The Influence of Estrogen Signaling on Male Reproduction in Medaka (*Oryzias latipes*)

by

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Environment in the Graduate School of Duke University

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ABSTRACT

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Abstract

Endocrine disrupting chemicals (EDCs) are ubiquitous and often act as xenoestrogens with the ability to disrupt estrogen signaling through differential binding to the various estrogen receptors. Exposure to these xenoestrogens has led to detrimental effects on male reproduction. In fish, observed effects include sex reversal, presence of testicular oocytes, altered courting behavior, vitellogenin synthesis in males, altered fertility and gonadal histopathology. Understanding how xenoestrogens exert their effects is complicated by the existence of multiple estrogen receptors (ESR1, ESR2a, ESR2b, and GPER), coupled with their ability for crosstalk and differential binding capability of selective estrogen receptor modulators (SERMS). Additionally, estrogen can signal through both classic genomic signaling and nongenomic signaling. Furthermore, the importance of estrogen signaling in normal male reproduction is just beginning to be understood. The primary goal of this dissertation was to assess the implications of aberrant estrogen signaling on male reproductive capacity, testicular morphology and gene expression changes in the small aquarium model fish, medaka, by investigating effects of a general estrogen receptor agonist, ethinylestradiol (EE2), and those of a G-protein estrogen receptor (GPER) specific agonist, G-1. This was assessed through breeding experiments, histological assessment of testicular morphology and microarray assessment of testicular gene expression changes following exposure to EE2 and G-1.
Finally, a comparison of altered testicular morphology between EE2 and G-1 induced changes was further assessed using a variety of histological techniques. The findings demonstrate that a 14-day exposure to EE2 impaired male reproductive capacity and altered testicular morphology and gene expression in a time- and dose-dependent manner. The testicular morphologic alterations observed include increased germ cell apoptosis, decreased germinal epithelium and thickening of the interstitium. These morphologic changes were highly associated with gene expression changes. A pathway analysis of the differentially expressed genes emphasized genes and pathways associated with apoptosis, cell proliferation, collagen production/extracellular matrix organization, and protein ubiquitination among others. Comparatively, a 14-day exposure to G-1 did not affect male reproductive capacity but did alter testicular morphology and gene expression. The histological analysis found an increased cellularity of the interstitium leading to thickened interstitium but no change in germinal epithelium. The microarray data indicate differential expression in genes most commonly involved in cell cycle, cell proliferation, apoptosis, transcription, translation, and ubiquitination. Finally, an assessment of the testicular histological phenotypes following EE2 and G-1 exposure indicate different morphologic changes led to thickened interstitium observed in the two exposures. In EE2 exposed fish, thickening of interstitium was associated with increased collagen deposition on the periphery of the organ while the interior thickening was primarily due to the collapse of intralobular
space associated with decreased germinal epithelium. In the G-1 exposed fish, the thickened interstitium was due to increased cellularity. A modest increase in cell proliferation was observed contributing to the increase in interstitial cells, however, it is also possible that there is a decrease in normal apoptosis and cell turnover as well. These findings highlight the importance of anchoring gene expression changes with morphology and ultimately proper tissue/organ function as well as the potential differences in effects that may occur with EDCs and SERMs.
Dedication

To my amazing husband, Javier, and parents, Doyle and Susan Thompson. Your unwavering support and encouragement mean more to me than words can say.
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1. Introduction

Undesired effects of endocrine disrupting chemicals (EDCs) on male fertility underscore need for understanding implications of estrogen signaling on male reproduction. Individual chemicals vary in their ability to disrupt estrogen signaling through direct binding with estrogen receptors (ESRs) leading to skepticism of findings and relevance of EDCs to the health of wildlife and humans. However, recent discoveries of ESR2 (ERβ) and selective estrogen receptor modulators (SERMs) may provide mechanistic underpinnings (O’Donnell et al. 2001). In addition, an abundance of data indicates nongenomic mechanisms of action of estrogens and ESRs as well. In nongenomic signaling, gene transcription occurs independent of ESRs directly binding to DNA but rather they proceed through protein-protein interactions leading to the modulation of transcription factors (Björnström & Sjöberg 2005, Sanchez et al. 2002, Acconcia & Kumar 2005, Daufeldt et al. 2003). Furthermore, a seven-transmembrane G protein-coupled receptor, GPER (formerly GPR30), was discovered to bind estrogen as its endogenous ligand likely mediating many of the nongenomic actions seen with estrogen signaling (Prossnitz et al. 2008a). These findings and the continued release of estrogenic compounds into our environment highlight the critical need to continue investigating the mechanistic actions of estrogen signaling and EDCs.

Estrogenic EDCs negatively impact male reproduction and impair reproductive organ development and function (Edwards et al. 2006, Hotchkiss et al. 2008).
Understanding the normal physiological role(s) of estrogen in the testis will be important to thoroughly understand the mechanisms of estrogenic EDCs. Recent data, largely from mammalian models, demonstrates estrogen to be a key endocrine component of spermatogenesis and testicular function (O'Donnell et al. 2001). Our ability to identify the functional role(s) of estrogen in the testis and determine how EDCs upset this delicate signaling balance are bolstered by 1) the discovery of ESR2 (ERβ), as well as differential cell localization compared to ESR1 (ERα) (Fasco 1998, Pfaffl et al. 2001, Hawkins et al. 2000); 2) the differential ligand binding affinity for each ESR subtype and distinct action (agonistic and antagonistic) of SERMs (Kuiper et al. 1997, Losel et al. 2003); 3) the nongenomic action of estrogen and ESRs regulating gene expression (Björnström & Sjöberg 2005, Sanchez et al. 2002, Acconcia & Kumar 2005, Daufeldt et al. 2003); 4) the discovery of GPER and its ability to mediate nongenomic estrogen signaling (Prossnitz et al. 2008a); 5) reproductive problems in ESR1, ESR2 and aromatase (CYP19) knockout mice (Hess 2003, Carreau et al. 2006); and 6) altered fertility associated with pharmaceutical and environmental chemicals acting as SERMs (Edwards et al. 2006, Cappon et al. 2004).

Regardless of source, the eventual fate of many EDCs is the aquatic environment (Campbell et al. 2006). Subsequently, fish are exposed to these environmental contaminants and effects have been seen in multiple wild populations (Aoki et al. 2010, Allen et al. 1999, Edwards et al. 2006, Harshbarger et al. 2000, Hinck et al. 2009, Jobling 2010, ...
et al. 1998, van Aerle et al. 2001, Viganò et al. 2001). With the recent molecular underpinnings of estrogen signaling discussed above and the molecular advances made in small fish models, such as sequenced genomes, these animal models are increasingly important for assessing effects of EDCs. Therefore, research in small aquarium fish models lends credence to the environmental implications of EDCs as well as relevant data for other vertebrate species.

1.1 Endocrine Disrupting Chemicals

Exogenous chemicals can disrupt the endocrine system leading to health effects in vertebrates (Guillette & Gunderson 2001, Edwards et al. 2006, Goksøyr 2006, Saradha & Mathur 2006). EDCs come from both synthetic and natural sources. Synthetic sources include plastics, detergents, cosmetics, flame retardants, herbicides, pesticides, drugs such as oral contraceptives, and other chemicals. They enter the environment through active application, runoff, and industrial and sewage discharges. Natural sources include human and animal hormones, phyto- and mycoestrogens, animal husbandry and livestock runoff and purposefully or unintentionally placed hormones in food and feed ingredients.

EDCs have been found in surface- and wastewater, sediment, groundwater, aquatic life and the atmosphere (Campbell et al. 2006). Many EDCs are released into the environment through surface non-point source runoff, such as agricultural land runoff, industrial waste discharge, wastewater treatment facility discharge, and atmospheric
deposition. However, the aquatic environment is the ultimate sink of EDCs (for reviews see Campbell et al. 2006, Scholz & Mayer 2008).

EDCs can alter endocrine signaling through the following mechanisms: 1) mimicking endogenous hormones by binding to receptors causing an agonistic effect, 2) blocking the binding sites of endogenous hormones inhibiting normal signaling and causing an antagonistic effect, 3) altering expression of receptors in cells, 4) altering steroidogenic enzymes involved in hormone synthesis and metabolism. Because these pathways are complex and interdependent, careful analysis and mechanistic understanding of EDCs is essential.

Observed effects of EDCs on wildlife, including fish, range from subtle and reversible to permanent including altered sex determination with feminized or masculinized organs, impaired reproduction and impaired sexual behavior (for review see Vos et al. 2000). While other modes of action of EDCs are currently being investigated, to date a majority of studies on EDCs have focused on xenoestrogens. Specific effects of xenoestrogens on fish have been well documented and include: sex reversal, intersex, altered courting behavior, induction of the egg yolk precursor protein vitellogenin in males, altered fertility in both male and female, altered spermatogenesis and oogenesis, and gonadal histopathology (Vos et al. 2000).
1.2 Estrogen Signaling

Our understanding of estrogen signaling has increased tremendously in the past decade. Estrogen production is part of a complex metabolic steroid synthesis pathway that begins with the precursor, cholesterol, and produces a variety of steroids. Ultimately, testosterone is converted to estradiol through the cytochrome P450 enzyme, aromatase (CYP19). Until 1995, ESR1, a nuclear receptor, was assumed to be the only estrogen receptor responsible for mediating all estrogen signaling. However, a second nuclear receptor, ERS2, was discovered in mammals (Kuiper et al. 1996). Subsequently, there have been two ESR2 genes described in teleosts, ESR2a and ERS2b, due to an apparent genome duplication event in teleosts (Amores et al. 1998, Halm et al. 2004, Menuet et al. 2002, Meyer & Van de Peer 2005, Pinto et al. 2005, Tchoudakova et al. 1999). Most recently a seven-transmembrane G protein-coupled receptor, GPER, was also determined to moderate estrogen signaling (Filardo et al. 2000). Furthermore, estrogen signaling can occur through multiple mechanisms including the “classical or genomic” pathway and “nongenomic” pathways (Cheskis et al. 2007).

“Classical” estrogen signaling occurs when estrogen binds to an ESR which then dimerizes with another estrogen bound ESR. The dimer pair then binds to the estrogen response elements (ERE) leading subsequently to transcription (Nilsson et al. 2001). However, other mechanisms of estrogen signaling are recognized including: 1) gene expression modulation independent of direct binding to DNA but through the
interaction of the estrogen activated ER with other transcription factors, 2) ligand independent ER activation through phosphorylation by other signaling pathways, and 3) rapid effects on signal transduction pathways, including second messenger production, ion channels, and protein kinase cascades (Edwards 2005, Nilsson et al. 2001).

While the mechanistic understanding of nongenomic estrogen signaling is evolving, research is also continuing to assess the physiological contribution of this steroid signaling and identify those receptors that mediate the response. GPER, considered an orphan receptor until recently, is activated by estrogen and mediates MAP kinase signaling through transactivation of the epidermal growth factor receptor (EGFR) (Filardo et al. 2000). Subsequent studies show estrogen-mediated GPER signaling to be involved in other cell signaling pathways as well, including PI3K/AKT, cAMP/PKA and Ca$^{2+}$ (Maggiolini & Picard 2010, Prossnitz et al. 2007). Ligand binding studies indicate that GPER has high affinity for 17β-estradiol but little affinity for estrone, estriol, and non-physiological estrogen such as 17α-estradiol as well as other steroids including progesterone, cortisol and testosterone (Prossnitz et al. 2008b). The synthetic estrogenic ligands tamoxifen and ICI 182 780 show substantial GPER binding affinity and activation while diethylstilbestrol (DES) does not. Phytoestrogens and xenoestrogens also differentially bind to GPER. For example, the phytoestrogen genistein, which has high binding affinity to ESR2, has high binding affinity for GPER
resulting in activation of MAPK. The phenolic estrogens, such as bisphenol A (BPA) and nonylphenol, which are weakly estrogenic, exhibit greater affinity for GPER compared to ESR1, while DDT, DDE and kepone show low affinity to GPER similar to that of ESR1. However, questions remain regarding GPER mediated nongenomic signaling, its physiological role and balance between it and all other potential estrogen signaling pathways.

1.3 Estrogen and Male Reproduction

Despite the critical role of spermatogenesis for species survival, significant gaps in our understanding of regulatory pathways exist. Recently, the importance of estrogen as a key regulatory endocrine component of spermatogenesis was established (for reviews see Akingbemi 2005, Carreau & Hess 2010, O’Donnell et al. 2001). However, details of molecular and physiological roles of estrogen signaling in male reproduction are still unknown. This makes it difficult to identify how EDCs can disrupt estrogen-influenced endocrine function leading to impaired reproduction. This is further complicated by the intricate hypothalamic-pituitary-gonadal (HPG) axis where signaling can also go awry.

Data indicates ESRs are localized to the testis of multiple vertebrate species, including medaka (Carreau & Hess 2010, Chakraborty et al. 2011, Schulz et al. 2010). However, varied localization of ESRs to specific cell types has been found. It is suggested that this is due to species variability as well as experimental technique
The majority of studies localizing ESRs and aromatase to the different testicular cell types have been carried out in rodents and primates, including humans (for reviews see Akingbemi 2005, Carreau & Hess 2010, O’Donnell et al. 2001). Overall, ESR2 is more abundant in the testis than ESR1. While there are discrepancies, most gene expression data indicates a potential role of estrogen in all the major cells types of the testis. Overall, ESR1 is primarily expressed in Leydig, Sertoli, myoid and efferent duct epithelial cells, while ESR2 is expressed in Sertoli cells and most prominently in germ cells. Aromatase is localized to all testicular cell types indicating the potential for estrogen production in all major cells of the testis. In the adult rat, aromatase levels in germ cells are equal or greater than that of both Leydig and Sertoli cells, suggesting an important role of estrogen production in germ cells (Carreau & Hess 2010, Levallet et al. 1998, O’Donnell et al. 2001). These findings indicate that the testis is capable of synthesizing and responding to estrogens and of importance for proper function. Furthermore, there is a far higher concentration of estrogen in the testis and rete testis compared to circulating male serum levels (Hess 2000). Additionally, aromatase activity in germ cells is equal to or higher than that of Leydig cells indicating, contrary to previous ideas, that germ cells may be contributing substantially to the high estrogen levels within the testis (Hess 2000, Carreau et al. 2001, Carreau et al. 2002). This local estrogen synthesis and response in germ cells suggests that paracrine and intracrine actions of estrogens may be important in male germ cell development.
From the above review, we see that the involvement and role of estrogen in the initiation and maintenance of testicular function and spermatogenesis may be due to the action of estrogen at multiple sites and organizational levels in the male reproductive system including the hypothalamic-pituitary-gonad (HPG) axis, and testicular cells (Leydig, Sertoli, germ and efferent duct epithelial cells) (for reviews see Carreau & Hess 2010, O'Donnell et al. 2001). The majority of studies assessing the physiological relevance of estrogen in the testis and reviewed here were performed in mammals unless otherwise specified. The involvement of androgen in controlling the release of gonadotropins from the pituitary through negative feedback thus balancing the HPG axis is well known. However, it is now clear that the aromatization of androgens to estrogens is a major component of this feedback action (Pitteloud et al. 2008, Hayes et al. 2001, Hayes et al. 2000, Hayes et al. 1998, O'Donnell et al. 2001). Additionally, during pubertal development in the rodent, studies have shown that neonatal exposure to estrogens or xenoestrogens can alter the organization of the HPG axis, an effect that can persist into adulthood (Atanassova et al. 2000, Sharpe et al. 1998, Tena-Sempere et al. 2000, Atanassova et al. 1999). In adult rats, estradiol exposure significantly decreased circulating levels of FSH and LH, which subsequently lead to reduced serum and testicular testosterone levels (O'Donnell et al. 2001). Interestingly, low doses of estradiol stimulated FSH in neonates and in adult hypogonadal mice indicate that estrogen can have both negative and positive effects on the male pituitary. This evidence indicates an
active role of estrogen in regulating the HPG axis and its ability to disrupt the feedback mechanism if signaling is aberrant.

Because Leydig cells are responsible for androgen production, they are considered the endocrine cells of the testis. Normal Leydig cell generation, differentiation and function are critical for male reproductive development and steroidogenesis during puberty and adulthood. Multiple studies indicate that estrogens act to control Leydig cells through inhibiting Leydig cell proliferation and development (for review see Abney 1999). Furthermore, there is evidence that estrogen is involved in the balance of Leydig cell steroidogenesis. Estrogen can inhibit steroidogenic enzymes required for testosterone synthesis, such as P450 17α-hydrolase/C17,20 lyase, in Leydig cells and thus decrease testosterone production (Onoda & Hall 1981, Saunders et al. 1997, Brinkmann et al. 1980). Estradiol has also been shown to inhibit testosterone production by altering Leydig cell LH receptors suggesting estrogen is involved in Leydig cell responsiveness to LH (Huhtaniemi et al. 1980, Vanbeurden et al. 1978). Interestingly, androgens have been shown to regulate Leydig cell production of estrogen sulfurylation and subsequently the inactivation of estrogens indicating that a delicate balance exists between these hormones (Qian & Song 1999). As Leydig cell function is important in male sexual development, testicular steroidogenesis and normal fertility, the demonstration of estrogen modulation of Leydig cells suggests that abnormal estrogen signaling may pose important consequences for male fertility.
As the “nurse cells” to male gametes, proper Sertoli cell function and signaling is essential for spermatogenesis; and furthermore are important for full spermatogenic potential in adult organisms. It is hypothesized that estrogen has a stimulatory effect on Sertoli cell division but an inhibitory effect on differentiation and development of Sertoli cells (O’Donnell et al. 2001). Estrogen production is high in proliferating Sertoli cells, particularly during development, and lower in differentiating Sertoli cells (Papadopoulos et al. 1986, Papadopoulos et al. 1993, Dorrington & Khan 1993). Near the end of proliferation, FSH-induced aromatase activity declines. This coincides with the decrease in estrogen production and mitotic activity of Sertoli cells (Dorrington et al. 1993, Dorrington & Khan 1993). Sertoli cell differentiation is stimulated by thyroid hormone, which also decreases aromatase activity in prepubertal Sertoli cells (Cooke et al. 1994, Bunick et al. 1994, Ulisse et al. 1994). Additionally, androgens from maturing Leydig cells may also help down-regulate aromatase levels during the switch from Sertoli cell mitosis to differentiation (Hardy et al. 1991, Verhoeven & Cailleau 1988b, Verhoeven & Cailleau 1988a). This is also the time that germ cells start to develop which may also contribute to decreasing aromatase activity in Sertoli cells (Lemagueresse & Jegou 1988).

Germ cell development is a series of highly controlled mitotic and meiotic divisions and differentiation, i.e., from immature spermatogonia to mature specialized elongated spermatid or spermatozoa. While it is well known that spermatogenesis is
dependent on FSH and testosterone, there is now evidence of a direct role of estrogen in mediating germ cell proliferation, viability and function (O’Donnell et al. 2001). Given the localization of aromatase, ESR1, ESR2 and GPER (discussed below) to Sertoli cells and germ cells, paracrine action of estrogen from Sertoli cells on germ cells as well as intracrine action of estrogen produced within germ cells is possible. In fish, estrogen has been shown to cause mitotic proliferation of testicular germ cells in Japanese huchen (Hucho perri), japanese eel (Anguilla japonical) and medaka (Amer et al. 2001, Miura et al. 1999, Song & Gutzeit 2003). This stimulatory effect of E2 on spermatogonial proliferation also occurs in amphibia and reptiles (Chieffi et al. 2000, Chieffi et al. 2001, Chieffi et al. 2002, Minucci et al. 1997). In vitro organ culture and primary cell culture of medaka testis found a proliferative effect on spermatogonia at low concentrations of EE2 but an inhibitory effect at high concentrations (Song & Gutzeit 2003). In testes of hypogonadal mice, congenitally deficient in GnRH and therefore lacking FSH and LH, spermatogenesis is arrested at the early stages of germ cell development. Administration of estradiol to these animals initiates all stages of spermatogenic cell development suggesting that estrogen is capable of inducing spermatogenesis (Ebling et al. 2000). However, this may have been due to an indirect effect of estrogen stimulating low levels of FSH. Overall, estrogen appears to play more of a proliferative, survival role in germ cells. It has a stimulatory effect on gonocyte proliferation, germ cell development (Li et al. 1997a, Ebling et al. 2000) and possibly a direct role in preventing
germ cell apoptosis (Pentikainen et al. 2000). A complete understanding of molecular signaling mechanisms and concentrations by which estrogen exerts these effects on germ cells has yet to be determined.

Transgenic knockout mouse models have helped reveal the importance of estrogen in male reproduction. The ERαKO (ESR1 knock-out) and ArKO (aromatase knock-out) mice show decremental fertility, while ERβKO (ESR2 knock-out) mice do not (O’Donnell et al. 2001, Akingbemi 2005). Surprisingly, the phenotype of each of these knockout animals is different. ERαKO mice show altered spermatogenesis primarily due to an indirect effect of defective absorption of the efferent ductules causing fluid build-up. ArKO mice show severe spermatogenic disruption including the presence of multinucleated cells, suggesting early disruption of spermatogenesis, degenerated round spermatids, a lack of elongated spermatids, and hypertrophy and hyperplasia of Leydig cells (Murata et al. 2002). This observation suggests that estrogen is important in spermatogenic cell development; however, ERβKO mice are fertile. Closer histological examinations of ERβKO animals show an increase in spermatogonia but no change in the composition of any other germ cell stage (Gould et al. 2007). There is also an increase in Leydig cells but a decrease in Leydig cell volume and no change in testosterone levels. The drastic differences in phenotypes and findings of the knockout animal models emphasize the complexity of estrogen signaling in male reproduction and the need for further investigation. It is important to consider the fact that the organization of the
HPG axis is likely disrupted in these animals. It is also important to note that the ERβKO animals have a deletion in the DNA binding region of ESR2 preventing it from directly binding to DNA. It has not been determined whether this deletion prevents other ESR2 signaling such as interaction with other transcription factors. Furthermore, the action of estrogen on germ cells through nongenomic mechanisms has not been ruled out.

