement in regulating *StAR* mRNA expression, 15 – 25 mg pieces of testis tissue were cultured *ex vivo* from four individual male LMB and treated with vehicle, E2, or ICI, under basal and hCG-stimulated conditions. Cultures were pre-treated for 2 hours with vehicle, 100 nM E2, or 10 µM ICI, followed by supplementation with fresh medium containing the same doses of vehicle, ICI, or E2, alone or in combination with hCG ([final] = 1 U/mL). Cultures were incubated for 6 and 20 hours and *StAR* mRNA levels were quantified by qPCR (Figure 3-8, A and B, respectively). 6 hour exposures were carried out to examine the acute response of the chemicals, whereas the 20 hour exposures were conducted to characterize the longer-term, more genomic responses. 6 hour exposure to hCG revealed a 2-fold induction of *StAR* mRNA levels and E2 slightly stimulated *StAR* mRNA levels above levels observed in the vehicle controls under basal conditions only. 6 hour E2 and ICI exposure did not alter *StAR* mRNA expression under hCG-stimulated conditions. As expected, the induction of *StAR* mRNA by hCG exposure was diminished after 20 hours. Interestingly, concomitant exposure of ICI with hCG for 20 hours induced *StAR* mRNA levels greater than 2-fold relative to control levels. Altogether, these experiments examined the testis-specific changes in *StAR* mRNA expression upon exposure to chemicals known to impact ER signaling, and changes in
StAR mRNA expression indicate that ER pathways may be involved in controlling transcription of the StAR gene in LMB.

**In Silico Analysis of the LMB StAR Gene Promoter Reveals Putative ER responsive transcriptional elements**

Using computer software designed to analyze DNA sequences for putative transcriptional elements, multiple putative estrogen responsive elements (EREs) were identified in the 5.6 kb LMB StAR gene promoter. With greater than 80% homology to consensus vertebrate core element sequences, 3 ER binding elements and 8 AP-1 elements were identified in the StAR gene promoter, raising the possibility that the StAR promoter may be regulated by ER signaling.

**EMSA Analysis of ERE/-2678 in the LMB StAR Promoter**

The presence of bands seen by EMSA analysis using probes designed against the ERE/-2678 element in the LMB StAR promoter (putative ERE located at -2678 bp from the start of the coding sequence for the gene; Table 2-4) and nuclear fractions isolated from MA-10 mouse Leydig tumor cells under basal and E2-treated conditions suggests that a protein binds to the ERE/-2678 in the LMB StAR promoter (Figure 3-9). Probes designed against a perfect mouse ERE were used as a positive control. A distinct band is present in lanes where ERE/-2678 is represented; the band intensifies when bound to E2-treated nuclear extracts. When a cold unlabeled probe was added in excess of the labeled ERE/-2678 probe, the band disappeared, confirming specificity of the probe. Addition of an ERβ antibody to the binding reaction (last lane) displaced the specific band, suggesting that ERβ is capable of binding the ERE/-2678 element in the LMB StAR promoter.

**Discussion**

This study investigated the seasonal expression of ER, AR, and StAR transcripts throughout the reproductive season by month and by reproductive stage in female and male LMB, and
searched for possible relationships between the expression of these genes and other physiological parameters. The distal portion of the 5.6 kb LMB StAR gene promoter was cloned and multiple putative EREs were identified in the DNA sequence. As confirmed by EMSA, mammalian ERβ is capable of binding to the ERE/-2678 element in the LMB StAR promoter in vitro.

The reproductive cycle of LMB is complex and is controlled by many environmental and physiological cues, so gene expression was analyzed by both month and by reproductive stage. LMB in Florida typically reproduce in late spring; reproduction is greatly dependent upon water temperature. Throughout the summer months when water temperatures are high, female LMB reabsorb follicles (atresia) and male LMB testes regress, stopping spermatogenesis. It was observed that gene expression analyzed from later reproductive stages in both female and male LMB was very similar to the expression levels observed in February and March (Spring). Grouping the individuals by reproductive stage distinguished trends observed in gene expression by month into significant observations dependent upon gonad stage, therefore all subsequent discussion will focus on stage-specific gene expression.

In both female and male LMB, the expression profiles of all ER isoforms were highly correlated with one another throughout the various stages of each reproductive cycle (Tables 3-1 and 3-2, respectively); these data parallel previous work conducted using pond-reared LMB (44). In the ovary, the ERβ isoforms were highly expressed during the early stages of reproduction and minimally expressed during the late vitellogenic and maturation stages. In male LMB, expression of the ERβa and ERβb remained fairly steady throughout each stage, except during the β reproductive stage when spermatocytes were predominantly present and spermatids and spermatozoa were not. Few studies have been conducted characterizing ER expression in the teleost testes, however, a recent study in sea bass by Vinas and Piferrer examined ER mRNA
expression in specific testicular cell types using laser microdissection (spermatogonia, spermatocytes, spermatids, spermatozoa) by stage, and all ER isoforms decreased throughout spermatogenesis (168). In LMB, spermatogenesis proceeds in a cystic mode; specifically, as spermatogenesis advances through late reproduction, the cells populating the testes and their ratios vary greatly (169), and our data encompass all cell types present in the testis at a given stage. When spermatids and spermatozoa are present (late stages), it is apparent that the area occupied by different cell types varies greatly upon examination of histological micrographs of the LMB testes (Figure 3-3). Altogether, our data suggest that all ER isoforms are likely to play unique and complex roles in regulating genes involved in female and male reproductive progression and cycling.

In this study, AR expression was analyzed in the gonads of wild female and male LMB. Multiple studies have indicated that there are two isoforms of ARs in teleosts, though the functionality, ligand binding properties, and tissue distribution of both isoforms vary greatly between individual species; however, both isoforms of AR receptors have been detected in the gonads in multiple fish species (76-78, 80, 81). While LMB are likely to contain two isoforms of AR as shown for other fish, so far, only a fragment of one isoform which is in a region that is highly homologous among all AR isoforms has been cloned. In rainbow trout, it was reported that AR-A is more highly expressed in the ovaries whereas AR-B is a more testes-specific isoform (80). The potential for differential regulation of genes by different AR isoforms is likely and it would be interesting to further investigate this in LMB. Overall, the roles that ARs play in controlling genes in the LMB testes and ovary are intricate, and further research is warranted to better understand complex AR signaling pathways.
In the LMB ovary, \( AR \) mRNA was highly abundant (> 2 x 10^6 copies/µg total RNA throughout all stages), however, transcripts did not vary significantly between stages or by month in the females. In the LMB testes, \( AR \) mRNA was notably less abundant (> 2X less copies than observed in females throughout all stages). \( AR \) transcripts were significantly less abundant during the \( \beta \) stage, when spermatocytes were present and spermatids and spermatozoa were not, as observed with \( ER{\beta}a \) and \( ER{\beta}b \) mRNA. Again, this could be due, in part, to the different populations of different cell types, as discussed earlier regarding \( ER \) mRNA levels in the testis.

\( StAR \) mRNA abundance in the LMB ovary and testes peaked markedly during late reproductive stages (late vitellogenesis and maturation in female and \( \gamma{\varepsilon} \) and \( \gamma{\lambda} \) (spermiogenic stages) in the testes), as expected. Gonadal \( StAR \) expression has been characterized throughout the reproductive stages of multiple fish species (97, 106, 167), and all studies report an increase in \( StAR \) expression during the later reproductive stages in both sexes, as observed in our study. \( StAR \) mRNA was less abundant in the ovary than in the testes. In the females, \( StAR \) increased nearly 3-fold during early vitellogenesis and 9-fold during maturation from levels observed during the other reproductive stages. Notably, there is a significant peak in February when \( StAR \) expression is plotted by month, giving strong indication that at least one round of spawning likely occurred in late February or early March, given that \( StAR \) levels are the highest during maturation. In the males, \( StAR \) increased > 2.5-fold during spermiation from the levels seen when no spermatids or spermatozoa were present. The acute upregulation of \( StAR \) mRNA during the beginning stages of reproduction reinforces the idea that the StAR protein plays a critical role in steroid hormone synthesis and reproduction in LMB. \( StAR \) mRNA levels increase dramatically in the later stages of reproduction as well, indicating that the expression of \( StAR \)
mRNA must increase several fold in order to accommodate the surge of sex steroid hormones required for spermiation and ovulation.

Physiological parameters (body length, gonad weight, GSI, and age) within each sex were predominantly positively correlated with one another. All ER isoforms and AR mRNA levels were significantly positively correlated with one another in both sexes as well. In the ovary, ER\textsubscript{βa} and ER\textsubscript{βb} were negatively correlated with StAR expression, whereas in the testis, ER\textsubscript{βa} and AR were positively correlated with StAR expression. Studies have shown that E2 and T exposure causes a decrease in StAR gene expression (102, 114, 124), and it is possible that ER and AR signaling may mediate the reported repression.

To investigate the potential for ER regulation of LMB StAR mRNA, LMB testis tissue was treated with E2 and ICI 182,780 under basal and hCG-stimulated conditions. Treatment for 6 hours with hCG elicited a 2-fold induction of StAR mRNA and treatment with E2 slightly stimulated StAR mRNA levels under basal conditions. Interestingly, 20 hour exposure of testis tissue to hCG in combination with ICI 182,780 was the only treatment that stimulated StAR mRNA levels. Although ICI 182,780 has been characterized as an ER antagonist, recent studies have shown that the compound can actually act as a selective ER modulator and agonize estrogenic pathways by activating AP-1/Sp1 signaling (170). The membrane-bound G protein-coupled receptor 30, a potential membrane-bound ER, is activated by ICI 182,780 and other ER antagonists (171). In addition, it has been reported that ER\textsubscript{β} has the potential to activate the promoter for retinoic acid receptor by binding Sp1 sites when bound to ICI 182,780 and other antagonists, but not when bound to E2 (172). The slight stimulation of StAR mRNA upon treatment with E2 and the interesting results obtained with ICI 182,780 and hCG indicate
potential for ER regulation of the StAR gene promoter, either by nuclear ERs or by pathways controlled by membrane-bound ERs.

