THE RELEVANCE OF TOXICANT-INDUCED APOPTOSIS TO REPRODUCTIVE IMPAIRMENT IN FISH AND MAMMALS

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Introduction

Pollution of aquatic environments and widespread reproductive impairment in fish: Evidence of endocrine disruption

An increasing body of scientific evidence suggests that contamination of aquatic environments with a wide range of synthetic chemicals has adversely affected reproduction in fish (Kime, 1998). Effects such as delayed age to maturity, and decreases in egg size, ovary weight, fecundity, and circulating levels of steroid hormones (Hose et al., 1981, 1989; Spies et al., 1985; Johnson et al., 1988; Singh, 1989; Thomas 1988, 1990; Thomas and Khan, 1997) have been well documented in many fish populations exposed to water contaminants.

The endocrine system is composed of complex cell signaling pathways that are responsible for regulation and coordination of physiological functions during development and adulthood in all vertebrates. The reproductive endocrine system consists of glands, hormones, receptors, transport proteins, neurosecretory products, and feedback mechanisms that are crucial in maintaining reproductive

physiological homeostasis. Pollutants such as polychlorinated biphenyls (PCBs), polyaromatic hydrocarbons (PAHs), heavy metals, and many pesticides have been shown to affect reproductive endocrine homeostasis in vertebrates. These compounds are collectively called endocrine disrupting chemicals (EDCs).

Most EDCs mimic endogenous ligands for hormone receptors and can modulate intracellular signaling events. EDCs can block receptor sites and prevent binding of the appropriate ligand, act as agonists or partial agonists, alter timing of a signal, interfere with hormone feedback mechanisms which are responsible and contribute to maintaining homeostasis. These feedback mechanisms maintain hormone concentrations at a physiologically appropriate range to maintain system homeostasis and to prevent undesirable fluctuations in hormone levels. EDCs can alter biosynthesis and metabolism of hormones by altering activity of key enzymes that are involved in hormone production and/or degradation, thus affecting circulating hormone levels. As a result, endocrine active compounds can also affect hormone-dependent behavior of the exposed animal. The obvious complexity of this system and the interconnected nature of the endocrine system are such that interference with any single factor can cause

dysfunction of a range of biological responses. Therefore, a major concern of EDCs exposure is their potential to alter intracellular-signaling pathways, thereby affecting reproductive function in exposed organisms (Kime, 1996, 1998; Hoyer, 1999; Harrison et al., 1997; Ashby et al., 1997; Guillette, 2000).

Many components of the reproductive endocrine system are surprisingly similar in fish and humans. The similarity in reproductive endocrine function of vertebrates and the frequent occurrence of reproductive disorders in diverse classes of organisms has brought attention to the potential harmful effects of these compounds on human fertility (Sharpe and Skakkebaek, 1993). Effects of EDCs become apparent only after exposure of the individual to very low levels of the toxicant over a long period of time. Hence, the problem with aquatic pollution is no longer viewed as simply a matter of counting dead fish, but a serious concern for long-term effects of low-level exposure to EDCs. It is therefore quite possible that many industrial products that are in widespread use today may have harmful future manifestations in both human and wildlife species (Colborn et al., 1993).

Sources and toxicity of common aquatic pollutants: Polyaromatic hydrocarbons (PAHs) and metals

Aquatic environments receive pollution from almost all current human activities and innovations in technology that are aimed at satisfying an ever growing increase in consumer demand for manufactured goods on which our economy is based. These activities range from mining, electroplating, combustion of fossil fuels, transportation industries, heating and cooling devices, electricity generating plants, agricultural use of pesticides, food processing, industrial detergents, as well as disposal of plastics and other industrial processes. The focus of this dissertation will be on the ubiquitous and highly persistent and bioavailable PAHs and metals.

PAHs are a major class of industrial pollutants, and an estimated 230,000 metric tons of PAHs enter aquatic environments each year through surface run-off, oil spills, recreational and industrial boating, municipal waste and atmospheric deposition (Neff, 1979). The majority of PAHs are believed to enter aquatic environments from atmospheric deposition of hydrocarbons produced by combustion of fossil fuels (McElroy et al., 1989). PAHs are persistent organic lipophilic chemicals and a major pollutant of aquatic and

marine environments. They accumulate in lipid rich tissues such as the gonads (Varanasi et al., 1982; Meador et al., 1995) and biologically relevant concentrations (i.e. concentrations that can generate a signal / be recognized by endocrine tissues and glands) of PAHs have been reported in fish from the North Sea, the Baltic and the Great Lakes (Kime, 1999).

Many studies have measured concentrations of sex steroid hormones in blood serum as an indicator of gonadal health. Some studies (Jobling et al., 1995; Arukwe et al., 1997) refer to PAHs as estrogenic xenobiotics while others provide evidence for their ability to inhibit steroidogenesis in ovarian tissue of fish (Thomas, 1988, Thomas and Khan, 1997; Singh, 1989; Monteiro et al., 2000). Although there is conflicting evidence as to the exact mechanism(s) of PAH action in gonadal tissue, their potential for altering the hormonal environment of the ovary is undisputed.

Many PAHs are also agonists of a ligand activated transcription factor, the aryl hydrocarbon receptor (AhR) (Gonzalez and Fernadez-Salguero, 1998). Ligand binding and loss of 2 heat shock protein 90 molecules (HSP90) from the AhR allows this transcription factor to translocate to the

nucleus, where it interacts with DNA of target genes and initiates synthesis of proteins that are involved in many cellular biochemical pathways including biotransformation (Rowland and Gustafsson, 1997), cell proliferation and differentiation, and apoptosis (Hoffer et al., 1996; Zaher et al., 1998).

Metals such as cadmium, copper, mercury, zinc, and lead are another major class of aquatic contaminants and commonly enter aquatic environments through combustion of fossil fuels, mining and smelting operations, as well as pesticide applications (Kime, 1998). Airborne metals eventually reach aquatic environments by rainfall or by direct atmospheric deposition. While some metals are essential elements in a number of biochemical processes (i.e. zinc, copper) others such as cadmium, lead and mercury occur rarely in nature and have no known biological function.

Cadmium is a well-known teratogen, hepatotoxicant, and carcinogen. Many recent studies describe cadmium as a potent inducer of apoptosis in soft tissue such as liver and kidney (Habeebu et al., 1998; Ishido et al., 1999). Furthermore, cadmium has been shown to interfere with oogenesis in fish (Brown et al., 1994; Pascoe and Shazili,

1986). Cadmium toxicity can be the result of altered oxidation/reduction balance in cells that cause a decrease in the level of endogenous antioxidants (Robertson and Orrenius, 2000). Cadmium can also affect calcium homeostasis in the cells and alter a wide variety of cellular functions including transcription and gene expression, calcium-dependent protein phosphorylation, and proteolytic processes that are implicated in cellular signal transduction pathways (Sen, 2000). Intracellular calcium homeostasis is controlled by redox state of the cell and it plays a crucial role in regulation of calcium-dependent enzyme activities (Iryo et al., 2000).

Patterns of ovarian development in fish

Fishes represent a class of vertebrates with greatly diverse reproductive strategies (oviparity, ovoviviparity, viviparity, hermaphroditic, intersexual, ambisexual). Despite this diversity in reproductive strategies, the control of ovarian development is surprisingly well conserved in fishes. Environmental cues, especially photoperiod and temperature, play important roles in fish reproduction and ovarian cyclicity (Baggerman, 1990). Signals from the environment are received in the

hypothalamus of the brain where they are integrated to form neurosecretory signals that ultimately affect the hormonal milieu of the ovary. These environmental signals initiate gonadal growth, development, and eventual ovulation (Van Der Kraak et al., 1997). Most species of fish are gonochoristic (dioecious) with male and female individuals that have distinct phenotypes. Less prevalent are the hermaphroditic species where male and female gonads emerge in the same individual fish (Redding and Patino, 1993). In gonochoristic teleosts synchronous and asynchronous patterns of primordial germ cell development have been described (Tyler and Sumpter, 1996). Under optimal environmental and hormonal conditions in the ovary of synchronous fish, a cohort of primordial germ cells enter meiosis and form the clutch of primary oocytes for the breeding season. In synchronous spawners, egg production and therefore ovulation may be either a yearly occurrence, or an event of a lifetime (Salmonidaes) (Dye et al., 1986). In contrast, in asynchronous spawners, eggs are selected from a heterogeneous population of developing oocytes that are ovulated in several batches during each spawning season. This asynchronous pattern of piscine ovarian development is analogous to that seen in birds and mammals (Etches and Petitte, 1990; Monniaux et al., 1997).

Oogenesis

Fish oogenesis is the complete cycle of egg development and maturation during which a select group of oogonia form a clutch for the breeding season. The primordial germ cells (i.e. oogonia) of fish must overcome two meiotic blocks during oogenesis. The first meiotic block, in all vertebrates, is imposed at the prophase I stage on all oogonia present in the ovary. The brain lifts this first block upon receipt of appropriate environmental cues (i.e. changes in photoperiod and subtle changes in water temperature). As soon as the first meiotic block is lifted, the oogonium becomes a growing oocyte. At this stage the somatic cells of the ovary (granulosa and thecal cells) encircle the oocyte and continue to grow and synthesize sex steroids that will maintain the life of the follicle throughout the process of oogenesis.

The oocyte will continue to grow and mature but it is once again arrested when it reaches the metaphase II stage of cell division. After ovulation, when sperm fertilize the fully matured oocyte, or ovum, it is released from the second block to complete the second meiotic division and give rise to a new individual of the species.

Although piscine oogenesis is a continuous process, it can be divided into four stages on the basis of morphological and physiological changes in the ovary (Wallace and Selman, 1981): (1) A cohort of oogonia are selected to form a clutch for the breeding cycle. (2) During previtellogenesis a single layer of granulosa cells develops around each oocyte and ovarian interstitial cells encircle the granulosa layer and form the theca cell layer (Nagahama et al., 1994). This assemblage of cells constitutes an ovarian follicle. Granulosa and theca cells are the sites of sex steroid hormone production (steroidogenesis) (Nagahama et al., 1994) as well as synthesis of several other biologically active molecules such as growth factors, cytokines and prostaglandins (Van Der Kraak et al., 1997). The zona radiata is a proteinacious non-cellular layer that forms between the granulosa cells and the oocyte of each ovarian follicle. (3) Vitellogenesis is the period of enormous oocyte growth. (4) Oocyte maturation and ovulation follows vitellogenesis. The coordinated actions of ovarian hormones and growth factors principally control these sequential events of oogenesis.

Environmental cues and reproductive endocrine homeostasis

Environmental signals (i.e. photoperiod, temperature) are received and integrated in the brain where they modulate hypothalamus-pituitary secretions that control reproductive hormone levels in the blood. Most important of these are pituitary gonadotropins that are secreted as a response to release of hypothalamic gonadotropin releasing hormone (GnRH). Although there has been significant debate in regards to the number of gonadotropins in fish (Tyler et al., 1991), it is currently accepted that two gonadotropin hormones are present in most, but not all, teleosts. These are generally called GtH-I and GtH-II, which are analogous to follicle-stimulating hormone (FSH) and luteinizing hormone (LH), respectively. Concentration of GtH-I is highest during early stages of oocyte development and during vitellogenesis, and for this reason GtH-I is thought to be involved both in steroid biosynthesis and in uptake of vitellogenin by the oocyte. In contrast, GtH-II dominates later stages of development and hence may stimulate synthesis of maturation inducing hormone. A major role of gonadotropins is to stimulate the gonads and their secretion of steroids. Secretion of sex steroids in turn initiates changes in secondary sexual characteristics,

behavior, development of gametes and their maturation, which leads to spawning (Tyler and Sumpter, 1996).

Vitellogenesis in many teleosts entails production of testosterone by thecal cells upon GtH-I stimulation. Testosterone then diffuses into the granulosa cell layer where it is aromatized to 17β-estradiol, which is released into the circulation and stimulates hepatocytes to produce vitellogenin. Vitellogenin travels back to the ovary where it passes through the thecal and granulosa cell layers, binds to vitellogenin specific receptors on the surface of the oocyte and is taken up by the receptor-mediated process of endocytosis (Specker and Sullivan, 1994). Subsequent to vitellogenesis, the oocyte enters a maturational phase prior to ovulation (Idler and Ng, 1983; Van Der Kraak and Donaldson, 1986).