Much work is needed to further understand the role of GPER and nongenomic signaling of estrogen in male reproduction and spermatogenesis. Recent preliminary studies have localized GPER to the testis and, in particular, to dividing spermatogonia in adults, potentially having utility as an indicator of spermatogonial cells committed toward meiotic division (Prossnitz et al. 2008a, Paduch et al. 2007). Two distinctive signaling pathways in the testis that have been shown to be activated with estrogen: ERK1/2 (extracellular signal-regulated protein kinases), a mitogen activated protein kinase (MAPK), and the PI3K/AKT pathway (Bouskine et al. 2008, Chieffi et al. 2002, Lee et al. 2007, Li et al. 1997a, Luconi et al. 2002, Moe-Behrens et al. 2003, Russo et al. 2005, Stabile et al. 2006, Vicini et al. 2006b). These pathways are commonly involved in nongenomic estrogen signaling and GPER signaling (Prossnitz et al. 2008). These signaling pathways mediate cellular activities such as proliferation, differentiation, growth, survival, cell cycle, transcription and translation (Katso et al. 2001, Murphy & Blenis 2006). Both ERK1/2 and PI3K/AKT signaling have been shown to mediate spermatogonial cell proliferation (Sette et al. 1999, Lee et al. 2007, Moe-Behrens et al.
2003, Inselman & Handel 2004, Dolci et al. 2001, Feng et al. 2000, Li et al. 1997b, Bouskine et al. 2008, Chieffi et al. 2002). However, the role, mechanism, and implications of nongenomic estrogen signaling and the possible mediation by GPER in the process of male reproduction and spermatogenesis need to be further explored and elucidated.

1.4 Medaka

Fish are at high risk for exposure to EDCs as the aquatic environment is the sink for contaminants. Effects of EDC exposure has been documented in multiple wild fish populations (Aoki et al. 2010, Allen et al. 1999, Harshbarger et al. 2000, Hinck et al. 2009, Jobling et al. 1998, van Aerle et al. 2001, Viganò et al. 2001). Furthermore, evidence shows that EDCs can cause long term reproductive effects leading to population decline in natural fish populations (Kidd et al. 2007, Palace et al. 2006). Additionally, sex determination in many fish is malleable.

Medaka is a small animal model fish commonly used for assessing EDCs. They spawn daily with embryos hatching in 7-10 days and sexual maturation by 3 months (Kinoshita et al. 2009). This short life cycle makes for a relatively cost-effective, efficient means of assessing EDCs. They are gonochoristic, genetically sex-determined fish with secondary sex characteristics. Furthermore, the medaka genome is sequenced allowing for methodical molecular evaluation (Kasahara et al. 2007). This makes medaka a valuable laboratory model for reproductive and molecular assessment of EDCs.
1.5 Structure of the Medaka Testis

1.5.1 Development

The medaka is genetically sex-determined in which the expression of the male sex-determining gene DMY/dmrt1bY is detected in XY somatic cells surrounding the primordial germ cells (PGCs) (Kobayashi et al. 2004, Matsuda 2005, Matsuda et al. 2002). Initially there are two populations of PGCs that develop on the left and right lateral sides of the hindgut. During stages 31-33, PGCs and gonadal somatic cells from both sides of the hindgut merge at the dorsal aspect of this structure forming a single gonadal primordium. At stages 33-35, the sox9b-expressing gonadal primordium is separated into left and right lobes (Nakamura et al. 2008, Kinoshita et al. 2009). This bilobed configuration, which is connected by the central efferent duct that runs centrally and longitudinally through the testis, persists through development and into the adult testis. From stage 35 on, germ cells develop in a sexually dimorphic pattern with DMY expressed in Sertoli cells in males (Kinoshita et al. 2009, Kobayashi et al. 2004). DMY is continually expressed in Sertoli cells throughout adulthood.

1.5.2 Adult Structure

The lobular and interstitial compartments are the two main constituents that make up the testis.
1.5.2.1 Lobular Compartment

The lobular compartment consists of Sertoli cells and germinal epithelium. The medaka testis has a restricted spermatogonial lobular structure meaning it is composed of lobules that blindly end at the periphery of the testis (Grier 1976, Grier 1981, Parenti & Grier 2004, Schulz et al. 2010). In fish with this testicular structure, spermatogonia are only present at the periphery of the lobule. Sertoli cells surround a single spermatogonia forming an isogenic cyst that undergoes synchronized development through spermatogenesis (Pudney 1995, Loir et al. 1995, Schulz et al. 2010, Nobrega et al. 2009).

There are few quantitative histological and stereological investigations of fish spermatogenesis, particularly of spermatogonia. In addition, there are currently no unique molecular markers of the various spermatogonial stem cells in fish to help identify cell types, although there are a few promising candidates (RET, OCT4, PLZF, GPR125, notch1) (Nobrega et al. 2009, Schulz et al. 2010). Leal et al. (2009) standardized a morphological classification of male germ cells using zebrafish to establish a categorization of spermatogonia in fish. Furthermore, they intentionally paralleled mammalian nomenclature to simplify comparative approaches. Two types of spermatogonia have been described: Type A (or primary) and Type B (or secondary). Type A spermatogonia are the largest cells of the spermatogenic process with a clear, large nucleus with distinct nucleoli, poorly differentiated cytoplasm with many nuages (Miura et al. 1999, Pudney 1995, Schulz et al. 2010, Leal et al. 2009, Johnson et al. 2009).
Type B spermatogonia are smaller in size compared to Type A spermatogonia and have a smaller, denser nucleus with a more prominent nuclear envelope and heterochromatin but continue to have distinct nucleoli (Miura et al. 1999, Pudney 1995, Schulz et al. 2010, Leal et al. 2009, Johnson et al. 2009).

In medaka, it has been estimated that type B spermatogonia divide clonally 9-10 times before entering meiosis (Shibata & Hamaguchi 1988). However, a detailed comprehensive study of spermatogonia in medaka has not been completed as it has in other fish species such as zebrafish, Nile tilapia and African catfish (Leal et al. 2009, Schulz et al. 2010, Schulz et al. 2005). Type B spermatogonia differentiate into primary spermatocytes at which point meiosis begins. Primary spermatocytes have a larger, rounder nucleus compared to type B spermatogonia with the chromosomes appearing as bold lines or dots throughout the nucleus (Leal et al. 2009, Schulz et al. 2010, Johnson et al. 2009). Secondary spermatocytes, resulting from meiosis I, are rare as they quickly enter meiosis II. They are smaller than primary spermatocytes and have a round nucleus with dense chromatin (Leal et al. 2009, Schulz et al. 2010). Meiosis II results in spermatids which have a highly condensed nucleus. These cells undergo spermiogenesis eliminating excess organelles and cytoplasm and form their flagellum developing into spermatozoa (Leal et al. 2009, Schulz et al. 2010, Johnson et al. 2009). In medaka, spermatogenesis from primary spermatocyte to spermatozoa takes 12 days (Nobrega et al. 2009, Nagahama 1983).
Sertoli cells are commonly differentiated from germ cells by their position toward the wall of lobules. They have a well-defined elongated or triangular nucleus and variably evident nucleoli (Johnson et al. 2009). Commonly, higher magnification with greater resolution is needed to definitively identify cells. Our light microscopic observations have been supported by electron microscopic observations (not shown). In teleosts, the number of Sertoli cells per cyst increases as germ cells divide in order to continue to support the increasing cyst volume and level off during meiosis (Leal et al. 2009, Schulz et al. 2005, Vilela et al. 2003, Sàbat et al. 2009, Schulz et al. 2010). Unfortunately, there is little medaka specific research in regard to Sertoli cell proliferation associated with spermatogenesis and cyst volume (Grier 1976). As spermatogenesis progresses, the cyst of germ cells is moved toward the central efferent duct of the testis. Mature sperm are released into the central efferent duct lumen and eventually exit through the urogenital cloaca (Kinoshita et al. 2009).

The literature regarding the fate of the Sertoli cells once they release their sperm into the efferent duct system is scarce. Studies speculate that after release of sperm, Sertoli cells may degenerate or become integrated into the efferent duct epithelium (Grier 1976, Leal et al. 2009). Perhaps lending credence to the latter fate, a close inspection of Sertoli cells with morphological characteristics of apoptosis were not observed in either tilapia or zebrafish (Leal et al. 2009, Schulz et al. 2005, Vilela et al. 2003, Sàbat et al. 2009, Schulz et al.). The theories regarding this lack of apoptosis
suggest a high level of phagocytic ability of surviving Sertoli cells to achieve the rapid removal of their apoptotic neighbors (Leal et al. 2009). Secondarily, it is thought that Sertoli cells establish the efferent duct epithelial lining (Leal et al. 2009, Schulz et al. 2005, Vilela et al. 2003, Sàbat et al. 2009, Schulz et al. 2010, Pudney 1995). It has been shown that medaka coexpress DMRT1, a putative transcription factor, and its homolog DMY, the medaka sex determining gene, in Sertoli cells (Kobayashi et al. 2004, Nakamoto et al.). This expression is also seen in the efferent duct epithelial cells, further indicating that in medaka Sertoli cells play a role in formation of the efferent duct system.

1.5.2.2 Interstitial Compartment

The interstitial compartment is readily distinguished by shape, location, and staining characteristics (more eosinophilic than the lobular contents). We have found this to be broad, more easily seen toward the central portion of the testis and distinguished by its eosinophilic staining. With higher resolution, specific cell types reside in the interstitium. These include Leydig cells, peritubular myoid cells, connective tissue, efferent duct epithelial cells, endothelium of blood vessels and blood cells (Koulish et al. 2002, Schulz et al. 2010, Lo Nostro et al. 2004, Grier 1981, Loir et al. 1995, Nobrega et al. 2009).

Leydig cells are usually found in small groups or clusters and have an increased density near the efferent duct system (Takahashi & Iwasaki 1973). Identifying characteristics include: their round shape, distinct cell boundaries, dense and round
nucleus, generally prominent nucleoli and prominent nuclear membrane (Loir et al.
 ultrastructure of their cytoplasm show many free ribosomes, smooth endoplasmic
 reticulum, numerous mitochondria with evident cristae. Lipid droplets can sometimes
 be seen but lipid negative cells are also observed (Grier et al. 1989). Leydig cells are
 considered the main stereodogenic cell of the testis. They express 3β-hydroxysteroid
dehydrogenase (3β-HSD), an important enzyme in the steroid synthesis pathway (Loir
 et al. 1995, Takahashi & Iwasaki 1973, Lo Nostro et al. 2004). This is frequently used as a
 biomarker of Leydig cells, which has also been demonstrated in Leydig cells of medaka
 (Takahashi & Iwasaki 1973).

Myoid cells line the lobules of the teleost testis but are not contiguous (Grier
 ultrastructure is similar to such cells of the mammalian testis. The teleost myoid cell
cytoplasm has fine smooth muscle-like microfilaments oriented parallel to the long axis
of the cell along with dense bodies associated with the plasma membrane. They have an
 elongated nucleus and cells are joined by focal contacts to collagen fibers. They have
 been suggested to have a role in collagen synthesis (Lo Nostro et al. 2004, Grier 1981,
 Grier et al. 1989). It is hypothesized that they form a contractile network and thereby
 help move spermatocysts toward the efferent duct system.
The efferent duct epithelial cells are columnar in shape (Grier et al. 1980, Pudney 1995, Sàbat et al. 2009). As discussed above, it is thought that the Sertoli cells release their sperm into the efferent duct system and become integrated into the duct system. The Sertoli cell will hypertrophy and slowly acquire a columnar shape (Pudney 1995, Sàbat et al. 2009).

1.6 Dissertation Objectives and Outline

Estrogenic chemicals are ubiquitous in the environment. Some of these chemicals have the ability to differentially interact with and bind ESRs and disrupt estrogen signaling. A variety of effects due to estrogenic EDCs have been observed in fish. However, the underlying molecular signaling and gene expression changes associated with the observed effects are not thoroughly understood.

The primary goal of this dissertation is to assess the implications of aberrant estrogen signaling on reproduction, testicular morphology and gene expression changes in medaka. As discussed above, there are multiple ESRs that moderate estrogen signaling through “classic” genomic signaling as well as nongenomic signaling. Both of these signaling mechanisms are important to maintain proper endocrine signaling and testicular physiology. The major goal of this work is to discern the differences in effects caused by a general estrogen receptor agonist versus those caused by nongenomic signaling through a GPER specific agonist.
This dissertation is organized into three research chapters that contribute to understanding the estrogenic effects on male reproduction through different estrogen signaling:

- In Chapter 2, I assessed the effects of ethinylestradiol (EE2), an environmentally relevant, general estrogen receptor agonist, on medaka reproduction, testicular morphology and gene expression through a medaka microarray.

- In Chapter 3, utilizing the same experimental design as in Chapter 2, I used G-1, a GPER specific agonist, to assess the implication of GPER mediated nongenomic signaling on medaka reproduction, testicular morphology and gene expression.

- In Chapter 4, I assessed the differences in the altered testicular histology following exposure to EE2 and G-1. Here I emphasized the interstitium of the testis.

Finally, the findings of this dissertation and their implications are summarized in Chapter 5.
2. Anchoring 17α-ethinylestradiol induced gene expression changes with testicular reproductive function and morphology in medaka

This is a collaborative effort between Hilary D. Miller, Bryan W. Clark, David E. Hinton, Andrew Whitehead, Stan Martin, and Seth W. Kullman

2.1 Introduction

Endocrine disrupting chemicals (EDCs) impair reproductive function in diverse wildlife populations (Guillette & Gunderson 2001, Hotchkiss et al. 2008). Because the eventual sink for many EDCs is the aquatic medium, fish have been frequently studied. Multiple wild fish populations with altered gonads, in particular testicular oocytes, have been observed around the world including: roach (Rutilus rutilus), gudgeon (Gobio gobio) and flounder (Platichthys flesus) from rivers in the United Kingdom, barbel (Barbus plebejus) from Italy, grey mullet (Mugil cephalus) in coastal waters of Japan and Korea, and shovelnose sturgeon (Scaphirhynchus platorynchus) from the Mississippi River and bass species from rivers within the United States (Aoki et al. 2010, Allen et al. 1999, Harshbarger et al. 2000, Hinck et al. 2009, Jobling et al. 1998, van Aerle et al. 2001, Viganò et al. 2001). Many of these gonadal alterations have been attributed to EDCs, which are often associated with agricultural and municipal effluents. In male fish exposed to estrogenic EDCs, gene expression is altered and reproductive physiology and morphology are subsequently impacted as evidenced by: synthesis of the yolk protein, vitellogenin; altered spermatogenesis; testicular fibrosis; development of testicular

Ethinylestradiol (EE2), the synthetic estrogen in oral contraceptives, is of particular interest from an environmental standpoint due to its high estrogenic potency and its detection in aquatic systems receiving sewage treatment water (Thorpe et al. 2003, Desbrow et al. 1998, Kuch & Ballschmiter 2000, Larsson et al. 1999, Tabak et al. 1981). A 7-year study in northwestern Ontario, Canada in which an entire lake was regularly dosed with environmentally relevant concentrations of EE2 (4.8-6.1 ng/L) for three years demonstrated impaired spermatogenesis and testicular oocytes in both pearl dace (*Margariscus margarita*) and fathead minnow (*Pimephales promelas*) populations (Kidd et al. 2007, Palace et al. 2006). Furthermore, there was a collapse of the fathead minnow population after the second year due to loss of young-of-the-year individuals (Kidd et al. 2007). Laboratory studies of model fish, such as zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*), have also shown decreased reproductive capacity following EE2 exposure (Santos et al. 2007, Seki et al. 2002).

Surprisingly, few studies have assessed global gonadal gene expression changes associated with EE2 exposure in fish. The majority of studies, for example, have targeted specific genes involved in steroid hormone biosynthesis or known estrogen-responsive genes (Filby et al. 2006, Filby et al. 2007, Islinger et al. 2003, Santos et al. 2007). The endocrine system is a complex, integrated system. Proper reproduction and steroid
hormone signaling is dependent on the hypothalamus-pituitary-gonadal (HPG) axis. The HPG axis is a feedback loop and imperative for proper regulation of endocrine signaling. EDCs can influence multiple points within the HPG axis (Ankley et al. 2009). However, in this study, we are specifically focused on overall gonadal gene expression to obtain a more complete understanding of how EDCs exert their effects at the organ level. The development of microarrays provides a powerful tool to assess potentially important changes in testicular gene expression following EE2 exposure.

The aim of this study was to further understand the molecular mechanisms of estrogen toxicity by associating testicular gene expression pathways with altered testicular morphology and reproductive function. To achieve this aim, we used a sub-chronic 14 day exposure to EE2 as our treatment regimen and followed with three assessments: (1) a breeding study to assess the reproductive effect of a 14 day exposure on the male fish, in which the histology of the testis was linked to fertilization success; (2) a histological examination of the testis on day 1, 7 and 14 of the EE2 exposure as well as after a 14 day depuration period following the exposure; and (3) testicular gene expression analysis through a medaka specific microarray on day 1, 7 and day 14 of the EE2 exposure.
2.2 Materials and Methods

2.2.1 Chemicals

EE₂ (98% purity, Fluka, St. Louis, MO) was used to prepare nominal stocks (0.004, 0.04, and 0.4 mg/ml) in dimethyl sulfoxide (DMSO). The stocks were stored at room temperature in the dark.

2.2.2 Medaka

Orange-red (OR) outbred-medaka fish (Oryzias latipes) were maintained at the Duke University Aquatic Research Facility under standard recirculating water conditions following approved animal care and maintenance protocols (Duke University Institutional Animal Care and Use Committee). Water temperature and pH were monitored daily and maintained at ~25°C and ~7.4, respectively, and broodstock were under a strict light:dark cycle of 16:8 hours. Dry food (Otohime B1, Reed Mariculture, Campbell, CA) was fed several times per day via automated feeders with once daily supplementation of newly-hatched Artemia nauplii. Adults reared under the above conditions were used in all aspects of this study including gene expression, histology and reproduction (see below).

2.2.3 Reproductive Assessment

This consisted of three time periods: pre-exposure, male exposure, and post-exposure. Throughout all three time-periods, egg production and fertilization rate were monitored.
2.2.3.1 Pre-exposure

Reproductively active, 5-6 month old adult medaka were placed randomly in breeding groups consisting of three females and one male (25 breeding groups total). Each breeding group was maintained in a 2-L glass beaker with 1800 mL of embryo rearing medium (ERM; Kirchen & West 1976). Daily renewal of 75% of the medium was performed. Each breeding group received approximately 2 mg/day dry food (Otohime, Reed Mariculture, Campbell, CA, USA) and artemia nauplii twice per day. Beakers were arranged randomly in isolation and maintained under a 16:8 light:dark cycle for 12 days. Eggs were collected and fecundity of each breeding group was established daily. Fertilization rate of each group was determined under a dissecting microscope according to procedures previously described (Iwamatsu 2004, Kinoshita et al. 2009). After the pre-exposure period, each breeding group was randomly assigned to one of three treatments groups.

2.2.3.2 Male Exposure

Each male was removed and placed individually in a designated 500-mL beaker for the male exposure period. Males were treated with 500 mL of spiked ERM with either DMSO (control) 0.001 µg/L EE2 or 10.0 µg/L EE2 for 14 days with a 50% renewal every 48 hours. Males were fed approximately 0.5 mg/day dry food and artemia nauplii daily and maintained under a 16:8 light:dark cycle. Throughout the male exposure, females were maintained in 2-L glass beakers with 1800 mL ERM as described in the
pre-exposure period. Fecundity and fertilization rates of females were monitored during this time.

2.2.3.3 Post-exposure

Individual males were returned to their original beaker for breeding to maintain consistency of breeding groups. For each breeding group, fecundity and fertilization rate were recorded on days 1-14, 17 and 20 of the post exposure period. Water conditions were maintained in the same manner as above, including the daily 75% water renewal. This design made it possible to relate morphology of testis to each individual’s fertilization success prior to and following exposure. On day 20 post-exposure of the breeding experiment, males were anesthetized in ice-cold ERM and the testis was removed and processed for paraffin based histological analysis as described below.

2.2.3.4 Statistics

One-way analysis of variance, followed by Tukey HSD post-hoc test was performed to assess treatment effects of EE2 exposures using JMP 8 (SAS Institute Inc.). The number of eggs produced/day, number of fertilized eggs/day and the fertilization percentage of eggs laid/day were used to analyze differences between treatment groups. All data in figures are presented as the mean ± SEM. \( P < 0.05 \) was considered significant.
2.2.4 Histological Analysis and Gene Expression

2.2.4.1 Exposures

Treatment exposures were completed in triplicate including DMSO (vehicle control, 1.0 µg/L EE2, and 10.0 µg/L EE2. Fish were sampled for histology and gene expression on days 1, 7 and 14 of exposure and for histology only on day 14 post-exposure depuration (recovery). For histologic analysis, 2-liter beaker replicates received 23 individuals each for exposures for the days 1, 7 and 14 time point and a separate set of beakers received 11 fish for the post-exposure day 14 depuration time point. For gene expression analysis, 5 male fish were placed in 2-liter beaker replicates for each treatment and sampling time point. ERM was spiked with the appropriate EE2 stock (0.0025% of total volume) and equally distributed between the 2-L beakers for a total of 2 L spiked ERM per beaker with a 50% renewal of spiked ERM every other day for 14 days. Following the 14 day exposure, fish for histologic analysis were placed into fresh ERM for 2 days and then moved into a flow-through system until time of sacrifice, i.e. 14 days post-exposure. The fish were maintained under a 16:8 hour light:dark cycle and fed *ad libitum* the dry diet as above on alternate days.

2.2.4.2 Histological Analysis

This was performed as follows: high resolution on control animals for general testicular anatomy and morphology; analysis of males on day 1, 7 and 14 of EE2 exposure and day 14 post-exposure depuration (recovery); and analysis of actively
breeding medaka following EE2 exposure as described above in the reproductive assessment.

2.2.4.3 High Resolution Methods

Adult male 6 month old fish collected from our colony were anesthetized in ice-cold ERM. The testis was removed and fixed in a cocktail of 0.5% glutaraldehyde, 2% paraformaldehyde, 1% sucrose and 1% CaCl₂ in Histochoice (Amresco, Solon, Ohio) for 24 hours at 4°C followed by immersion in Holt’s gum sucrose solution (1% aqueous gum arabic and 30% aqueous sucrose) at 4°C for ≥24 hours (Kong et al. 2008). Tissue samples were then embedded in glycol methacrylate, sectioned at 2.5 μm thickness, mounted on glass histological slides and stained with hematoxylin and eosin.