Our laboratory previously cloned a 2.9 kb segment of the LMB StAR promoter (89) and an additional 2.6 kb of the distal region of the promoter was cloned in this study. Upon examination of the promoter for putative ER, Sp1, and AP-1 sites, several putative elements that may be involved in mediating the effects observed in our experiments were identified using MatInspector TF Search software. No Sp1 sites were identified, but several putative AP-1 and ER sites were identified, including one located -2678 bp upstream from the transcriptional start site (ERE/-2678). The ERE/-2678 site exhibited the highest homology with the core mammalian ERE sequence (~87%). Interestingly, upon examination of the StAR promoter sequences from brook trout and multiple other vertebrates, only the fish promoters contained a putative canonical ER binding site. Because ERβ transcript levels were highly correlated with StAR transcript levels in both male and female LMB gonads and the putative ERE located in the LMB StAR promoter was most homologous to the core mammalian ERE sequence, the potential for ERβ to bind to the ERE/-2678 site was analyzed by EMSA/supershift. The presence of multiple bands indicated that proteins and/or protein complexes can, indeed, bind to the ERE/-2678 site \textit{in vitro}. When an antibody against ERβ was added to the reaction, one of the bands was completely displaced, implicating that ERβ is capable of binding to the element. The antibody selected was designed against a peptide that spanned into the DNA binding domain of ERβ, so displacement of the band verifies specificity of ERβ for the ERE/-2678 probe. The presence of putative AP-1 sites and other putative ER sites in combination with the results observed in our \textit{ex vivo} experiments implicate that ERs may interact with a number of different elements in the StAR promoter; however, the binding of ERβ to the ERE/-2678 element in the LMB StAR promoter \textit{in}}
vitro is supportive of the idea that ERs can directly bind to the StAR gene promoter and control its activation, though further functional studies are warranted.

In summary, this study describes transcript levels for genes important for reproduction in the gonad of wild female and male LMB throughout the year, and characterizes the seasonal and stage-specific differences between the mRNA expression of the ER isoforms, AR, and StAR. Our in vitro work suggests that ERs may play a role in regulating the LMB StAR gene promoter; however, further research investigating the functionality of the putative regulation is warranted. Altogether, this study provides novel information regarding the relationship between ER/AR and StAR mRNA expression in teleost fish and implicates that reproductive signaling pathways in LMB are extremely complex.
Table 3-1: Pearson correlations for seasonal transcript levels in the gonad of female LMB. (p<0.05*, p<0.01**, p<0.001***)

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<th>GSI</th>
<th>Age</th>
<th>StAR</th>
<th>ERα</th>
<th>ERβα</th>
<th>ERβb</th>
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Table 3-2: Pearson correlations for seasonal transcript levels in the gonad of male LMB. (p<0.05*, p<0.01**, p<0.001***)

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Figure 3-1. Variations in gonadosomal indices of wild LMB and water temperature in Welaka, FL. A) Female LMB and B) male LMB. Gonadosomal indices are represented by the black lines and water temperatures are represented by the gray lines. Each point (GSI) is representative of the mean GSI ± SEM (n=3-4 individuals per month).
Figure 3-2: Representative histological micrographs of female LMB ovarian stages. Female stages were classified based on the predominant stage and are 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 μm. 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).
Figure 3-3: Representative histological micrographs of male LMB testicular stages. Male stages were classified based on the predominant stage and are as follows; A) spermatagonia (SG); B) spermatocytes (SC); C) spermatids (SD) and <50% spermatozoa (SZ); and D) SD and >50% SZ. Scale bars correspond to 50 μm.
Figure 3-4: Monthly *StAR*, *AR*, and *ER* mRNA expression in the female LMB gonad as determined by qPCR. Each bar represents the mean copy number ± SEM of untransformed data followed by ANOVA with a Tukey’s post hoc test (n=3-4 individuals/month). Graphs are as follows; A) *StAR* mRNA; B) *AR* mRNA; C) *ERα* mRNA; D) *ERβα* mRNA; and E) *ERββ* mRNA. All mRNA expression data were normalized to ribosomal 18S rRNA copy number. Different letters indicate statistical differences among groups (P <0.05).
Figure 3-5: Monthly steroidogenic \textit{StAR}, \textit{AR}, and \textit{ER} mRNA expression in the male LMB gonad as determined by qPCR. Each bar represents the mean copy number ± SEM of untransformed data followed by ANOVA with a Tukey’s post hoc test (n=3-4 individuals/month). Graphs are as follows; A) \textit{StAR} mRNA; B) \textit{AR} mRNA; C) \textit{ER}\alpha mRNA; D) \textit{ER}\beta\alpha mRNA; and E) \textit{ER}\beta\beta mRNA. All mRNA expression data were normalized to ribosomal \textit{18S} rRNA copy number. Different letters indicate statistical differences among groups (P < 0.05).
Figure 3-6: Stage-specific StAR, AR, and ER mRNA expression in female LMB gonad as determined by qPCR. Each bar represents the mean copy number ± SEM of untransformed data followed by ANOVA with a Tukey’s post hoc test (n=3-9 individuals/month). Graphs are as follows; A) StAR mRNA; B) AR mRNA; C) ERα mRNA; D) ERβα mRNA; and E) ERββ mRNA. All mRNA expression data was normalized to ribosomal 18S rRNA copy number. Different letters indicate statistical differences among groups (P < 0.05).
Figure 3-7: Stage-specific StAR, AR, and ER mRNA expression in male LMB gonad as determined by qPCR. Each bar represents the mean copy number ± SEM of untransformed data followed by ANOVA with a Tukey’s post hoc test (n=7-17 individuals/month). Graphs are as follows; A) StAR mRNA; B) AR mRNA; C) ERα mRNA; D) ERβα mRNA; and E) ERββ mRNA. All mRNA expression data was normalized to ribosomal 18S rRNA copy number. Different letters indicate statistical differences among groups (P <0.05).
Figure 3-8: Changes in testicular StAR mRNA expression upon exposure to vehicle, ICI 182,780 or E2 under basal and hCG-stimulated conditions for (A) 6 hours and (B) 20 hours. 15-25 mg slices of testis tissue were cultured in L-15 medium in duplicate in a 24 well plate and pre-exposed to 0.1% vehicle, 10 µM ICI 182,780, or 100 nM E2 for 2 hours, followed by a change in medium containing the same treatment, under both basal and hCG-stimulated conditions ([final] = 1 U/mL) for 6 hours (A) and 20 hours (B). RNA was isolated from the tissue and reverse transcribed for analysis by qPCR. All data was normalized to 18S rRNA. Each bar represents mean copy number ± SEM collected from two individual males (cultured in duplicate).
Figure 3-9: ERβ from MA-10 mouse Leydig cells binds to ERE/-2678 in the LMB StAR gene promoter in vitro. Probes designed against the ER element located -2678 bp upstream from the start of the gene coding sequence (ERE/-2678) were combined with nuclear extracts isolated from MA-10 mouse Leydig tumor cells isolated from either basal (arrow A) or E2 treated conditions (arrow B). Non-biotinylated probes (cold) were used to confer specificity of DNA-protein interaction. An ERβ antibody was added in the last lane (arrow C) to determine which isoform of ER bound the LMB ERE/-2678 element in the LMB StAR promoter in vitro.

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Figure 3-9: ERβ from MA-10 mouse Leydig cells binds to ERE/-2678 in the LMB StAR gene promoter in vitro. Probes designed against the ER element located -2678 bp upstream from the start of the gene coding sequence (ERE/-2678) were combined with nuclear extracts isolated from MA-10 mouse Leydig tumor cells isolated from either basal (arrow A) or E2 treated conditions (arrow B). Non-biotinylated probes (cold) were used to confer specificity of DNA-protein interaction. An ERβ antibody was added in the last lane (arrow C) to determine which isoform of ER bound the LMB ERE/-2678 element in the LMB StAR promoter in vitro.
CHAPTER 4
REGULATION OF THE STEROIDOGENIC ACUTE REGULATORY PROTEIN BY ORPHAN NUCLEAR RECEPTOR SIGNALING PATHWAYS IN LARGEMOUTH BASS

Introduction

Identification of the StAR protein in 1994 significantly advanced the field of cholesterol metabolism (52). It has now been well characterized in multiple mammalian species that StAR transports cholesterol across the mitochondrial membrane and controls the rate-limiting step for steroidogenesis (56, 92). Regulation of steroidogenesis occurs in a tissue-specific manner and involves multiple signaling pathways, including PKA and PKC, amongst others, and this appears to be conserved across most vertebrate species (57, 92, 109, 173, 174). It is known that ACTH, an upstream regulator of the cAMP response, induces StAR mRNA expression in rainbow trout and in eel (175-177). Binding elements for transcription factors known to mediate cAMP responses, such as SF-1 and CREB, have been identified in the mammalian StAR gene promoter, however the promoter is very complex with many transcriptional elements for which the functions are still unknown (178). Although genome sequencing and expressed sequence tag (EST) projects are underway for many fish species, including LMB, there are no previous publications citing in silico or functional promoter analysis of the StAR gene in any fish model.

Mammals and lower vertebrates are likely to exhibit similar transcriptional regulatory mechanisms for the StAR gene. It is well established that the StAR gene is highly regulated by the cAMP/PKA pathway across multiple species and that this pathway is important in reproduction. The transcriptional mechanisms controlling this gene in lower vertebrate animals

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Kocerha RJ*, Prucha MS*, Kroll KJ, Steinhilber D, and Denslow ND. 2009. Regulation of the Steroidogenic Acute Regulatory (StAR) Protein by Orphan Nuclear Receptor Signaling Pathways in Largemouth Bass (*Micropterus salmoides*). Submitted to *Endocrinology*, under review. *Both authors contributed equally to the preparation of this manuscript.
such as in fish have not been investigated and could provide much needed insight into the complex networks involved in the regulation of steroidogenesis.