Oocyte maturation is a hormonally controlled process and is technically referred to the period between the release of oocyte from the first meiotic arrest at prophase I, and the start of the second block at metaphase II of meiosis. Maturation of oocytes involves GtH-II, which predominates final stages of oocyte development (Suzuki et al., 1988; Swanson and Dickhoff, 1990; Swanson, 1991; Prat et al., 1996). Morphological characteristics of maturation

include germinal vesicle migration (GVM) to the animal pole (yolk free end of the oocyte) and its subsequent breakdown (GVB). Both GVM and GVB have been suggested to involve microtubular alterations and cellular cytoskeletal changes. In addition to a number of steroids that appear to be involved in GVB *in vitro* (Nagahama et al., 1994) two steroid hormones have been described as endogenous maturation inducing hormones (MIH) in fishes. 17α,20β-Dihydroxy-4-pregnen-3-one (17α,20β-DP) is found in most so far studied fishes, while 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-P) has been reported less frequently (Trant and Thomas, 1989; Thomas and Trant, 1989). It must be noted that both granulosa and thecal cells are required for gonadotropin-stimulated MIH biosynthesis by ovarian tissue (Young et al., 1986; Nagahama et al., 1994). During maturation of the oocyte, thecal cells produce 17α-hydroxyprogesterone which diffuses into granulosa cell layer where it is converted to 17α,20β-DP. Just prior to ovulation, a shift in steroidogenic pathway occurs which results in an increase in production of 17α, 20β-DP and a significant decrease in synthesis of 17β-estradiol. It is suggested that this shift in the steroidogenic pathway is regulated by gonadotropins (Van Der Kraak et al., 1997).

An optimal hormonal environment for growth, development, and maturation of oocytes is achieved by harmonious interactions of granulosa and theca cells, which have been shown to be sensitive targets for the action of many environmental contaminants (McMaster et al., 1996).

Steroidogenesis

Steroidogenesis is the process of sex steroid production, and in the ovary the primary sites for steroid biosynthesis are the theca and granulosa cells of the ovarian follicle (Fostier, et al., 1983). In the ovary the initial precursor for steroid biosynthesis is cholesterol, which is derived from animal fat of the diet or from *de novo* synthesis. Cholesterol arrives in the ovary via the bloodstream bound to low-density lipoproteins that have specific receptors on the plasma membrane of ovarian cells. After the receptor complex is internalized, cholesterol is released for biosynthesis of the major ovarian steroids (i.e. progesterone and estradiol) (Ojeda, 1996). Steroid synthesis begins in the mitochondrial membrane of thecal cells where side-chain cleaving enzymes of the cytochrome P-450 system (P450scc) convert cholesterol (C-27) to pregnenolone (C-21) in the rate-limiting step of the

steroid synthetic pathway. Pregnenolone is then converted to progesterone by the action of 3β-hydroxysteroid dehydrogenase. Progesterone is then further metabolized by the P-450 system to yield first 17α-hydroxyprogesterone and next androstenedione. As previously mentioned, close interaction between theca and granulosa layers plays an important role in regulation of steroidogenesis and ovarian development.

Other cellular proteins may also affect the process of steroidogenesis. Recent studies suggest that a variety of environmental stressors that cause increased expression of cellular stress proteins (i.e. heat shock proteins HSP) may also affect steroidogenesis. The 70 kDa stress protein (HSP70) functions as a cellular chaperone, participating in biosynthesis, repair, and transport of proteins through various cellular compartments (Mosser et al., 2000), and has an inhibitory effect on hormone-sensitive steroidogenesis (Khanna et al., 1994; Kime et al., 1996). In addition, HSP70 is involved in luteolysis in humans and rats (Khanna et al., 1995a, 1995b). Increasing evidence suggests that HSP70 may play a major role in protecting cells against apoptotic cell death at multiple steps in the

apoptotic process (Mosser et al., 1997, 2000, Creagh et al., 2000, Mallouk et al., 1999).

Apoptosis

It is now widely accepted that apoptosis or physiological cell death plays a crucial role in the development and homeostasis of biological systems (Steller, 1995). Apoptotic cell death is different from necrotic cell death both physiologically and biochemically. Necrosis results from physical damage or trauma to cells and involves random loss of cell structure and finally lysis of the cell. Unlike necrosis, apoptosis is an active, orderly, and sequential process, which most often requires biosynthesis of new proteins. Apoptosis takes place in individual cells scattered in the tissue and it involves cell shrinkage, nuclear and cytoplasm condensation, release of cytochrome *c* from the mitochondria, caspase activation, plasma membrane blebbing, externalization of phosphatidylserine (a plasma membrane phospholipid that is normally on the inner surface of the lipid bilayer), DNA fragmentation, and formation of small membrane bound vesicles (i.e. apoptotic bodies) that contain cellular organelles and debris that can be taken up and degraded by

surrounding cells (Wilson, 1998; Allen et al., 1997; Thompson, 1995). Apoptosis is normally not associated with an inflammatory response since plasma membrane integrity is sustained throughout the process and cytosolic contents of the cell are not released into the interstitial space (Hsueh et al., 1996).

A late apoptotic event is the activation of a family of calcium / magnesium dependent nuclear endonucleases (Samali et al., 1998). Endonucleases specifically cleave DNA at distinct internucleosomal sites and generate fragments of DNA in size multiples of 185-200 base pairs (bp). The hallmark of apoptotic death is a ladder pattern of these 200 bp long DNA fragments that can be visualized by gel electrophoresis (Wyllie, 1998). The ladder pattern of apoptotic DNA degradation is different than a "smear" of low molecular DNA on gels that results from the random breakdown of DNA occurring during necrosis. This difference in DNA cleavage pattern can be used to distinguish cells undergoing apoptosis from normal cells or necrotic cells.

In a developing organism, apoptosis is the mechanism by which an individual is carved out of a large cell mass (Vaux et al., 1994; Saunders, 1966, Hammar and Mottet,

1971). This suggests that apoptosis may have evolved to regulate exact cell numbers in an organ by eliminating superfluous ones (Raff, 1992; Raff et al., 1993). Apoptosis may also be viewed as a cellular adaptive mechanism for removal of unwanted and potentially harmful cells such as those that react with self-antigens (i.e. immunoreactive lymphocytes) or infected cells (Vaux et al., 1994). Although the exact mechanisms controlling apoptotic cell death are unknown, it appears that different intracellular signal transduction pathways converge to activate a common highly conserved death program in all animal cells from worms to humans.

The participation of mitochondria in apoptosis has recently gained much attention. The initial release of cytochrome *c* from mitochondria and formation of an apoptosome complex is followed by activation of caspases. Caspases are special proteases that act during the "execution" stage of apoptosis (Green and Reed, 1998; Li et. al., 1997). Cytochrome *c* is a component of the mitochondrial electron transport system and is involved in cellular ATP production by shuttling electrons between complexes. Cytochrome *c* is synthesized in the cytosol and is transported into the mitochondrial intermembrane space in an unfolded configuration. In the intermembrane space

of mitochondria a heme group is covalently at tached to the cytochrome, which causes a conformational change in the molecule, where it can then function in the respiratory chain. It is this holo-cytochrome *c* that can start the cascade of caspase activity. As indicated previously, the exact molecular mechanism(s) behind cytochrome *c* release from mitochondria in response to an apoptotic stimulus is (are) not clear. Furthermore, the precise way in which caspases achieve cell death is not known simply because of the variety of intracellular substrates for these enzymes and their ability to move across sub-cellular compartments after they are activated (Robertson and Orrenius, 2000).

Apoptosis related proteins

Early understanding of the molecular mechanism of apoptotic cell death came from embryogenesis of the nematode *Caenorhabditis elegans* (Ellis and Horvitz, 1986; Ellis et al., 1991; Gumienny et al., 1999). These studies specifically identified two pro-apoptotic (Ced-3, Ced-4), and one anti- apoptotic (Ced-9) proteins. In conjunction with biochemical studies in mammalian cells, it is now clear that the molecular mechanism of apoptosis is evolutionarily highly conserved in diverse animals. For

example, in mammals, interleukin-1 β-converting enzyme and apoptosis activating factor (Apaf) are the equivalents of Ced-3 and Ced-4 and Bcl-2 proteins are the counterparts of Ced-9 (Hsu and Hsueh, 2000). The Bcl-2 family of proteins was the first anti-apoptotic proteins discovered. Increased expression of this family of proteins blocks apoptosis in many cell types. It has now become clear that Bcl-2 proteins can homodimerize or heterodimerize with anti- apoptotic factors in the cell to regulate channel formation in the outer mitochondrial membrane, an event that stabilizes cytochrome *c* in the intermembrane space of mitochondria. In contrast to anti-apototic members, the Bcl-2 family of proteins also contain a pro-apoptotic group that when over-expressed in a cell will contribute to cell apoptosis (Hsu and Hsueh, 2000). A well studied pro-apoptotic member is Bax protein that in the presence of an apoptotic signal moves to the mitochondria where it becomes incorporated into the mitochondrial membrane and stimulates release of cytochrome *c* (Eskes et al., 1998; Finucane, et al., 1999). The Bcl-2 family of gene products plays an important role in ovarian tissue homeostasis and/or death, and alterations in their expression can rescue cells from death or induce cell death machinery (Mosser et al., 2000; Mosser et al., 1997).

Hormonal control of apoptosis and atresia in the ovary

Mammalian embryos hold a finite collection of oocytes encircled by a few somatic cells for oocyte maintenance and support. The vast majority (75%-99.9%) of mammalian embryonic oocytes are eliminated from the ovarian tissue before ovulation in a degenerative process known as atresia (Byskov, 1978; Hsueh et al., 1994). Atresia is a highly regulated and hormonally controlled process within the vertebrate ovary and is considered to be crucial for maintenance of ovarian homeostasis and cyclicity. Current available data strongly suggest that apoptosis is the molecular mechanism for follicular atresia in mammals and birds. In mammals, atresia occurs during any stage of follicular development and it is usually initiated by apoptosis of granulosa cells (Tilly et al., 1991; Kaipia and Hsueh, 1997). Much less is known about apoptosis in teleost ovarian function and a direct link between apoptosis and atresia in teleosts is yet to be demonstrated. Our knowledge from mammalian models should be applied to teleosts with care (Wood and Van Der Kraak, 2001). These data suggest that while in teleosts apoptosis is involved in normal ovarian growth and postovulatory regression, it may not be as important an event in teleost ovarian follicle atresia.

Regulation of follicular development and atresia is a complex, multifactorial process that involves interactions between endocrine factors (gonadotropins) and intraovarian regulators (sex steroids, growth factors and cytokines). Redundant intracellular pathways act to either rescue follicles from apoptotic demise (survival factors) or to promote atresia (atretogenic factors) in individual follicles. Most of our knowledge about the hormonal control of apoptosis comes from the mammalian *in vitro* culture model of isolated preovulatory follicles (Tsafriri and Braw, 1984; Hirshfield, 1991; Hsueh et al., 1996). The preovulatory follicular model is physiologically relevant because paracrine interactions (i.e. factors produced in one cell that act on the neighboring cell) between follicular cells are not disrupted (Chun and Hsueh, 1998). Gonadotropins are the major survival factors in ovarian tissue and suppress apoptosis and atresia through activation of the cyclic AMP-dependent protein kinase pathway. Gonadotropins bind to their membrane receptor on granulosa cells and activate adenylyl cyclase (a membrane bound enzyme), which leads to production of the second messenger cAMP and subsequent activation of cAMP-dependent protein kinase. Another pituitary hormone, growth hormone, also suppresses the onset of apoptosis in follicular cells

(Hsueh et al., 1996). A number of locally produced paracrine signals (IGF, EGF, TGF-α, KGF, HGF) are also involved in suppression of apoptosis in granulosa cells. These growth factors bind specific receptors on granulosa cells and inhibit cell death. The actions of these survival factors in turn are counteracted by atretogenic factors such as TGF-β, and gonadotropin-releasing hormone (GnRH) (Kaipia and Hsueh, 1997; Hsueh et al., 1994, 1996; Tilly, 1996). Estrogens and androgens are also potent endocrine players in the ovary with estrogens acting as follicle survival factors and androgens as atretogenic agents (Billing et al., 1993).

In addition to hormones, cytokines are also involved in regulation of apoptosis in the ovary (Andreu-Vieyra and Habibi, 2000). Cytokines are soluble protein products of many immune cells and are important in both immune regulation and communication between the immune and endocrine system (Casey, 1996). Interleukin-1β (IL-1β) is a cytokine that affects ovarian function in many cell types. IL-1β induces synthesis of nitric oxide (NO), which in turn activates guanylyl cyclase, and has been shown to be a follicle survival factor pathway (Kaipia and Hsueh, 1997). Other cytokines, including transforming growth factor-β, and

macrophage colony-stimulating factor (M-CSF) as well as the pituitary adenylyl cyclase-activating polypeptide (PACAP), a novel neuropeptide with significant homology to vasoactive intestinal peptide and growth hormone releasing hormone (GhRH), have also been reported to act as follicular survival factors (Lee et al., 1999).