2.2.4.4 Paraffin Embedment

At each sampling time-point during exposure, 1-2 fish from each replicate beaker were anesthetized in ice-cold ERM, and the testis was collected for histology. Testes were fixed in 2% paraformaldehyde/phosphate buffered saline (PBS) for a minimum of 72 hr at 4°C and stored in 6% sucrose/PBS at 4°C until time of processing. Following dehydration in a graded series of ethanol (70% (x1), 85% (x1), 95% (x1) and 100% (x3) for 10 minutes each) and clearing in xylene (10 min x 3) tissues were embedded in paraffin (Paraplast® Plus, McCormick Scientific, St. Louis, MO). 5-μm thick, longitudinal serial sections were cut using a Microm HM 355 S microtome (Thermo Fisher Scientific,
Walldorf, Germany) through the entire organ, mounted on histological slides and stained with hematoxylin and eosin for analysis.

### 2.2.4.5 RNA Isolation

For gene expression, the testis of three fish per replicate beaker were removed as previously described (Volz et al. 2005, Volz et al. 2006), pooled and immediately frozen in liquid nitrogen for RNA isolation. In short, three pooled testes were homogenized with 1 ml RNA Bee (TelTest, Friendswood, Texas) using a Polytron homogenizer (Kinematica, Bohemia, New York) cleaned with RNaseZAP (Sigma, St. Louis, Missouri), diethylpyrocarbonate (DEPC) treated water, and sterile de-ionized water. Total RNA was purified from the homogenate using RNeasy Mini Kit (Qiagen, Valencia, California) followed by an on-column-digest with DNase to eliminate DNA contamination using RNase-free DNase Set according to manufacturer’s instructions (Qiagen, Valencia, California). The sample was then eluted with 30 µl RNase-free water (52°C). Total RNA samples were stored at -80°C. RNA concentrations were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California).

### 2.2.4.6 Array Production

Probes were produced by mining the medaka Ensembl Genome Browser using the biomart function for all annotated medaka genes based on the MEDAKA1 (October 2005) assembly provided by the National Institute of Genetics (NIG) and the University of Tokyo. This resulted in 15,207 predicted gene sequences. All medaka genes were
assigned homologs to the Human Ensembl Genebuild 36 (http://useast.ensembl.org/Homo_sapiens/Info/Index?db=core). Seventy-mer oligo probes were designed using the eArray portal with defined quality control parameters for both cross hybridization and base composition score. RNA was amplified using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent); and was annealed with a primer containing a polydT and a T7 polymerase promoter for reverse transcription and first and second strand cDNA synthesis. cRNA was produced using T7 RNA polymerase and incorporated cyanine-5 (Cy5) labeled CTP. The quality of the labeled cRNA was verified and concentration was measured spectrophotometrically. Control or experimental cRNA (0.75 µg) was hybridized to each array as a single channel hybridization. Hybridization was conducted on a custom 15K x 8 Agilent medaka array using the “In situ Hybridization Kit-Plus” (Agilent) at 60 °C for 17 h. The arrays were washed according to Agilent's SSPE wash protocol using a solution of 6× SSPE, 0.005% N-lauroylsarcosine, followed by a solution of 0.06× SSPE, 0.005% N-lauroylsarcosine, and Agilent's Stabilization and Drying Solution. The arrays were scanned on an Agilent G2565BA Microarray Scanner and data from the scans were compiled with Agilent Feature Extraction Software 8.1.

2.2.4.7 Microarray Statistical and Pathway Analysis

Analysis of the microarray data was performed using JMP Genomics 4.1(SAS Institute Inc, Cary, North Carolina). Data was log2 transformed during the import
process and normalized using the standard normalization routine implemented in JMP Genomics 4.1. A distribution analysis was conducted for quality control purposes prior- and subsequent to normalization and alignment of the overlay plots was used as an indicator of high quality data. Data analysis was performed by conducting a Principal components analysis (PCA) by day and treatment using time-matched treatment-to-control differences calculated from standard least-square mean. An ANOVA was performed to test for statistical differences between treatment and control groups on a day by day basis. The False Discovery Rate (FDR) at alpha 0.05 was used to account for the multiple testing problem. Hierarchical clustering was performed using the significant gene sets derived from the ANOVA analysis data set.

Genes for which differential expression was significant were further analyzed through the use of Ingenuity Pathway Analysis (IPA; Ingenuity® Systems, www.ingenuity.com). A data set containing significantly different genes based on our ANOVA analysis with their corresponding gene identifiers was uploaded into the application and used for molecular network and canonical pathway generation. Each identifier was mapped to its corresponding object in Ingenuity’s Knowledge Base and molecular networks were generated based on their connectivity. Canonical pathways most significant to the data set were identified, from Ingenuity Pathways Analysis library. The significance of the association between the data set and the canonical pathway was measured in 2 ways: 1) A ratio of the number of molecules from the data
set that map to the pathway divided by the total number of molecules that map to the canonical pathway; and 2) Fisher’s exact test, to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

2.3 Results

2.3.1 Reproductive Assessment

During the pre-exposure, all groups were reproductively active with no statistically significant difference in daily or total fecundity or fertility (p>0.05) (Figures 1-3). Upon removal of males, females continued to produce eggs but egg production was sporadic and greatly reduced through the entirety of the exposure period. All fertilization of eggs ceased 8 days following removal of males indicating residual sperm was retained in the breeding chambers following removal of males (Figure 3).

Following the male exposure and the designed return of males to their respective breeding chambers, there was a significant decrease in the total number of eggs/day in both EE2 treatments. While control beakers averaged 54.4 (±6.4) eggs/day (n=6), the 1.0 (n=6) and 10.0 (n=7) µg/L EE2 beakers averaged 29.2 (±6.9) and 19.7 (±3.8) total eggs/day respectively (Figure 1). Both the 1.0 and 10.0 µg/L EE2 treatments were significantly different from control (p<0.05) but not from each other (p=0.16). The number of fertilized eggs/day also exhibited a decrease with statistically significant differences between the control and EE2 exposed groups. The control breeding groups produced 50.4 (±7.0)
fertilized eggs/day while 1.0 and 10.0 µg/L EE2 groups produced 21.7 (±5.7) and 7.6 (±2.5) fertilized eggs/day, respectively (Figure 2). The fertilization rate of eggs laid/day exhibited a dose-response. The average percent of eggs fertilized/day for control, 1.0 and 10.0 µg/L EE2 was 91.3% (±4.4), 62.8% (±8.3) and 28.8% (±5.8), respectively (Figure 3). The 1.0 µg/L EE2 treatment group was significantly different from control and the 10.0 µg/L EE2 treatment group was significantly different from both control and 1.0 µg/L EE2. At the end of the post-exposure breeding period fertilization rates and fecundity remained at decreased levels suggesting recovery of reproductive capacity was not achieved during the time frame of our study.

2.3.2 Histology

Histological assessment of our control medaka demonstrates that the testis is bilobed with a restricted spermatogonial lobular structure composed of lobules that end blindly at the periphery of the organ. This is similar to testicular organization of medaka reported in the literature (Grier 1976, Grier 1981, Parenti & Grier 2004, Schulz et al. 2010). In fish with this testicular architecture, spermatogonia are only present at the periphery of the lobule. Based on our results and those described by others, Sertoli cells surround a single spermatogonia forming an isogenic cyst that undergoes synchronized development through spermatogenesis (Pudney 1995, Loir et al. 1995, Schulz et al. 2010, Nobrega et al. 2009). Further analysis revealed that the testis is divided into two compartments: the lobular compartment, consisting of germ cells and Sertoli cells, and

Our observations using high resolution approaches with plastic (GMA) embedment sectioned at 2.5 µm thickness confirmed and extended observations of routine paraffin embedment. In control animals, both in transverse and longitudinally (frontal) oriented sections, the testis was bilobed with each connected by a central efferent duct that ran centrally and longitudinally through the testis (Figure 4a and b). Under low magnification, the interstitium stained with a pronounced eosinophilia while germinal epithelium showed more prominent basophilic staining. As is shown in Figure 4a and b, the mature spermatozoa showed greatest amount of basophilia seen particularly well in the central efferent duct. Lumen of the lobules were nearly completely filled with rounded cells whose morphology was consistent with germinal epithelium. As the lobular lumens were followed centrally, they were occupied by cells whose nuclei stained extremely basophilic (i.e., dark purple). Curvy elongated eosinophilic structures marked the interstitium. This material provided sufficient resolution to identify specific compartments and cell types and spermatogonial stages in the testis.
Animals exposed to EE2 specifically for microscopic analysis of the testis demonstrated both time- and dose-dependent alterations in testicular structure including: thickening of the interstitial tissue, increased apoptosis of germ cells, and altered spermatogenesis as evidenced by decreased proportion of spermatocytes and spermatids in germinal epithelium (Figure 4C-F). Apoptotic germ cells were identified by their shrinkage, nuclear condensation, and fragmentation into spherical, membrane-bound bodies. The latter are often phagocytized by neighboring cells as previously described (Johnson et al. 2009). No change in testicular morphology was observed on day 1 of exposure. On day 7, one individual from 1.0 µg/L treatment group (n=3, 2 testes per sample for a total of 6 organs) exhibited thickening of the interstitial tissue and decreased spermatozoa. However, testes from the remaining five organs did not differ from controls (Figure 4C). The 10.0 µg/L treatment (n=3) after 7 days of exposure exhibited a similar thickening of the interstitial tissue as well as increased apoptosis of spermatocytes and spermatids (Figure 4D). At day 14 of exposure, all individuals in the 1.0 µg/L (n=3) treatment exhibited thickening of interstitial tissue and increased apoptotic spermatocytes and spermatids. In addition, one replicate also displayed a decrease in spermatozoa (Figure 4E). The 10.0 µg/L (n=3) treatment at day 14 displayed increased thickening of the interstitial tissue as well as altered spermatogenesis and a loss of germinal epithelium (Figure 4F). Following 14 days of depuration (recovery), individuals exposed to 1.0 µg/L EE2 (n=3) exhibited signs of recovery (Figure 4G). Two
of these replicates had an increased proportion of spermatocytes with concomitant decreased proportion of spermatozoa, indicative of initial resumption of spermatogenesis. However, the thickened interstitium persisted. The third replicate showed presence of testicular oocytes, thickened interstitium and many spermatozoa. Following 14 days of recovery, individuals exposed to 10.0 µg/L EE2 exhibited continued thickening of interstitial tissue, eosinophilic change in the interstitium (likely proteinaceous fluid), altered spermatogenesis, and a decrease in germinal epithelium (Figure 4H). Testicular oocytes were observed in one of the 10.0 µg/L replicates.

Testicular morphology of EE2 treated male breeders following the reproductive assessment demonstrated alterations similar to those of testes from males on day 14 of exposure. In some instances, however, further degeneration was observed in the testis of the breeding males versus the testis of males immediately following the 14 day EE2 exposure. The 1.0 µg/L EE2 breeding males (n=4) had altered germ cell epithelium and mild to severe thickening of the interstitium compared to controls (n=5). This ranged from an increase in intermediate stage germ cells (spermatocytes and spermatids) and a decrease in spermatozoa associated with mild to moderate thickening of the interstitium to a severe loss of germ cell epithelium which was associated with maximal thickening of the interstitium (Figure 5B & 5C). Testicular oocytes and karyomegalic germ cells were found in three of the four replicates. The gonad without testicular oocytes had severe depletion of the germ cell epithelium and severe thickening of the interstitium.
The 10.0 µg/L EE2 breeding males (n=6) showed more pronounced alterations than did those of the 1.0 µg/L EE2 males. All animals had a moderate to severe thickening of the interstitium which was also associated with a significant loss of germ cell epithelium (Figure 5D & 5E). Testicular oocytes and karyomegalic germ cells were present in four of the six gonads assessed. As seen in the 1.0 µg/L EE2 treated breeding animals, gonads without testicular oocytes had severe thickening of the interstitium and significant loss of germ cell epithelium.

2.3.3 Gene Expression

Significant differences in gene expression were observed between control and treatment groups at all time-points analyzed (Supplementary Data; Table A). Analysis of data sets for 1.0 µg/L EE2 and 10.0 µg/L EE2 exposures on days 1, 7, and 14 demonstrated time- and dose-dependent alterations. Time and dose response trends were evident in both PCA (Figure 6) and hierarchical clustering (Figure 7). Assessment of principal components demonstrated a clear temporal trend. The first principal component accounted for 47.7% of the data variability and was largely influenced by the strong temporal response in each EE2 treatment. After considering the temporal contribution to the variation in gene expression data, the second (16.3%) and third (13.8%) principal components appeared to be influenced by dose accounting for a combined 77.8% of the total data variability. Evident in both PCA and hierarchical clustering, gene expression alterations on days 1 and 7 appeared more similar than gene
expression changes on day 14. Within each day, dose effects are observed with the 10µg/L dose resulting in more pronounced alteration in gene expression levels and pattern. This was particularly evident in day 14.

Examination of data on a gene by gene basis demonstrates that relative to day one control there were 198 and 819 significant transcripts differentially expressed in 1.0 µg/L EE2 and 10.0 µg/L EE2 doses, respectively (Figure 8a and 8b). Of these responses 87 of the 198 transcripts were unique in the 1.0 µg/L EE2 dose and 448 of the 819 transcripts were unique in the 10.0 µg/L dose. Similar patterns were observed for days 7 and 14 with 391 total transcripts in the 1.0 µg/L EE2 dose on day 7 (200 unique), and 2365 transcripts in 1.0 µg/L EE2 dose on day 14 (2141 unique) (Figure 8a). The 10.0 µg/L EE2 dose on day 7 had 792 total transcripts (235 unique) and day 14 had 5306 total transcripts (4546 unique) (Figure 8b). Of the significantly expressed transcripts, there were 54 genes common to days 1, 7 and 14 in the 1.0 µg/L EE2 dose (Figure 8a) and 92 common genes expressed in the 10.0 µg/L dose (Figure 8b).

To assess the functionality of gene expression changes using IPA, gene lists representing the following differentially expressed transcripts were generated including: 1) genes common to all sampling days in the 1.0 µg/L treatment group (54 genes); 2) genes common to all sampling days in the 10.0 µg/L treatment group (92 genes); 3) genes unique to day 14 of exposure in the 1.0 µg/L treatment group (2141 genes); and 4) genes unique to day 14 of exposure in the 10.0 µg/L treatment group (4546 genes) (Figure 8a)
and b). The top gene networks for the significantly different genes common to all days in the 1.0 µg/L and 10.0 µg/L treatment groups and the top networks and canonical pathways for the significantly different genes unique to day 14 in the 1.0 µg/L and 10.0 µg/L treatment groups are listed in Table 1 and 2. The IPA networks for the genes unique to day 14 in the 10.0 µg/L treatment group appear the most different of any of the network assessments.

A comparison of the top 3 IPA molecular networks generated from differentially expressed genes common to all days in the 1.0 µg/L and 10.0 µg/L EE2 treatments indicate multiple network molecules identified in both treatments. Ontology of these network genes indicate involvement of angiogenesis and wound healing (EFEMP2, Fibrin, FYN, Histone H3, ID1, P38 MAPK, PDGF BB, Pka, Smad), apoptosis and cell death (CXXC5, FOLR1, ID1, NFκB complex, P38 MAPK, TGF beta, TGM2), cell adhesion (CDH5, TGM2), cell cycle and proliferation (CCND1, CD81, ERK1/2, MYOCD, PDGF BB, Pka, SOX9, Tgf beta), collagen (COL4A1, COL4A2), cytoskeletal organization (ACTA1, ACTG1, ACTT1, CALD1, EPB41, F actin, FLNC, PDGF BB, PDLIM3), hormone signaling (CYP19A1, FSH, GPER, Insulin, Lh, NR0B1, NR0B2, NR5A1, PTPRN), inflammatory response (BMP6, CXXC5, NFκB complex, P38 MAPK, Smad), male gonad function (BMP6, CCND1, NR0B1, NR0B2, NR5A1, Smad, SOX9), response to stimulus (GPC1, P38 MAPK, TULP1), telomere maintenance (Histone H4), transcription (ID1, VENTX), and undetermined (SLP1). A comparison of the top 5 IPA molecular networks generated
from differentially expressed genes unique on day 14 in the 1.0 µg/L and 10.0µg/L EE2 treatments also indicate multiple network molecules identified in both treatments. Gene ontology of these genes indicate involvement in glycolysis (DLAT, PDHA1, PDK2, PDK4), transcription and angiogenesis (RBM15), translation (EEF1B2), ubiquitination (DUB, USP30, USP37, USP46, WDR20), and undetermined (SURF2). It is important to bear in mind that while not all of the network genes are differentially expressed, the generated networks show relationships and connectivity between the genes suggesting involvement and possible perturbation of these signaling processes.

2.4 Discussion

The purpose of the present investigations was to achieve an integrated understanding of the effects of a 14 day exposure of EE2 on male reproductive parameters and to relate these to alterations in testicular structure. Our data indicate 1) fertility decreased in a dose dependent manner upon EE2 exposure, 2) morphologic alterations were time and dose dependent and included altered spermatogenesis and thickening of the interstitium, and 3) gene expression changes were time and dose dependent with IPA networks indicating involvement in multiple molecular pathways and signal transduction cascades.

Our reproductive assessment was designed to allow us to specifically assess male reproductive capacity without the compounding factor of female interaction. Fertility of adult males decreased in a dose dependent manner with fertilization rates of
62.8% and 28.8%, after exposure to 1.0 µg/L and 10.0 µg/L EE2, respectively. Other studies in medaka, zebrafish and fathead minnow that assessed effects of estradiol exposure on reproduction in fish also showed a decrease in male fertility (Balch et al. 2004, Foran et al. 2002, Hashimoto et al. 2009, Kang et al. 2001 Parrott, 2005 #541, Santos et al. 2007, Seki et al. 2002, Tilton et al. 2005, Weber et al. 2003, Xu et al. 2008). Interestingly, in addition to decreased fertility we observed that following reintroduction of males post-exposure to their unexposed female breeding group there was a decrease in the number of eggs laid per day in both treatments. The exact cause of this phenomenon is unknown, however, a previous study observed altered male courtship behavior and decreased copulation of EE2 exposed male medaka although no changes in female egg production was observed (Balch et al. 2004). Furthermore, the decreased egg production is unlikely due to stress on females by reintroduction of males to the breeding chamber because control breeding groups do not have decreased egg production post-exposure.

With the ability of EDCs to disrupt reproduction it is important that we understand the potential ecological implications. The reproductive impairments demonstrated in this study highlight the potential ecological impact of estrogen exposure at the population level. Whole lake exposure studies demonstrated ecotoxicological effect of EE2 on fish populations (Kidd et al. 2007, Palace et al. 2006). These environmentally relevant exposures of EE2 led to population collapse in multiple
fish species due to altered reproduction and the subsequent lack of young of the year needed to maintain the populations. Our study adds further weight to the male role in impaired reproduction by demonstrating that estrogen exposure compounded reproductive effects through decreased egg laying in females, possibly due to behavioral alterations as well as the decrease in fertilization rates.

The histologic analysis of testes from the exposure-only males found a decrease in early stage germ cells (spermatogonia, spermatocytes), an increase in apoptotic germ cells and a generalized thickening of the interstitium. Testicular function is highly dependent on a tightly controlled balance between cell proliferation and apoptosis. Our gene expression/IPA analysis suggests a perturbation of these important mitogenic and apoptotic signaling pathways throughout the EE2 exposure. Interestingly, an increased prevalence of differentially expressed genes within these pathways was associated with increased exposure dose and time. In the testicular morphology assessment, observations of decreased germ cells and increased apoptotic cells further suggest these signaling perturbations impacted the testis, particularly the germinal epithelium found in the lobular compartment of the testis. And like gene expression changes, altered morphology increased with exposure dose and time. Modest changes in spermatogenesis and the germinal epithelium are first observed in the 1.0 µg/L treatment group and moderate changes in 10.0 µg/L treatment group on day 7 including an increase in apoptotic germ cells. More advanced change was observed on day 14 of
exposure including severe alterations in the 10.0 µg/L group evident by continued germ cell apoptosis and decreased early stage germ cells. This pattern of expression and morphologic change is also observed with genes involved in angiogenesis, wound healing and collagen production and the histological observation of the thickening of the interstitium. Following the 14 day depuration period, the 1.0 µg/L treatment group showed signs of recovering in both the lobular and interstitial testicular compartments while the 10.0 µg/L treatment group continued to exhibit a worsening morphology.

Findings of the histologic assessment of the testis following the breeding experiment indicate similar alterations. However, these actively breeding males had an accentuated testicular morphology compared to their EE2 exposed, nonbreeding counterparts. Even the control breeding males had changes in the gonad including an increase in early stage germ cells and a slight thickening of the interstitium. This is likely due to an overall decreased volume of mature sperm indicating active reproduction. The testis from a single control male in the breeding experiment contained testicular oocytes, karyomegalic germ cells, and disorganization of the lobular space. While spermatogenesis was occurring in this animal (i.e., spermatozoa were produced), the altered morphology and general disorganization of the lobular space was significant. Surprisingly, the EE2 treated breeding males had a furthered morphologic change compared to males depurated for 14 days in the absence of females and stress of breeding. It is important to keep in mind that breeding males actively expelled mature
sperm, evident by successful fertilization, albeit decreased in the EE2 treated breeding males. Active spermatogenesis is needed to replenish spermatozoa reserves that fill the efferent duct space. We hypothesize that the active dispensing of spermatozoa from the breeding males plays a role in the appearance of accentuated thickened interstitium observed in both control and treated animals due to a decrease of late stage germ cells occupying lobule and efferent duct space. In the 1.0 µg/L treatment group, males allowed to depurate unaccompanied by females showed signs of recovery but males in the presence of females had the most significant testicular changes compared to all other sampling points of this treatment, including a further increase in interstitial thickening and a decrease in germinal epithelium. In the 10.0 µg/L treatment, following the depuration period there was no sign of recovery. However, in the presence of females there was further morphologic degeneration with, again, severe thickening of the interstitium, and significant depletion of germinal epithelium accompanied by altered spermatogenesis. Previous studies report similar findings to ours regarding altered testicular histology following estradiol exposure in fish, including fathead minnow, zebrafish and medaka (Balch et al. 2004, Kang et al. 2001, Seki et al. 2002, van der Ven et al. 2003, Xu et al. 2008). While the exposure time and doses vary, the histological alterations observed are similar to the changes reported here including altered spermatogenesis, testicular disorganization, testicular oocytes, thickening of the interstitium and fibrosis.
Findings from the reproductive assessments of estrogen exposed medaka breeding pairs also indicate impaired reproductive success (i.e. decreased mating behavior, fertilization and egg production) and associated altered testicular morphology including testicular oocytes and thickened interstitium (Balch et al. 2004, Kang et al. 2001, Seki et al. 2002, Tilton et al. 2005). These studies indicate that medaka hatchlings exposed during development may be more sensitive to estrogen than adults. Our findings regarding decreased reproductive success and altered morphology are in agreement with these prior findings.