Circadian rhythm plays an important role in reproduction in vertebrates, and it exists centrally, in peripheral tissues, and even within individual cells (179). Control of gene expression at the cellular level is important in regulating reproductive processes, including steroid hormone production. It is known that RORα and rev-erbα are two signaling proteins that play integral roles in controlling genes central to the circadian cascade (85-87). RORα and rev-erbα both bind to similar core sequences (ROR element – RORE), however they induce opposing effects on the transcription of target genes (61).

The aims of this study were to clone and characterize the LMB StAR gene and promoter at the tissue and cellular levels, respectively. We also attempted to identify and characterize elements in the LMB StAR promoter that are putatively involved in the transcriptional activation of the LMB StAR promoter. Our results show that RORα and rev-erbα are both capable of binding a core sequence in the LMB StAR promoter and interestingly, that rev-erbα binds to a core sequence in the mouse StAR promoter. Altogether, our study presents a novel mechanism through which the StAR promoter is controlled.

Results

Previous Studies Reveal Multiple Putative Transcriptional Elements may be Involved in Mediating cAMP-Induced Activity of the StAR Promoter (89, 90)

Dr. Jannet Kocerha, a former graduate student that completed her Ph.D. in our laboratory, cloned the coding region for the LMB StAR gene and optimized a qPCR assay using primers designed against the LMB StAR coding region. It was shown that ex vivo treatment of LMB ovarian follicles with 1 mM dbcAMP stimulated StAR mRNA levels nearly 10-fold above basal levels. In addition, it was also reported that StAR mRNA levels vary in the ovaries of female
LMB throughout reproduction, paralleling variations in plasma E2 levels observed in the same individuals (44).

To investigate the transcriptional regulation of the LMB StAR gene, a 2.9 kb segment of the LMB StAR gene promoter was previously cloned. Using MatInspector TF Search software, in silico analysis of the 2.9 kb LMB StAR promoter for transcriptional elements revealed a number of putative sites located throughout the promoter sequence, including elements for 2 SF-1, 6 GATA, 1 ER, 3 RORα/rev-erbα, 1 YY-1, and 1 SREBP (Figure 4-1). Promoter sequences (including the 5’ UTR) for the StAR gene from LMB (Acc. No. DQ166819), brook trout (Acc. No. AY308064), rat (Acc. No. AB006007), mouse (180), and human (Acc. No. U29098) were aligned based on the transcriptional start sites for the gene. The sites selected for mapping had ≥80% homology to the core mammalian transcription factor sequence.

Using transient transfection assays in Y-1 mouse adrenocortical cells, Dr. Kocerha showed that the 2.9 kb LMB StAR promoter was dose-responsive to dbcAMP and that there were several putative elements in the 5’ distal segment of the promoter that may be involved in regulating this response. To investigate the potential role that the distal 1 kb of the LMB StAR promoter might play in dbcAMP-induced activity of the promoter, Dr. Kocerha generated a 1.86 kb promoter construct that lacked the 5’ distal region of the promoter and examined its response to dbcAMP in Y-1 cells. It was observed that the deletion of the 5’ distal region of the promoter segment significantly impaired induction of promoter activity nearly 80% of that observed using the 2.9 kb promoter construct, implicating that the distal 1 kb may be important in mediating dbcAMP response.

Dr. Kocerha generated point mutation constructs to investigate the potential roles that some of the putative elements may play in regulating the dbcAMP-induced activity of the LMB
StAR promoter. Mutation of a putative RORE located in the distal segment of the promoter, ROR/-1969, yielded a similar impairment of dbcAMP-induced activity when the construct was transfected in Y-1 cells. Alteration of the ROR/-1969 site reduced the dbcAMP induction by approximately 80%. The data reported by Dr. Kocerha implicated that the putative ROR/-1969 element could be involved in mediating the LMB StAR promoter response to dbcAMP in Y-1 cells.

**ChIP Verification of RORα/rev-erbα Proteins binding to the ROR/-1969 Element in the LMB StAR Promoter and to the ROR/-634 in the Murine StAR Promoter.**

To verify that RORα and rev-erbα bind to the ROR/-1969 element in the LMB StAR promoter, ChIP assays were run with Y-1 cells transfected with the StAR promoter and cultured under both basal and dbcAMP-induced conditions. The transfected cells were fixed with formaldehyde and chromatin was prepared as per protocol. After immunoprecipitation with a polyclonal antibody against either mouse IgG (non-specific control), RORα, or rev-erbα, DNA was purified and qPCR was run on each sample using primers encompassing either the ROR/-1969 element in the LMB StAR promoter (Figure 4-2A) or the mROR/-634 element in the murine StAR promoter (Figure 4-2B). Note: asterisks in Figure 6B indicate that DNA was below detection limits.

In both species, the DNA encompassing each of the ROREs was highly enriched under basal conditions when pulled down with the antibody for rev-erbα, implicating that rev-erbα binds to the LMB ROR/-1969 element and to the mROR/-634 element in the StAR promoter of each species under basal conditions. Upon treatment with dbcAMP, the enrichment of DNA bound to rev-erbα observed under basal conditions diminished to non-specific levels observed with the IgG antibody with both the LMB and mouse elements. Concomitantly with this decrease, a slight increase was seen for the LMB element when the RORα antibody was used,
whereas there was no detectable enrichment above the non-specific IgG control with the mROR/-634 element.

**EMSA Analysis of RORα4 Binding to the ROR/-1969 Element**

In order to assess whether recombinant RORα4 protein could bind to the ROR/-1969 transcriptional site in the LMB *StAR* promoter, an EMSA was performed using the specific probes for this promoter site and recombinant human RORα4 protein (Fig 4-3). The recombinant protein bound to the ROR/-1969 probe, producing a single band. Addition of both the RORα antibody and the cold unlabeled probe verified the specificity of the ROR/-1969-RORα4 interaction. Further verification of specificity was exhibited by the use of a scrambled probe, which did not bind the protein. A perfect human RORE probe was run as a positive control, yielding a band that ran at the same size as that seen with ROR/-1969.

**EMSA analysis of ROR/-1969 Activity in Y-1 Adrenocortical Cells**

To further verify the activity of the ROR/-1969 site in the LMB *StAR* promoter, protein-DNA interactions were examined by EMSA using Y-1 nuclear extracts obtained from both basal and dbcAMP-induced cells and a probe encompassing the ROR/-1969 site in the LMB *StAR* promoter (Fig 4-4). A similar banding pattern was observed between the basal versus dbcAMP-induced nuclear fractions, however, one band appeared more prominent (denoted by the arrow) under basal conditions when compared to dbcAMP-induced conditions. The presence of multiple bands indicates that the ROR/-1969 site is capable of binding proteins found in Y-1 cells. The addition of a cold unlabeled probe quenched the bands seen with ROR/-1969, verifying that proteins/complexes are capable of binding specifically to the site in the LMB *StAR* promoter.
Discussion

We report the first in-depth study on transcriptional regulation of the *StAR* gene in a fish model, LMB. The *StAR* cDNA and a large portion of its promoter were cloned to study its regulation *in vivo* and *ex vivo* in LMB ovarian follicle cultures, and then, ultimately, for more comprehensive examination of regulation of *StAR* transcription with transfection assays. Our data show that LMB *StAR* is transcriptionally regulated by dbcAMP and that rev-erbα and RORα play critical roles in the activation of the LMB *StAR* promoter.

*In vivo* and *ex vivo* examination of LMB *StAR* established a functional correlation of *StAR* mRNA expression with steroidogenesis in LMB. An increase in *StAR* mRNA levels *in vivo* paralleled the levels of E2 detected in the plasma, suggesting that the connection between *StAR* mRNA synthesis and the biosynthesis of steroids from cholesterol occurs in LMB as it does in mammals. The regulation and metabolism of steroid hormones is very complex in all vertebrates and, although we associate an increase in *StAR* mRNA abundance with increased plasma E2, the activities of other key enzymes, such as P450 aromatase and enzymes involved in Phase II metabolism in the liver are important factors that control plasma levels of steroid hormones. The upregulation of LMB *StAR* mRNA by dbcAMP in *ex vivo* cultures of LMB ovarian follicles implicates that important signaling mechanisms which transactivate *StAR* may be conserved across species.

To identify the transcriptional elements involved in the regulation of *StAR* transcription in lower vertebrates, a 2.9 kb portion of the LMB *StAR* promoter was cloned for sequence analysis and used in transfection assays. *In silico* analysis revealed multiple putative transcriptional elements that appeared to be conserved in the promoters of fish and mammals, although the specific positions in the sequence didn’t always correspond. The professional web-based
programs identified various potential response elements in the LMB \textit{StAR} promoter, including sites for 2 SF-1, 6 GATA, 1 ERE, 3 ROR\(\alpha/\text{rev-erb}\alpha\), 1 YY-1, and 1 SREBP (Fig 4-1), all with >80% homology with core sequences for mammalian transcription factors. Other sequences that predict binding by CRE and AP1 transcription factors as shown in the mammalian \textit{StAR} promoter were predicted but with less than 80% homology and these are not included in the figure. The putative elements plotted in Figure 4-1 aren’t necessarily functional elements involved in the regulation of the StAR promoter; determination of their functionality will require further experimentation.

Critical elements involved in dbcAMP-regulation of LMB \textit{StAR} transcription are located in the distal portion of the promoter. The 2.9 kb LMB \textit{StAR} promoter was induced >2-fold in response to 1 mM dbcAMP, which mirrored results published for the human \textit{StAR} promoter in Y-1 cells (129). However, deletion of 1 kb from the 5’ end of the LMB 2.9 kb promoter significantly diminished the induction by dbcAMP. There are putative AP-1, SF-1, ROR/\text{rev-erb}, and ERE sites as well as others within this region, which could be critically involved in regulating \textit{StAR} transcription. SF-1 and AP-1 sites have been well studied and their roles in cAMP activation have been well defined (129, 137, 181). We focused our investigation on characterizing the potential roles that ROR\(\alpha\) and \text{rev-erb}\(\alpha\) could play in regulating transcriptional activity of the \textit{StAR} promoter.