Recent studies indicate that gonadotropin inhibition of apoptosis involves upregulation of oxidative stress response genes, whose protein products (i.e. superoxide dismutase, catalase) act to guard cells against deleterious effects of reactive oxygen species (Dharmarajan et al., 1999). Granulosa cells and the oocyte also produce cytokines such as TNF-α, and IL-6 that promote cell death. Moreover, other local hormone binding proteins such as IGF binding protein-4 (IGFBP-4) have been shown to inhibit apoptosis in the ovary (Wandji et al., 1998).

Ovarian function and apoptosis

Both germ cells and somatic cells of the vertebrate ovary may undergo apoptotic cell death and their survival and/or demise is solely determined by the hormonal factors that regulate ovarian function. The vast majority of information on ovarian function pertains to mammals. In

mammals apoptosis regulates follicular atresia and luteolysis which results from lack of sufficient exposure to follicular survival factors (i.e. gonadotropins and estradiol) or conversely, from increased exposure to follicular atretogenic compounds (i.e. testosterone, prostaglandins). During atresia in the fish ovary, much like mammals, granulosa cells that provide hormonal support for oocyte growth and development undergo rapid apoptotic cell death (Hsueh et al., 1994; Janz and Van Der Kraak, 1997). Ovarian follicles that escape atresia will continue to grow and eventually will be ovulated.

The apoptotic pathway may also be stimulated in response to environmental stressors such as malnutrition, temperature extremes, and exposure to many classes of environmental contaminants (Robertson and Orrenius, 2000). Whether a cell escapes an environmental insult or dies because of it is determined by its ability to induce proteins that either promote or inhibit cell death. To maintain regulation and selectivity of apoptosis, tight control must be exerted over the expression of many cellular factors. Although a wide variety of signals initiate apoptosis, the stepwise molecular path to death is highly evolutionarily conserved (McConkey, 1998). Recent data suggests that toxicant induced cellular stress may be

contributing to loss of reproductive tissue by apoptosis and decreased reproductive capacity in exposed individuals (Janz et al., 1997, 2001; Heimler et al., 1998; Savabieasfahani et al., 1999; Mann et al., 1999).

HSPs as biochemical indicators of environmental stress in aquatic organisms

A highly conserved cellular adaptive response, which allows cells to tolerate normally lethal conditions, is expression of a family of intracellular proteins called the heat-shock proteins (HSPs) (Morimoto et al., 1994). HSPs function as cellular chaperones, participating in biosynthesis, repair, and transport of proteins through various compartments of a cell (Mosser et al., 2000). HSPs are induced by a variety of stressors, including toxicants (Ryan and Hightower, 1994; Williams et. al., 1996; Janz et. al., 1997, 2001; Vijayan et. al., 1998) and have been used as biochemical indicators of environmental stress in aquatic species (Feder and Hofmann 1999; Iwama et al., 1998). Amongst HSPs that have so far been studied the HSP70 family are best defined with a constitutive 73 kDa form (HSP70), and an inducible 72 kDa form (HSP72). The two forms of HSP70 have similar functions, which involve

refolding of misfolded proteins and escorting irreversibly damaged proteins to lysosomes for breakdown and recycling. Recent investigations in mammals have shown that both forms of HSP70 play a crucial role in protection of cells against stress-induced apoptosis (Mosser et al., 1997, 2000; Mallouk et al., 1999). Furthermore, HSP70 induction has been associated, *in vitro*, with impaired ability of steroidogenic cells to synthesize steroids in response to acute stimulation (Stocco and Clark, 1996) possibly via suppression of steroidogenic acute regulatory protein (StAR) by HSP70 (Liu and Stocco, 1997). In addition to *in vitro* studies that link induction of HSP70 with suppressed steroidogenesis, HSP70 has also been associated with reduced steroidogenesis and luteal regression in rats (Khanna et al., 1994, 1995a). Field studies in wild fish exposed to bleached pulp mill effluents reported increases in ovarian cell apoptosis and HSP70 expression that were associated with reduced ovary size and alterations in circulating sex steroid hormone levels (Janz et al., 1997, 2001). Because of their roles in ovarian function, altered HSP70 expression may be relevant to the ovarian dysfunction observed in fishes exposed to toxicants. The dynamics of the relationship between HSP70 expression, steroidogenesis, and apoptosis in response to toxicant exposure warrants

investigation. The overall goal of this thesis was to examine mechanisms of toxicant-induced apoptosis in the ovary and to identify possible linkages between apoptosis, HSP70 expression, and steroidogenesis in fish.

Mechanisms by which HSP70 inhibits apoptosis

It has become increasingly clear that some HSPs, especially HSP70, can inhibit apoptosis (Mosser et al., 1997, 2000; Liossis et al., 1997; Samali and Orrenius, 1998). The molecular machinery that drives the apoptotic program consists of a family of cysteine proteases (caspases) that cut their substrate at specific sites (aspartic acid residues) on the protein polypeptide chain. Under normal physiological conditions, caspases are constitutively expressed in inactive forms known as procaspases. Procaspases are activated by specific proteolytic cleavage (Cohen, 1997; Thornberry and Lazebnik, 1998). Active caspases can augment apoptotic events by cleaving precursor forms of themselves and other caspases (Stennicke et al., 1998). Mitochondria play an important role in the initiation of the action of caspases in response to various apoptotic stimuli (Green and Reed, 1998). Rupture of the mitochondrial outer membrane by

apoptotic signals causes the release of cytochrome *c* into the cytoplasm (Li, et al., 1997; Saleh, et al., 1999). Cytochrome *c*, in the presence of dATP/ATP, interacts with and triggers oligomerization of the cytosolic apoptotic-protease-activating factor (Apaf-1). The resulting structure is called an apoptosome, which recruits and activates procaspase-9, which in turn recruits, cuts and activates caspase-3 and caspase-7 (Cain et al., 2000; Hu et al., 1999). HSP70 has been shown to inhibit Apaf-1-mediated activation of procaspase-9 and apoptosis (Saleh et al., 2000; Beere et al., 2000; Li et al., 2000). These findings strongly suggest that HSP70 is an important anti-apoptotic regulator in cells and that it functions at a very early stage in the apoptotic pathway (Creagh et al., 2000). Because of the role HSP70 plays in inhibition of apoptosis and its involvement in steroidogenesis it was of keen interest in our investigation. This study was designed to examine possible linkages between three highly conserved biochemical pathways (apoptosis, HSP70 induction, steroidogenesis) that play crucial roles in ovarian homeostasis and have been shown to be targets for the actions of many ubiquitous environmental contaminants such as PAHs and heavy metals.

Environmental pollution and the oxidative stress hypothesis

The interest in apoptosis has been growing in toxicology as more studies inspect the ability of environmental contaminants to induce apoptosis in various biological systems. Increasingly, laboratory and field studies report the ability of common environmental pollutants to exert their toxicity, at least in part, by promoting apoptosis (Goldsmath et al., 1998; Janz at al., 2001, 1997; Heimler et al., 1998; Mann et al., 1999; Marty et al., 1997; Savabieasfahani et al., 1999; Hiura et al., 2000; Zhai et al., 2000). Many reported toxicants exert their apoptotic effect by generating reactive oxygen species (ROS) that damage mitochondria, and current literature on apoptosis strongly suggests the involvement of mitochondria in apoptotic cell signaling by various stimuli (O'Brien and Salacinski, 1998; Thevenod and Friedman, 1999; Kunimoto, 1994; Lin et al., 1997; Stridh et al., 1999; Bratton et al., 2000; Ye, et al., 1999; Huang et al., 2000; Szuster-Ciesielska et al., 2000). It is therefore conceivable that a common mechanism for the toxic actions of many environmental pollutants may be their ability to damage mitochondria and induce release of cytochrome *c* from the mitochondrial membrane; an event that initiates the cascade of caspase action (Green and Reed,

1998). The oxidative stress hypothesis has been emerging from a wealth of studies that link stress-induced generation of ROS to alteration of normal physiological functions in a cell. This hypothesis states that severe damage to cells causes an increase in concentration of ROS in the cell, which overwhelms cellular machinery responsible for their proper discarding. This increase in ROS concentration triggers cellular signaling pathways that direct cells to undergo apoptotic death.

Reactive oxygen species are ubiquitous in all aerobic organisms and may have endogenous and exogenous sources. Mitochondrial oxidative phosphorylation reactions are a continuous source of ROS formation in cells. Common free radicals of oxygen ($^{\bullet}OH$, $^{\bullet}NO$, and $O_2^{\bullet -}$) are short lived but can react with non-radicals to form a new radical species and perpetuate a chain of free radical formation (Shackelford et al., 2000). Hydrogen peroxide (H_2O_2) is also produced in the mitochondria and is relatively non-reactive towards DNA but can be involved in formation of the highly reactive hydroxyl radicals ($^{\bullet}OH$) that rapidly react with DNA and can cause various DNA modifications (Chance et al., 1979; Boveris and Chance, 1973; Forman and Boveris 1982; Michalik et al., 1995). Mitochondrial DNA is more prone to

damage by ROS because of its close proximity to enzyme systems of the electron transport chain that generate high concentrations of $^{\bullet}OH$ and H_2O_2. To escape deleterious effects of mitochondrial DNA damage, and to maintain genomic integrity, aerobic organisms have developed redundant adaptive mechanisms to protect both mitochondrial and nuclear DNA from damage by ROS. These mechanisms include constitutive and inducible levels of cellular antioxidants that effectively neutralize ROS, and enzyme defense mechanisms (i.e. catalase and superoxide dismutase (SOD)) that isolate and metabolize ROS (Halliwell and Gutteridge, 1986). In addition there are DNA repair enzymes to remove mutations and mechanisms for genomic inspection (i.e. cell cycle check point system) to monitor DNA fidelity. To maintain genomic integrity and avoid mutations, cells activate a genome surveillance pathway or a cell cycle checkpoint response (Hartwell and Weinert, 1989; Kaufmann and Paules, 1996). A cell cycle checkpoint response is a pause in the progress of cell cycle, which will allow the cellular DNA repair machinery to correct mistakes and avoid further damage. Toxicant insult to a cell activates detoxification enzymes, many of which are also located in the inner mitochondrial membrane. Increased metabolic activity in the cell invariably

contributes to increased production of ROS and can exhaust cellular machinery to remove these radicals. Increased oxidative stress has been shown to cause the release of mitochondrial cytochrome *c* into the cytosol thus initiating apoptosis (Feng Gao et al., 2001).

Despite the overwhelming rate of continual basic research on apoptosis, relatively little is known about mechanisms of toxicant-induced apoptosis. A wealth of current scientific literature substantiates the hypothesis that toxicant-induced apoptosis may accelerate atresia in the ovary of many vertebrates and contributes to reproductive impairment in diverse organisms. For this reason, there is an urgent need to (1) characterize environmental toxicant induced apoptosis and (2) develop a screening model in which to assess ovotoxicity of currently used xenobiotics. Moreover, since certain cell types are naturally more prone to apoptosis, potential target sites are easy to select for further investigations. Currently there is both the need and the technical ability to study, in detail, the role of apoptosis in cell death caused by environmental contaminants.

Apoptosis in hematopoetic, immune, and ovarian cells of wildlife exposed to environmental contaminants

Immune and hematopoeitic cells represent highly organized and regulated populations of cells with the ability to communicate and maintain physiological integrity and homeostasis of an organism. Maintenance of homeostasis in immune and hematopoetic systems involves continuous cell proliferation, differentiation, and death by apoptosis (Williams, 1994; Boorman et al., 1982). A number of environmental toxicants such as PAHs and metals have been shown to be immunotoxic and compromise immune function (Tomar and Kekvliet, 1991; Davila et al., 1995; Lochmiller et al., 1999). In addition, recent studies have demonstrated induced rates of apoptosis in immune and ovarian cells following *in vivo* and *in vitro* exposure to toxicants in laboratory animal models. For example, polycyclic aromatic hydrocarbons (Hinoshita et al., 1992; Yamaguchi et al., 1996, 1997; Holladay and Smith, 1995; Holladay et al., 1998; Lutz et al., 1998; Hardin et al., 1992; Mann et al., 1999), halogenated aromatic hydrocarbons (McConkey et al., 1988; Kamath et al., 1997) and heavy metals (Shanker et al., 2000; Tsangaris and Tzortzatou-Stathopoulou, 1998; Guo at al., 1998) have been shown to induce apoptosis in immune cells. Moreover, toxicants such

as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 4-vinylcyclohexene diepoxide have been reported to induce apoptosis in ovarian cells *in vivo* and *in vitro* (Springer et al., 1996; Heimler et al., 1998). We hypothesized that the highly proliferating cell types of immune, hematopoetic, and ovarian tissues are most at risk for toxicant induced apoptosis. Therefore we examined rates of cell death by apoptosis in bone marrow, thymus, spleen and ovarian tissues in wild vertebrate inhabitants of sites contaminated with PAHs and heavy metals.