It is likely that the altered testicular morphology and perturbation of proper cell signaling by EE2 are connected with altered gene expression. The IPA networks allowed us to identify functional testicular gene networks associated with the EE2 exposure. When comparing IPA networks for genes common to all days, multiple genes and molecules involved in hormone signaling and male reproduction were mapped to the networks including CYP19a (aromatase), FSH (follicle stimulating hormone), Insulin, LH (leutinizing hormone), NR0B1 (DAX1), NR0B2 (SHP), NR5A1 (SF1), SOX9. CYP19a, FSH, Insulin and LH are all integral to the endocrine system, proper Leydig and Sertoli cell function and subsequently reproduction (for reviews see Carreau & Hess 2010, Lu et al. 2005, O'Donnell et al. 2001, Schulz et al. 2010). NR0B1, NR0B2 and NR5A1 are members of the nuclear receptor family, important to Leydig and Sertoli cell function, and partner together as regulators of steroidogenesis through regulation of
steroidogenic regulatory protein (STAR) and biosynthesis of hormones (Luo et al. 1994, McCabe 2007, Niakan & McCabe 2005, Sekido & Lovell-Badge 2008). NR5A1 also helps regulate SOX9, essential in sex determination, gonadal development and Sertoli cell function (Sekido & Lovell-Badge 2008). There are many feedback mechanisms involved with hormone homeostasis and therefore it is anticipated that hormone signaling would be altered through the entirety of the EE2 exposure. In addition, hormone signaling is intimately related to reproduction and development and genes involved with male reproduction are identified in the IPA networks as well.

As previously mentioned, testicular function is dependent on tightly controlled cell proliferation and apoptosis. Interestingly, when comparing the IPA networks of significant genes common to all days in to 1.0 µg/L treatment to the IPA networks of significant genes common to all days in the 10.0 µg/L treatment, there were multiple genes associated with cell proliferation, cell cycle and apoptosis mapped to the networks in both treatments. In particular, genes of established signaling pathways that were noted in the IPA networks include ERK1/2 MAPK, p38 MAPK, NFκB, PDGF, TGFβ and AKT. These gene and their associated pathways are interdependent with cross-talk between pathways that are critical to coordinating cellular responses. Interestingly, KEGG (www.genome.jp/kegg/) indicates various combinations of these genes found together in multiple pathways but all are found in the MAPK pathway (map04010; http://www.genome.jp/kegg-bin/show_pathway?map04010). The MAPK and PI3K/Akt
ERK/MAPK pathway also appeared in the IPA assessment of significantly different genes unique to day 14 in both treatments. In the 10.0 µg/L treatment group, the ERK/MAPK pathway was the second ranked canonical pathway with multiple associated transcription factors differentially expressed including ESR, Ets, Stat1/3, Elk-1, c-Myc, and N-Myc. Of particular interest is the central role of MYC family of oncogenes in the top molecular network suggesting that the disruption of the MYC gene family has an important role in changes seen at day 14 in the 10.0 µg/L treatment group. MYC broadly influences genes important to functions such as cell proliferation, cell growth, apoptosis, differentiation, stem cell renewal, metabolism, ribosome biogenesis, protein synthesis and mitochondrial function, all of which are critical for proper testicular function (for review see Dang et al. 2006). The involvement of MYC-related pathways has proven to be complex, far reaching and vital for cell function, yet only superficially understood. Transcription targets of MYC or other transcription regulators with which MYC partners were differentially expressed in our study. They include: TERT, BRCA1, CDKs (cyclin dependent kinases), EIF4s (eukaryotic translation initiation factors; the top canonical pathway on day 14 in the 10.0 µg/L treatment assessment), EEFs (eukaryotic translation elongation factors), E2Fs (transcription factors), histone H4, histone deacetylases (HDACs), RPLs and RPSs (ribosomal protein large and small subunits), NCOR, NCL, APEX1, and DDX18 (Dang et al. 2006, Leone et al. 2001, Levens 2002, O'Donnell et al. 2005, Ruggero 2009, Wu et al. 1999, Zeller et al. 2003). MYC is
activated with various mitogenic signals including the MAPK/ERK and PI3K/AKT pathways (for review see Hann 2006), and proteins involved in these pathways have transcripts that are significantly different in multiple treatments and days in our study. The MYC family of genes is also important in stem cell biology including spermatogonial stem cell proliferation. Disruption of this can lead to significant problems with spermatogenesis (Braydich-Stolle et al. 2007, Ewen et al. 2010, Kieffer et al. 2010). An emphasis on MYC genes further suggests that the MAPK and PI3K/AKT pathways are important to the testicular effects seen from the EE2 treatment.

The second top molecular network of genes unique to day 14 in the 10.0 µg/L treatment group centers around G protein-coupled receptors (GPCRs). Many significantly different GPCRs are part of this network, including the G protein estrogen receptor (GPER). GPCRs are a large family of proteins important in multiple signal transduction pathways whose agonists include neurotransmitters, hormones, chemokines, and bioactive lipids (Goldsmith & Dhanasekaran 2007, Rozengurt 2007). Furthermore, cross-talk between GPCRs and EGFRs are important in regulating steroidogenesis (Evaul & Hammes 2008). Not surprisingly GPCRs are important regulators of cell survival, apoptosis, movement, proliferation, differentiation and growth. Additionally, all four classes of G proteins are able to regulate MAPK and PI3K/AKT pathways and subsequent transcription factors through multiple mechanisms of action (Goldsmith & Dhanasekaran 2007, Rozengurt 2007).
In the classical (genomic) estrogen signaling pathway, estrogen and estrogen like ligands bind to the nuclear estrogen receptors (ESR1 and/or ESR2a, ESR2b). Ligand bound estrogen receptors homodimerize and bind with canonical estrogen response elements (EREs) within regulatory regions of target genes. Subsequent recruitment of nuclear receptor co-regulators to the estrogen receptor complex facilitates chromatin acetylation/deacetylation and initiation/repression of gene transcription (Osborne & Schiff 2005). However, estrogen is also able to function through nongenomic signaling or membrane initiated steroid signaling (MISS) in which estrogen rapidly activates protein kinases (MAPK, PI3K, and PKC), adenylate cyclase, calcium and cAMP (Björnström & Sjöberg 2005). This has been shown to be regulated through membrane bound ESR and the aforementioned GPER (Hammes & Levin 2007, Prossnitz & Maggiolini 2009). Additionally, there has been extensive data, particularly in breast cancer research, demonstrating crosstalk between estrogen signaling and ErbB2/HER2/neu, a membrane tyrosine kinase epidermal growth factor receptor, regulating MAPK and AKT signaling (Arpino et al. 2008). ErbB2 is differentially regulated on day 14 in both the 1.0 and 10.0 µg/L treatments. Our gene expression data suggests perturbation of both genomic and nongenomic estrogen signaling networks.

One study with zebrafish is similar in design to the present study (Santos et al. 2007). A major difference, however, is the simultaneous exposure of males and females. Reproductive assessment of exposed animals revealed similar findings to ours including
a decrease in egg production, fertilization success and viable embryos in the 5 ng/L EE2 treatment. A histological analysis found that zebrafish testes exhibited all stages of spermatogenesis but further histologic detail was not given. However, a sperm quality analysis indicated decreased sperm quality in the EE2 exposed males. A microarray approach assessed effects on zebrafish gonadal gene expression and the associated reproductive impairment following exposure of 0.05, 0.5 and 5 ng/L EE2 to adult breeding colonies for 21 days. Transcriptional analysis of males indicated over-represented differentially expressed genes consistent with protein modification and metabolism. In particular, they underscored the differentially expressed genes found in the ubiquitin system. Our microarray analysis also found that ubiquitin genes were differentially regulated and identified in the IPA analysis of significant genes common to all days as well as IPA analysis of unique genes from day 14 in the 1.0 and 10.0 µg/L treatment groups. Additionally, we found more ubiquitin genes differentially regulated with time and dose. Ubiquitination is important in protein modification, metabolism and recycling (for reviews see Sutovsky 2003, Toshimori 2003). It is particularly important in maintaining normal apoptosis in the testis. In mammals, approximately 20-30% of theoretically expected germ cells mature to spermatozoa due to regulation spermatogonia density and removal of cells with chromosomal damage (de Rooij & Russell 2000, Leal et al. 2009). In fish, 60% of theoretical germ cells reach maturity (Leal et al. 2009). In fish, the stage at which the majority of apoptosis occurs is species
dependent. Additionally, during spermatogenesis chromatin rearrangement involving histones and protamines is essential for proper cell structure modification from large primordial germ cells to the minimized, efficient spermatozoa. Ubiquitin-dependent proteolysis is important in this cellular condensation (Baarends et al. 1999). Perturbation of this ubiquitin process has been implicated in the degradation of sperm quality (Sutovsky 2003). Santos et al. (Santos et al. 2007) suggest the EE2 induced alterations of gene expression profiles in the ubiquitin system and in glutathione peroxidase are likely mechanisms that led to changes in biological processes key to testis homeostasis. This subsequently caused lower sperm quality, and decreased fertilization success observed in the males. Similar to our work, this study finds altered gene expression connected to testis function and subsequently reduced fertility following EE2 exposure. However, since both males and females were exposed simultaneously in the Santos et al study, the male’s contribution to decreased fertility cannot be deciphered. For example, while one may infer that decreased in egg production was due to female impairment, it may have arisen by a combination of male and female effects. Our findings of decreased egg production after male only exposure signifies the role played by males in this alteration.

The goal of this study was to link reproductive impairments and altered testicular morphology induced by EE2 exposure in male medaka to testicular gene expression changes. Decreased fertility in both treatment groups was observed along with morphologic changes including thickened interstitium and altered
spermatogenesis. These alterations were both time and dose dependent with the most severe effects seen in the 10.0 µg/L EE2 treatment group on day 14. This treatment group also had a decreased capacity to recover from the EE2 exposure as well as significantly impaired reproduction. Pathway analysis of significantly different genes from the microarray suggests differential expression of genes involved in angiogenesis and wound healing, apoptosis, cell death, cell adhesion, cell cycle and proliferation, collagen, cytoskeletal organization, glycolysis, hormone signaling, inflammatory response, male gonad function, response to stimulus, telomere maintenance, transcription, translation, and ubiquitination.

In summary, we have demonstrated that EE2 exposure in male medaka impaired reproduction in previously verified, robustly reproducing males. Further, the physiological alterations were manifested in altered testicular morphology. Finally, analysis of testis specific gene expression and pathway analysis anchored the alterations in structure and function of this male reproductive organ. There is more to be done, especially in regard to a further detailed understanding related to the altered signaling pathway and subsequent sequence of events that led to an altered testis in exposed individuals. However, the groundwork is now established for an integrative understanding of the role of EE2 on testis structure and function.
Table 1: Top associated molecular networks function generated by IPA using the corresponding list of significant genes.

Significantly different genes listed in bold.

<table>
<thead>
<tr>
<th>Significant Gene List</th>
<th>Top Associated Molecular Network Functions</th>
<th>Network Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 ug/L EE2 common to all days</td>
<td>1. Embryonic Development, Organismal Development, Organ Development</td>
<td>ACTA1, ACTG1, Actin, Alpha actin, BMP6, CALD1, COL4A1, CYP19A1, EPB41, F ACTIN, Fibrin, FLNC, FSH, ID1, Insulin, Histone h3, Histone h4, Lh, MYOCD, NFκB (complex), NR0B1, NR0B2, NR5A1, PDLIM3, Pka, PTPRN, TAGLN, Tgf beta, TGM2, PDGF BB, SLPI, Smad, SOX9, TPM1, TROPOMYSIN,</td>
</tr>
<tr>
<td>1.0 ug/L EE2 common to all days</td>
<td>2. Connective Tissue Disorders, Genetic Disorder, Dermatological Diseases and Conditions</td>
<td>C1QL1, CD81, COL4A1, COL4A2, COL4A3, COL5A3, COL7A1, COL8A1, COL12A1, COL15A1, collagen, CRHR2, CTSS, CXXC5, EFEMP2, EGFR, EMID1, ERK1/2, FGF7, FN1, FOS, FYN, GPER, GPC1, GPR56, IFNG, ITGBB, LINGO1, MAN1A2, P38 MAPK, PTGER1, PAX3, SMAD9, ST8SIA2, TULP1</td>
</tr>
<tr>
<td>3. Lipid Metabolism, Liver Cholestasis, Molecular Transport</td>
<td></td>
<td>ABCB11, AFP, AKAP13, Cadherin (E,N,P,VE), C14orf156, CADM4, CAV1, CCND1, CDH5, CJB2, Ctnna, CTNNB1, CYP7B1, CYP8B1, CYP19A1, DYNC1LI1, ERBB3, ESR1, FHL1, FOLR1, GJB1, HNF4A, LDB1, NR0B1, PHB2, PKP2, PNPO, PTPRB, SLC10A1, SLP1, SOX9, SVIL, Thymidine Kinase, TYMS, VENTX</td>
</tr>
<tr>
<td>10.0 ug/L EE2 common to all days</td>
<td>1. Cancer, Reproductive System Disease, Drug Metabolism</td>
<td>ACTA1, ACTA2, ACTG1, Actin, Alpha actin, BMP6, CALD1, CXXC5, CYP17A1, CYP19A1, DTNA, EPB41, F Actin, FLNC, FSH, hCG, ID1, INHA, Lh, MCAM, MYOC, NR0B2, NR0B1, NR5A1, NR5A2, NFκB (complex), PDLIM3, PNPT1, PTPRN, RGS4, RNF7, RNA polymerase II, SLP1, Smad, SMAD7</td>
</tr>
<tr>
<td>1. Post-Translational Modification, Protein Degradation, Protein Synthesis</td>
<td>AHNAK, APBB2, ARIH1, CDC34, c-Src, EGFR, GOT1, GPM6B, GRLF1, MGRN1, MXI1, NDFIP2, PLRG1, PRCC, RNF13, RNF25, RNF46, RNF167, RNF181, RNF185, RUSC1, SCAMP3, SLC11A2, SLC22A18, SPARCL1, SPDEF, SPG20, SOX17, UBE2D1, UBE2D1B, UBE2H, UBE2J2, UBE2L3, UBE2N, UBE2V2</td>
<td></td>
</tr>
<tr>
<td>2. Lipid Metabolism, Nucleic Acid Metabolism, Small Molecule Biochemistry</td>
<td>ABC9, Aconitase, ACTN1, C12orf44, CSTF2, DLAT, EEF1B2, EEF1G, ERK1/2, FXN, JDP2, KCND2, KSR1, LONP1, MGAT3, MICALL1, MOBKL1B, P13A1, PDHA1, PDK2, PDK4, PLEK, RALGPS2, RBM15, SAMD4B, SLC12A4, STK38L, SURF2, VARS, VLDL, VRK2, WDR20, UCN3, USP46, YWHAB,</td>
<td></td>
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<tr>
<td>3. Post-Translational Modification, Gene Expression, Metabolic Disease</td>
<td>ARNT, ARNT2, BAG4, BST1, CALCOCO1, CDC371, CDKN2D, CRKL, DNAJC13, DNAJC16, DUB, ERBB2, Estrogen Receptor, HSD17B12, Hsp22/Hsp40/Hsp90, Hsp90, NKX6-2, P4HA2, PQLC1, SCG5, SELENBP1, SLELE, TBC1D17, TMEM43, UCHL1, USP4, USP7, USP30, USP37, USP45, USP47, WSB2, ZDHHC14, ZFHX3, ZFP36L1,</td>
<td></td>
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<tr>
<td>2. Cell Cycle, Cellular Development, Hematological System Development and Function</td>
<td>ADM2, beta estradiol, CCND1, CXCL14, DDAH1, EFEMP2, FGF2, FOLR2, FYN, GAB3, GPC1, GPCR, GPER, GPR101, GPR182, GPRC6A, HOXA3, HS3ST2, HS3ST3A1, NBEA, P2RY12, PPARA, retinoic acid, RIN3, RXFP4, SC5DL, SGTB, SRC, sulfotransferase, thyroid hormone, TP53, TRHR, TULP1, Ubiquitin, VENTX,</td>
<td></td>
</tr>
<tr>
<td>3. Embryonic Development, Tissue Morphology, Cardiovascular System Development and Function</td>
<td>ADCY, Akt, Ap1, CD81, CD151, CDH5, COL4A1, COL4A2, Collagen type IV, Creb, ERK1/2, FBLN5, Fibrin, FSHR, GDF6, Gpcr, Histone h3, Histone h4, HSF1, IL1, Insulin, LDL, LPL, Mapk, P38 MAPK, PDGF BB, Pka, PI3K complex, RXFP4, SORL1, SOX9, Tgf beta, TGM2, Vegf</td>
<td></td>
</tr>
</tbody>
</table>

1.0 ug/L EE2 unique to Day 14
<table>
<thead>
<tr>
<th>1. Cell Cycle, Connective Tissue Development and Function, Carbohydrate Metabolism</th>
<th>ANLN, CDR2, CEBPZ, CEP57, CHD5, CTPS, DDX18, DIAPH3, FANC1, GAMT, HEATR1, KLHL5, METTL13, MRPL12, MUM1, MXD4, MYC, MYCN, MYCT1, NCOA5, NOSIP, OMG, PLA1A, RPL5, RFX3, RPL13, RPS12, RPS23, SCAMP1, SCPEP1, SLC25A19, TPP2, WAC, XPO5, YME1L1</th>
</tr>
</thead>
<tbody>
<tr>
<td>2. Cell Signaling, Nutritional Disease, Psychological Disorders</td>
<td>BAI1, CELSR2, DRD5, FFAR2, FZD3, Gpcr, GPER, GPR1, GPR18, GPR22, GPR37L1, GPR75, GPR101, GPR125, GPR144, GPR146, GPR172A, GPRC5B, GRM6, MC4R, MCHR1, MTNR1A, NPBWR2, OPN1LW, OPN1SW, P2RY13, QRFPR, RXFP1, RXFP3, SCTR, SSTR5, TAAR5, TAS1R2, UTS2R</td>
</tr>
<tr>
<td>3. Cellular Assembly and Organization, DNA Replication, Recombination, and Repair, RNA Post-Transcriptional Modification</td>
<td>ABR, ACP6, AP2S1, BANF1, C2ORF18, CD2BP2, CDK5RAP3, DDX21, DHC7, DHX36, EWSR1, GMCL1, HMX3, IFT52, IFT55, IRE2B,IFT8B, KHK, KIFAP3, LEMD3, LMNA, PREX1, PRPF8, Rac, RANBP3, RPL35, ROR1, SDHB, SEPHS1, SMNDC1, SNRPA1, SRPRB, STAMBPL1, SUN2, TOR1A</td>
</tr>
<tr>
<td>10.0 ug/L EE2 unique to Day 14</td>
<td></td>
</tr>
<tr>
<td>4. Cell Cycle, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair</td>
<td>AIFM1, AKTIP, APEX2, AURKB, BRD4, C7orf25, CDCA8, CENPN, CYTSB, DLG5, EEF1B2, ESPL1, FRA10AC1, GNB2, HOOK2, IMM17A, INCENP, KIF20A, NCAF2, NPB1, PDS5B, P-TEFb, RAD21, RCN1, RIBC2, SMC2, STAG2, STAG3, TCP11L1, TIMM10, TIMM50, TOMM34, TOMM70A, TRIM37, VDAC2</td>
</tr>
<tr>
<td>---</td>
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</tr>
<tr>
<td>5. Post-Translational Modification, Lipid Metabolism, Nucleic Acid Metabolism</td>
<td>ACAP1, Akt, CRTAP, DHX8, DLAT, DLD, DUB, NAF1, PAIP1, PDHA1, PDK1, PDK2, PDK3, PDK4, PDP1, RBM15, SGK2, SIRT6, SNX27, SURF2, THEM4, UBXN1, USP1, USP12, USP24, USP30, USP37, USP38, USP40, USP46, USP48, UXS1, WDR20, WDR48, ZBTB2</td>
</tr>
</tbody>
</table>
Table 2: Top canonical pathways generated by IPA using the corresponding list of significant genes.

Significantly different genes listed in bold.

<table>
<thead>
<tr>
<th>Significant Gene List</th>
<th>Top Canonical Pathways</th>
<th>Significant Genes In Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 ug/L EE2 unique to Day 14</td>
<td>1. Leukocyte Extravasation Signaling</td>
<td>ACTN, CRK, GNAI, F-Actin, Fer, ITGA4, ITGB1, JAM2, JAM3, JNK, MEKK4, MMP, MMP9, NCF2, NCF4, NOX, PI3K, PKC, Rac1, RAPL, RhoGAP, SDF-1, TIMP, VASP, Vav</td>
</tr>
<tr>
<td></td>
<td>2. VDR/RXR Activation</td>
<td>CEBPB, CKII, Cyclin C, CYP24A1, GADD45A, HES1, HOXA10, NCOR2, PDGFα, PKC, PPARD, Runx2, RXR, SERPINB1, WT1</td>
</tr>
<tr>
<td></td>
<td>3. Production of Nitric Oxide and Reactive Oxygen Species in Macrophages</td>
<td>AKT, CAT, GP91, HoxA10, IκB, JAK, MEKK, P40phox, P67phox, PI3K, PKC, PKCβ, PP1, PP2A, PU.1, Rac1, Rho,</td>
</tr>
<tr>
<td></td>
<td>4. RAR Activation</td>
<td>AC, AKT, CDK7, CK2, COUP-TF, CRABP2, CSK, JNK, NIX1, PKA, PKC, PI3K p110, PNRC, Rac1, RALDH, RBP, RDH, RXR, SMAD, SMRT, TFIIH, TGF-β, TRUP, Vinexin,</td>
</tr>
<tr>
<td></td>
<td>5. Reelin Signaling in Neurons</td>
<td>ApoE, CRKL, Fe65, Integrinα3β1, JNK, LIS1, NudeL, PI3K, RhoGEF</td>
</tr>
<tr>
<td>10.0 ug/L EE2</td>
<td>1. Regulation of eIF4 and p70S6K Signaling</td>
<td>AKT, c-Raf, eIF3, eIF2, eIF4α, eIF4E, eIF4EBP, Integrin, MNK1, PAIP1, PI3K, PP2A, Ras, RPS6, SOS</td>
</tr>
<tr>
<td>unique to Day 14</td>
<td>2. ERK/MAPK Signaling</td>
<td>3. Protein Ubiquitination Pathway</td>
</tr>
<tr>
<td>------------------</td>
<td>------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td></td>
<td>14-3-3 (β, γ, η, θ, ζ), 4E-BP1, AFT-1, C3G, c-Myc, cPLA2, c-Raf, CREB, CRK, eIF4E, Elk-1, EPAC, ER, Ets, FAK, Integrin, KSR, MNK1/2, N-Myc, PKA, PP1, PP2A, PKC (α, β, γ, δ, ε, ι), SOS, STAT1/3, Talkin, VRK2</td>
<td>DUB, E2, E3 cofactor, E3 HECT, E3 RING, E4, HSP, MHC class I, PSMA2, PSMA6, PSMB7, PSMB10, PSME1, PSMID, TAP,</td>
</tr>
</tbody>
</table>
Figure 1: Number of eggs produced per day for medaka breeding groups during the pre- and post-exposure period following a 14 day exposure to EE2 period.