Site-directed mutation of the putative ROR/\text{-1969} element (located between the -1.86 and -2.9 kb region of the LMB \textit{StAR} promoter) diminished the response of the promoter to dbcAMP, suggesting potentially new signaling mechanisms for dbcAMP regulation of \textit{StAR} transcription.

Direct roles for ROR\(\alpha\) and \text{rev-erb}\(\alpha\) in steroidogenesis have not previously been reported; therefore, we pursued a functional analysis of their regulation and binding interaction with the
LMB *StAR* promoter. RORα is a nuclear factor involved in the transcriptional activation of genes involved in multiple physiological processes, including those central to controlling peripheral circadian rhythm (182). There are four isoforms of RORα that arise from alternative promoter usage and alternative splicing. RORα1 and 4 are ubiquitously expressed. It is notable that cholesterol has been identified as a ligand for RORα in recent studies (88, 183), suggesting that it may be a key player in steroid production. In addition, rev-erbα has been reported to competitively bind the same element as RORα, disallowing the activation of target genes by RORα (184). The possibility that RORα and rev-erbα regulate steroidogenesis is strongly supported by the presence of the element at position -1969 in the LMB *StAR* promoter. Indeed, bioinformatic analysis also revealed high-affinity sites for RORα in several mammalian species, including human, rat, and mouse.

Site-specific mutation of the putative RORα/rev-erbα element in the LMB promoter repressed the dbcAMP activation in transfection assays by greater than 80%. Noting that RORα and rev-erbα bind to the same core sequence, mutation of the ROR/-1969 site in the LMB *StAR* promoter disallowed the binding of either protein under basal and dbcAMP-stimulated conditions. This may account for the loss of response to dbcAMP observed upon mutation, especially if RORα’s presence mediates the activation of the promoter induced by dbcAMP.

Additionally, endogenous binding of RORα and rev-erbα to the LMB ROR/-1969 element and of rev-erbα to the mouse mROR/-634 element were verified by ChIP, implicating that these proteins play significant roles in controlling the transcriptional activation of the promoters in both species. The mouse *StAR* promoter has been very well characterized (185) and it has been reported that negative regulatory elements may lie between base pairs -966 and -254 relative to the transcriptional start site (180). Our studies have shown that rev-erbα binds to the mouse
ROR/-634 element (located approximately -634 bp upstream of the transcriptional start site) under basal conditions and that the protein does not associate with the element under dbcAMP-stimulated conditions, signifying that rev-erbα may play an important role in the basal control of the promoters in LMB and mice. Interestingly, RORα is involved in the control of circadian rhythms and these experiments implicate a causal link between circadian clock and control of steroidogenesis through RORα/rev-erbα signaling pathways.

Because the enrichment of RORα in the ChIP studies on the LMB ROR/-1969 element was not as profound when compared to the results with rev-erbα, we chose to conduct EMSA experiments to further investigate the putative role of RORα in regulating promoter activity. We verified that human RORα4 protein is capable of binding to the LMB ROR/-1969 site; these studies further advocate that the ROR and rev-erb members of the orphan nuclear receptors family play an integral role in modulating steroidogenesis. Putative identification and subsequent functional analysis of the RORE site further support our findings that the distal region of the StAR promoter is important in regulation of the StAR gene and is controlled by potent signaling molecules.

The combination of ovarian follicle, promoter deletion, and site-directed mutation data implies that transcriptional elements between -1.86 kb to -2.9 kb of the promoter are required for cAMP-induced activation of the StAR promoter in LMB. The mutation data revealed that over 80% loss in transcriptional activity of the LMB StAR promoter can be attributed to an RORE upstream from -2 kb of this region. ChIP and EMSA analyses in our studies reveal that both rev-erbα and RORα may play integral roles in the control of the activation of the LMB StAR promoter. Additionally, it appears that regulation of StAR transcription is conserved from
mammals to lower vertebrates, and that non-classical species such as LMB are increasingly pertinent model systems for comparative studies.
Figure 4-1: In silico comparison of the StAR promoter across species. Promoter sequences (including the 5' UTR) for the StAR gene promoters from LMB and brook trout (Acc. No. AY308064), and the StAR gene promoters from rat (Acc. No. AB006007), mouse (180), and human (Acc. No. U29098) were aligned based on the transcriptional start sites for the gene. Putative transcriptional elements were identified using Genomatix MatInspector online software. The sites selected for mapping had ≥80% homology to the core mammalian transcription factor sequence. (1) cAMP-response element binding protein site, (2) AP-1 site, (3) SF-1 site, (4) GATA binding factor site, (5) RORα/rev-erbα site, (6) ER site, (7) Yin and Yang 1 site, (8) Sterol regulatory element binding protein, (9) CCAAT enhancer binding protein.
Figure 4-2: Functional analysis of RORα and rev-erbα by ChIP. A) qPCR results of ChIP using primers designed against the ROR/-1969 element in the LMB StAR promoter. B) qPCR results of ChIP using primers designed against the RORE in the mouse StAR promoter. Plates of Y-1 cells were transfected for 18 hours and treated either without or with 1 mM dbcAMP for 20 hours. ChIP was run on each sample with an antibody specific to mouse IgG (non-specific control), RORα, or rev-erbα and following incubation with Protein G agarose beads and multiple washes, complexes were eluted and DNA was purified. qPCR was run on each sample and results are reported graphically as % enrichment to a 1:10 dilution of each input control. Note: asterisks (*) indicate that the DNA was below detectable limit by qPCR. Each figure is representative of one of three replicated experiments.
Figure 4-3: Functional analysis of RORα by EMSA with recombinant RORα4 protein. ROR/1969 and a perfect human RORE were added to recombinant human RORα4 protein in 1X binding buffer (A and D, respectively). Addition of an antibody specific to RORα4 to the reaction diminished the banding pattern observed in the lanes containing RORα4 protein with the LMB probe and human probe (B and E, respectively). A scrambled probe was combined with the RORα4 protein and yielded no band (C), verifying specificity of the protein-DNA interaction in vitro.
Figure 4-4: EMSA with basal and dbcAMP-induced nuclear fractions. The putative LMB RORE (ROR/-1969) was added to either basal or cAMP-induced Y-1 nuclear fractions in 1X binding buffer. The reactions were separated on a native gel and transferred to a membrane for EMSA analysis using chemiluminescence.
CHAPTER 5
STERIDOGENIC ACUTE REGULATORY PROTEIN AS A TARGET FOR ORGANOCHLORINE PESTICIDES IN LARGEMOUTH BASS

Introduction

EDCs can negatively impact reproduction and development in wildlife and in humans by mimicking or altering the actions of endogenous steroid hormones, or by disrupting the synthesis or metabolism of circulating steroid hormones. There is substantial evidence that links exposure of humans and wildlife to EDCs with changes in steroidogenic capacity, secondary sex characteristics, gonad development, and production and size of eggs and sperm (3-15).

EDCs are ubiquitous in the environment and include many different types of compounds, including pesticides, fungicides, and their metabolites (16-20), plasticizers (21-23), papermill effluent (24, 25), pharmaceuticals in sewage wastewaters (26), and others. Hundreds of studies report that many different species exhibit reproductive and developmental abnormalities upon exposure to EDCs, including humans, mammals, amphibians, reptiles, birds, invertebrates, and fish (reviewed in (9)).

Many EDCs are very stable and persist in the environment, and the main sink for many EDCs is in freshwater lakes and rivers; soluble compounds aggregate in the surface waters, whereas less soluble compounds collect in the sediments. It is for this reason that numerous aquatic vertebrates, including multiple species of fish, are at high risk for exposure to EDCs and susceptible to reproductive abnormalities (reviewed in (27)). LMB are a freshwater teleost species and have been reported to exhibit altered gene expression and distorted circulating steroid hormone levels in response to exposure to EDCs, both in a controlled laboratory setting and in the wild. (2, 3, 16, 25, 28, 65, 67, 113, 144, 158, 186-188).

Multiple OCPs have been associated with endocrine disruption in LMB, including DDE, DIEL, MXC, and TOX, amongst others. Many studies have shown that these OCPs have the
capacity to disrupt ER and AR expression in LMB (65, 113, 144, 188, 189); however, select studies have shown that other genes involved in the production and metabolism of steroid hormones are subject to altered expression levels, including the gene encoding the StAR protein (3, 113, 144).

StAR protein controls one of the rate-limiting steps in steroid hormone production in all vertebrates and is responsible for the delivery of cholesterol to the outer mitochondrial membrane prior to conversion into steroid hormones in steroidogenic tissues (52). In mammals, the StAR gene has been identified as a target of a number of pesticides, including Roundup (19), dimethoate (115), and lindane (20). Additionally, it has been reported that in vivo exposure of LMB to DDE, DIEL, and MXC resulted in altered StAR mRNA levels in the gonads; however, the mechanisms through which the chemicals disrupted StAR mRNA levels have not yet been elucidated.

StAR protein is an integral player in the steroid biosynthetic pathway; however, there are multiple enzymes that are important in producing sex steroid hormones, including the enzyme P450 aromatase (Cyp19); Cyp19a is the isoform of P450 aromatase responsible for the metabolism of androgens into estrogens in the gonads of vertebrates, including LMB. In mammalian studies, Cyp19a gene expression has been shown to be disrupted in the gonad upon ex vivo exposure to DDE (150, 190). Interestingly, Cyp19a has been reported to be a target of some of the same pesticides reported to disrupt the StAR gene in mammalian systems, including both lindane (191) and Roundup (glyphosate) (192).

The aim of our current study was to examine gonad-specific gene expression changes in the genes encoding StAR, ER’s βa and βb (highly expressed in the gonad), AR, and Cyp19a, in LMB gonads in response to ex vivo exposure to DDE, DIEL, MXC, and TOX, under basal and
tropic hormone-induced conditions. We attempt to isolate tissue-specific responses to gain a better understanding of the mechanisms of action of OCPs in changing StAR gene expression and steroid hormone levels in LMB.