Hypotheses

Based on studies that correlate reproductive impairment to environmental toxicant exposure (e.g. Van Der Kraak et al. 1992, McMaster et al. 1996, Janz et al. 1997, Savabieasfahani, et al., 1999) the following hypotheses were generated to be investigated:

1. Fish ovarian follicles exposed to PAHs and trace metals *in vitro* will exhibit increases in apoptotic cell death when compared to unexposed fish follicles.
2. Follicular survival factors will suppress the rate of spontaneous apoptosis in cultured fish follicles, and these

factors will also suppress toxicant induced apoptotic DNA fragmentation in fish follicles.

3. Exposure to toxicants will reduce steroid hormone production in cultured follicles.
4. Cellular stress proteins (i.e.HSP70) will be induced in toxicant exposed follicles.

Based on studies that demonstrate induction of apoptosis in hematopoetic, immune, and ovarian tissues as a result of exposure to environmental toxicants we hypothesized:

1. Apoptotic cell death will be higher in bone marrow, thymus and spleen tissue of cotton rat inhabitants of toxic waste sites compared to those living on reference sites.
2. Ovarian apoptosis and atresia will be higher in rats living on contaminated sites in comparison to rats inhabiting reference sites.

METHODS

General outline of experiments

We used ovarian follicle culture method to determine effects of hormones, growth factors, and toxicants on apoptosis, HSP70 expression, and sex steroid hormone production in two fish species, fathead minnow (*Pimephales promelas)* and channel catfish (*Ictalurus punctatus*) with contrasting reproductive strategies (asynchronous Vs synchronous spawnners). After incubation of follicles in serum-free media for 48 (fathead minnow) and 24 (channel catfish) hours, follicles and culture media were collected. DNA extracts from follicles were used to assess extent of apoptotic fragmentation by 3′ End labeling of the DNA. Expression of HSP70 in the same follicles was measured by western blotting, and steroid hormone concentration in culture media was measured using an enzyme-linked immunosorbent assays (ELISA).

We also examined extent of apoptotic DNA fragmentation in ovaries of cotton rats (*Sigmodon hispidus*) from presumed petrochemical contaminated sites and ecologically matched reference sites. We used ovarian tissue slices of cotton

rats from contaminated and reference sites to examine apoptosis on cellular level by Tdt-mediated dUTP nick end labeling (TUNEL) assay. We aimed to correlate molecular data (3′ End labeling data) with cellular level data (TUNEL) to investigate the specific cell type in which apoptosis more commonly occurred.

Experimental animals

Fathead minnows

Mature female fathead minnows (*Pimephales promelas)* were acquired from colonies of the Ecotoxicology and Water Quality Research Laboratory at Oklahoma State University. Female fathead minnows were kept in aquaria with continuous flow water at 23°C until they were brought to the laboratory for experimental use.

Channel catfish

Four channel catfish (*Ictalurus punctatus)* were obtained in June 1998 from Durant and Tishamingo hatcheries in Oklahoma where they were kept in cement outdoor raceways. The fish were transported to Oklahoma State University in a large aerated water tank with cover to minimize heat exposure and were kept in 1000-liter raceways for later use.

During May and June 1999, three catfish were caught by gill netting from Lake Carl Blackwell in Oklahoma and brought to our laboratories live in containers of lake water. Fish were maintained, overnight, in our facilities for later use.

In May, June and July of 2000, three female catfish were obtained; one from Lake Carl Blackwell and two from a local pond on the property of Mrs. Elaine Stebler (Route 3 Box Number 375, Perkins, Oklahoma 74059, USA). Animals were brought back to the laboratory alive, in buckets of lake water, and were killed soon after arrival.

Cotton rats and collection sites

Landfarming sites

In the summer of 1998 wild female cotton rats were live-trapped from populations living on five land farming sites in Oklahoma. Processing of crude oil uses many chemicals and generates up to 270 billion pounds of hazardous waste each year (Schroder, 2000). Land farming is a method of disposal for petrochemical contaminants and consists of application of waste to soil followed by addition of fertilizer and tillage to enhance biodegradation. Four contaminated landfarmins sites, which we used as our study sites in this investigation,

were located on an abandoned oil refinery in Duncan, Oklahoma. These sites were used for landfarming practices, which were discontinued in the early 1980s. The fifth site was located in Ponca City, Oklahoma on an active oil refinery where wastes were land treated for rapid biodegradation. These sites were presumed to be contaminated with heavy metals and PAHs.

Reference sites

Cotton rats were also collected from five ecologically matched nearby reference sites (i.e. biotic and abiotic components of the landfarming (toxic) and reference sites resembled each other very closely). Concentration of heavy metals averaged 30.0 ± 3.3 ppm on reference sites while total PAHs were low to non-detectable on these sites.

The predominant plant on both contaminated and their matched reference sites were Johnson Grass (*Sorghum halapenes*), Little Bluestem (*Schizachyrium scoparium*), Big Bluestem (*Andropogon gerardii*), and Bermuda grass (*Cynodon dactylon*). All study sites were early successional, tall grass prairie ecosystems.

Cotton rats

Cotton rats were anesthetized, killed by cervical dislocation, and the thymus and one ovary removed, weighed,

and immediately frozen in liquid nitrogen. The other ovary was immersed in buffered 4% paraformaldehyde for subsequent histological analyses. The following summer (1999), wild female cotton rats were collected from the same sites in a similar manner. Animals were anesthetized and killed by cervical dislocation and this time the bone marrow content of the left femur was washed into a 3-ml cryo-tube with a 15-gauge sterile syringe. Approximately 1/8 of the total spleen from each individual was also collected for apoptosis studies while the rest of the tissue was used for studies of immune competence.

Preparation of stock solutions

9, 10 Dimethyl- 1, 2, benz[a]anthracene (D3254) was purchased from Sigma Chemicals (St. Louis, MO) and a stock solution of 100 mM in acetone was prepared and kept at -20 °C. The stock was diluted 1000 times (100 μM DMBA) for use in incubation wells. Phorbol 12-myristate 13-acetate (PMA) (Sigma, P-8139) was dissolved in DMSO, 1g/ 4.05 ml. The 0.4 mM concentration was attained by serially diluting PMA in DMSO and the final concentration of 4 μM was achieved by adding 1 μl of this dilution to 1 ml of medium 199 in each well for follicle treatment. A stock solution of 100 mM cadmium chloride (Fisher Scientific, MW = 228.4 anhydrous)

in ultra pure water was made. A final concentration of 100 μM cadmium chloride in culture media was achieved by addition of 1 μl of this stock solution to 1 ml of medium 199 in each well. Subsequently, one ml of the above stock solution (100 mM) was added to 9 ml of ultra pure water. A final concentration of 10 μM cadmium chloride in culture media was achieved by addition of this dilution in 1 ml of medium 199 in each well. Testosterone propionate anhydrous (TES) (MW = 344.5, Sigma, St. Louis, MO) was dissolved in ethanol (100 mg/ml) and kept at -20 °C. Aliquots of 1 μl TES were added to each well prior to incubation.

17β-Estradiol (E-2758) was also prepared in ethanol (100 mg/ml) and added to 1 ml of media for a final concentration of 100 ng/ml in incubations. A 20 μg/ml stock solution of epidermal growth factor (Sigma), was made in M199 with 1% BSA. The final concentration of 0.1 μg/ml was achieved by adding 5 μl of this stock to 1 ml of M199 in each incubation well. Forskolin (Sigma) was made in DMSO at 4.11 mg/ml and was refrigerated (-20 °C) for use in incubations. Forskolin stock was thawed and 1 μl was added to 1 ml of media (M199) to attain a final concentration of

10 μM in incubations and 3-isobutyl-1methylxanthine (IBMX; Sigma, 0.222 mg/ml M199) was prepared fresh each day.

Follicle collection and treatments

Ovaries from 2-3 mature female fathead minnows were removed and placed in a perti dish with medium 199 such that the ovaries were completely submerged in the media. We used a collection of ovaries from different fish (i.e. pooled) because the ovary of an individual fathead minnow in most cases did not contain sufficient number of preovulatory follicles to be used in setting up a trial. Preovulatory follicles (0.85 ± 0.1 mm, n = 13) were separated from ovarian tissues and mixed well to provide a homogeneous pool of follicles. Thirty intact follicles were placed in each well of 24-well polystyrene tissue culture plates with 1 ml of medium 199 (M199; GibcoBRL) supplemented with HEPES (6g/l), bovine serum albumin (1g/l), sodium bicarbonate (0.35g/l) and streptomycin sulfate (0.1g/l). Catfish ovaries were removed from each female channel catfish and preovulatory follicles (1.98 ± 0.06 mm, n = 6) were separated from ovarian tissue with fine forceps. Eight intact follicles were placed in each well of 24-well polystyrene tissue culture plates with 1 ml of medium 199 with similar supplementation. Immediately

prior to the start of incubations, media in each well was replaced with 1 ml M199 containing one of the following treatments: 100 μM dimethylbenz[a]anthracene (DMBA, a prototypic polycyclic aromatic hydrocarbon and aryl hydrocarbon receptor (AhR) agonist), 0.4 μM phorbol myracetate (PMA, a protein kinase C activator), 10 and 100 μM $CdCl_2$, 4 μM 17β-estradiol, 3 μM testosterone, 0.1 μg/ml epidermal growth factor (EGF) and 10 μM forskolin + 0.22 mg/ml isobutylxanthine (forskolin/IBMX, activator of cAMP-dependent protein kinase). Concentrations of treatments are reported as nominal levels. Control follicles received 1 ml of medium only. After incubation at 22°C, media were collected and follicles were immediately frozen in liquid nitrogen. Follicles were used to determine apoptotic DNA fragmentation and HSP70 expression, while media were used to measure concentrations of testosterone and 17β-estradiol.

Biochemical analysis of apoptosis

DNA extraction

Total genomic DNA was extracted and phenol/chloroform purified as described in Gross-Bellard et al. (1973). Briefly, ovarian follicles were thawed on ice and placed in

1.5-ml micro centrifuge tubes containing 400 µl of homogenization buffer (0.1 M NaCl, 0.01 M EDTA, 0.3 M Tris-HCl, 0.2 M sucrose, pH 8.0), and gently homogenized with a hand-held homogenizer. After adding 25 µl of 10 % SDS to each sample, they were incubated at 65°C for 30 minutes. 70 µl of 8 M potassium acetate was then added to each sample and mixed well prior to one-hour incubation on ice. Following this incubation tubes were microcentrifuged at 8000*g* for 10 minutes at 4°C in an Eppendorf Model 5415C microcentrifuge. The supernatant was collected in a new microcentrifuge tube and extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) until no protein contamination was apparent. A single extraction with chloroform:isoamyl alcohol (24:1, v/v) followed. Nucleic acids were precipitated with 2.5X volume ice-cold 100% isopropanol, and incubated overnight at -20°C to allow small molecular weight DNA to precipitate. The next morning tubes were microcentrifuged at 14,000*g* for 30 minutes; the pellets were air-dried for 20 min at room temperature before they were resuspended in 40 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). After addition of 2 µl of DNase-free RNase (Boehringer Mannheim) samples were incubated for 1 hour at 37°C and extracted once with an

equal volume of phenol:chloroform:isoamyl alcohol followed by extraction with an equal volume of chloroform:isoamyl alcohol. Sample DNA was precipitated by adding 4 µl of 3 M sodium acetate and 100 µl of ice-cold isopropanol and incubation at -20°C overnight to ensure total recovery of small DNA fragments. The following day DNA was collected, pellets were washed with 175 µl 80% ice-cold isopropanol, and air-dried for one hour before they were resuspended in 30 µl of sterile water. The yield of DNA from approximately 30 fathead follicles, which was measured by spectrophotometry (GeneQuant), was 15.7 ± 1.38 µg (mean ± SEM, n = 50 individual samples of 30 follicles each), and 8 catfish follicles had a yield of 14.15 ± 1.86 µg (n = 50). DNA purity was determined to be 91.5 ± 1.1 (mean ± SEM, n = 45) for fathead minnow samples. Similarly for catfish follicle, DNA purity was 94.7 ± 1 (n = 53). DNA was quantified by measurement of sample absorbency at 260 nm.