Number of eggs produced per day for medaka breeding groups during the pre- and post-exposure period following a 14 day exposure to EE2 period (mean ± SEM). No statistical difference was found during the pre-exposure period. One-way ANOVA of the post-exposure time period found significant differences between the control and treatment groups. Different letters indicate statistical difference in the post-exposure time period (p < 0.05).
Figure 2: Number of fertilized eggs produced per day for medaka breeding group following a 14 day exposure to EE2 during the 20 day post-exposure period.

Number of fertilized eggs produced per day for medaka breeding group following a 14 day exposure to EE2 during the 20 day post-exposure period (mean ± SEM). No statistical difference was found during the pre-exposure period. One-way ANOVA of the post-exposure time period found significant differences between the control and treatment groups. Different letters indicate statistical difference in the post-exposure time period (p < 0.05).
Figure 3: Percentage of eggs fertilized for medaka breeding groups during three periods: pre-exposure, male exposure, and post-exposure.

Percentage of eggs fertilized for medaka breeding groups during three periods: pre-exposure, male exposure, and post-exposure. Dashed lines indicate the mean percentage fertilized during the 20-day post-exposure period for each treatment. Different letters indicate a statistical difference (p < 0.05).
Figure 4: Sections of testis from 6 month old male medaka stained with H&E following EE2 exposure.

Sections of testis from 6 month old male medaka stained with H&E. A: Transverse section of testis showing both lobes joined by the central efferent duct (CED). Spermatogonial germ cells are at periphery of the organ. B: A longitudinal section of a DMSO control animal showing normal testicular morphology. Dark basophilic staining central region is comprised of spermatozoa filling lumen of efferent duct. As in A (above) spermatogonial cells are restricted to the periphery of the organ. Between the periphery and the CED various stages of spermatogenesis are encountered. C: Animal exposed to 1.0 µg/L EE2 for 7 days. When interstitium of animals from this group were compared to controls only one animal showed enhanced thickening of the interstitium and this was limited in extent. D: Animal exposed to 10.0 µg/L EE2 for 7 days. Thickened interstitium was apparent in a zone half way between the periphery and the CED. E: Animal exposed to 1.0 µg/L EE2 for 14 days. At this time, moderate thickening of the interstitium and increased area of clear space devoid of germ cells characterized the lobular lumen. There is a decrease in the proportion of intermediate staged germ cells as evident by the smaller area occupied by these cells (i.e. spermatocytes and spermatids) and a general decrease in germinal epithelium. F: Animal exposed to 10.0 µg/L EE2 for 14 days has a severe thickening of the interstitium, increased vacuolization, and an overall decrease in germinal epithelium. G: Animal exposed to 1.0 µg/L EE2 for 14 days followed by 14 days of recovery showed return toward control morphology. The thickening of the interstitium has diminished compared to E or F and there is an abundance of intermediate stage germ cells indicating active spermatogenesis. The CED does not have many spermatozoa but there are spermatids and spermatozoa in the efferent duct system preparing to enter the central duct. H: Animal exposed to 10.0 µg/L EE2 for 14 days followed by 14 days of recovery. There is continued thickening of the interstitium and significantly altered spermatogenesis. Only mature sperm and a few spermatogonia are present but intermediate stages of germ cells are absent or greatly reduced indicating little active spermatogenesis. There is also eosinophilic change in the interstitium.
Testis of medaka following a 14 day EE2 exposure followed by active breeding with 3 females for 20 days A: DMSO control male. B: Male exposed to 1.0 µg/L EE2. There is thickening of the interstitium, an increase in proportion of spermatocytes, minimal spermatozoa. C: Male exposed to 1.0 µg/L EE2. This organ has severe thickening of the interstitium with a focal area of basophilic cells center of field and a severe decrease in germinal epithelium. D: Male exposed to 10.0 µg/L EE2. Severe thickening of interstitium and severe loss of germinal epithelium are. There are, however, spermatocytes and spermatids present suggesting active spermatogenesis. E: Male exposed to 10.0 µg/L EE2. There is severe thickening of the interstitium with loss of germinal epithelium.
Principle components analysis based on significant genes from 1.0 µg/L EE2 treatment group (circles) and 10.0 µg/L EE2 treatment group (diamond) on day 1 (purple), day 7 (blue), and day 14 (green). The first principal component accounted for 47.7% of the data variability and was largely influenced by the strong temporal response in each EE2 treatment. The second (16.3%) and third (13.8%) principal components appeared to be influenced by dose accounting for a combined 77.8% of the total data variability.
Figure 7: Hierarchical cluster diagram of significantly different genes following exposure to EE2 in males.

Testicular gene expression pattern changes in male medaka following a 14-day exposure to EE2. Hierarchical cluster of significantly different genes based on mean fold change as determined by one-way ANOVA. Red indicates up regulation; blue indicates down regulation.
Figure 8: Venn diagrams of significantly different genes on day 1, day 7 and day 14 of EE2 exposure for A) the 1.0 µg/L EE2 treatment group and B) the 10.0 µg/L EE2 treatment group.

The 54 common genes in the 1.0 µg/L EE2 treatment group, the 92 common genes in the 10.0 µg/L EE2 treatment group, the 2141 genes unique to day 14 1.0 µg/L EE2 treatment group, and the 4546 unique genes to day 14 10.0 µg/L EE2 treatment group were used for Ingenuity Pathway Analysis.
3. Influence of GPER signaling on fertility, testicular morphology and gene expression changes in medaka

This is a collaborative effort between Hilary D. Miller, Andrew Whitehead, Stan Martin, David E. Hinton and Seth W. Kullman

3.1 Introduction

The physiological role of estrogens in male reproduction and the implications of aberrant estrogen signaling from endocrine disrupting chemicals (EDCs) have long been studied (for review see Edwards et al. 2006, Goksøyr 2006, Hotchkiss et al. 2008). While the majority of studies assessing estrogen signaling have focused on classic estrogen signaling, current data indicates nongenomic mechanisms of estrogens signaling must be considered as well (for review see Acconcia & Kumar 2005, Björnström & Sjöberg 2005, Sanchez et al. 2002).

In the classical (genomic) estrogen signaling pathway, estrogen and estrogen like ligands bind to the nuclear estrogen receptors (ESR1 and/or ESR2a, ESR2b). Ligand bound estrogen receptors homodimerize and bind with canonical estrogen response elements (EREs) within regulatory regions of target genes. Subsequent recruitment of nuclear receptor co-regulators to the estrogen receptor complex facilitates chromatin acetylation/deacetylation and initiation/repression of gene transcription (Osborne & Schiff 2005). Estrogen and some estrogen like compounds also function through activation of nongenomic (non-classical) mechanism involving membrane initiated steroid signaling (MISS). Activation of these pathways is mediated through purported
membrane associated ESRs, G-protein coupled receptors (GPCRs) and/or epidermal growth factor receptors (EGFRs) which rapidly activates protein kinases including ERK1/2 MAPK, PI3K, PKA and PKC (for review see Björnström & Sjöberg 2005). Initiation of such rapid signaling pathways modulates downstream transcription factors that facilitate gene expression regulation and pleiotropic effects.


GPER, formerly GPR30, is a GPCR that has been demonstrated to bind estrogen as an endogenous ligand and mediates nongenomic estrogen signaling (Filardo et al. 2002, Filardo & Thomas 2005, Prossnitz et al. 2008a). GPER activation leads to the following signal transductions: SRC-dependent release of heparin-bound epidermal growth factor (HB-EGF) and subsequent activation of EGFR and phosphorylation of ERK1/2; SRC-independent activation of EGFR and phosphorylation of PI3K/AKT; stimulation of adenylyl cyclase and subsequent increase in cAMP; and an increase in intracellular Ca^{2+} (for review see Maggiolini & Picard 2010). In the testis, estrogen (17β-
estradiol) bound GPER mediates nongenomic signaling pathways, through adenylyl cyclase stimulation and phosphorylation of ERK1/2 and Akt (Bouskine et al. 2008, Chimento et al. 2010a, Chimento et al. 2010b, Lucas et al. 2010, Sirianni et al. 2008).

GPER has also been localized to the testis in two fish species, croaker and zebrafish (Pang et al. 2007, Liu et al. 2009). And importantly in zebrafish, in situ hybridization followed by laser capture microdissection and RT-PCR localized GPER expression to spermatogonia, spermatocytes and somatic cells including Sertoli cells, but no expression was shown in haploid spermatids (Liu et al. 2009). However, data regarding GPER signaling and its function in the testis of fish is lacking.

Mammalian data on the role of GPER in the testis, although still incomplete, is more extensive. In vitro studies suggest an intricate balance between ESRs and GPER in regulating nongenomic estrogen signaling that is important to both cell proliferation and apoptosis. In germ cells, data indicate the ability of both ESR1 and GPER to moderate nongenomic estrogen signaling with subsequent effects on increased cell proliferation and decreased apoptosis, while ESR2 inhibits these effects. Using the JKT-1 cell line, derived from human testicular germ cell seminoma expressing GPER and ESR2, estrogen activates ERK1/2 and protein kinase A (PKA) (Bouskine et al. 2008). GPER activation resulted in cell proliferation while, estrogen binding to ESR2 in this cell line inhibited cell proliferation (Chevalier et al. 2011, Roger et al. 2005). Interestingly, in GC-1, an immortalized mouse spermatogonial cell line that expresses GPER and ESR1 but
not ESR2, the EGFR/ERK/cfos signaling cascade was rapidly activated through GPER and ESR1 crosstalk leading to cell proliferation (Sirianni et al. 2008). Primary cultures of rat pachytene spermatocytes and round spermatids expressing ESR1, ESR2 and GPER activated the EGFR/MAPK signaling cascade through each receptor independently (Chimento et al. 2010a, Chimento et al. 2010b). In the primary rat pachytene spermatocytes, independent stimulation of the ESR1 and GPER led to decreased cyclin A1 and B1 expression and increased BAX expression, which are associated with apoptosis (Chimento et al. 2010a). Rat primary round spermatids demonstrated similar results with independent activation of ESR1 and GPER that also led to decreased cyclin B1 and an increase in Bax while ESR2 activation had the opposite effect (Chimento et al. 2010b). These findings indicate that estrogen signaling involving all estrogen receptors, including GPER, contributes to the intricate balance between cell proliferation and apoptosis in testicular germ cells. Interestingly, there are similar findings in Sertoli cells.

An assessment of primary cultures of rat Sertoli cells, which express ESR1, ESR2 and GPER, indicate estrogen exposure induced ERK1/2 phosphorylation through ESRs and GPER (Lucas et al. 2010). Furthermore, ESRs mediated estradiol induced increases in cyclin D1, important in regulation of Sertoli cell proliferation while GPER mediated ERK1/2 phosphorylation through EGFR transactivation via $G_{i/\gamma}$ subunits that promoted SRC-mediated metalloprotease-dependent release of EGFR ligands. Subsequently, there was increased expression of the anti-apoptotic protein BCL2 and decreased expression
of pro-apoptotic protein BAX. This suggests that ESRs influence Sertoli cell proliferation whereas GPER play a role in antiapoptotic effects in Sertoli cells. Thus, the above makes it imperative that detailed studies are necessary if we are to interpret effects of various estrogenic EDCs on the testis.

Environmental estrogens can act as selective estrogen receptor modulators (SERMS) and may differentially bind to the various estrogen receptors (McDonnell et al. 2002, Paige et al. 1999, Bulayeva & Watson 2004, Watson et al. 2010). We now know that environmental estrogens can differentially bind both ESRs and GPER (Thomas & Dong 2006). However, the physiological role of GPER in male reproduction and spermatogenesis, and implications of environmental estrogens on GPER signaling and their subsequent effects on male reproduction constitute important gaps in our understanding.

In a previous study, we assessed male medaka reproductive function following a male-only 14-day exposure to ethinylestradiol (EE2) and found impaired reproduction with altered testicular morphology including: altered spermatogenesis, increased apoptotic germ cells and thickened interstitium (Chapter 2). Follow up microarray analysis during the 14-day EE2 exposure revealed differential expression of genes associated with cell proliferation and apoptosis including the ERK1/2 and PI3K/Akt pathways. Testicular function is highly dependent on tightly controlled balance between cell proliferation and apoptosis and this data suggests that estrogen induced
perturbation of these important mitogenic and apoptotic signaling pathways contributes to altered testicular morphology and impaired male reproduction. With data indicating a role for nongenomic estrogen signaling through GPER in these signaling pathways, we then sought to assess the implications of aberrant GPER signaling. Accordingly, we exposed adult male medaka to G-1, a GPER specific agonist, and subsequently found alterations in testicular morphology and gene expression changes.

### 3.2 Materials and Methods

#### 3.2.1 Chemicals

G-1 (98% purity, Cayman Chemical, Ann Arbor, Michigan)) was used to prepare nominal stocks (0.004, 0.04, and 0.4 mg/ml) in dimethyl sulfoxide (DMSO). The stocks were stored at -20°C in the dark.

#### 3.2.2 Medaka

Orange-red (OR) medaka fish were maintained at the Duke University Aquatic Research Facility under standard recirculating water conditions. Animal care and maintenance protocols, approved by the Duke University Institutional Animal Care and Use Committee (DUIACUC) were used. Water temperature and pH were monitored daily and maintained at ~25°C and ~7.4, respectively, and broodstock were maintained under a strict light:dark cycle of 16:8 hours. Dry food (Otohime B1, Reed Mariculture, Campbell, California) was fed several times per day via automated feeders with once daily supplementation of newly-hatched artemia nauplii. Adults reared under the above
conditions were used in all aspects of this study including gene expression, histology and reproduction (see below).

### 3.2.3 Predicted Medaka GPER Sequence Analysis

Ensembl Genome Browser ([http://www.ensemblgenomes.org/](http://www.ensemblgenomes.org/)) was used to mine the medaka genome databases for GPER. The predicted medaka GPER amino acid sequences were compared with GPER amino acid sequences of other species mined from Ensembl Genome Browser and GenBank (NCBI, [http://www.ncbi.nlm.nih.gov/genbank/](http://www.ncbi.nlm.nih.gov/genbank/)) including rat (*Rattus norvegicus*) (*ENSRNOP0000001732*), mouse (*Mus musculus*) (*ENSMUSP00000080370*), human (*ENSP00000380281*), Atlantic croaker (*Micropogonias undulates*) (GenBank: EU274298.1), zebrafish (*Danio rerio*) (*ENSDARP00000070486*), stickleback (*Gasterosteus aculeatus*) (GPER1of2, *ENSGACP00000001736*, GPER2of2, *ENSGACP000000018482*), medaka (GPER1of2, *ENSORLP00000006336*, GPER2of2, *ENSORLP00000009642*), tetraodon (*Tetraodon nigroviridis*) (GPER1of2, *ENSTNIP00000001979*, GPER2of2, *ENSTNIP000000021763*, *ENSTNIP000000039827*), fugu (*Takifugu rubripes*) (*ENSTRUP000000039827*). Multiple alignment of amino acid sequences were performed using ClusalW2 ([http://www.ebi.ac.uk/Tools/msa/clustalw2/](http://www.ebi.ac.uk/Tools/msa/clustalw2/)).
3.2.4 Breeding Experiment

This consisted of three time periods: pre-exposure, male exposure, and post-exposure. Egg production, fertilization rate, and hatching success were monitored for each period.

3.2.4.1 Pre-exposure

Procedures were the same as in Chapter 2. Briefly, reproductively active, 6 months old adult medaka were placed randomly in breeding groups consisting of three reproductively active females and one male (26 breeding groups total). Each breeding group was maintained in a 2-L glass beaker with 1800 mL of ERM, renewed by 75% daily. Fish were fed ad libitum a dry diet Otohime B1 aquarium feed (Reed Mariculture, Campbell, California) x1/day and freshly hatched artemia nauplii from lab culture x2/day. Beakers were arranged randomly in isolation and maintained at ~24°C on a 16:8 light:dark cycle for 8 days. Eggs were collected daily from each breeding, counted and fertilization rate determined under a dissecting microscope. After the pre-exposure period, each breeding group was randomly assigned to one of four treatments (DMSO control, 0.1 µg/L G-1, 1.0 µg/L G-1, or 10.0 µg/L G-1).

3.2.4.2 Male exposure

The male from each breeding group was removed and placed individually in designated 500-mL beakers for a 15 day exposure to G-1. During the exposure, the females were maintained as described above during the pre-exposure. Females were
continuously monitored for fecundity and fertilization rate in the absence of males. Each male exposure beaker was filled with 500 mL of ERM spiked with the appropriate treatment in the manner described previously. The males were fed the dry diet as above x1/day and freshly hatched artemia nauplii x2/day. The spiked water was renewed by 50% every other day.

3.2.4.3 Post-exposure

Following the 15-day exposure, males were returned to their appropriate beaker and conditions were maintained in the same manner as in the pre-exposure period. For each breeding group, egg production and fertilization rate were recorded for 14 days in the post-exposure period.

3.2.4.4 Statistics

One-way analysis of variance was performed to assess treatment effects using JMP 8 (SAS Institute Inc.). The number of eggs produced/day, the total number of eggs produced, number of fertilized eggs/day or the fertilization percentage of eggs laid/day were used to analyze differences between treatment groups. All data in figures are presented as the mean ± SEM. P < 0.05 was considered significant.
3.2.5 Histologic Analysis and Gene Expression

3.2.5.1 Exposures

In this separate study, four exposure groups were exposed as follows: DMSO vehicle control, 0.1 µg/L, 1.0 µg/L, and 10.0 µg/L G-1. Fish were sampled for histology on day 14 of exposure. For each treatment, the 2-liter beaker replicates received 7 individuals each for exposures for the day 14 time-point. Embryo rearing medium (ERM) was spiked with 75 µl of the appropriate stock (0.0025% of total volume). The spiked ERM was equally distributed between the 2-L beakers for a total of 1.5 L spiked ERM per beaker with a 50% renewal of spiked ERM every other. The fish were maintained under a 16:8 light:dark cycle and fed the dry diet x1/day and freshly hatched artemia nauplii from lab culture x2/day. At the end of the 14 day exposure, fish were anesthetized in ice-cold ERM for histology and gene expression analysis.

3.2.5.2 Histology

For each beaker replicate 2-3 fish were used for histological analysis. The testis was dissected and placed in a cocktail of 0.05% glutaraldehyde, 2% paraformaldehyde, 1% sucrose and 1% CaCl₂ in Histochoice (Amresco, Solon, Ohio) for ≥24 hr at 4°C and stored in Holt’s gum sucrose solution (30% aqueous sucrose and 1% aqueous gum arabic) at 4°C until paraffin embedment (Kong et al. 2008). Serial longitudinal sections of 5-µm thickness were made through the entirety of tissue, mounted on slides, cleared,
rehydrated and stained with hematoxylin and eosin for qualitative and morphometric analyses as below.

3.2.5.3 Morphometric Analysis

NIS Elements Basic Research (Nikon, Melville, NY) software was used for morphometric analysis. Four non-overlapping fields per testis were analyzed using a total of two sections/testis and two fields/section. Individual sections from an organ were a minimum of 20 µm apart and care was taken to ensure that sections used were midway through the testis, near the central efferent duct thereby avoiding natural thickening of the interstitium and efferent duct system occurring near the distal ends of the organ. Fields for analysis were selected by first establishing a field containing the peripheral edge of the testis at 200x magnification. Then, this field was maintained while the objective providing 400x magnification was moved into position and an image taken for analysis. A grid of intercept points 25 µm apart (108 intercept points total) was then overlaid on the image and each cell at each intercept point was categorized as germ- or non-germ cell. The point counts for all fields were then averaged and used for statistical analysis. To establish a solid baseline for controls, 3-4 testes/beaker replicate were analyzed. For the treatments, one randomly selected testis per replicate was analyzed.

The average percentage of non-germ cells per replicate was used to analyze differences between control and treatment groups. One-way analysis of variance followed by Dunnett’s test was performed to the assess treatment effects using JMP 8
(SAS Institute Inc., Cary, North Carolina). All data in figures are presented as the mean percentage ± SEM. \( P < 0.05 \) was considered significant.

### 3.2.5.4 RNA Isolation

Following the 14-day exposure, three testes per replicate beaker were removed (Volz et al. 2005), pooled and immediately frozen in liquid nitrogen for total RNA isolation as previously described (Volz et al. 2005, Volz et al. 2006). In short, three pooled testes were homogenized with 1 ml RNA Bee (TelTest, Friendwood, Texas) according to the manufacturer's protocol. Tissue was homogenized using a Polytron homogenizer (Kinematica, Bohemia, New York) cleaned with RNaseZAP (Sigma, St. Louis, Missouri), DEPC-treated water, and sterile de-ionized water. Total RNA samples were stored at -80°C. RNA quality was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California).