Results

Gene Expression Changes in Ovarian Tissue upon Ex Vivo Exposure to OCPs under Basal Conditions

To examine ovarian-specific gene expression responses to OCP exposure, 15 – 25 mg pieces of LMB ovary from recrudescent LMB were cultured for ex vivo analysis. This experiment represents the combined data from four individual LMB cultured in duplicate. Cultures were pre-exposed to medium containing vehicle or 100 µM doses of DDE, DIE1, MXC, or TOX for 2 hours, followed by a change to fresh culture medium and subsequent incubation for 20 hours. RNA was extracted from each sample and gene expression analysis of StAR, ERβa, ERβb, AR, and Cyp19a mRNA levels was performed using qPCR (Figure 5-1). No statistically significant changes were detected among treatments. Results are represented as fold change from basal vehicle-treated control values.

Gene Expression Changes in Ovarian Tissue upon Ex Vivo Exposure to OCPs under hCG-Stimulated Conditions

Since hCG is a tropic hormone known to stimulate steroidogenic pathways in vertebrates, the impact of OCP exposure in ovarian-specific gene expression was examined by qPCR under hCG-stimulated conditions. 15 – 25 mg pieces of LMB ovary were cultured from the same individuals used in the female basal experiments and used for ex vivo gene expression analysis. This experiment represents the combined data from the same four individuals used to investigate basal gene expression responses. Cultures were pre-exposed to medium containing vehicle or 100 µM doses of DDE, DIE1, MXC, or TOX for 2 hours, followed by a subsequent change to
fresh medium containing 10 U/mL hCG. Following RNA purification, \textit{StAR}, \textit{ER\_\beta\_a}, \textit{ER\_\beta\_b}, \textit{AR}, and \textit{Cyp19a} mRNA levels were examined by qPCR (Figure 5-2). Results are reported in fold change from the respective basal vehicle control. Pre-treatment with vehicle followed by exposure to 10 U/mL hCG revealed a nearly 12-fold induction of \textit{StAR} mRNA and a 2.5-fold induction of \textit{AR} mRNA. Pre-treatment with both DDE and DIEL yielded a nearly 25-fold induction of \textit{StAR} mRNA expression in response to hCG; nearly double the induction observed in controls. \textit{ER\_\beta\_a} and \textit{ER\_\beta\_b} mRNA expression profiles were not significantly changed upon exposure to hCG in combination with vehicle or any contaminant. \textit{AR} mRNA expression was significantly stimulated ~2-fold by hCG upon pre-exposure to vehicle, DIEL, and TOX, whereas pre-treatment with DDE and MXC diminished the hCG-induction to insignificant levels. \textit{Cyp19a} mRNA levels showed no significant response to any treatment.

**Gene Expression Changes in LMB Testis Tissue upon \textit{Ex Vivo} Exposure to OCPs under Basal Conditions**

To elucidate testis-specific responses to OCP exposure, 15 – 25 mg slices of testis tissue dissected from spermiating male LMB were cultured for \textit{ex vivo} analysis. This experiment represents the combined data collected from four individual recrudescent male LMB cultured in duplicate. Cultures were pre-exposed to medium supplemented with either vehicle or with 100 µM doses of each OCP for 2 hours, followed by a change to fresh culture medium and subsequent incubation for 20 hours. RNA was isolated from each sample and qPCR analysis was conducted to determine the tissue-specific expression of \textit{StAR}, \textit{ER\_\beta\_a}, \textit{ER\_\beta\_b}, \textit{AR}, and \textit{Cyp19a} mRNA levels in response to OCP exposure (Figure 5-3). Treatment with DDE and DIEL stimulated \textit{StAR} mRNA and \textit{ER\_\beta\_b} mRNA expression levels nearly 3-fold and 2-fold above the levels observed in the controls, respectively. However, due to inter-individual variation, only DIEL exposure resulted in significant stimulation of \textit{StAR} mRNA expression.
above basal levels. Only treatment with DDE significantly stimulated 
Cyp19a mRNA levels above those observed in the vehicle control. No significant changes were detected in ERβa mRNA expression in response to exposure to any OCP.

**Gene Expression Changes in LMB Testis Tissue upon Ex Vivo Exposure to OCPs under hCG-Stimulated Conditions**

To investigate the testis-specific gene expression responses to OCP exposure under stimulated conditions, 15 – 25 mg pieces of LMB slices of testis tissue were cultured from the same individuals used in the basal experiments. This experiment represents the combined data from the same four LMB used to investigate basal gene expression responses. Cultures were pre-exposed to medium containing vehicle or 100 µM doses of DDE, DIEL, MXC, or TOX for 2 hours, followed by a subsequent change to fresh medium containing 10 U/mL hCG. Following RNA purification, StAR, ERβa, ERβb, AR, and Cyp19a mRNA levels were examined by qPCR (Figure 5-4). Results are reported in fold change from the respective basal vehicle control. Tissues treated with vehicle and hCG revealed a 2-fold induction of StAR mRNA, although results were not significant upon comparison to basal control levels. However, pre-treatment with both DDE and DIEL showed a significant induction of StAR mRNA upon exposure to hCG that increased ~4-fold above basal control levels. Treatment with all OCPs and hCG yielded no significant changes to the expression of AR, ERβa, ERβb, or Cyp19a transcripts.

**Changes in Testosterone (T) Production by Ex Vivo Ovarian and Testis Cultures Following Exposure to OCPs under Basal and hCG-Stimulated Conditions (193)**

To complement the gene expression profiles of the ovarian and testis tissues exposed to OCPs under both basal and hCG-stimulated conditions, T levels in the culture medium that the pieces of tissue were cultured in were analyzed by RIA (data not shown).
In ovarian tissues, treatment with DDE under both basal and stimulated conditions yielded a slight reduction in T production relative to controls. Treatment with DIEL yielded little change in basal T production, however, under stimulated conditions DIEL stimulated T production ~120% above the hCG control. Interestingly, M XC exposure severely impaired T production by the cultured ovarian tissue under basal and stimulated conditions, reducing levels to nearly 20% of respective controls. Exposure to TOX also critically impaired ovarian T production to only 60% of that observed in the basal controls and 20% of the levels observed in the hCG controls.

In testis exposed to DDE, a marked decrease in T production was observed. T produced by the DDE-treated tissue was reduced to 80% and 55% with respect to controls, under basal and hCG-stimulated conditions, respectively. DI EL exposure did not alter T production more than 10% relative to controls under both conditions. Under basal conditions, MXC exposure did not affect T production by the testis; however, under stimulated conditions, T levels were only ~75% of those observed in the hCG controls, though the results were quite variable. TOX exposure did not affect T production under basal conditions, whereas T production was decreased to ~60% of that observed in the stimulated controls.

Transfections with the 2.9 kb LMB StAR Promoter in MA-10 Leydig Cells

In order to determine whether the promoter for the LMB StAR gene was targeted directly by the OCPs investigated in this study, steroidogenic cells were transfected with a 2.9 kb portion of the StAR gene promoter and subsequently exposed to DMSO (vehicle), 1 µM or 10 µM doses of each OCP for 20 hours, followed by exposure to fresh growth medium alone (Figure 5-5) or medium supplemented with 10 U/mL hCG (Figure 5-6) for 4 hours. In addition, ICI 182,780, a potent ER antagonist, was added ([final]: 10 µM) in addition to a 10 µM dose of each individual OCP to determine if ER signaling may be involved in mediating any effects observed in StAR.
promoter activity. No significant change was observed upon treatment with any OCP; however, the LMB StAR promoter did appear to be stimulated by hCG by approximately 2-fold.

**Discussion**

Reproduction, gonadal growth, and steroidogenesis in vertebrates, including LMB, are highly controlled by the hypothalamus-pituitary-gonadal (HPG) axis. Because OCPs can disrupt circulating steroid hormone levels and gonadal StAR mRNA expression in LMB and the mechanisms through which this occurs are not well understood, the current study attempted to simplify things and examined gonad-specific changes in StAR mRNA expression in response to acute *ex vivo* exposure to DDE, DIEL, MXC, and TOX. Gonadotropins released from the pituitary, including LH (teleost GHT-II) and FSH (teleost GHTI), tightly control gonadal steroidogenesis; therefore, in order to mimic quiescent and stimulated *in vivo* stages, *ex vivo* exposures to OCPs were conducted, under both basal and hCG-stimulated conditions. StAR mRNA levels varied significantly among treatments, so the capacity of OCPs to disrupt the activity of LMB StAR promoter was investigated using transient transfections. Surprisingly, none of OCPs disrupted transcriptional activation of the 2.9 kb LMB StAR promoter in transfections in MA-10 cells. DDE, DIEL, MXC, and TOX have different modes of action (MOA) and as expected, differentially impacted gene expression in the LMB gonad.

Several studies have characterized the MOA of DDE. DDE has been reported to be a potent AR antagonist and weak ER agonist (4, 11, 194). In addition, DDE can activate non-genomic signaling pathways; DDE has been shown to inhibit the generation of cAMP in granulosa cells (17), activate membrane ER-related pathways (195), and to activate extracellular signaling kinase 1 and 2 (ERK1/2) pathways (195, 196). In our study, *ex vivo* gonadal treatment with DDE caused a moderate decrease in T production by the ovary and testis and a moderate increase in Cyp19a mRNA in the testis under basal conditions. The induction of StAR mRNA by
hCG was substantially exacerbated in the ovary and testis exposed to DDE. Transfections with the 2.9 kb StAR promoter in MA-10 cells suggested that the elevated levels of StAR mRNA were not a result of increased activity at the gene promoter level.

In vertebrates, ERK phosphorylation is required for the activation of a number of proteins and transcription factors, including the StAR protein; it is also known that ERK docks itself on the StAR protein at the mitochondria, critically enhancing the transport of cholesterol across the mitochondrial membrane (197, 198). Intuitively, because DDE has been shown to activate ERK signaling, it is possible that DDE disrupts proper ERK function in LMB gonads, which could mediate the observed accumulation of StAR mRNA in the LMB gonads treated with DDE.

DIEL, although very different structurally from DDE, can also potently activate ERK1/2 pathways (196). In this study, DIEL exposure, like DDE, highly enhanced the levels of StAR mRNA in the LMB gonads; however, this was observed under both basal and hCG-induced conditions.