3' End labeling of DNA

The DNA 3'-end labeling assay is described by Tilly and Hsueh (1993). Briefly, 2 µg of DNA was added to a 1.5 ml microcentrifuge tube and the volume was brought up to 30 µl with sterilized water. To each tube containing sample

DNA the following reagents were added: 10 µl 5XTdt enzyme reaction buffer (0.2 M potassium cacodylate, 25 mM Tris-HCl, 0.25 mg / ml bovine serum albumin, pH 6.6), 5 µl $CoCl_2$ (2.5 mM $CoCl_2$ final concentration), 4 µl [α-^{32}P]ddATP (20 µCi, 13.3 pmole, Amersham Pharmacia), and 1.25 µl (30 U) terminal transferase. Tubes were vortexed, microcentrifuged briefly and then incubated in a 37 °C water bath. After one hour, the reaction was terminated by addition of 5 µl of 0.5 M EDTA (pH 8). Two microliters of tRNA solution (50 µg) as carrier was added to samples and DNA was precipitated with 0.2 vol (12 µl) of 10 M ammonium acetate and 3 vol (180 µl) ice-cold isopropanol. Samples were mixed well by repeated inversions then incubated for one hour at -20 °C after which they were microcentrifuged at 14,000 g for 20 minutes at 4 °C to pellet the DNA. The radioactive supernatant was discarded and the DNA pellet was resuspended in 60 µl 1XTE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). DNA was precipitated once more by adding 12 µl of 10 M ammonium acetate and 180 µl of ice-cold isopropanol followed by incubation at -70 °C for one hour. Samples were then microcentrifuged at 14,000 g for 20 minutes at 4 °C to pellet the DNA. The radioactive supernatant was discarded,

tubes were inverted on a paper towel and the pellets were air-dried for one hour with an electric fan at room temperature. The fully dried pellets were resuspended in 30 µl 1XTE buffer and left at room temperature for 2 hours before overnight incubation at 4°C to resuspend DNA. The following morning samples were brought to room temperature and 6 µl of DNA gel-loading buffer (0.25% bromophenol blue, 0.25% xylene, and 30%glycerol) was added to each tube to be loaded on to a 2% agarose gel. Agarose gel (2%) was prepared by adding 3 g of agarose to 150 ml of 1XTAE buffer (40 mM Tris-acetate, 1 mM EDTA) and heating the mixture in a microwave oven for 30-second intervals until complete dissolution of agarose. Gels were poured when agarose was at 80°C and left to solidify with combs for 30 minutes at room temperature. Twenty-three microliters of sample was loaded onto the gel and resolved by electrophoresis (110 volts) for approximately 3 ½ hours using 1XTAE as running buffer. Following electrophoresis gels were dried in a slab-gel dryer (*without* heat) for 45 minutes. Dried gels were wrapped in plastic and exposed to X-ray film at -70 °C for 24 hours. Autoradiographs were used to guide in the excision of the low molecular weight DNA from the gel with a sharp scalpel. Cerenkov counting without scintillation

fluid was used to measure radioactivity. These readings were used as a quantitative estimate of the degree of DNA labeling. Data were expressed as nmol ddATP incorporated/μg DNA.

Detection of heat shock protein expression (HSP70)

Western immunoblotting and densitometry were used to quantify expression of HSP70 (Janz et al., 1997). Most of the yolk proteins from follicles were removed by gently disrupting eggs in Tris-KCl buffer (50 mM Tris-HCl, pH=7.5 and 150 mM KCl) with a microtube pestle and yolk proteins were allowed to diffuse and dissolve in the buffer. The suspension was centrifuged at 2000Xg for 10 min at 4°C. The supernatant was discarded and the pellet containing follicular cells was homogenized in a 1 ml glass-teflon Potter-Elvehjem homogenizer (5 X 10 sec at 2000 rpm) in 125 μl of ice-cold buffer (50 mM HEPES (pH=7.5), 150 mM NaCl, 1% Triton-X-100, 10% glycerol, 1 mM EGTA, 1.5 mM $MgCl_2$, 0.1 mg/ml AEBSF, 20 μg/ml soybean trypsin inhibitor, 4.26 μg/ml leupeptin and 1.9 μg/ml aprotinin). The homogenization tube was washed twice (2 X 62.5 μl) to minimize loss of cellular proteins and homogenate was transferred into a 1.5 ml microcentrifuge tube and left to rotate for 1 hr at 4°C. After centrifugation at 10,000Xg for 25 min at 4°C, protein

content of the supernatant was measured using a DC protein assay (BioRad) and samples were stored at -80°C. Total follicular protein (200 μg/lane) was separated using SDS-PAGE with 10% acrylamide gels (3½ hours at 70mA) and transferred onto 0.45 μm nitrocellulose membranes (Bio-Rad) for 16 hours at 30V and 4°C. Membranes were blocked with 5% skim milk in Tris-buffered saline (TBS) (20 mM Tris (pH=7.5), 500mM NaCl) for one hour while on a shaker. A monoclonal anti-mouse IgG-HRP antibody (Sigma H-5147) at 1:5000 dilutions, in TBS was used to blot samples for HSP70 for 1 hour at room temperature. Membranes were washed for a total of 30 minutes (three successive washes of 5, 10, and 15 minutes) with TBS (with Tween-20, 0.05% final concentration) to remove excess antibody followed by a 30-minute incubation with anti-mouse IgG-horseradish peroxidase conjugate (1:5000 dilution). Membranes were exposed x-ray film (Molecular Technologies, St. Louis MO) for one second in the dark to visualize protein bands. NIH Image software was used to determine the optical density of the bands.

Sex steroid hormone determinations

Steroid hormones were ether extracted from culture media (McMaster et al., 1992) and 17β-estradiol and

testosterone concentrations were determined using enzyme-linked immunosorbent assays (ELISA) (Cayman Chemicals Ann Arbor, MI and Oxford Biochemical Research Inc. Oxford MI). ELISA is based on competition between steroid hormone present in the sample (testosterone or 17β-estradiol) and a hormone molecule conjugated with acetylcholinesterase (hormone-tracer) for a limited number of hormone specific rabbit antiserum binding sites. The concentration of hormone-tracer is kept constant and since the concentration of hormone in each sample varies; the amount of tracer that is able to bind to the rabbit antiserum will be inversely proportional to the amount of sex steroid molecules present in the sample. This rabbit antiserum-hormone (free or tracer) complex binds to the mouse monoclonal anti-rabbit antibody that lines each well of a plate. The plate is emptied of the reaction mixture and washed to remove any unbound reagents or molecules from the well. After washing, the Ellman's Reagent, which contains the substrate for acethylcholinesterase, is added to each well. The resulting enzymatic reaction has a distinct yellow color with a strong absorbency at 412 nm. A spectrophotometric measurement of the intensity of this color is proportional to the amount of hormone-tracer bound to the wells of the

plate, which is inversely proportional to the amount of sex steroid hormone present in each sample.

For 17β-estradiol, extraction efficiency was 97% and intra-assay and inter-assay coefficients of variation were 13.2% and 17.2% respectively. For testosterone, extraction efficiency was 99% and the intra and inter-assay coefficients of variation were 8.2% and 12% respectively. Parallelism between extracted samples and the standard provided by the commercial kit was observed for each hormone assay.

Tdt-mediated dUTP nick end labeling (TUNEL)

Cotton rat ovaries were fixed and paraffin embedded, then cut in 5 μm thick sections and mounted on microscope slides (Fisher, Pittsburg, PA) for use in a modified version of TUNEL assay as described in Gavrieli et al. (1992). DNA in ovarian sections from two land farming sites and corresponding reference sites was 3'-end labeled using a commercial In Situ Cell Death Detection, AP kit (Boehringer-Mannheim, Indianapolis, IN). Tissue sections were deparaffinized and rehydrated prior to incubation with 40 μg/ml Proteinase K (Boehringer-Mannheim) for one hour in a humidified incubator at 37°C. A positive control slide was included in every TUNEL assay, which was incubated with

500 ng/mL of DNase for 1 hour at 37°C. A negative control slide was also included in each assay, which was incubated with deoxyUTP-fluorescein without terminal transferase. Apoptotic nuclei in ovarian cells were 3'-end labeled by terminal transferase and deoxyUTP-fluorescein. The labeled nuclei of follicular cells were identified by incubating slides with alkaline phosphatase- conjugated anti-fluorescein antibody for 30 minutes at 37°C. Slides were then incubated with tetrazolium (Boehringer-Mannheim) for 15-20 minutes until distinct purple nuclei were evident in the positive control slide. Slides were then dehydrated and mounted with Permount (Fisher Scientific). Blinded slides were analyzed for apoptotic cells using a light microscope.

Morphological identification of atretic follicles

Mid-ovarian sections from cotton rats collected from two land farm and two reference sites were stained with hematoxylin and eosin, and examined under a light microscope. Follicles were classified as either primary (<150 μm with fewer than 20 somatic cells) or antral/preovulatory (>400 μm with at least four layers of granulosa cells). Primary follicles with greater than 3

pyknotic nuclei and antral follicles with greater than 5 pyknotic nuclei were considered atretic (Tsafriri and Braw, 1984). The number of primary and antral follicles exhibiting atresia was expressed as a percentage of the total number of ovarian follicles (atretic + healthy) at each developmental stage. Corpora lutea were also counted in each section.

Statistical analysis

Fish data

Data was tested for homogeneity of variances (Bartlett's test) and normality (Kolmogorov-Smirnov test, InStat Statistical software, 1992-1998 Graph Pad InStat Statistical Software). A one-way analysis of variance (ANOVA) was used to test for treatment effects and was followed by a modified Bonferroni test as an a posteriori test for the comparison of differences between treatment means. This test compares each treatment to the control.

Paired t-tests were used for comparisons other than to the control.

Cotton rat data

Ovary, thymus, bone marrow and spleen data were tested for homogeneity of variance (Bartlett's test) and normality (Kolmogorov-Smirnov test). These conditions were met and

the data was subsequently analyzed using a two-tailed Student t-test. Statistical significance was set at $p < 0.05$. An unpaired t-test was used to analyze follicular atresia data combined from sites 1 and 5. Statistical significance was also set at < 0.05.

Results

To determine an incubation time that would correspond to maximum apoptotic DNA fragmentation of fathead minnow ovarian cells in follicles cultured in serum-free media, a preliminary time course experiment was conducted. There was no significant difference in the rate of apoptosis between uncultured follicles immediately snap frozen in liquid nitrogen (1.81 ± 0.26 nmol/μg DNA, n = 7) and follicles cultured for 24 hours (2.16 ± 0.71 nmol/μg, n = 4). However there was a four-fold increase ($p = 0.0003$) in apoptosis when comparing 24-hour cultures with those incubated for 48 hours (7.01 ± 0.44 nmol/μg, n = 7). The incubation time for all subsequent treatments was set at 48 hours based on these results.

Fathead minnow follicle time course

There was no significant increase in the extent of ovarian follicular cell apoptosis until 48 hours (7.01 ± 0.44, n = 7) past incubation in serum free media (Fig. 1). The 12-hour cultures exhibited a significantly lower rate of apoptosis when compared to follicles incubated for 72 hours ($n = 2$, $p < 0.01$). Apoptotic cell fragmentation was

similar in 24 (7.7 ± 2.4), 48 (7.0 ± 0.4) and 72-hour (10.83 ± 2.3) incubations and only differed when comparing these time points to the *in vivo* rate of apoptosis (follicles that were immediately frozen in liquid nitrogen after fish dissection) ($n = 7$, $p < 0.001$).

Figure 2 depicts a time course of HSP70 expression in fathead follicles incubated in serum free media for 0 hours (6.2 ± 1.2, $n = 4$), 12 hours (1.18 ± 0.16, $n = 3$), 24 hours (2.7 ± 0.11, $n = 3$), 48 hours (6.82 ± 1.2, $n = 5$), and 72 hours (1.85 ± 1.28, $n = 3$). HSP70 levels significantly declined during the first 12 hours of follicle incubation in serum free media, however, these levels increased again and reached initial levels after 24 hours of incubation.