### 3.2.5.5 Array Production and Analysis

Probes were produced by mining the medaka Ensembl Genome using the biomart function for all annotated medaka genes based on the MEDAKA1 (October 2005) assembly provided by the National Institute of Genetics (NIG) and the University of Tokyo. This resulted in 15,207 predicted gene sequences. All medaka genes were assigned homologs to the Human Ensembl Genebuild 36 (http://useast.ensembl.org/Homo_sapiens/Info/Index?db=core). 70-mer oligo probes were designed using the eArray portal with defined quality control parameters for both
cross hybridization and base composition score. Testis RNA was amplified using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies, Santa Clara, California); and was annealed with a primer containing a polydT and a T7 polymerase promoter for reverse transcription and first and second strand cDNA synthesis. cRNA was produced using T7 RNA polymerase and incorporated cyanine-5 (Cy5) labeled CTP. The quality of the labeled cRNA was verified and concentration was measured spectrophotometrically. Control or experimental cRNA (0.75 µg) was hybridized to each array as a single channel hybridization. Hybridization was conducted on a custom Agilent 8x15K medaka array using the “In situ Hybridization Kit-Plus” (Agilent Technologies, Santa Clara, California) at 60 °C for 17 h. The arrays were washed according to Agilent's SSPE wash protocol using a solution of 6× SSPE, 0.005% N-lauroylsarcosine, followed by a solution of 0.06× SSPE, 0.005% N-lauroylsarcosine, and Agilent's Stabilization and Drying Solution. The arrays were scanned on an Agilent G2565BA Microarray Scanner and data from the scans were compiled with Agilent Feature Extraction Software 8.1.

Analysis of the microarray data was performed using JMP Genomics 5 (SAS Institute Inc, Cary, North Carolina). Data was log2 transformed during the import process. Normalization was done using the JMP Genomics 5 standard normalization routine. A distribution analysis was done for quality control purposes prior to and subsequent to normalization. A Principal components analysis (PCA) was performed by
treatment using treatment-to-control differences calculated from standard least-square mean. To test for statistical differences, a one-way ANOVA was performed with treatment used as the defining variable for the one-way classification. The False Discovery Rate (FDR) at alpha 0.05 was used to correct for multiple testing.

Genes for which differential expression was significant were further analyzed through the use of Ingenuity Pathway Analysis (IPA; Ingenuity® Systems, www.ingenuity.com). For each treatment, a data set containing all significantly different genes based on our one-way ANOVA analysis with their corresponding gene identifiers was uploaded into the application and used for molecular network and canonical pathway generation. Each identifier was mapped to its corresponding object in Ingenuity’s Knowledge Base and molecular networks were generated based on their connectivity. Canonical pathways most significant to the data set were identified, from Ingenuity Pathways Analysis library. The significance of the association between the data set and the canonical pathway was measured in 2 ways: 1) A ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway; and 2) Fisher’s exact test, to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.
3.3 Results

3.3.1 Predicted Medaka GPER Sequence Analysis

Ensembl Genome Browser (http://www.ensembl.org/index.html) was used to mine the medaka genome database. A search query for GPER identified two putative GPERs genes in medaka, GPER 1 of 2 and GPER 2 of 2. Analysis of gene organization and structure within the medaka genome demonstrated that GPER sequences represent two distinct gene paralogs each containing a defined loci, intron-exon boundaries and 5’ UTRs within the medaka genome consistent with gene duplicates found in this species (Howarth et al. 2008). mGPER 1 of 2 is located on medaka chromosome 8 position 8,225,078-8,226,391 (+ direction) and mGPER 2 of 2 is located on chromosome 19 position 8,234,754-8,236,052 (- direction). Each gene contained a single open reading frame encoding a putative protein sequence of 360 (mGPER 1 of 2) and 318 (mGPER 2 of 2) amino acids. An ATG initiation codon was only located in mGPER (1 of 2) and not identified for GPER 2 of 2. A TAG termination signal was not found in either predicted gene sequence. The predicted translation products of each GPER are shown in Figure 9.

Based upon putative sequence provided in Ensemble, both medaka GPERs were sequenced from medaka testis cDNA to confirm. Comparisons of the two medaka GPER protein sequences demonstrate minimal (56%) amino acid homology between each paralog. The mGPER 1 of 2 contains the aspartic acid, arginine and tyrosine or DRY region triplet sequence highly conserved in GPCRs, whereas mGPER 2 of 2 does not
contain the DRY sequence. However, both contain the conserved cysteine residues. Both
GPERs displayed a high degree of homology within the highly conserved 7tm_1 protein
domain (PF00001). Previous phylogenetic assessment of GPERs including mGPER 1 of 1
found a high homology and conservation of GPER in vertebrates with GPERs teleosts
examined grouping together in the phylogenetic tree (Liu et al. 2009). Our amino acid
comparison indicates higher amino acid between mGPER 1 of 2 and the mammalian
GPERs and croaker (Micropogonias undulates), zebrafish (Danio rerio), stickleback
(Gasterosteus aculeatus) (GPER 1 of 2) and tetraodon (Tetraodon nigroviridis) (GPER 1 of 2)
(Table 3). mGPER 2 of 2, on the other hand, has minimal homology with the
aforementioned but higher homology with stickleback (GPER 2 of 2), tetraodon (GPER 2
of 2), and fugu (Takifugu rubripes).

3.3.2 Breeding Experiment

During the pre-exposure, all groups were reproductively active with no
statistically significant intergroup difference in the number of eggs produced/day, or the
fertilization rate of eggs laid/day (p>0.05; Figure 10 and 11). The females continued to
produce eggs during the absence of the males throughout the exposure period but egg
production was sporadic and greatly reduced. During the post-exposure period, there
was also no difference in the average number of eggs laid/day in the treatment groups
compared to the DMSO control group. The DMSO control beakers averaged 35.7 (±1.1)
eggs/day (n=5), the 0.1 µg/L treatment group averaged 30.1 eggs/day (±4.7) (n=6), the 1.0
μg/L treatment group averaged 36.4 eggs/day (±4.2) (n=4) and 10.0 μg/L treatment group averaged 33.6 eggs/day (±3.9) (n=7) μg/L (Figure 10). The fertilization percentage of eggs/day also demonstrated no change from the DMSO control group during the post-exposure period (p>0.05). The average percent of eggs fertilized/day for control, 0.1, 1.0 and 10.0 μg/L treatment groups was 88.0% (±3.0), 85.5% (±6.4), 83.0% (±8.4) and 81.7% (±6.7), respectively (Figure 11).

3.3.3 Histology

Qualitative analysis of testicular histology demonstrated increased cellularity within the interstitial tissue (Figure 12). The principal interstitial cell types increased by G-1 exposure were the Sertoli and efferent duct epithelial cells associated with later stages of spermatogenesis, i.e., those associated mainly with spermatids and spermatozoa. Morphology suggested no alterations with respect to spermatogenesis. This response was seen in all of the treatment groups and appears to be due to an increased number of the Sertoli and efferent duct epithelial cells evident by increased nuclei observed. Point count morphometric analysis indicated an increase in area of non-germ cells (i.e. Sertoli cells, Leydig cells, efferent duct epithelial cells, peritubular myoid cells, connective tissue, endothelium of blood vessels and circulating blood cells). In the DMSO control group, an average of 22.9±0.65% (mean ±SEM; n=6) of intercepting points overlaid non-germ cells, the 0.1 μg/L G-1 treatment group averaged 31.4% (±3.5) (n=7), the 1.0 μg/L G-1 treatment group averaged 36.5% (±3.5) (n=7), and the 10.0 μg/L G-1
treatment group averaged 35.3% (±4.1) (n=6) (Figure 13). While the 0.1 µg/L G-1 treatment group exhibited an increase in non-germ cell area, it was not significantly different from the DMSO control group (p=0.2). However, both the 1.0 and 10.0 µg/L G-1 treatment groups were significantly different from controls (p<0.05).

### 3.3.4 Gene Expression

Analysis of our microarray data demonstrated that significant differences in gene expression occurred between the DMSO control and all treatment groups (Figure 14; Supplementary Data, Table B). Dose trends are evident in both PCA (Figure 15), and hierarchical clustering (Figure 16). PCA indicated the first principal component accounted for 15.47% of the variability and was influenced by dose, while the second principle component accounted for 12.7% and the third principal component accounted for 10.6%. Interestingly, the 0.1 µg/L and 10.0 µg/L treatment groups cluster together away from the 1.0 µg/L treatment. One possibility is a suggesting a U-shaped dose response. Initial quality assessment of microarray data indicates that the 1.0 µg/L treatment group is acceptable; however, further quality assessment is needed to determine if dose-response is accurate. There were 432 differentially expressed genes in the 0.1 µg/L treatment group, 192 in the 1.0 µg/L treatment group, and 595 in the 10.0 µg/L treatment group (Figure 17).

To assess the functionality of gene expression changes using IPA, gene lists containing all significantly different genes for each treatment were generated for
assessment. The top five molecular networks and canonical pathways and their
associated genes for the treatment groups are shown in Tables 4 and 5. Genes or gene
families identified in at least one of the top networks in each treatment included beta
actin (ACTB), ATPase (ATP), eukaryotic translation elongation factor (EEF), eukaryotic
translation initiation factor (EIF), extracellular signal-regulated kinase 1/2 (ERK1/2),
human chorionic gonadotropin (hCG), histone, high mobility group-box (HMGB1),
interferon alpha/beta/gamma (IFNα/β/γ), mitochondrial encoded cytochrome c oxidase
II (MT-CO2), nuclear factor κB (NFKB), proteosome subunit (PSM), ribonucleotide
reductase (Rnr), ribosomal protein LP0 (RpLP0), ribosomal protein S (RPS), telomerase
reverse transcriptase (TERT), ubiquitin-conjugating enzyme (UBE), ubiquitin specific
peptidase (USP), exportin 1 (XPO1), zinc finger protein (ZFP) (Table 4). These genes
function in apoptosis, cell cycle and proliferation, chromatin organization, cytoskeletal
organization, steroid signaling, transcription, translation, transport, and ubiquitination.
It is important to bear in mind that while not all of the network genes are differentially
expressed, the generated networks show a relationship and connectivity between the
genes suggesting involvement and possible perturbation of these signaling processes.
Interestingly, significant genes common to all treatments (Table 5) were involved in
similar functions including: apoptosis, cell cycle, cell proliferation, cell migration,
chromatin modification, cytoskeletal organization, DNA repair metabolism,
transcription, translation, transport.
3.4 Discussion

In the present study, we investigated the role of GPER signaling in the testis. Our results demonstrated that these ranges of G-1 exposure did not impact the overall adult male reproductive capacity but did result in altered testicular morphology and testicular gene expression.

Our analysis of medaka GPER indicates two putative genes mapped to distinct genomic locations with unique exon-intron organization. Identification and comparison of multiple GPERs from other teleosts suggests paralogous origins from a gene duplication event prior to teleost speciation. As previously reported, numerous teleosts, including medaka, have multiple copies of single-copy mammalian genes (Howarth et al. 2008, Postlethwait 2007). In medaka and the other teleosts assessed, GPER 1 of 2 has higher homology to the mammalian GPER than that of GPER 2 of 2. While both GPER 1 of 2 and GPER 2 of 2 contain the conserved cysteine residues, only GPER 1 of 2 contains the highly conserved DRY region characteristic of GPCRs. A functional assessment of GPER 2 of 2 is needed to confirm its signaling capability.

Our morphological investigation indicates an increase in Sertoli cells and efferent duct epithelial cells following the 14 day G-1 exposure. Sertoli cell function in the testis is a delicate balance between proliferation and apoptosis in medaka (Leal et al. 2009, Pudney 1995, Sàbat et al. 2009, Schulz et al. 2005, Schulz et al. 2010, Vilela et al. 2003). In the medaka testis, following the release of mature germ cells into the efferent duct the
Sertoli cell transition into efferent duct epithelial cells (Leal et al. 2009, Schulz et al. 2005, Vilela et al. 2003, Sàbat et al. 2009, Schulz et al. 2010, Pudney 1995, Kobayashi et al. 2004, Nakamoto et al.) Sertoli cells have a very high phagocytotic activity and remove other apoptotic Sertoli cells quickly (Leal et al. 2009). Disruption of this process could account for the increased cellularity of these cells. With no inhibitory changes in spermatogenesis and the germinal epithelium observed, it is not surprising that reproductive function remained intact following the G-1 exposure.

Our microarray data indicate differential expression in genes most commonly involved in apoptosis, cell cycle, cell proliferation, cell migration, chromatin modification, cytoskeletal organization, DNA repair, metabolism, transcription, translation, transport and ubiquitination. This can be seen in the significantly different genes common to all treatments, and in the genes associated with the molecular networks as well as the canonical pathways (Table 4 and 5). Research shows that the ERK1/2 and AKT signaling pathways are central to GPER signaling. Both of these pathways have a presence in the molecular networks and canonical pathways analysis indicating involvement and/or signaling perturbation of these genes and pathways. AKT was also significantly different in the 10.0 µg/L treatment group. Genes and signaling pathways involved in cell proliferation and apoptosis are strongly emphasized in the pathways analysis, which both ERK1/2 and AKT play important roles in these biological functions. It is important to remember that nongenomic estrogen signaling is a fast
protein phosphorylation process while our gene expression data is following a 14 day exposure. Therefore the gene expression is likely a reflection of long term stimulation of these pathways and/or representative of the morphologic changes induced by long term G1 exposures as opposed to short term signaling that is frequently used to assess GPER signaling.

Interestingly, NFκβ is found as a central point to one of the top molecular pathways in each treatment, although not significantly different. NFκβ signaling is regulated by multiple mechanisms including: cytokines such as TNF-α and IL-1; a number of growth factors including platelet derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF) and insulin like growth factors (IGFs); the cAMP/protein kinase A (PKA) pathway; and steroid hormones including estrogen (for review see Delfino & Walker 1999, Schmitz et al. 2004). Many of these growth factors are important in GPER signaling. They are also present in many of the molecular networks and canonical pathways from our study. NFκβ is a transcription factor that contributes to cell proliferation, development and apoptosis and has a high connectivity to other signaling pathways (Schmitz et al. 2004). It also plays a role in testicular function through involvement in cell proliferation and apoptosis (Choo et al. 2011, Starace et al. 2005, Delfino & Walker 1999, Vaithinathan et al. 2010). With the many common pathways found between GPER and NFκβ it may be interesting to further investigate the interactions of these signaling mechanisms.
Understanding how GPER signaling cooperates with ESRs and the other signaling networks is going to be important for future assessments of SERMs. Few studies have assessed the potential signaling ability of SERMs in relation to GPER activity. An *in vitro* GPER binding assay found relatively high binding affinities for genistein, bisphenol A, nonylphenol and Kepone (Thomas & Dong 2006). Additionally, these SERMs had similar ability as E2 to up-regulate adenylyl cyclase activity via GPER. An assessment of xenoestrogens on JKT-1 cells, a human testicular pure seminoma cell line expressing aromatase, ERS2 and GPER but not ESR1, found that bisphenol A (BPA) stimulated cell proliferation by PKA pathway activation and subsequent phosphorylation of the transcription factor CREB through GPER (Bouskine et al. 2008, Bouskine et al. 2009, Chevalier et al. 2011). Interestingly, the JKT-1 cell proliferation dose-response curve for BPA had an inverse U-shape. This lends credence to the potential hormetic dose response observed in the gene expression data. Furthermore, in this cell line E2 signaling alone had a suppressive effect on cell proliferation due the presence ESR2. However, BPA, which has 8-50 times higher binding affinity for GPER than for ESRs, induced a proliferative effect due to GPER signaling. These findings demonstrate the complex nature of estrogen signaling and the potential ability of SERMs to interfere with the proper balance of this signaling through aberrant GPER activation.

Our study is the first to assess the implications of targeted GPER activation *in vivo* finding a response in testicular morphology and associated testicular gene
expression changes. It is unclear from this study whether the increased cellularity of the interstitium is due to an increased proliferation of Sertoli cells/efferent duct epithelial cells or due to a decrease in apoptosis of these cells. However, previous *in vitro* work on primary cultures of rat Sertoli cells indicate an anti-apoptotic role for GPER and our gene expression data indicates involvement in cell proliferation, cell cycle and apoptosis. As previously stated, in medaka Sertoli cells transition into the efferent duct system following germ cell maturation and this suggests the potential for GPER to disrupt the balance of cell proliferation and apoptosis in Sertoli/efferent duct epithelial cells. However, further work is needed to assess whether effects are due to increased proliferation or decreased apoptosis. Furthermore, our gene expression data reflects only day 14 of exposure whereas exploring earlier signaling events may help shed light on the processes leading up to the resultant morphologic change. The decision to focus on day 14 was driven by our desire to compare EE2 results to those seen after GPER.

The emphasis of the present- and our companion studies was at the level of the organ and yields organ specific information. We are aware that the total *in vivo* signaling in the testis at any point in time likely reflects testicular as well as endocrine driven events from the hypothalamic-pituitary-gonadal axis. However, by comparing morphologic data to those derived from gene expression analyses and relating them to male reproductive function we are provided with information that can be analyzed in the future by more refined tools to determine changes in cells/tissues at specific sites.
In summary, our study demonstrates that in adult medaka exposure to GPER specific agonist, G-1, does not lead to impaired reproductive ability but to increased cellularity in the interstitium tissue of the testis following the 14 day exposure. In addition, our microarray data demonstrated differentially expressed genes involved in apoptosis, cell cycle, cell proliferation, cell migration, chromatin modification, cytoskeletal organization, DNA repair, metabolism, transcription, translation, transport and ubiquitination.
Table 3: Amino acid homology of medaka GPER to compared to other GPERs.

Amino acid homology of medaka GPER to compared to other GPERs. ClusalW (EMBL-EBI; [http://www.ebi.ac.uk/Tools/msa/clustalw2/](http://www.ebi.ac.uk/Tools/msa/clustalw2/)) multiple sequence alignment software was used to assess medaka GPER 1 of 2 and GPER 2 of 2 against rat (*Rattus norvegicus*) (ENSRNOP00000001732), mouse (*Mus musculus*) (ENSMUSP00000080370), human (ENSP00000380281), Atlantic croaker (*Micropogonias undulates*) (GenBank: EU274298.1), zebrafish (*Danio rerio*) (ENSDARP000000070486), stickleback (*Gasterosteus aculeatus*) (GPER1of2, ENSGACP00000018482; GPER2of2, ENSGACP00000011856), and medaka (GPER1of2, ENSORLP00000006336; GPER2of2, ENSORLP00000009642) tetraodon (*Tetraodon nigroviridis*) (GPER1of2, ENSTNIP00000021763; GPER2of2, ENSTNIP0000001979), fugu (*Takifugu rubripes*) (ENSTRUP00000039827) GPER mined from Ensemble Genome Browser or GenBank.

<table>
<thead>
<tr>
<th>AA homology</th>
<th>MEDAKA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GPER1of2</td>
</tr>
<tr>
<td>humanGPER</td>
<td>52%</td>
</tr>
<tr>
<td>ratGPER</td>
<td>61%</td>
</tr>
<tr>
<td>mouseGPER</td>
<td>61%</td>
</tr>
<tr>
<td>croakerGPER</td>
<td>88%</td>
</tr>
<tr>
<td>zebrafishGPER</td>
<td>83%</td>
</tr>
<tr>
<td>tetraodon 1of2</td>
<td>86%</td>
</tr>
<tr>
<td>tetraodon 2of2</td>
<td>65%</td>
</tr>
<tr>
<td>Stickleback 1of2</td>
<td>88%</td>
</tr>
<tr>
<td>Stickleback 2of2</td>
<td>57%</td>
</tr>
<tr>
<td>Fugu</td>
<td>51%</td>
</tr>
</tbody>
</table>
Table 4: Top molecular networks generated by IPA using the corresponding list of significant genes.

Significantly different genes listed in bold.