The results observed in our experiments with MXC and TOX were quite different than those seen with DDE and DIEL. In the ovary, there was no change in StAR mRNA levels but the response of AR mRNA stimulation by hCG was blocked by MXC. Treatment with MXC and TOX severely impaired T production by the ovary and testis (data not shown). Stimulation of StAR mRNA by hCG was completely compromised upon pre-exposure to the two contaminants in the testis. Transfections with the LMB StAR promoter revealed that neither MXC nor TOX affect the activity of the StAR promoter under basal or hCG-induced conditions.

It is evident that MXC and TOX disrupt pathways distal to StAR transcriptional activation by hCG. The MOA of MXC has been extensively characterized; however the MOA of TOX is not well understood. MXC, very similar in structure to DDE, has been characterized as an AR-
antagonist and ER-agonist in mammals and fish (65, 151, 194). In addition, MXC has been reported to inhibit steroid hormone production at a point distal to cAMP synthesis in gonadal cells (17). Recent work in mammalian granulosa cells has shown that expression of the LH receptor is significantly decreased upon *in vitro* exposure to the MXC metabolite HPTE (199); it has also been reported that *in vivo* exposure to MXC and HPTE in male rats caused a significant decrease in plasma T, but no change in the plasma levels of LH and FSH (200). Additional research in rats reported that *in vivo* MXC exposure resulted in decreased circulating progesterone and increased circulating LH levels, as well as decreased LH receptor levels in large antral follicles (201).

hCG, a potent analog of LH, activates LH receptor signaling which is highly involved in the activation of expression of the *StAR* gene. Recent studies have examined LH and FSH receptor expression in the gonads of LMB and sea bass, and it was observed that expression of the FSH receptor was connected with early stages of gonadal development, and also with the spermiation/maturation-ovulation periods, whereas LH receptor expression was highly associated with the final stages of gamete maturation and spermiation/ovulation (106, 202). The male LMB used in our study were spermiating (mature) and the females were still undergoing vitellogenesis (SG). Based upon reproductive stage-specific expression of the gonadotropin receptors, it is possible that the testis tissue expressed higher ratio of LH/FSH receptors than the ovaries did in our *ex vivo* studies. Intuitively, it is possible that LH/FSH metabolism and/or LHR/FSHR expression in the LMB gonad may be targeted by MXC. It is likely that MXC may disrupt gonadal LHR or FSHR expression and/or ligand activation. In addition, it is possible that TOX may disrupt similar pathways; however, further studies are warranted to validate these hypotheses.
A study that was conducted previously by Garcia-Reyero et al. examined gene expression and plasma steroid hormone levels in LMB fed food laced with DDE and DIEL for 120 days (113). Many of the results observed in our studies with DDE and DIEL parallel the results observed in the feeding study. A summary of both studies is listed in Table 5-1. Garcia-Reyero et al. reported that expression of \textit{StAR} mRNA in the ovary and testis was stimulated several fold higher in LMB exposed to the contaminants when compared to the levels observed in control animals. Our study showed a very similar stimulation in the gonads treated \textit{ex vivo} with DDE and DIEL, suggesting that \textit{StAR} expression in the gonad is one gene that is truly targeted by DDE and DIEL. Garcia-Reyero et al. also reported that circulating plasma 11-KT and E2 levels were altered from control levels. T production by the LMB ovarian and testis tissue cultured in these experiments was decreased upon exposure to DDE. \textit{Ex vivo} DIEL exposure did not change T production by the testis and stimulated T production in the ovaries under hCG-stimulated conditions. The biosynthesis of steroid hormones is highly controlled by the HPG axis and regulation of sex steroid hormone levels depend upon a number of hormones released by the pituitary, including LH and FSH, among others.

The study by Garcia-Reyero et al. reported a several fold decrease in \textit{ER} and \textit{Cyp19a} mRNA in DDE-fed LMB, and also showed that \textit{Cyp19a} was stimulated in the ovary and repressed in the testis of fish fed DIEL. In our study, it was observed that \textit{ER\^{\beta}a} and \textit{ER\^{\beta}b} mRNA levels were not affected by DDE and DIEL exposure and \textit{Cyp19a} levels were significantly increased only in testis tissue treated with DDE under basal conditions. The study by Garcia-Reyero et al. was a long-term study that examined the effects of chronic exposure to the contaminants in the whole animal, whereas this study examined gonad-specific effects upon acute exposure to the OCPs. It is no surprise that the changes observed are not the same,
however the elevated levels of $StAR$ mRNA in both studies is suggestive of the idea that the two OCPs are capable of disrupting $StAR$ expression directly at the gonadal level.

Collectively, these studies indicate that $StAR$ mRNA levels are targeted in the gonad upon $ex vivo$ exposure to DDE and DIEL, whereas hCG induction of $StAR$ mRNA was targeted upon $ex vivo$ exposure to MXC and TOX. Transfections with the 2.9 kb $StAR$ promoter construct in mouse MA-10 Leydig cells revealed no alteration in transcriptional activity in response to treatment with any of the OCPs; however, it must be noted that transactivation of gene promoters is extremely complex, and, although it is unlikely, it is possible that the 2.9 kb fragment does not encompass the entire functional region of the LMB $StAR$ promoter. Numerous studies on the mammalian $StAR$ promoter have confirmed that the proximal 300-1000 bp of the $StAR$ promoter is ample in activating the $StAR$ gene (126, 128, 178, 180, 185, 203); thus, it is likely that the effects on $StAR$ transcript levels observed in the $ex vivo$ cultures treated with DDE and DIEL are due to the disruption of signaling pathways involved in modulating the activity of $StAR$ mRNA or protein. These experiments suggest that MXC and TOX disrupt hCG-induced activation of $StAR$ mRNA by altering expression of gonadotropin receptors or disrupting the binding of gonadotropins to receptors in the LMB gonad. Further studies are warranted to investigate these hypotheses.

Altogether, my data on $StAR$, $ER$, $AR$, and $Cyp19a$ gene expression in response to $ex vivo$ LMB gonad exposure yields some insight as to the tissue-specific mechanisms underlying the alteration of plasma sex steroid hormone levels in LMB exposed to these EDCs. While the original proposal was that OCPs directly regulate LMB $StAR$ promoter activity, it now seems more likely that the mechanisms through which the OCPs disrupt $StAR$ gene expression are
found further upstream in gonadotropin signaling and/or further downstream in post-transcriptional and post-translational regulatory cascades.
Table 5-1: Summary of changes observed in LMB gonads in *ex vivo* versus *in vivo* DDE and DIEL exposure. The *in vivo* data summarized here was published previously by Garcia-Reyero, et al. (113)

<table>
<thead>
<tr>
<th>DDE Exposure</th>
<th>Ex Vivo Response</th>
<th>In Vivo Response</th>
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<tbody>
<tr>
<td></td>
<td>Ovary</td>
<td>Testis</td>
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<tr>
<td></td>
<td>Basal</td>
<td>hCG</td>
</tr>
<tr>
<td><strong>StAR mRNA</strong></td>
<td>N.C.</td>
<td>↑</td>
</tr>
<tr>
<td><strong>ERβα mRNA</strong></td>
<td>N.C.</td>
<td>N.C.</td>
</tr>
<tr>
<td><strong>ERβb mRNA</strong></td>
<td>N.C.</td>
<td>N.C.</td>
</tr>
<tr>
<td><strong>AR mRNA</strong></td>
<td>N.C.</td>
<td>N.C.</td>
</tr>
<tr>
<td><strong>Cyp19a mRNA</strong></td>
<td>N.C.</td>
<td>N.C.</td>
</tr>
<tr>
<td>Hormone synthesis</td>
<td>↓</td>
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**DIEL Exposure**