Follicular testosterone biosynthesis at selected time points were compare to the 12-hour cultures because this was the earliest time point in this set of experiments. Testosterone biosynthesis was similar at 12 (50 ± 1.9, $n = 3$) and 24 hours (53 ± 0.22, $n = 3$, $p > 0.05$), but it was significantly higher when comparing media extracts from 48-hour cultures (122.82 ± 12, $n = 6$, $p < 0.001$) to 12 and 24-hour cultures. Testosterone levels in culture media were

not different at 72 hours compared to other time points (80 ± 16.4, n = 3, $p > 0.05$)(Fig.3A).

17β -estradiol production was similar in 12 (44 ± 4.76, n = 4) and 24-hour (39 ± 6.76, n = 3) cultures and it increased significantly at 48-hour (149.5 ± 12.3, n = 6, $p < 0.001$)(Fig. 3B). There was no difference in 17β -estradiol levels when comparing 72-hour media extracts to samples that had been incubated for 12 or 24-hours only ($p > 0.05$) (Fig.3B).

Fathead minnow follicle treatments

Epidermal growth factor (4.36 ± 0.6, n = 6, p = 0.01), 17β-estradiol (1.3 ± 0.3, n = 5, p = 0.0003) and PMA (1.7 ± 0.4, n = 6, p = 0.0004) significantly suppressed DNA fragmentation in ovarian follicles cultured for 48 hour compared to follicles incubated in serum-free medium (Fig. 7A). Forskolin / IBMX (4.8 ± 1.5, n = 5) did not significantly suppress apoptosis. DMBA (4.44 ± 1.8, n = 4), testosterone (3.75 ± 1.35, n = 6), and 10 μM $CdCl_2$ (7.65 ±2.34, n = 4) had no effect on ovarian cell apoptosis when compared to follicles cultured in serum-free medium (Fig.7B). However, addition of 100 μM $CdCl_2$ to the medium

(26.55 ± 4, n = 5) significantly (p = 0.0008) increased apoptotic DNA fragmentation in follicular cells. A two-tailed student t-test determined that treatment of follicles with a combination of forskolin / IBMX and 100 μM $CdCl_2$ (3.2 ± 1, n = 7) suppresses (p = 0.015) cell death, when compared to the 100 μM $CdCl_2$ treatment alone (Fig. 7B). Apoptotic DNA fragmentation was also significantly suppressed (p = 0.02) when comparing this treatment with control follicles.

HSP70 expression was similar when comparing 0-hour (snap frozen)(6 ± 1.22, n = 4) follicles with folliles that were cultured for 48 hours in serum-free medium (4.4, ± 1, n = 12, Fig. 2). Treatment of follicles with EGF significantly induced HSP70 expression (8.44 ± 0.82, n = 11, p = 0.02) in comparison to follicles cultured in serum-free media (Fig.8A). Forskolin / IBMX (8.3 ± 1.6, n = 8) and 17β-estradiol (6.77 ± 1.07, n = 11), and PMA (5.38 ± 0.82, n = 9) treatments did not significantly induce HSP70 expression (Fig. 8A). Testosterone treatment (7.76 ± 0.53, n = 12) significantly (p = 0.03) increased expression of HSP70 in follicular cells (Fig. 8B). DMBA (7.57 ± 2.66, n = 5), and 100 μM $CdCl_2$ (7.25 ± 0.87, n = 14), and forskolin /

IBMX and 100 μM $CdCl_2$ (4.65 ± 0.75, n = 12) had no effect on HSP70 levels when compared to the control group.

Testosterone production was significantly suppressed by forskolin / IBMX treatment (59.37 ± 7.44, n = 10, p = 0.001), and PMA (18.64 ± 1.35, n = 5, p = 0.0005), and increased by 17β-estradiol treatment (337.65 ± 54, n = 5, p = 0.01) when compared to follicles cultured in serum-free medium (Fig. 9A). Treatment of follicles with EGF had no effect of testosterone biosynthesis by follicular cells (91.4 ± 22.55, n = 10). 10 μM $CdCl_2$ (18.2 ± 3.22, n = 5, p = 0.0005), 100 μM $CdCl_2$ (42.86 ± 12.85, n = 9, p = 0.004), a combination of forskolin / IBMX and 100 μM $CdCl_2$ (9.13 ± 1.74, n = 7, p = 0.0004), and DMBA (19 ± 2, n = 4, p = 0.0005), significantly decreased testosterone production by follicular cells (Fig. 9B).

Treatment of follicles with forskolin / IBMX significantly increased 17β-estradiol concentration in culture media (334.6 ± 72.74, n = 6, p = 0.03) in comparison to the control group (118.12 ± 18.1, n = 7) (Fig. 10A). EGF (122 ± 18.56, n = 7) and PMA (45.05 ± 7.38, n = 4) had no effect on 17β-estradiol levels. Exposure to DMBA (265.31 ± 82.22, n = 7), 10 μM $CdCl_2$ (239.26 ± 81.85, n = 4), 100μM

$CdCl_2$ (86.1 ± 8.92, n = 6), or testosterone (110.12 ± 23.2, n = 6) did not affect 17β-estradiol levels in the media when compared to follicles cultured in serum-free media alone (Fig. 10B).

Channel catfish time course

There was a significant increase ($p < 0.05$) in apoptotic DNA fragmentation when comparing 24-hour cultures of catfish follicles in serum free media (14.36 ± 2.09 nmol/μg DNA, n = 10) with that of snap frozen follicles (0 hour) (3.03 ± 0.80 nmol/μg DNA, n = 13). However, the extent of DNA fragmentation did not differ between 24-hour cultures and those that were incubated for 48 hours (29.22 ± 13.58 nmol/μg DNA, n = 5) (Fig. 4). Based on this observation the incubation time for all subsequent experiments was set at 24 hours.

Figure 5 depicts a time course of HSP70 expression in catfish follicles incubated in serum free media for 0 hours (5.11 ± 1.81, n = 5), 3 hours (0.31, n = 1), 6 hours (0.49, n = 1), 12 hours (1.63, n = 1), 18 hours (1.44, n = 1), 24 hours (0.85 ± 0.42, n = 4)(Fig. 5). Levels of HSP70 in follicular cells did not differ ($p = 0.08$) when comparing

0-hour (snap frozen) follicles (5.11 ± 1.81, n = 5) with follicles that were cultured for 24 hours in serum-free medium (0.85 ± 0.42, n = 4).

Testosterone levels were similar at 12 (19.04 ± 0.1, n = 2), 18 (20.8 ± 1.5, n = 3), and 24 (13.52 ± 0.66, n = 3) hour cultures (Fig. 6A). 17β -estradiol levels were the same when comparing 12 (3505 ± 15.5, n = 2), 18 (63.5 ± 33.5, n = 2), 24 (192.8 ± 87.2, n = 5), and 48-hour (77.7 ± 35, n = 3) culture media extracts (Fig. 6B).

Channel catfish follicle treatments

Amongst survival factors tested, epidermal growth factor (16.21 ± 5, n = 5), 17β- estradiol (15.52 ± 5.25, n = 7), PMA (23.32 ± 6.4, n = 10), and forskolin / IBMX (26.25 ± 5.56 nmol/μg DNA, n = 8) had no effect on apoptotic DNA fragmentation in follicular cells (Fig. 11A). DMBA (16.59 ± 2.69, n = 8), testosterone (16.58 ± 3.42, n = 7), 10 μM $CdCl_2$ (1.89 ± 1.65, n = 2), 100 μM $CdCl_2$ (36 ± 15.5, n = 6), or a combination of forskolin / IBMX and 100 μM $CdCl_2$ (26.53 ± 20.44, n = 4) had no effect on ovarian cell apoptosis when

compared to follicles cultured in serum-free medium (Fig.11B).

Treatment of follicles with EGF (1.76 ± 0.68, n = 4), 17β- estradiol (1.59 ± 0.6, n = 4), PMA (2.17 ± 0.9, n =3), or forskolin / IBMX (1.5 ± 0.55, n = 4) had no effect on HSP70 expression (Fig.12A). When compared to the control group, DMBA (7.89 ± 0.09, n = 2) and 100 μM $CdCl_2$ (10.2 ± 1.45, n = 6) caused an increase ($p < 0.001$) in HSP70 levels (Fig. 12B). Other atretogenic factors, 10 μM $CdCl_2$ (3.9 ± 1.3, n = 3), and testosterone (2 ± 1, n = 4), did not affect levels of HSP70 in follicular cells.

Testosterone biosynthesis was unaltered by any of the survival factors including forskolin / IBMX treatment (201.6 ± 74.25, n = 6), 17β-estradiol (54.5 ± 13, n = 5) and EGF (59.4 ± 23.3, n = 8) when compared to follicles cultured in serum-free medium alone (Fig. 13A). Testosterone production was also unaffected by DMBA (88.8 ± 32.45, n = 7), PMA (128.1 ± 49.32, n = 7), 10 μM $CdCl_2$ (327.6 ± 202.67, n = 4), and 100 μM $CdCl_2$ (77.2 ± 33.2, n = 7) in follicular cells. Treatment of follicles with a combination of forskolin / IBMX and 100 μM $CdCl_2$ (194.9 ± 136.5, n = 5) had

no effect on testosterone concentrations in the media (Fig. 13B).

Treatment of follicles with forskolin / IBMX significantly increased 17β-estradiol concentration in culture media (877 ± 222.6, n = 7, $p < 0.05$) in comparison to the control group (Fig. 14A). EGF (212.5 ± 67.44, n = 6) and PMA (327.55 ± 80.4, n = 7) had no effect on 17β-estradiol levels. Exposure to DMBA (262.9 ± 77.2, n = 7) did not affect 17β-estradiol levels in the media and there was no evidence that other apoptogens including 10 μM $CdCl_2$ (314.6 ± 85.5, n = 5), 100μM $CdCl_2$ (152.4 ± 37, n = 8), or testosterone (677.66 ± 171.7, n = 6) altered 17β-estradiol levels in the media when compared to follicles cultured in serum-free media alone (Fig. 14B).

Cotton rat

In comparison to ecologically matched reference sites, the rate of ovarian cell apoptosis was significantly elevated in two of the five petrochemical-contaminated sites we examined (Fig. 15, A and B). Apoptosis was significantly increased (48% greater) in ovarian cells of cotton rats inhabiting contaminated sites ($p = 0.009$). The

percentage of atretic follicles was determined in a subsample of ovaries from cotton rats collected from sites 1 and 5 (Table 1).

Atresia was evaluated in histological sections of ovaries using both standard morphological indices and *in situ* 3'-end labeling of apoptotic DNA fragments. No significant differences in the percentage of primary and antral (i.e. with an antrum, antrum is a large cavity located in the center of the follicle and is filled with proteins and factors that nourish the growing follicle in mammals) atretic follicles between animals from contaminated and reference sites was observed. However, the number of uterine scars, a measure of successful previous implantation of zygote, was significantly lower in animals from contaminated sites in comparison to matched reference sites. There were no significant differences in corpora lutea or ovary to body weight ratios among animals residing on contaminated and reference sites.

The rate of apoptosis in thymocytes was elevated at one of five contaminated sites in comparison to the matched reference site (Fig. 15, C and D) ($p < 0.05$).

There were no marked differences in thymus to body weight ratios among animals collected from contaminated and reference sites (Table 1).

The rate of apoptosis in bone marrow progenitor cells ($p = 0.06$) and splenocytes ($p = 0.06$) was not significantly different between animals from contaminated and reference sites.

TABLE 1. Morphological measurements, number of uterine scars and corpora lutea, and the percentage of primary (immature) and antral (mature) ovarian follicles undergoing atresia in female cotton rats.

Parameter	Reference Sites	Contaminated Sites
Body weight (g)	117.4 ± 3.9	125.1 ± 6.1
Thymus to body weight ratio	0.29 ± 0.04	0.24 ± 0.02
Ovary to body weight ratio	0.46 ± 0.03	0.47 ± 0.02
Number of uterine scars	12.6 ± 1.4	7.4 ± 1.3 [a]
Atretic primary follicles (% of total follicles)	53 ± 9	63 ± 7
Atretic antral follicles (% of total follicles)	58 ± 1	77 ± 9
Corpora lutea (% of total follicles)	17 ± 6	16 ± 3

[a] Significantly different from reference sites ($p < 0.01$)

Data are presented as overall means ± SEM of rats collected from five petrochemical-contaminated and five reference sites (n=27-28). Atresia and corpora lutea data are pooled from sites 1 and 5 (n = 11).

FIGURE 1. Apoptotic DNA fragmentation in preovulatory fathead minnow follicles cultured for 0, 12, 24, 48, and 72 hours in serum-free medium. DNA was radiolabeled and fractionated on agarose gel by electrophoresis. Low-molecular-weight DNA (<15kb) was excised from the gel and incorporated radioactivity was quantified using Cerenkov counting. An asterisk indicates significantly different from control.