<table>
<thead>
<tr>
<th>Significant Gene List</th>
<th>Top Associated Molecular Network Functions</th>
<th>Network Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 ug/L G-1</td>
<td>1. Drug metabolism, genetic disorder, metabolic disease</td>
<td>Adaptor protein 2, Ap1 gamma, AP1M1, Ap2 alpha, ARR3, ATP1A1, CCDC50, CD82, CFTR, Clathrin, CLINT1, Cytochrome c, Cytochrome c oxidase, HBP1, IBTK (includes EG:25998), MAP1LC3A, MT-CO2, NBR1, NFkB (complex), NFkB (family), OTUB2, Pkg, POT1, PRDX3, RALBP1, RGS3, RIPK4, SQSTM1, TLR5, TOM1L2, TPMT, TRAPPc9, TRPC4AP, TXN2, Ubiquitin</td>
</tr>
<tr>
<td></td>
<td>2. DNA replication, recombinaiton, and repair, cancer, gastrointestinal disease</td>
<td>AMBP, Basc, C14orf1, Cdc2, CHAF1B, CNP, CUX1, Cyclin A, Cyclin B, Cyclin D, Cyclin E, Dscc1, E2f, Erk1/2, FEN1, Hdac, Ifrd1, Kdm2b, Lig1, Mbd1, Mll3, Msh6, Ncoa6, Rad50, Rb, Rb1, Rbbp4, Rbbp5, Rfc4, Suv39h1, Tk1, Tob1, Tox4, Tyms, Wee1</td>
</tr>
<tr>
<td></td>
<td>3. Cellular assembly and organization, cellular compromise, gene expression</td>
<td>Actb, Actl6a, Akt, ARL2, Beta Tubulin, Casp12 (includes EG:120329), Caspase, Chek1, Col18a1, Cse1l, Eif4g2, Fkbpl, Fsh, Gde1, Hcg, Histone h4, Hsp90, Hyou1, Incenp, Ktn1, Lh, Mek, Mll, NPC2, Pknox1, Pp2a, Prc1, Prkaa1, SmarcC1, Sptan1, Stk38l (includes EG:23012), Tubulin, Ube2a, Vegf, Wdr61</td>
</tr>
<tr>
<td>1.0 ug/L G-1</td>
<td>1. Cellular Development, Hematological System Development and Function, Hematopoiesis</td>
<td>ACTB, AXIN1, BRD2, CCDC76, CDK1, Cyclin A, E2f, E2F4, EEF1A2, EEF1D, EIF2C2, ERK1/2, GNAS, GTF2H4, hCG, Histone h3, ILF3, ITGAL, LTBP1, NFYA, PCYT1A, PIM1, PNO1, POLD1, PP2A, PPM1L, Rb, Rnr, RPLP0 (includes EG:6175), RPS8, STK38L (includes EG:23012), TERF1, TIAL1, TRPM7, XPO1</td>
</tr>
<tr>
<td>2. Cancer, Gastrointestinal Disease, Genetic Disorder</td>
<td>19S proteasome, 26s Proteasome, ATP6V0B, ATPase, Cbp/p300, COPS2, CREBBP, CYLD, EP400, HISTONE, Histone h4, HMGB1 (includes EG:3146), ID1, IFIH1, IFN Beta, Ifn gamma, ING1, LAMA5, MLL, MLL3, MMP9, MT-CO2, NCOA6, NfkB (complex), PSMA, PSMA3, PSMC2, PSMC5, PSMD4, SP4, SUDS3, Tgf beta, Thyroid hormone receptor, TRIP4, Ubiquitin</td>
<td></td>
</tr>
<tr>
<td>3. Inflammatory disease, respiratory disease, cellular movement</td>
<td>20s proteasome, BCR, CASP3, CD200, CHMP5, DIDO1, EEF1D, EYA2, HMGB1 (includes EG:3146), Hsp70, IFN Beta, Ifn gamma, IKBKG, IKZF5, IL12 (complex), Immunoglobulin, Interferon alpha, Jnk, MAD1L1, MHC Class II (complex), NAT10, PARP, PAPR9, PSME1, Rnr, RPLP0 (includes EG:6175), RPS7, RPS8, TBC1D7, TERT, Tnf, TUBGCP4, TXNDC17, XPO1, ZFP106</td>
<td></td>
</tr>
<tr>
<td>4. DNA replication, recombination, and repair, cellular development, cancer</td>
<td>collagen, Collagen type I, Collagen type IV, DNA-directed DNA polymerase, DNA-directed RNA polymerase, Gm-csf, GPN1, GPN3, Growth hormone, GTF2A2, GTF2H4, Holo RNA polymerase II, MAD2L2, MED24, MMP9, NF2, NR5A2, Pdgf, PI3K (complex), PIM1, POLG2, POLR2C, POLR3A, PRIM2, REV3L, RNA polymerase II, SDHC, SETD2, SGSM3, STAT5a/b, SWAP70, TARBP2, TCEB2, Thyroid hormone receptor, WSB2</td>
<td></td>
</tr>
<tr>
<td>3. Cellular Assembly and Organization, RNA Damage and Repair, Cancer</td>
<td>BUB1, C1ORF9, C2ORF69, CIC, COIL, COMMD4, COMMD6, CYB5R2, EDC3, EGFL7, GPR137, GPR155, KLC4, LOX, LPIN3, LRRC47, MIR298 (includes EG:723832), NAT10, NCBP1, NIPBL, PARN, PPP6R3, PYCR2, RELA, SGOL1, SMG6, SPC24, ST18, TERT, TMEM9B, UBR5, UPF1, YWHAZ, ZFP36, ZGPAT</td>
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</tr>
<tr>
<td>4. Amino Acid Metabolism, Post-Translational Modification, Small Molecule Biochemistry</td>
<td>amino acids, ART1, B3GALT5, BMI1, C18ORF25, CSNK2A1, EYA1, GPCPD1, HNF1A, HNMT, INS1, KDM2B, KIF5C, LIF, MIRLET7B (includes EG:406884), NFKBIA, PCGF6, PDP1, PDP2, PLC, POM121, PPP1M1J, PSKH1, RABGGTA, REV3L, RNF26, RNF186, RWDD3, SPO11, TOX4, TRIM72, UBE2D2, UBE2I, ZNF646, ZP3</td>
<td></td>
</tr>
<tr>
<td>5. Post-Translational Modification, Cellular Assembly and Organization, Cellular Function and Maintenance</td>
<td>B9D2, C11ORF1, C1D, CHD1L, CTSZ (includes EG:1522), DNAH6, DNAH9, DNAH10, DNAH14, DNAI1, DNAL1, DPM1, DPM2, GGT6, GIPC2, GSTO1, HNF4A, KLC4, MRPL44, ORAI2, PAN2, PDE4DIP, SEC23IP, SETDB1, SF3A3, SLC30A7, SNRPA1, SNRPD2, TRDMD1, TSNAX, TSNAXIP1, TXNDC9, USP5, USP30, ZFP64</td>
<td></td>
</tr>
<tr>
<td>1. Protein Synthesis, Cell Cycle, Cellular Assembly and Organization</td>
<td>ACACA, ARHGAP21, ARNT, CTSD, DUB, EEF1A2, EEF1B2, EEF1D, ERCC6, HEXA, HIPK3, HIRIP3, Histone h3, IKBKG, KTN1, LARS, MBD1, Pkc(s), PNO1, RNA polymerase II, RPL11, RPL23, RPL12 (includes EG:6136), RPL21 (includes EG:6144), RPS6, RPS3A, SBF1, SEC61A1, STIM1, TFEB, USP3, USP19, USP37 (includes EG:57695), WDR1, ZFP106</td>
<td></td>
</tr>
<tr>
<td>2. Small Molecule Biochemistry, Cellular Compromise, Cell Death</td>
<td>HDGF, I kappa b kinase, IBTK (includes EG:25998), MGRN1, MT-CO2, NACA, NFkB (complex), NFkB (family), PAFAH1B2, PIGA, PIGC, PRDX2, PRDX3, PTPN2, RNF6, RNF115, RNF166, Rnr, RPLP0 (includes EG:6175), RPS7, RPS8, RPS9, RPS23, RPS2 (includes EG:6187), SBDS, SLC12A7, TNIP1, TPMT, TRAPPC9, TRIM23, UBE2, UBE2H (includes EG:7328), UBE2J2, UBE2W, ZHX1</td>
<td></td>
</tr>
<tr>
<td>3. Nucleic Acid Metabolism, Dermatological Diseases and Conditions, Genetic Disorder</td>
<td>19S proteasome, 20s proteasome, 26s Proteasome, AMBP, ATP5B, ATP6V1G1, ATPase, CCDC50, DDX1, DHX15, EIF4G2, ERK1/2, H+-transporting two-sector ATPase, LPP, MAP1LC3A, MKNK1, MT-ATP6, NBR1, NCBP1, POMP, PSMA, PSMA3, PSMA4, PSMC4, PSMD, PSMD3, PSMD4, PSMD11, RAB3GAP1, RALBP1, RAPGEF1, SQSTM1, SYNJ2BP, TBCE, Ubiquitin</td>
<td></td>
</tr>
<tr>
<td>4. Drug Metabolism, Lipid Metabolism, Molecular Transport</td>
<td>Adaptor protein 2, ADH5 (includes EG:128), ATP1A1, Collagen type I, DDX3X, EFTUD2, ERK, FAM167A, FAU, FBXO33, G3BP1, GOT2, GPN1, HMGB1 (includes EG:3146), HOXB3, Hsp70, Hsp90, HSPA9, ICK, IFN Beta, IL1, Interferon alpha, MSH6, MutS alpha, Nos, PPIL5, PUF60, SGSM3, SPIN1, TERT, TOPBP1, TROVE2, UGCG, XPO1, YTHDF1</td>
<td></td>
</tr>
<tr>
<td>5. Developmental Disorder, Genetic Disorder, Neurological Disease</td>
<td>ACAT2, ACTB, Actin, ACTL6A, BBS4, BCAM, CCNL1, CENPI, CEP290, Collagen(s), CYR61, FGFR1, FSH, hCG, HIP1R, HSD17B3, LAMA5, Laminin, Lh, Mapk, MAPKAPK3, Mek, MEPA1, PCYT1B, Pdgf, Pdgf BB, PTPN3, RAB2A, Rap1, RASSF4, RPRGRI1, RPL13, SMARCC1, SNAP23, SPTAN1</td>
<td></td>
</tr>
</tbody>
</table>
Table 5: Top canonical pathways generated by IPA using the corresponding list of significant genes and a list of significant genes common to all treatments.

Significantly different genes listed in bold.

<table>
<thead>
<tr>
<th>Significant Gene List</th>
<th>Top Canonical Pathways</th>
<th>Significant Genes in Pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 ug/L G-1</td>
<td>1. Role of BRCA1 in DNA damage response</td>
<td>Rb, Chk1, RFC, MSH6, Rad50</td>
</tr>
<tr>
<td></td>
<td>2. Pyrimidine metabolism</td>
<td>2.7.7.6, 2.7.4.20, 2.7.7.7, 3.1.3.5, 2.1.1.45, 3.1.3.5, 2.7.2.21</td>
</tr>
<tr>
<td></td>
<td>3. Mismatch repair in eukaryotes</td>
<td>MSH6, FEN1, RFC,</td>
</tr>
<tr>
<td></td>
<td>4. Hereditary breast cancer signaling</td>
<td>RNA Pol II, MSH6, RFC, RAD50, RFC,Wee1, CHK1, RB1</td>
</tr>
<tr>
<td></td>
<td>5. Antiproliferative role in TOB in T cell signaling</td>
<td>TOB, Rb, TGF-beta</td>
</tr>
<tr>
<td>1.0 ug/L G-1</td>
<td>1. Wnt/β-catenin signaling</td>
<td>Frizzled, Axin, UB, PP2A, CBP</td>
</tr>
<tr>
<td></td>
<td>2. Colorectal cancer metastasis signaling</td>
<td>Frizzled, Axin, Gαs, MMP, E2F4, Caspase 3</td>
</tr>
<tr>
<td></td>
<td>3. Tumoricidal function of hepatic Natural Killer cells</td>
<td>LFA1, Caspase 3/5/7</td>
</tr>
<tr>
<td></td>
<td>4. Protein ubiquitination pathway</td>
<td>UB, DUB, PSMD, PSMC, PSMA3</td>
</tr>
<tr>
<td></td>
<td>5. Cyclins and cell cycle regulation</td>
<td>PP2A, E2F, CDK1</td>
</tr>
<tr>
<td>10.0 ug/L G-1</td>
<td>1. Hereditary breast cancer signaling</td>
<td>UB, MSH6, Cdc2, Wee1, DDB2, CDK4/6, AKT</td>
</tr>
<tr>
<td></td>
<td>2. Cyclins and cell cycle regulation</td>
<td>SCK4/6, Wee1, HDAC, CDK7</td>
</tr>
<tr>
<td></td>
<td>3. Protein ubiquitination pathway</td>
<td>Ub, E2, HSP, E3RING, DUB, PSMC, PSMD, PSMA4, PSMA3</td>
</tr>
<tr>
<td></td>
<td>4. RAN signaling</td>
<td>RAN, Importin a, Exportin-1</td>
</tr>
<tr>
<td></td>
<td>5. Mismatch repair in Eukaryotes</td>
<td>PCNA, MSH6, Polo</td>
</tr>
<tr>
<td>Significant Genes Common To All Treatments:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------------------------</td>
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<td></td>
</tr>
<tr>
<td>ACTB, ARL2, CHRM5, DIDO1, ECHDC3, EEF1D, EGFL7, EYA1, GPCPD1, GTF2H4, HMGB1, ILF3, KDM2B, KIAA0020, KIAA0467, KIF21A, MLL, MMP9, MT-CO2, NCOA6, PIM1, PSMD4, REV3L, RPL15, RPL26, RPL4, RPLP0, RPS8, SEC23IP, SUDS3, TIAL1, TRAPPC10, TRAPPC2L, XPO1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 9: Multiple alignment of GPER amino acid sequences

Multiple alignment of GPER amino acid sequences acquired from Ensemble (http://www.ensembl.org/index.html) or GenBank (http://www.ncbi.nlm.nih.gov/genbank/) for rat (Rattus norvegicus) (ENSRNOP00000001732), mouse (Mus musculus) (ENSMUSP00000080370), human (ENSP000000380281), Atlantic croaker (Micropogonias undulates) (GenBank: EU274298.1), zebrafish (Danio rerio) (ENSDARP00000070486), stickleback (Gasterosteus aculeatus) (GPER1of2, ENSGACP00000018482; GPER2of2, ENSGACP00000011856), medaka (GPER1of2, ENSORLP00000006336; GPER2of2, ENSORLP00000009642), tetraodon (Tetraodon nigrioviridis) (GPER1of2, ENSTNIP00000021763; GPER2of2, ENSTNIP0000001979), and fugu (Takifugu rubripes) (ENSTRUP00000039827). The conserved amino acid residues are indicated by asterisks, conserved and semi conserved amino acid substitutions are indicated by semicolons or periods, respectively. Amino acid residues are color labeled as follows: red = small (small, hydrophobic); blue = acidic; pink = basic; green = hydroxyl, amine, basic; gray = others. The seven-hydrophobic transmembrane domains are shaded in gray. The characteristic DRY residues are enclosed by a rectangle. The triangles indicate the conserved cysteine residues.
Figure 10: Number of eggs produced per day for medaka breeding groups during the pre- and post-exposure period following the 15 day exposure to G-1 (mean ± SEM).

Number of eggs produced per day for medaka breeding groups during the pre- and post-exposure period following the 15 day exposure to G-1 (mean ± SEM). There were no significant differences between control and treatments in either the pre- or post-exposure time period.
Figure 11: Percentage of eggs fertilized for medaka breeding groups during three periods: pre-exposure, male exposure, and post-exposure.

Percentage of eggs fertilized for medaka breeding groups during three periods: pre-exposure, male exposure, and post-exposure. No significant differences in mean percentage fertilized were observed during the pre- or post-exposure period for each treatment.
Figure 12: Sections of testis from 6 month old male medaka stained with H&E.

Sections of testis from 6 month old male medaka stained with H&E. A: Section of a DMSO control testis showing normal testicular morphology. B: Animal exposed to 10.0 μg/L G-1 following a 14-day exposure demonstrated increased cellularity of the interstitium. SC = spermatocytes. ST = spermatids. L = Leydig cells. Arrow = Sertoli cells/efferent duct epithelial cells.
Figure 13: Average percentage of nongerm cells of the area point-counts of testis histology following the 14 day exposure to G-1 (mean ± SEM).

Average percentage of nongerm cells of the area point-counts of testis histology following the 14 day exposure to G-1 (mean ± SEM). Significant difference between control and treatment indicated with an asterisk (p < 0.05).
Figure 14: Volcano plots showing differences in testicular gene expression changes detected by microarray following a 14 day exposure to G-1.

Volcano plots showing differences in testicular gene expression changes detected by microarray following a 14 day exposure to G-1. The –log10 (p-value) is plotted against the fold change differences in expression between treated and control animals.
Figure 15: Principle components analysis of microarrays following a 14-day exposure to G-1.

Principle components analysis of microarrays following a 14-day exposure to G-1. Doses are distinguished by color as follows: 0.1 μg/L G-1, red; 1.0 μg/L G-1, green; and 10.0 μg/L G-1, blue. Principal components 1, 2, and 3 accounted for 15.4%, 12.7% and 10.6% of the data variability, respectively.
Testicular gene expression pattern changes in male medaka following a 14-day exposure to G-1. Hierarchical cluster of significantly different genes based on mean fold change as determined by one-way ANOVA. Red indicates up regulation; blue indicates down regulation.
Figure 17: Venn diagram of differentially expressed testicular genes following a 14-day exposure of G-1.

Venn diagram of differentially expressed testicular genes following a 14-day exposure of G-1. The red represents circle represents the 432 significant genes in the 0.1 µg/L treatment group; green represents 192 significant genes in the 1.0 µg/L treatment group; and blue represents the 596 significant genes in the 10.0 µg/L treatment group.
4. Comparison of altered testicular histology from a general estrogen agonist, ethinylestradiol, and a GPER specific agonist, G-1, in medaka

This is a collaborative effort between Hilary D. Miller, Seth W. Kullman and David E. Hinton

4.1 Introduction


Environmental estrogens have the ability to act as selective estrogen receptor modulators (SERMS) and thereby differentially bind to the various estrogen receptors, ESR1, ESR2 and GPER. This makes understanding the role and contribution of the different estrogen receptor signaling pathways to altered testicular morphology important for a complete comprehension of the potential effects environmental estrogens on the testis (McDonnell et al. 2002, Paige et al. 1999, Bulayeva & Watson 2004, Watson et al. 2010).
We recently found that in adult male medaka a 14 day exposure to either ethinylestradiol (EE2), a general estrogen receptor (ESR) agonist, or G-1, a GPER specific agonist, led to morphologic changes in the testis (Chapter 2 and 3). However, alterations differed between the individual responses to these receptor specific agonists. For example, EE2 exposure caused altered spermatogenesis, testicular oocytes and thickening of the interstitium, possibly due to interstitial fibrosis. G-1, on the other hand, caused thickening of the interstitium through an increased cellularity.

The interstitial compartment of the testis includes Leydig cells, peritubular myoid cells, connective tissue, efferent duct epithelial cells, endothelium of blood vessels and blood cells (Koulish et al. 2002, Schulz et al. 2010, Lo Nastro et al. 2004, Grier 1981, Loir et al. 1995, Nobrega et al. 2009). These cell types are important in a variety of functions including endocrine signaling, extracellular matrix formation and testicular structure and support for lobules. The above findings led us to extend and better define histological differences induced by these different estrogen agonists, with a particular emphasis on the interstitium. To achieve this, we used a pancytokeratin, AE1/AE3, antibody to detect a range of both high and low molecular weight keratins (Bunton 1993). Our laboratory has used this probe to describe epithelial cells of the medaka liver (Padilla et al. 2001) and have recently shown this to mark the interstitium of the medaka testis (unpublished results of this laboratory) including Sertoli cells and efferent duct epithelial cells but not Leydig cells. Proliferation of cells was assessed using an antibody
for the proliferating cell nuclear antigen (PCNA). Finally, alterations of specific connective tissue, i.e., collagen fibers, in the interstitium were conducted by use of the Gomori Trichrome stain.

4.2 Materials and Methods

4.2.1 Chemicals

EE2 (98% purity, Fluka, St. Louis, MO) and G-1 (98% purity, Cayman Chemical) were used to prepare nominal stocks (0.004, 0.04, and 0.4 mg/ml) in dimethyl sulfoxide (DMSO). The EE2 stocks were stored at room temperature in the dark and G-1 stocks were stored at -20°C in the dark.

4.2.2 Medaka

Orange-red (OR) medaka fish were maintained at the Duke University Aquatic Research Facility under standard recirculating water conditions. Animal care and maintenance protocols were approved by the Duke University Institutional Animal Care and Use Committee (DUIACUC). Water temperature and pH were monitored daily and maintained at ~25°C and ~7.4, respectively, and broodstock were under a strict light:dark cycle of 16:8 hours. Dry food (Otohime B1, Reed Mariculture, Campbell, CA) was fed several times per day via automated feeders with once daily supplementation of newly-hatched Artemia nauplii.
4.2.3 Exposures

Seven treatment groups were assessed: DMSO vehicle control, 1.0 µg/L EE2, 10.0 µg/L EE2, 0.1 µg/L G-1, 1.0 µg/L G-1, and 10.0 µg/L G-1. Fish were sampled for histology on day 14 of exposure. For each treatment, the 2-liter beaker replicates received 7 individuals each for the duration of the exposure. Embryo rearing medium (ERM) was spiked with 75 µl of the appropriate stock (0.0025% of total volume). The spiked ERM was equally distributed between the 2-L beakers for a total of 1.5 L spiked ERM per beaker with a 50% replacement of spiked ERM every other day for 14 days. The fish were maintained under a 16:8 hour light:dark cycle and fed *ad libitum* a dry diet Otohime B1 aquarium feed (Reed Mariculture) x1/day and freshly hatched brine shrimp from lab culture x2/day. On day 14 of exposure, 2-3 fish from each beaker replicate were anesthetized in ice-cold ERM. The testis was removed and fixed in a cocktail of 0.05% glutaraldehyde, 2% paraformaldehyde, 1% sucrose and 1% CaCl₂ in Histochoice (Amresco) for ≥24 hr at 4°C and stored in Holt’s gum sucrose solution (30% aqueous sucrose and 1% aqueous gum arabic) at 4°C until paraffin embedment (Kong et al. 2008). Serial longitudinal sections 5-µm thick were made through the entirety of testis, mounted on slides and stained with hematoxylin and eosin or reacted as follows.

4.2.4 Gomori Trichrome

Slides with deparaffinized sections were placed in preheated Bouin’s Solution (Sigma, HT10132) at 56°C for 15 minutes, cooled and washed in tap water. Sections were
stained with working Weigert’s Iron Hematoxylin Solution (Sigma, HT1079) for 5 minutes and rinsed in running tap water. Next, sections were stain in Trichrome Stain AB (Sigma, HT10516) for 5 minutes, placed in 0.5% acetic acid for 1 minute rinsed in water, dehydrated through an ethanol series, cleared using Clear-Rite 3 (Richard-Allan Scientific), and coverslipped using Permount (Fisher Scientific). This method stains collagen blue, nuclei purple/black, and cytoplasm and muscle fibers red.

4.2.5 Immunohistochemistry

Deparaffinized slides were immersed and boiled in 10mM citrate buffer (pH 6.0). After rinsing in water, endogenous peroxidase was quenched with 3% hydrogen peroxide and non-specific proteins were blocked with 10% normal goat serum (Zymed). The tissue sections were then immersed in 10% normal goat serum containing primary antibody, AE1/3 (1:100, Invitrogen, 08-0132) or PCNA (1:500, Dako, M0879) and incubated for 1 hour at room temperature in a humidified chamber. Sections were then rinsed in PBS and incubated with the secondary goat anti-mouse-HRP conjugated antibody (Dako S0809) for 30 minutes at room temperature in a humidified chamber. Samples were rinsed in PBS and signals detected with the substrate 3,3’-diaminobenzidine chromagen (DAB) (Dako, K3468). Samples were counterstained with hematoxylin and eosin and coverslipped using Permount (Fisher Scientific). Each slide also contained a negative control that was prepared by substitution of the primary antibody with 10% goat serum.
4.3 Results

Analysis of H&E stained testicular tissue indicated altered morphology following a 14 day exposure to EE2 and G-1. Animals exposed to 1.0 µg/L and 10.0 µg/L EE2 exhibited thickened interstitium and increased apoptotic germ cells as well as loss of germinal epithelium (Figure 18). Animals exposed to 0.1 µg/L, 1.0 µg/L and 10.0 µg/L G-1 also exhibited increased interstitium but no change in spermatogenesis. The thickened interstitium observed in both of the EE2 and G-1 exposed animals appeared to differ. For example, the EE2 thickened interstitium was observed both in the efferent duct space as well as at the periphery of the gonad. The G-1 induced alteration, on the other hand, appeared to be due to increased cellularity of interstitium affecting Sertoli and efferent duct epithelial cells mainly associated with spermatids and spermatozoa.