| | Ovary | Testis | Ovary | Testis |
| | Basal | hCG | Basal | hCG |
| **StAR mRNA** | N.C. | ↑ | ↑ | ↑ |
| **ERβα mRNA** | N.C. | N.C. | N.C. | N.C. | ↓ |
| **ERβb mRNA** | N.C. | N.C. | N.C. | N.C. | ↓ |
| **AR mRNA** | N.C. | N.C. | N.C. | N.C. | ↑ |
| **Cyp19a mRNA** | N.C. | N.C. | N.C. | N.C. | ↑ |
| Hormone synthesis | ↑ | N.C. | N.C. | ↓E2, ↓11-KT | ↓E2, ↓11-KT |
Figure 5-1: LMB ovarian gene expression changes in response to *ex vivo* OCP exposure under basal conditions. Minced ovarian tissue from four individual female LMB were cultured in duplicate in a 24 well culture plate and exposed to 100 µM doses of each OCP for 2 hours, followed by a change to fresh medium and incubated for 20 hours. RNA was isolated from the tissues and reverse transcribed for analysis by qPCR. Each bar represents the fold change from basal control of mean copy number ± SEM of untransformed data. One-way ANOVA followed by post hoc Dunnett’s pairwise multiple comparison were performed on log-transformed gene expression data and an asterisk (*) denotes a significant difference (P <0.05).
Figure 5-2: LMB ovarian gene expression changes in response to *ex vivo* OCP exposure under hCG-stimulated conditions. Minced ovarian tissue from four individual female LMB were cultured in duplicate in a 24 well culture plate and exposed to 100 µM doses of each OCP for 2 hours, followed by a change to fresh medium and incubated for 20 hours. RNA was isolated from the tissues and reverse transcribed for analysis by qPCR. Each bar represents the fold change from basal control of mean copy number ± SEM of untransformed data. One-way ANOVA followed by post hoc Dunnett’s pairwise multiple comparison were performed on log-transformed gene expression data and an asterisk (*) denotes a significant difference (P <0.05).
Figure 5-3: LMB testis gene expression changes in response to *ex vivo* OCP exposure under basal conditions. Testis tissue from four individual male LMB was sliced and cultured in duplicate in a 24 well culture plate and exposed to 100 µM doses of each OCP for 2 hours, followed by a change to fresh medium and incubated for 20 hours. RNA was isolated from the tissues and reverse transcribed for analysis by qPCR. Each bar represents the fold change from basal control of mean copy number ± SEM of untransformed data. One-way ANOVA followed by post hoc Dunnett’s pairwise multiple comparison were performed on log-transformed gene expression data and an asterisk denotes a significant difference (P <0.05).
Figure 5-4: LMB testis gene expression changes in response to *ex vivo* OCP exposure under hCG-stimulated conditions. Testis tissue from four individual male LMB was cultured in duplicate in a 24 well culture plate and exposed to 100 µM doses of each OCP for 2 hours, followed by a change to fresh medium and incubated for 20 hours. RNA was isolated from the tissues and reverse transcribed for analysis by qPCR. Each bar represents the fold change from basal control of mean copy number ± SEM of untransformed data. One-way ANOVA followed by post hoc Dunnett’s pairwise multiple comparison were performed on log-transformed gene expression data and an asterisk (*) denotes a significant difference (P <0.05).
Figure 5-5: Response of 2.9 kb LMB StAR gene promoter to treatment with OCPs under basal conditions. MA-10 mouse Leydig cells plated in 24 well plates were transfected with the 2.9 kb LMB StAR gene promoter incorporated into a Firefly Luciferase reporter plasmid and a controlled amount of Renilla Luciferase construct for data normalization. 24 hours post-transfection, transfected cells were exposed (in triplicate) to either 0.1 % DMSO, 1 mM OCP, 10 µM OCP, or 10 µM OCP + 10 µM ICI 182,780. After 20 hours, growth medium was changed and the same treatment was administered for 4 hours. Cells were lysed and luciferase quantities were analyzed. Data are plotted in Relative Luciferase Units (Firefly/Renilla Luciferase ratios).
Figure 5-6: Response of 2.9 kb LMB StAR gene promoter to treatment with OCPs under hCG-stimulated conditions. MA-10 mouse Leydig cells plated in 24 well plates were transfected with the 2.9 kb LMB StAR gene promoter incorporated into a Firefly Luciferase reporter plasmid and a controlled amount of Renilla Luciferase construct for data normalization. 24 hours post-transfection, transfected cells were exposed (in triplicate) to either 0.1 % DMSO, 1 mM OCP, 10 μM OCP, or 10 μM OCP + 10 μM ICI 182,780. After 20 hours, fresh growth medium was added containing the same treatment in combination with hCG ([final] = 10 U/mL) for 4 hours. Cells were lysed and luciferase quantities were analyzed. Data are plotted in Relative Luciferase Units (Firefly/Renilla Luciferase ratios).
CHAPTER 6
OVERALL DISCUSSION AND FUTURE DIRECTION

Endocrine disruption has been a topic of much controversy and debate throughout the past decade. Numerous EDCs have been identified, including many OCPs, and these chemicals not only have the capacity to harm wildlife, but also humans. Lake Apopka, a federally-appointed Superfund site in Florida, is known to be extensively contaminated with OCPs, including DDE, DIEL, MXC, and TOX. These OCPs are considered xenoestrogens (capable of disrupting ER and AR signaling) and their presence in Lake Apopka has been highly associated with the disruption of normal reproductive function and physiology in wildlife that inhabit the area. LMB are abundant in Lake Apopka and exhibit altered plasma sex steroid hormone levels upon in vivo exposure to a number of OCPs; sex steroid hormones tightly regulate reproduction in vertebrates, and disbalance of these hormones can lead to reproductive failure, or even death. This yields great concern, because the maintenance of biodiversity and life on earth is reliant upon successful reproduction. It is important to understand the mechanisms of action of OCPs mediating this disruption in order to gain a better understanding on the potential effects they may have on other wildlife and humans.

The StAR protein is a protein critically involved in steroid hormone biosynthesis in vertebrates, and likely in LMB, due to the conservation of gene regulation observed in these studies. Many OCPs found in the environment have been reported to disrupt the expression of this important protein in laboratory studies and in the wild. It is important to understand how the StAR gene is regulated under normal conditions in healthy LMB. It is also important to understand how OCPs disrupt the StAR gene in the gonad of LMB, because this is the main site of sex steroid hormone synthesis in LMB. The research presented and discussed in this dissertation encompasses the extensive characterization of the endogenous regulation of the
LMB StAR gene. In addition, the capacity for the LMB StAR gene to be regulated in LMB gonads by DDE, DIEL, MXC, and TOX was investigated in order to gain insight as to how these OCPs actually disrupt steroidogenesis in LMB.

In order to obtain understanding of the normal gene expression profiles in the gonads of healthy LMB, the expression of ER, AR, and StAR transcripts were characterized throughout the reproductive season by month and by reproductive stage in female and male LMB gonads collected from the St. John’s River in Welaka, FL. Grouping the individuals by reproductive stage distinguished trends observed in gene expression by month into significant observations dependent upon gonad stage. In both female and male LMB, all ER mRNA and AR mRNA levels were significantly positively correlated with one another. The data collected on gonadal ER and AR mRNA expression throughout the reproductive cycles of male and female LMB isoforms suggest that sex steroid hormone nuclear receptors play important and complex roles in regulating genes involved in female and male reproductive progression and cycling. To gain further insight into the importance of AR expression in the LMB gonad, it would be interesting to clone individual AR isoforms from LMB and characterize the isoform-specific variations in gene expression in the gonad.

In the seasonal study, StAR mRNA levels were also characterized in both sexes. The abundance of StAR mRNA in the LMB ovary and testes peaked markedly during late reproductive stages (maturation in female and spermiogenesis in the testes). This phenomenon parallels studies on StAR mRNA levels throughout mammalian reproduction, and the acute upregulation of StAR mRNA during the later stages of reproduction reinforces the idea that the StAR protein plays a critical role in steroid hormone synthesis and reproduction in all vertebrates, including LMB. In the ovary, \( ER\beta a \) and \( ER\beta b \) were negatively correlated with StAR
expression, whereas in the testis, \( ER\beta \) and \( AR \) were positively correlated with \( StAR \) expression. Studies have shown that E2 exposure causes a decrease in \( StAR \) gene expression (102, 114, 124), so in this work, \textit{ex vivo} gonad cultures were treated with E2 (potent ER agonist) and ICI 182,780 (a potent ER antagonist) and \( StAR \) mRNA expression was investigated in the gonad tissue. It was observed that concomitant treatment with ICI 182,780 and hCG caused a sustained elevation of \( StAR \) mRNA levels after 20 hours of treatment. Interestingly, it has been shown that ICI 182,780 can activate membrane-bound ERs, resulting in stimulation of downstream signal transduction pathways (171). In the LMB gonad, it is possible that ICI 182,780 agonizes membrane-bound ER pathways, acting as a selective ER modulator rather than a receptor antagonist. In additions, it has been reported that ER\( \beta \) has the potential to activate the promoter for some genes by binding Sp1 sites when bound to ICI 182,780 and other antagonists, but not when bound to E2 (172). Further experimentation using the \( StAR \) promoter and inhibitors of the MAPK pathways stimulated by membrane-bound receptors could yield insight as to which signal transduction pathways are involved in mediating the observations made in the \textit{ex vivo} experiments from my work. It is possible that membrane-bound ER signaling could be involved in the regulation of LMB \( StAR \) mRNA expression.

Our laboratory previously cloned 2.9 kb of the LMB \( StAR \) promoter (89, 90), and I cloned an additional 2.6 kb of the distal region of the promoter in an attempt to examine the distal region for putative elements that may be involved in the transcriptional regulation of the \( StAR \) gene. Several putative EREs were identified in the 2.9 kb \( StAR \) promoter using computer software, and, using EMSAs, I showed that mammalian ER\( \beta \) is capable of binding to an element in the LMB \( StAR \) promoter \textit{in vitro}. However, the presence of several other putative EREs (including sites for ER and AP-1) suggest that ERs could potentially interact with a number of
different elements in the \textit{StAR} promoter. The binding of ER\(\beta\) to the element in the LMB \textit{StAR} promoter \textit{in vitro} is supportive of the idea that ERs can directly bind to the \textit{StAR} gene promoter and control its activation, though further functional studies, including ChIP assays and investigation of the activity of other putative EREs, are warranted.

Because studies on mammalian \textit{StAR} promoters have indicated that the ample region for activation of the \textit{StAR} gene lies in the -300 – -1000 bp proximal promoter segment, upstream from the transcriptional start site (126, 128, 178, 180, 185, 203), I pursued all further characterization of the regulation of the LMB \textit{StAR} promoter using the 2.9 kb construct. When the 2.9 kb construct was analyzed, putative EREs were abundant; interestingly, so were hundreds of other putative binding sites, some of which were extremely interesting.

Dr. Jannet Kocerha, a former graduate student in our laboratory, made a significant contribution into the characterization of the 2.9 kb LMB \textit{StAR} promoter. She showed that, as observed in mammals, the LMB \textit{StAR} promoter is stimulated by cAMP in a dose-responsive manner. She also conducted transient transfections with variations of the 2.9 kb promoter construct, including point mutation and deletion constructs, examining cAMP response. She determined that point mutation of several elements located in the distal portion of the promoter diminished the activation of the promoter by cAMP. This is quite fascinating, considering that many researchers assert that the main regulatory regions of the mammalian \textit{StAR} promoter lie within < 1 kb of the transcriptional start site of the \textit{StAR} gene.

One of the sites that, upon mutation, ablated cAMP-response included a putative site for ROR\(\alpha\) and rev-erb\(\alpha\), ROR/-1969. It is notable that cholesterol has been identified as a ligand for ROR\(\alpha\) in recent studies (88, 183), suggesting that it may be a key player in steroid production. In addition, rev-erb\(\alpha\) has been reported to competitively bind the same element as ROR\(\alpha\),
disallowing the activation of target genes by RORα (184). Because RORα and rev-erbα have been associated in regulation of genes involved in peripheral circadian rhythm (85-87) and my study of StAR mRNA expression in LMB gonads throughout the reproductive cycle implicated that StAR mRNA expression is highly dependent on reproductive stage, I pursued further characterization of this site.