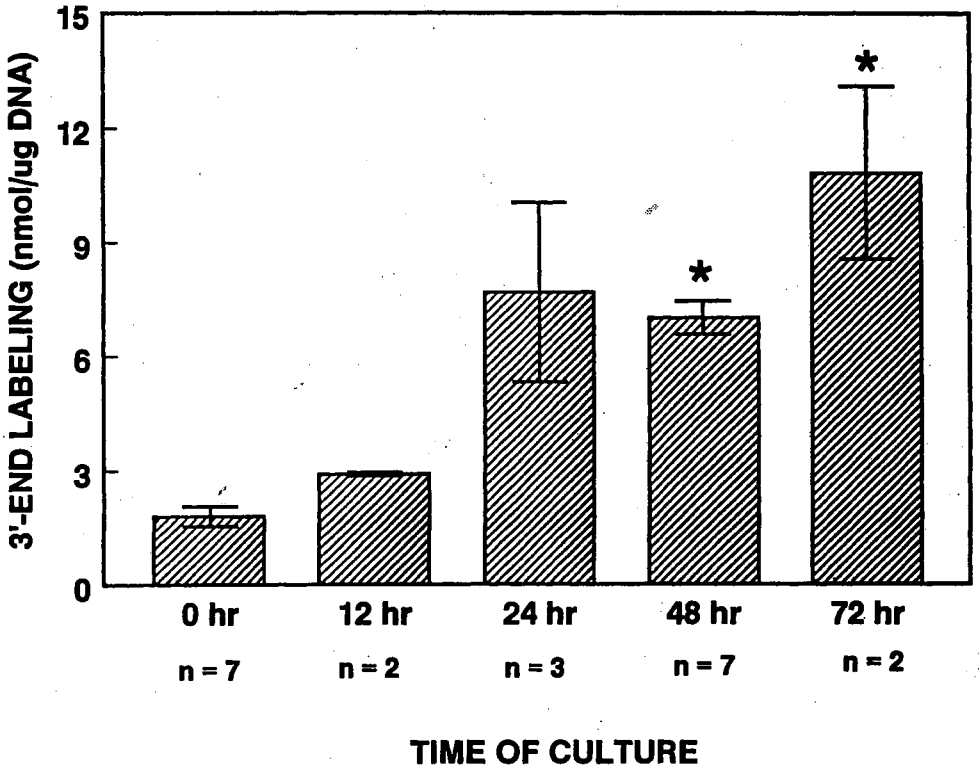

15
12
9
6
3
0
3'-END LABELING (nmol/ug DNA)
*
*
0 hr
n = 7
12 hr
n = 2
24 hr
n = 3
48 hr
n = 7
72 hr
n = 2
TIME OF CULTURE

FIGURE 2. Expression of HSP70 protein in preovulatory fathead minnow ovarian follicles as determined by Western blotting after 0, 12, 24, 48, and 72 hours of incubation in serum-free media. Data are expressed as means ± SEM (n = 3-5). An asterisk indicates significantly different from control.

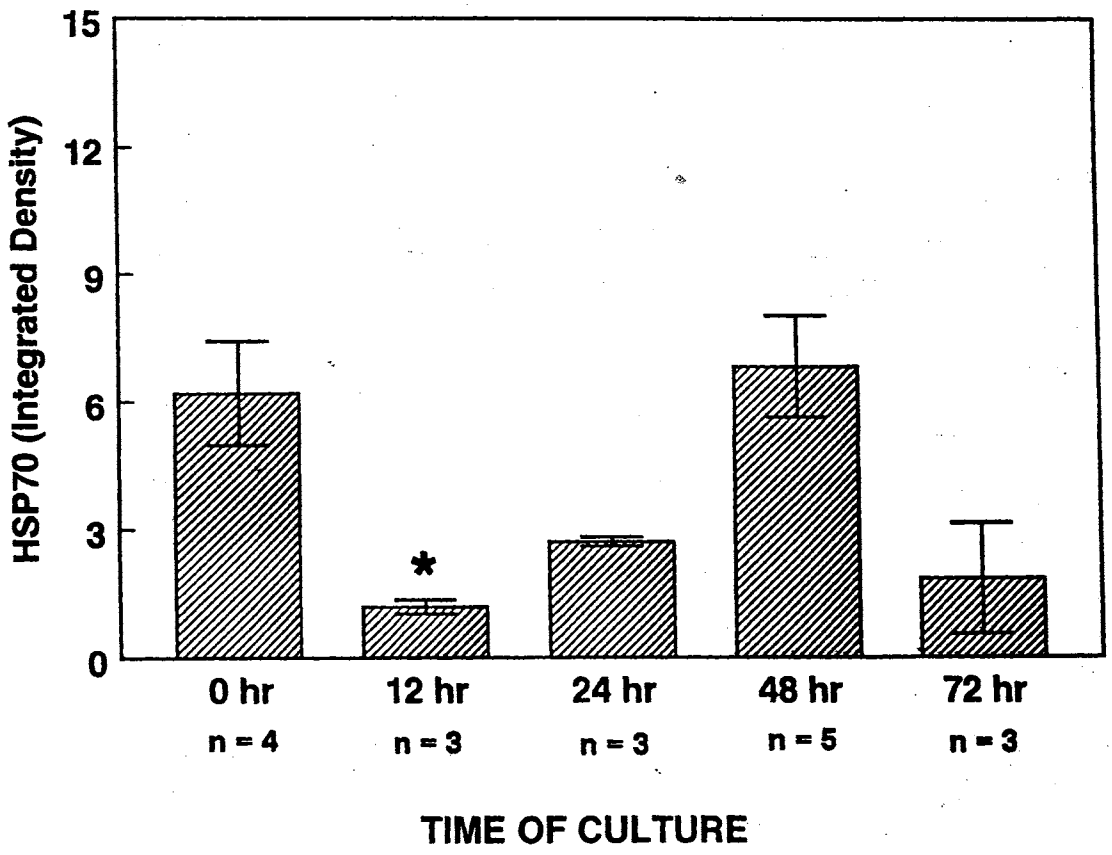
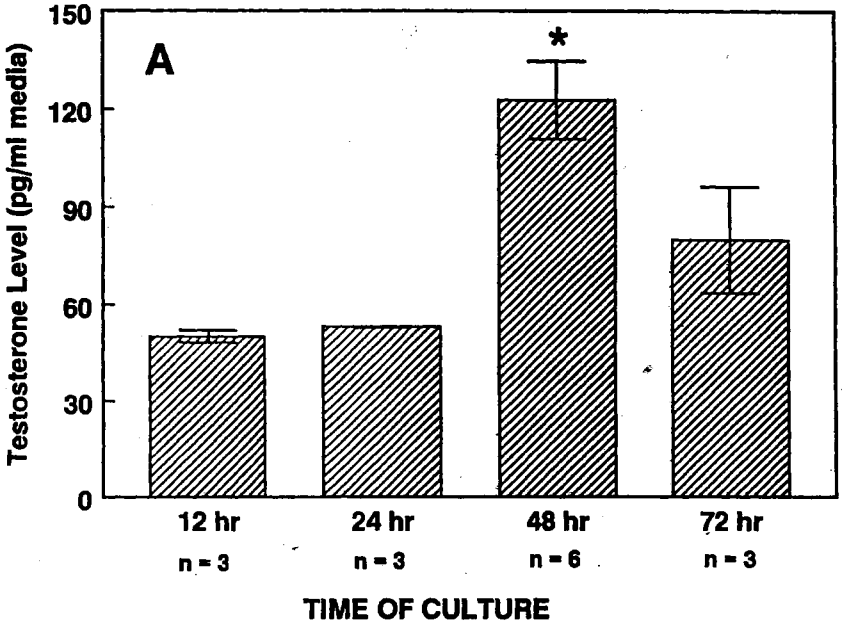

15
12
9
6
3
0
HSP70 (Integrated Density)
*
0 hr
n = 4
12 hr
n = 3
24 hr
n = 3
48 hr
n = 5
72 hr
n = 3
TIME OF CULTURE

FIGURE 3. (A) Testosterone production by fathead minnow follicles cultured for 12, 24, 48 and 72 hours in serum free media. Testosterone levels were determined using an enzyme-linked immunosorbent assay (ELISA). Data were expressed as means ± SEM (n = 3-6). (B) In vitro 17β-estradiol production by fathead minnow follicles was measured (A) at 12, 24, 48, and 72 hour of incubation in serum free media by an enzyme-linked immunosorbent assays (ELISA). Values are mean ± SEM of 3 to 6 samples per time point.


A
*
150
120
90
60
30
0
Testosterone Level (pg/ml media)
12 hr
n = 3
24 hr
n = 3
48 hr
n = 6
72 hr
n = 3
TIME OF CULTURE

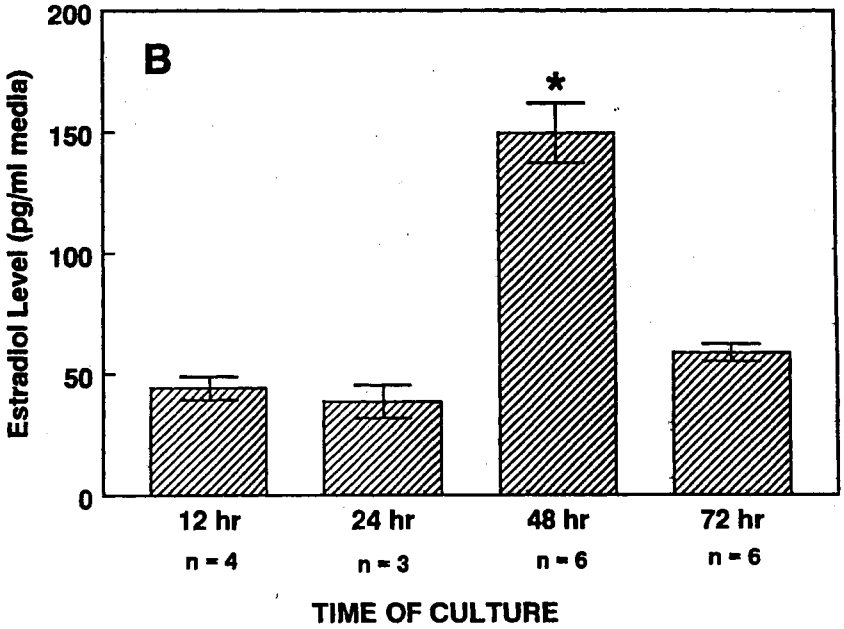

B
*
200
150
100
50
0
Estradiol Level (pg/ml media)
12 hr
n = 4
24 hr
n = 3
48 hr
n = 6
72 hr
n = 6
TIME OF CULTURE

FIGURE 4. Apoptotic DNA fragmentation in preovulatory catfish follicles cultured for 0, 3, 6, 12, 18, 24, and 72 hours in serum-free medium. DNA was radiolabeled and fractionated on agarose gel by electrophoresis. Low-molecular-weight DNA (<15kb) was excised from the gel and incorporated radioactivity was quantified using Cerenkov counting. Data are expressed as means ± SEM (n = 3-13).