4.3.1 AE1/AE2

To characterize changes in keratin deposition in medaka testis following EE2 or G-1 treatment we conducted immunohistochemistry (IHC) with the pancytokeratin antibody AE1/AE3. ISH detection of AE1/AE3 in DMSO control testis demonstrated strong labeling of interstitial tissues (Figure 19A). In particular, AE1/AE3 marked the cytoplasm of Sertoli cells and efferent duct epithelial cells. Following a 14 day exposure, both the 1.0 µg/L EE2 and 10.0 µg/L EE2 treated animals also exhibited increased AE1/AE3 staining in the interstitium (Figure 19B). In addition, these interstitial cells appeared more round and less attenuated in the EE2 treated samples compared to
DMSO controls. Animals treated for 14 days with 0.1 µg/L G-1, 1.0 µg/L G-1 and 10.0 µg/L G-1 also exhibited AE1/AE3 following the 14 day exposure (Figure 19C). These interstitial cells maintained their normal shape and were phenotypically similar to cells of DMSO controls. Overall, there was an increase in interstitial cells expressing AE1/AE3 in the EE2 treated animals group but no change in the G-1 treatment group. There was not an increase in staining intensity observed in either EE2 or G-1 treatments.

4.3.2 Gomori Trichrome

To determine whether exposure to EE2 or G-1 altered collagen deposition in the testis Gomori Trimchrome staining was used. Staining of DMSO control testes demonstrated collagen deposition in the efferent duct system predominately nearest the central efferent duct of the testis with no collagen staining toward the periphery of the testis (Figure 20A). By contrast, the 1.0 µg/L EE2 and 10.0 µg/L EE2 treatment groups demonstrated collagen deposition at the periphery of the testis, near the earlier staged germ cells (spermatogonia and spermatocytes) (Figure 20B). Comparatively, sections of testes from the G-1 treatment groups did not differ from controls with respect to collagen staining (Figure 20C).

4.3.3 PCNA

To determine if observed testicular morphological changes following EE2 or G-1 exposure are associated with alteration in cell proliferation, we conducted ISH with an antibody to PCNA. Sections of DMSO control testes reacted for PCNA demonstrated
proliferative activity within the majority of spermatogonia and spermatocytes (Figure 21A). Expression was also observed in the interstitium including Sertoli cells, efferent duct epithelial cells and Leydig cells. However, due to the high mitotic rate of spermatogonia and spermatocyte cell populations, PCNA was more frequent and prevalent in these versus other cell populations. In the 1.0 µg/L and 10.0 µg/L EE2 treatment groups, there was an overall decrease in spermatogonia and spermatocyte cell populations, but those present continued to exhibit PCNA staining (Figure 21B). There was a modest increase in PCNA staining within the interstitium of the EE2 treatment groups. In the G-1 treatment groups, there was no change in PCNA staining in germ cells and the overall appearance was similar to that of controls (Figure 21C). In all G-1 treatment groups a modest increase in PCNA expressing interstitial cells, primarily Sertoli and efferent duct epithelial cells, was observed.

4.4 Discussion

Environmental estrogens are able to act as SERMs (McDonnell et al. 2002, Paige et al. 1999, Bulayeva & Watson 2004, Watson et al. 2010). Our previous studies have shown that both EE2, a general estrogen agonist, and G-1, a GPER specific agonist, cause increases in the interstitium of the testis of adult medaka (Chapter 2 and 3). However, qualitative assessment of interstitial components using keratin, collagen and PCNA as indicators reveals differences between the two agonists. Marked change in either keratin or collagen can be indicative of abnormal cellular change and tissue damage and
therefore useful in assessing tissue alterations (Karantza 2011, Kisseleva & Brenner 2008).

Keratins are intermediate filaments and along with microfilaments and tubules comprise the cytoskeleton, including in Sertoli cells (for reviews see Aumüller et al. 1992, Karantza 2011, Schweizer et al. 2006). Keratins make up two of the five types of intermediate filaments, which are primarily expressed in epithelial cells. Intermediate filaments organize internal cellular structure and maintain cell shape by bearing tension and regulate a diverse array of cellular functions. Keratins are considered protectors of epithelial cell integrity under stress which can lead to altered expression and post-translational modification leading to keratin reorganization (Karantza 2011). However, their role in regulating normal cell physiology continues to emerge. Keratins are extensively used as histochemical markers for tumor pathology diagnosis. Estrogen influenced cancers, such as breast and ovarian cancer, display altered keratin patterns suggesting an interaction between estrogen and keratin. This and other data indicate endocrine control of keratin expression and function (Karantza 2011, Ramot et al. 2009).

Fibrosis is a pathophysiological stress response that begins with wound healing and tissue remodeling and repair to maintain functional organ integrity. However, continued exposure to the stressor leads to deregulation of normal healing resulting in fibrosis, excess formation and accumulation of extracellular matrix, scar tissue and ultimately organ failure (for review see Kisseleva & Brenner 2008). Collagen production
is a key event in fibrogenesis and increased collagen deposition is an indicator of its onset.

In the EE2 exposed fish, a significant decrease in germinal epithelium was observed. While PCNA was regularly expressed in cells that entered into spermatogenesis, the number of such cells was reduced by the EE2 exposure. This suggests that there is a decrease in germ cells entering into spermatogenesis as opposed to an overt cessation of spermatogenesis in cells committed to the process. This appears to lead to many of the morphologic changes observed in the interstitium. The biomarker for the interstitium, AE1/AE3, continues to be expressed following the EE2 exposure but interstitial cells appear more rounded, particularly toward the interior of the organ. This may be due the decrease in germ cells in the lobular and efferent duct space leading to a collapse of the space and therefore a decrease in the attenuation usually seen. Our assessment of testicular gene expression changes following EE2 exposure (Chapter 2) found differential expression of Keratin 17 (KRT17) and Trichoplein (TCHP; a keratin filament-binding protein). This suggests that some of the observed keratin staining may be due to changes in gene expression; however, the dramatic decrease in germinal epithelium and collapse of duct space likely contribute substantially. There is also a slight increase in PCNA staining in the interstitium of EE2 exposed animals. However, the AE1/AE3 and PCNA histological assessment does not address the interstitial changes at the periphery of the gonad. The Gomori’s Trichrome stain indicates that the
increased interstitium toward the periphery of the testis is due to increased collagen. The increased collagen staining found in EE2 exposed testis is supported by the testicular gene expression analysis (Chapter 2) which found differential gene expression of multiple genes involved in collagen production and regulation (CD36, COL1A1, COL1A2, COL4A1, COL4A2, COL5A1, COL6A1, COL6A2, COL6A3, COL11A1, COL12A1, COL13A1, COL17A1, COL18A1, COL21A1, CTHRC1, FMOD, ITGA1, MMP2, MMP9, MMP13, P4HA2, PCOLCE2, PLOD2, SCARB1, SERPINH1, TGFBI).

Findings of the present report suggest that in the EE2 treated testis there are two different morphologic changes in the interstitium, one type on the periphery of the testis and the second type toward the interior of the testis associated with the efferent duct system. On the periphery the testis, there is a loss of early stage germ cells and the associated interstitial space has increased collagen suggesting the onset of fibrosis. The interior of the gonad where the significant interstitial thickening occurred is likely due to the collapse of the lobular space due to a decrease in spermatids and spermatozoa. There is also an increase in PCNA expression suggesting interstitial cell proliferation also contributes.

In the G-1 animals, the morphologic changes in interstitium appear to be due to increased cellularity. AE1/AE3 continues to mark interstitial cells in this treatment group without any appreciable change. This finding is supported by testicular gene expression data of G-1 exposed medaka (Chapter3) which indicates no expression changes in
keratin associated genes. The Gomori Trichrome stain also shows no changes in collagen. Interestingly, testicular gene expression found far fewer differentially expressed genes associated with collagen production and regulation (COL18A1, MMP9) compared to EE2 exposed males. The PCNA results indicate a slight increase in proliferating interstitial cells but it is unlikely that this explains all of the increased cellularity observed. It is also possible that G-1 led to a decrease in apoptosis of these cells. Sertoli cell function in the testis is a delicate balance between proliferation and apoptosis. When Sertoli cells of the spermatocysts release their mature sperm into the efferent duct system they either undergo apoptosis or transition into efferent duct epithelial cells (Leal et al. 2009, Schulz et al. 2005, Vilela et al. 2003, Sàbat et al. 2009, Schulz et al. 2010, Pudney 1995, Kobayashi et al. 2004, Nakamoto et al.). Surviving Sertoli cells also have a very high phagocytic activity and remove other apoptotic Sertoli cells quickly (Leal et al. 2009). In vitro data of primary cultures of rat Sertoli cells indicated that GPER mediated cell proliferation and apoptosis (Lucas et al. 2010). Testicular gene expression data (Chapter 3) indicate involvement of pathways regulating both proliferation and apoptosis including ERK1/2, AKT as well as NFκβ. These cell cycle/proliferation and apoptosis pathways are highly interdependent. It is possible that there is a decrease in normal apoptosis and cell turnover of the Sertoli and efferent duct epithelial cells that is in addition to the increase in cell proliferation, as
indicated by PCNA. It may be the combination of these processes leads to the increased cellularity of the interstitium following the G-1 exposure.

Our assessment of the morphologic changes induced by EE2 and G-1 indicate a distinctive histological response to the different estrogen receptor agonists. The general estrogen agonist, EE2, caused a disruption in spermatogenesis and a decrease in germinal epithelium. This led to increased collagen deposition toward the periphery of the organ suggesting an onset of testicular fibrosis. The interstitial thickening in the central portion of testis toward the efferent duct system was due not to fibrosis but instead to decreased germ cells and as increase in interstitial cell proliferation. The GPER specific estrogen agonist, G-1, caused an increased cellularity in the interstitium that was associated with an increase in PCNA expression. However, the possibility of a decrease in apoptosis should also be addressed. Examining the differences in histological changes induced by these different estrogen agonists is important to understanding the potential effects of environmental estrogens.
Figure 18: Sections of testis from 6 month old male medaka stained with H&E from control animal (A), EE2 exposed animal (B), and G-1 exposed animal (C).

Sections of testis from 6 month old male medaka stained with H&E. A: Section from a DMSO control testis showing normal testicular morphology. B: Animal exposed to 10.0 µg/L EE2 for 14 days demonstrated a severe thickening of the interstitium, increased vacuolization, and an overall decrease in germinal epithelium. C: Animal exposed to 10.0 µg/L G-1 for 14 days demonstrated increased cellularity of the interstitium. SC = spermatocytes. ST = spermatids. L = Leydig cells. Arrow = Sertoli cells/efferent duct epithelial cells.
Figure 19: AE1/AE3 immunohistochemistry staining in the testis of 6 month old adult medaka from control animal (A), EE2 exposed animal (B), and G-1 exposed animal (C).

AE1/AE3 immunohistochemistry staining in the testis of 6 month old adult medaka. A: DMSO control animal. AE1/AE3 staining observed in the interstitial tissue, particularly Sertoli Cells and efferent duct epithelial cells. Leydig and germ cells are negative for AE1/AE3 staining. B: Animal exposed to 10.0 µg/L EE2. AE1/3 staining highlights the morphologic change in the thickened interstitial cells such as less attenuated and more rounded cells. C: Animal exposed to 10.0 µg/L G-1. While a thickened interstitium is observed there are no morphologic changes in interstitial cells identified by the AE1/AE3 staining. SG = spermatogonial; SC = spermatocytes; ST = spermatids; L = Leydig cells; CED = central efferent duct; scale bar = 100 µm.
Figure 20: Gomori trichrome staining in the testis of 6 month old adult medaka from control animal (A), EE2 exposed animal (B), and G-1 exposed animal (C).

Gomori trichrome staining in the testis of 6 month old adult medaka A: DMSO control animal. Minimal collagen, which stains blue, is observed in the interstitium. B: Animal exposed to 10.0 µg/L EE2. There is an increase in collagen at the periphery of the testis. C: Animal exposed to 10.0 µg/L G-1. No changes in collagen were observed. SG = spermatogonial; SC = spermatocytes; ST = spermatids; L = Leydig cells; CED = central efferent duct; arrow = collagen staining; scale bar = 100 µm.
Figure 21: PCNA immunohistochemistry staining in the testis of 6 month old adult medaka from control animal (A), EE2 exposed animal (B), and G-1 exposed animal (C).

PCNA immunohistochemistry staining in the testis of 6 month old adult medaka. A: DMSO control animal. Spermatogonia, spermatocytes, and interstitial cells including Leydig, Sertoli and efferent duct epithelial cells exhibit positive PCNA expression. Spermatids do not express PCNA. Spermatogonia and spermatocytes demonstrate a high frequency of PCNA expression. Interstitial cells display a more infrequent expression of PCNA in comparison to early stage germ cells. B: Animal exposed to 10.0 µg/L EE2. While there is a decrease in spermatogonia and spermatocytes, those present continue to demonstrate a consistent expression of PCNA. There is an increase in PCNA positive cells observed in the thickened interstitium. C: Animal exposed to 10.0 µg/L G-1. There is no change in germ cell epithelium or the PCNA positive germ cells. There is an increase in PCNA positive cells observed in the thickened interstitium. SG = spermatogonial; SC = spermatocytes; ST = spermatids; L = Leydig cells; arrow = PCNA expressing interstitial cell; scale bar = 100 µm.
5. Conclusion

5.1 Summary

In this dissertation I investigated the influence of estrogen signaling on male medaka reproduction by assessing reproductive capacity, organ, tissue and cellular morphology and testicular gene expression. My work focused on delineating the difference in testicular effects between a general estrogen receptor agonist, EE2, and a GPER specific agonist, G-1. This allowed me to explore the difference in testicular changes induced through aberrant signaling through both “classic” genomic and nongenomic estrogen signaling pathways versus only the nongenomic estrogen signaling pathway. The goals of this dissertation were to explore 1) the influence of a general estrogen agonist, EE2, on reproductive capacity, altered testicular morphology and testicular gene expression, 2) the influence of a GPER specific agonist, G-1 on reproductive capacity, altered testicular morphology and testicular gene expression, and 3) the differences in the altered testicular morphology induced by the two different estrogen agonists.

In Chapter 2, findings demonstrated that a 14-day exposure to EE2 induced time- and dose-dependent alterations in reproduction, testicular morphology and gene expression. Impaired reproduction and testicular morphologic alterations were most severe in the high dose of EE2. The increase in germ cell apoptosis, decrease in germinal epithelium and thickening of the interstitium observed proved to be highly associated
with changes in expression of certain genes. A pathway analysis of the differentially expressed genes emphasized genes and pathways associated with apoptosis, cell proliferation, collagen production/extracellular matrix organization, and protein ubiquitination among others. In particular, genes of established signaling pathways noted in the IPA networks include ERK1/2 MAPK, p38 MAPK, PDGF, TGFβ and AKT. These genes and their associated pathways are interdependent and cross-talk between these pathways not only exists but is critical to coordinating cellular responses.

Interestingly, the gene expression pathway analysis emphasized the MAPK/ERK and Akt pathways indicating the importance of mitogenic signaling which is critical in maintaining proper testicular physiology. These pathways are also involved in nongenomic estrogen signaling (Acconcia & Kumar 2005, Björnström & Sjöberg 2005, Daufeldt et al. 2003, Sanchez et al. 2002). Because GPER has a significant role mediating these nongenomic estrogen signaling pathways, I explored how exposure of male medaka to the GPER specific agonist, G-1, would compare to that of EE2 (Chevalier et al. 2011, Chimento et al. 2010a, Chimento et al. 2010b, Liu et al. 2009, McDonald et al. 2006, Sirianni et al. 2008, Vicini et al. 2006a).

The assessment of the G-1 experiment revealed no change in male reproductive capacity versus controls, but did find altered testicular morphology and differential testicular gene expression following the 14-day G-1 exposure (Chapter 3). The observation of unimpaired reproduction following the exposure suggests that
spermatogenesis was not affected. The histological analysis found an increased cellularity of the interstitium but no change in germinal epithelium. The microarray data indicated differential expression in genes most commonly involved in apoptosis, cell cycle, cell proliferation, cell migration, chromatin modification, cytoskeletal organization, DNA repair metabolism, transcription, translation, transport. Genes and signaling pathways involved in both cell proliferation and apoptosis were strongly emphasized in the pathways analysis, in which both ERK1/2 and AKT play important roles. Interestingly, NFκB was also a central point in the pathway analysis. NFκB is a transcription factor that contributes to cell proliferation, development and apoptosis and has a high connectivity to other signaling pathways (Schmitz et al. 2004). Its signaling is regulated by multiple mechanisms including: cytokines, growth factors, cAMP/protein kinase A (PKA) pathway and steroid hormones including estrogen (for reviews see Delfino & Walker 1999, Schmitz et al. 2004). Many of these growth factors were observed in the molecular networks and canonical pathways from our study. NFκB also plays a role in testicular function through involvement in cell proliferation and apoptosis (Choo et al. 2011, Starace et al. 2005, Delfino & Walker 1999, Vaithinathan et al. 2010). It is important to remember that nongenomic estrogen signaling is a fast protein phosphorylation process while our gene expression data followed a 14 day exposure. Therefore, the gene expression was a reflection of long term stimulation of these
pathways and subsequent morphologic changes as opposed to the short term exposure effects that are frequently analyzed in GPER signaling.

Finally, I compared the histological phenotype of the testis following the EE2 and the G-1 exposure (Chapter 4). As previously stated, the hematoxylin and eosin staining showed thickening of the interstitium and loss of germinal epithelium in EE2 exposed animals while the G-1 exposed animals have thickened interstitium with the appearance of increased interstitial cellularity. In the EE2 exposed fish, two different morphologic changes led to the thickened interstitium. On the periphery of the testis, there was an increase in collagen that was associated with a loss of early stage germ cells located there. This increased collagen production suggests tissue damage and the onset of fibrosis. In the interior of the gonad, there was also significant thickening of the interstitium. This was likely due to the collapse of the lobular space due to the loss of spermatids and spermatozoa as well as an increase in interstitial cell proliferation. In the G-1 exposed fish, there as an increased cellularity of the interstitium. A modest increase in cell proliferation was observed contributing to the increase in interstitial cells, however, it is also possible that there was a decrease in normal apoptosis and cell turnover of the Sertoli cells and efferent duct epithelial cells, too.

5.2 Implications

Few studies in fish assess the impacts of chemical exposure on a physiological endpoint, such as reproduction, as well as the associated morphologic response and
underlying global gene expression changes. These findings highlight the importance of anchoring gene expression changes with morphology and ultimately proper tissue/organ function. While no single study will ever answer all questions, linking molecular to apical endpoints helps give physiological significance to these sometimes intangible findings, such as gene expression changes.

The EE2 results further validate findings from other studies showing impaired reproduction and altered testicular morphology following estrogen exposure to male fish but with the addition of molecular underpinnings of gene expression. This assessment of resultant testicular gene expression changes implicates particular groups of genes and their associated biological functions in the resultant altered testicular morphology. It also helps further identify new estrogen responsive genes that may normally be overlooked; however, whether the gene expression response is direct or indirect will need to be further evaluated.

The G-1 exposure study is the first in vivo work to address the influence of aberrant GPER signaling on male reproduction. The findings demonstrate the ability of irregular GPER signaling to affect the testis as evident through changes in gene expression and altered morphology. However, the effects are not detrimental to reproductive function as seen with EE2 exposure. The observed changes were subtle but suggest that this signaling pathway cannot be disregarded. Investigating the ability of GPER signaling to induce change will need careful, critical assessment.
The comparison of the effects of a general estrogen agonist, EE2, and a GPER specific agonist, G-1, show different outcomes in reproductive effects and altered morphology but, interestingly, both similar and different signaling pathways that were altered. Gene expression analysis found more differentially regulated genes in the EE2 exposed animals than the G-1 exposed animals. A comparison of the EE2 and G-1 pathway analysis of differentially expressed gene indicate that apoptosis, cell cycle, cell proliferation, transcription, translation and ubiquitination were altered in both treatment groups. In particular, the ERK1/2 and AKT pathways were indicated to be important in the pathway analysis and are important to proper testicular function by influencing cell proliferation and apoptosis. Furthermore, these pathways are important in nongenomic estrogen signaling. However, even though similar signaling pathways were perturbed, significant differences in altered testicular morphology were found between the two agonists with EE2, the general estrogen agonist, showing the most detrimental changes. It is likely that EE2 exposure had a greater impact the HPG axis and general endocrine signaling. This is further supported by the gene expression pathway analysis which indicates involvement of multiple genes regulating hormone signaling. Additionally, ERK1/2 and AKT are influenced by a variety of signals including growth factors, hormones, cytokines among others. The modifications of these pathways by EE2 exposure are likely a culmination of changes in the endocrine system and subsequent alterations in growth factors and the like.
An interesting co-dependence between ESR1 and GPER also exists. Data indicates that the presences of ESR1, even in its unliganded form, can mediate GPER signaling and G-1 response (for review see Maggiolini & Picard 2010). There also appears to be positive and negative feedback mechanisms as well as signaling crosstalk regulating GPER expression. It is likely that estrogen signaling is dependent and mediated through both receptors and likely cell specific. Additional research is needed to fully understand the ins and outs of estrogen signaling. Estrogen signaling is further complicated by EDCs and SERMS with differential receptor binding capacity. These chemicals interfere with the designed balance of receptor interactions, signaling crosstalk and ultimately the intended estrogen response in that specific cell.

The developing story of estrogen signaling is complicated and the effect of EDCs on it is even more so. Overall, the results of this dissertation add to the understanding of the influence of estrogen signaling on male reproduction. We show that EE2, a general estrogen agonist, interferes with male reproduction and alters testicular morphology and gene expression. Interestingly, we show that G-1, a GPER specific agonist, does not cause impaired reproduction and leads to different effects on testicular morphology and gene expression changes. A comparison of morphologic changes observed in the testis found that EE2 induced increased collagen at the periphery of the testis and reduced spermatogenesis causing a collapse of lobular and efferent duct space causing thickening of the interstitium. Whereas G-1 caused an increased cellularity of the
interstitium due to a slight increase in cell proliferation of Sertoli and efferent duct epithelial cells and likely due to decreased apoptosis as well. These findings highlight the potential differences in effects that may occur with EDCs and SERMs.
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Biography

Hilary D. Miller was born in Fargo, North Dakota on September 8, 1979 to Doyle Glen Thompson and Susan Carol Thompson. She received her Bachelor of Science degree from the University of Florida in 2003.

Publications


Honors and Awards

National Science Foundation, Graduate Research Fellowship, Honorable Mention. 2007.