Functional assessment of the ROR/-1969 site was warranted, so using EMSAs and ChIP assays, I confirmed that, in mouse Y-1 adrenocortical cells transfected with the LMB StAR promoter, rev-erbα and RORα bind to ROR/-1969 under basal and cAMP-stimulated conditions, respectively. In order to investigate if the role these proteins play in regulating the StAR promoter is conserved between higher and lower vertebrates, I also pursued functional analysis of a putative RORE located in the mouse StAR promoter (mROR/-634). It was determined that rev-erbα, and possibly RORα, bind to this element, indicating that these orphan nuclear receptors may play an evolutionarily conserved role in regulating the activity of the StAR promoters in vertebrates.

In the EMSAs conducted examining the in vitro binding activity of the ROR/-1969 site with Y-1 nuclear fractions, there were several bands present. The presence of bands implies that several proteins and/or complexes have the capacity to bind the element. Future work is necessary to identify the proteins that are binding the element. Using the same probes used in the EMSAs, it is possible to add the nuclear fractions directly to the probe and to “pull-down” the proteins that bind to the probe. Following rigorous washes, mass spectrometry could be used to identify the proteins that are binding to the ROR/-1969, helping identify novel proteins that may be involved in regulating the StAR promoter.
There is no doubt that the LMB $\text{StAR}$ promoter is regulated in an acute and highly complex manner, involving a number of different transcription factors. Because the $\text{StAR}$ gene is a known target of OCPs, the complexity surrounding the regulation of the $\text{StAR}$ promoter only further complicates our understanding of how OCPs disrupt the $\text{StAR}$ gene in intact LMB. To add even more complication, reproduction, gonadal growth, and steroidogenesis are highly controlled by the hypothalamus-pituitary-gonadal HPG axis in intact organisms, opening up many pathways that could be targeted that mediate the effects of OCPs. Because OCPs disrupt circulating steroid hormone levels and gonadal $\text{StAR}$ mRNA expression in LMB and the mechanisms through which this occurs are not well understood, I examined gonad-specific changes in $\text{StAR}$ mRNA expression and hormone production in response to acute $ex \ vivo$ exposure to DDE, DIEL, MXC, and TOX under basal and hCG-induced conditions.

$\text{StAR}$ mRNA levels varied significantly among treatments, so we examined the capacity of OCPs to disrupt the activity of LMB $\text{StAR}$ promoter in transient transfections. Surprisingly, none of the OCPs disrupted transcriptional activation of the 2.9 kb LMB $\text{StAR}$ promoter in transfections in MA-10 cells. It must be noted that transcriptional activation of gene promoters is extremely complex, and, although it is unlikely based upon mammalian studies of the $\text{StAR}$ promoter, it is possible that the 2.9 kb fragment does not encompass the entire functional region of the LMB $\text{StAR}$ promoter. Further investigation using the expanded 5.6 kb LMB $\text{StAR}$ promoter would help clarify if OCPs impact $\text{StAR}$ promoter activity.

DDE, DIEL, MXC, and TOX all employ different modes of action and as expected, each of the OCPs differentially impacted $\text{StAR}$ mRNA expression and T production in the female and male LMB gonads. Collectively, my $ex \ vivo$ studies indicate that $\text{StAR}$ mRNA levels are
targeted in the gonad upon *ex vivo* exposure to DDE and DIEL, whereas hCG induction of *StAR* mRNA was targeted upon *ex vivo* exposure to MXC and TOX.

Based on extensive review of the literature, it is likely that the elevated levels of *StAR* transcript observed in the *ex vivo* cultures treated with DDE and DIEL are likely due to the disruption of signaling pathways involved in modulating the activity of *StAR* mRNA or protein. Future work analyzing StAR protein levels (phosphorylated and unphosphorylated) in *ex vivo*-treated tissues could help clarify if the protein levels are affected by the contaminants. Recent studies have shown that both DDE and DIEL can disrupt ERK1/2 signaling pathways pathways (195, 196), which play a role in controlling StAR protein activity (197, 198). If it is determined that StAR protein expression is also elevated, the use of MAPK/ERK inhibitors could help determine if MAPK pathways are directly targeted by DDE and DIEL, and if they mediate the effects on *StAR* mRNA levels observed in this study.

Because MXC and TOX disrupt hCG-induced activation of *StAR* mRNA in LMB gonads, it is likely that they disrupt pathways that are distal to *StAR* promoter activation. Although research on the modes of action of TOX is minimal, many studies on MXC and its metabolites have shown that LH receptor expression is significantly downregulated in granulosa cells treated with the contaminants (199). It is possible that MXC, and potentially TOX based on similar results from this study, alter the expression of gonadotropin receptors in the gonad or disrupt the binding of gonadotropins to receptors in the LMB gonad. Further work characterizing the expression of LH receptors in the gonads of LMB is warranted. qPCR analysis of the levels of LH receptor in gonad tissues exposed to MXC and TOX could yield insight into if these compounds are acting at the gonadotropin receptor level. If it is observed that LH receptor
expression is impaired, further experimentation would be warranted investigating the potential for the compounds to downregulate LH receptor activity and expression.

Altogether, the data presented and discussed throughout this dissertation provide valuable information on how the *StAR* gene is regulated by nuclear receptor signaling pathways in LMB and how this regulation may be evolutionarily conserved between higher and lower vertebrates. In addition, the characterization of the normal reproductive cycles of a subset of healthy wild LMB provided a baseline for gene expression profiles and how they vary throughout reproductive stages in healthy male and female LMB. This is important when one tries to assess the mechanisms through which EDCs, such as those present in Lake Apopka, act in disrupting circulating steroid hormone levels and potentially reproduction in LMB. The studies examining the tissue-specific response of LMB gonads to OCPs provides new information on how DDE, DIEL, TOX, and MXC function to disrupt *StAR* mRNA in LMB gonads, although further experiments are warranted in verifying the pathways suspected. Figure 6-1 is depicts the projected model of the regulation of the LMB *StAR* gene by nuclear receptor signaling pathways and by EDCs in the environment.
Figure 6-1: Figure depicting projected model of the regulation of StAR protein in LMB gonads. The interaction of LH/FSH with membrane receptors results in G-coupled protein receptors (G) activation, which activates adenyl cyclase (AC), catalyzing cAMP formation. Arachidonic acid (AA) is produced by phospholipase C (PLC); AA and cAMP activate PKA and PKC. PKA/PKC regulate transcriptional activity of the StAR promoter via transcription factors. Ca$^{2+}$ signaling has been shown to be involved in modulating LH/FSH-stimulated steroidogenesis. Epidermal growth factor (EGF), insulin-like growth factor (IGF), prolactin (PRL), and gonadotropin releasing hormone (GnRH) activate protein kinase cascades (Ras/Raf/others; MAPK/ERK1/2) and may function in regulating steroid biosynthesis. Red boxes outline projected targets of disruption by the OCPs studied in my work.
APPENDIX A
SUPPLEMENTARY DATA AND FIGURES

Miscellaneous figures: this appendix contains graphs and figures that, although were only briefly mentioned in the body of the text of this document, were excluded from the main body of this dissertation. Optimization of any scientific protocol is a very daunting task, and there are many points at which a researcher must assess the quality and integrity to ensure that a given experiment is running optimally.
Figure A-1: Representative scan of a 1% agarose/ethidium bromide gel confirming RNA integrity. For each experiment requiring the use of high quality purified RNA, following spectrophometric analysis to determine concentration, multiple samples were selected at random and electrophoresed on a 1% agarose/ethidium bromide gel. Approximately 0.5 µg of purified RNA was diluted in RNA loading dye and loaded per lane. The presence of RNA is confirmed by the two distinct bands (18S and 28S ribosomal RNA), and integrity and purity of the RNA is confirmed by the absence of smearing and extra banding.
Figure A-2: Verification of oligonucleotide annealing for EMSA. A 6% TBE DNA gel was run and stained with SYBR green to examine the results of the annealing reaction for EMSA.
Figure A-3: Verification of high transfection efficiency in Y-1 mouse adrenocortical cells transfected using FugeneHD and a GFP construct. (A) White light image and (B) fluorescent image captured of same field by microscope.
Figure A-4: Verification of optimal sonication of chromatin used in ChIP assays. A 1% agarose/TBE/ethidium bromide gel was run and the image of the gel was captured under UV light to ensure optimal shearing conditions for ChIP.
Figure A-5: Verification of high transfection efficiency in MA-10 mouse Leydig tumor cells transfected using FugeneHD and a GFP construct. (A) White light image and (B) fluorescent image captured of same field by microscope.
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BIOGRAPHICAL SKETCH

Melinda S. Prucha was born in the greater metropolitan area of Detroit, Michigan, USA. Her zest for science commenced early in her childhood as she attended grade school and quickly excelled in the physical sciences throughout middle and high school. Melinda graduated high school in the top 3% of her class in 1998, receiving departmental awards in both environmental sciences and in orchestra. Melinda, although presented with an opportunity to pursue musical studies, decided to pursue a career in the natural sciences. In order to fund her undergraduate education, she worked full-time as an assistant chief financial officer for a small business for several years while pursuing her Bachelor of Science degree in Environmental Health. In her final year, she performed duties as both a teaching and research assistant, and she graduated with honors from Oakland University in Rochester Hills, Michigan in May 2003. Immediately following her undergraduate degree, Melinda decided to continue her education and she moved to Gainesville, Florida where she began graduate studies in the Interdisciplinary Program in Biomedical Sciences in the College of Medicine at the University of Florida. She pursued course studies in the Department of Pharmacology and Therapeutics, and added on an additional specialization in Toxicology when she joined the laboratory of Dr. Nancy D. Denslow, where she completed her doctoral research. Working with Dr. Denslow, Melinda has furthered the understanding of the molecular signaling pathways involved in regulating the expression of key genes involved in steroid hormone biosynthesis in both fish and mammals. Melinda received her Ph.D. in Biomedical Sciences from University of Florida in August 2009 and will be continuing her training as a post-doctoral fellow under the direction of Dr. Nasser Chegini in the Department of Obstetrics and Gynecology in the College of Medicine at University of Florida.