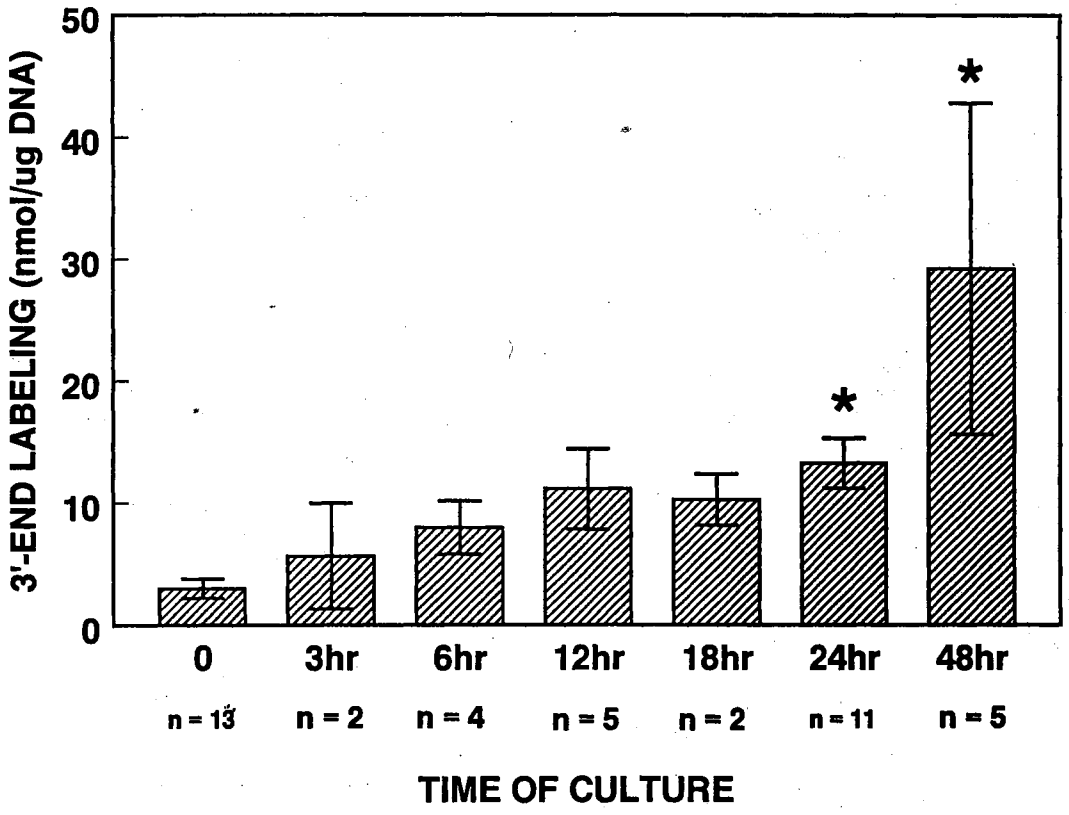

3'-END LABELING (nmol/ug DNA)
50
40
30
20
10
0
*
*
0
3hr
6hr
12hr
18hr
24hr
48hr
n = 13
n = 2
n = 4
n = 5
n = 2
n = 11
n = 5
TIME OF CULTURE

FIGURE 5. Expression of HSP70 protein in preovulatory follicles of channel catfish as determined by Western blotting after 0 and 24 hours of incubation in serum-free media. Data are expressed as means ± SEM (n = 1-5).

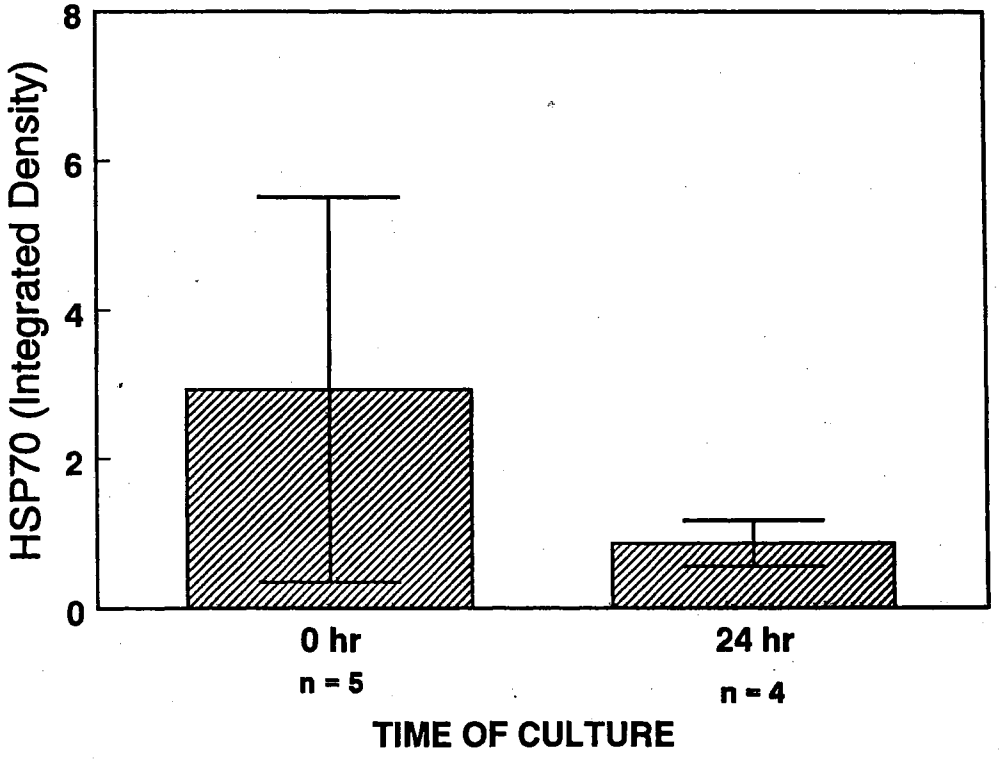
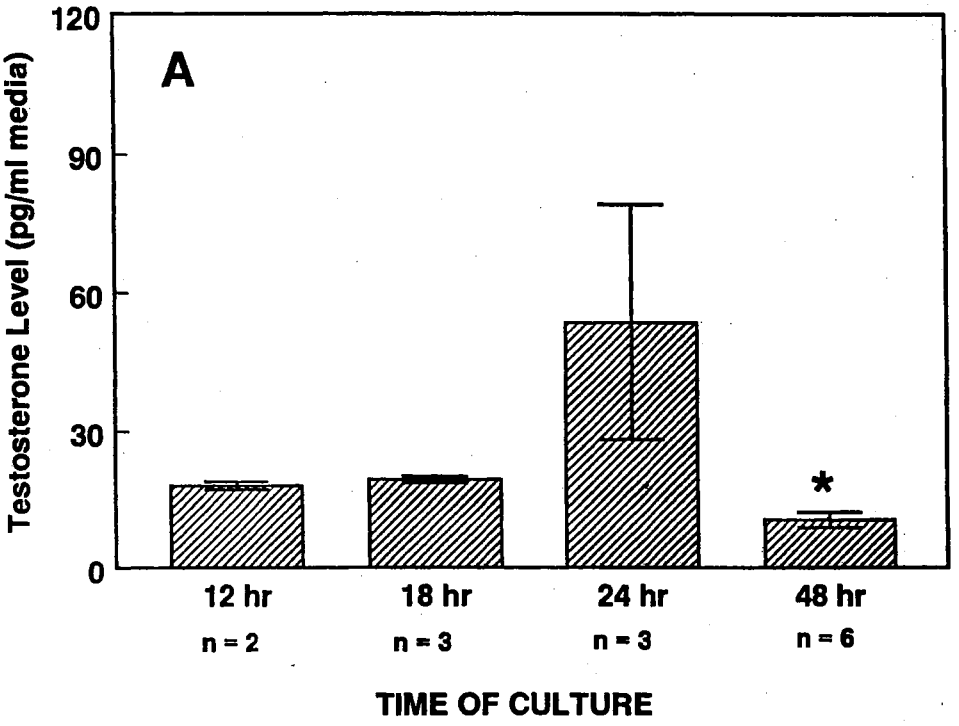

HSP70 (Integrated Density)
8
6
4
2
0
0 hr
n = 5
24 hr
n = 4
TIME OF CULTURE

FIGURE 6. (A) Testosterone production by channel catfish follicles cultured for 12, 18, 24, and 48 hours in serum free media. Testosterone levels were determined using an enzyme-linked immunosorbent assay (ELISA). Data were expressed as means ± SEM (n = 3-6). (B) In vitro 17β-estradiol production by channel catfish follicles was measured (A) at 12, 18, 24, and 48 hours after incubation in serum free media by an enzyme-linked immunosorbent assays (ELISA). Values are mean ± SEM of 2 to 5 samples per time point.


A
Testosterone Level (pg/ml media)
120
90
60
30
0
*
12 hr
n = 2
18 hr
n = 3
24 hr
n = 3
48 hr
n = 6
TIME OF CULTURE

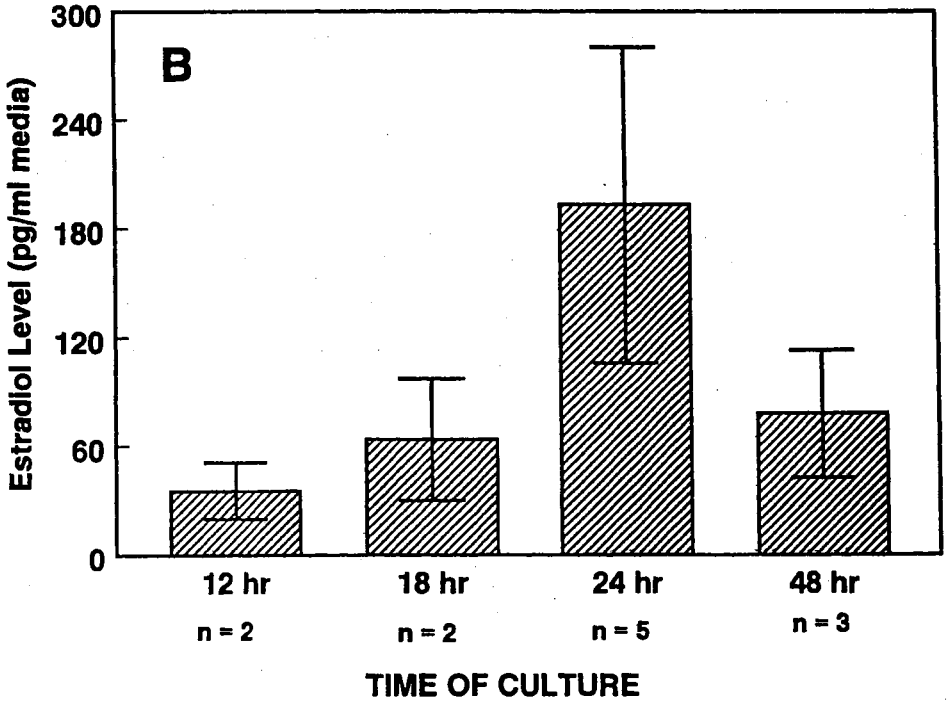
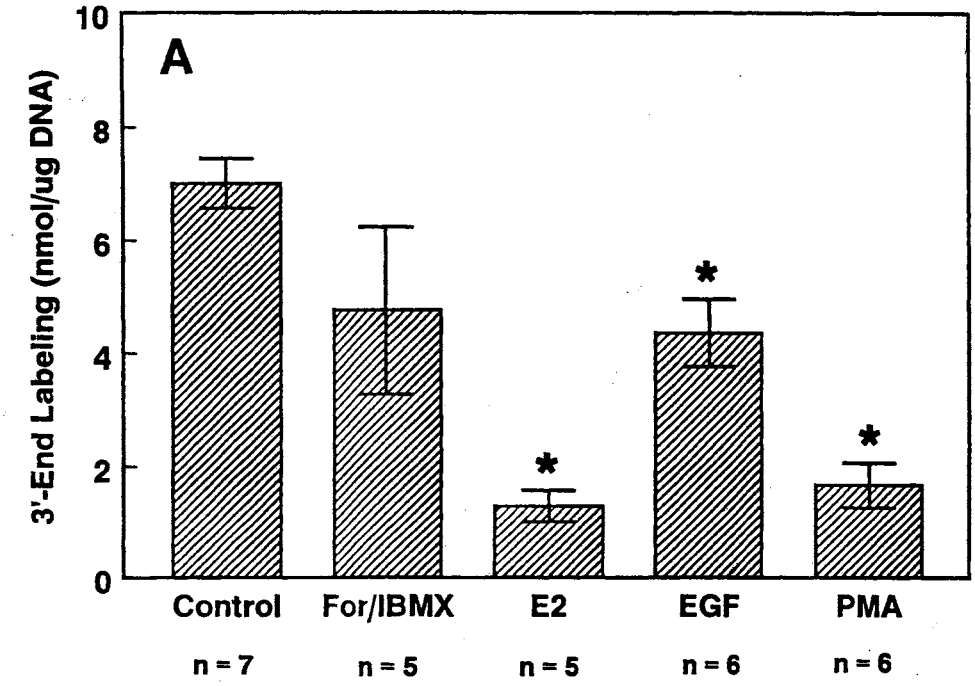

B
Estradiol Level (pg/ml media)
300
240
180
120
60
0
12 hr
n = 2
18 hr
n = 2
24 hr
n = 5
48 hr
n = 3
TIME OF CULTURE

FIGURE 7. Apoptotic DNA fragmentation in preovulatory fathead minnow follicles cultured for 48 hours in serum-free medium (control), in presence of (A) putative survival factors forskolin (10 μM) and isobutyl-methylxanthine (0.1 mM) (For / IBMX treatment), epidermal growth factor (EGF; 0.1 μg/ml), phorbol myracetate (PMA; 0.4 μM), or 17β-estradiol (E_2; 4μM), and (B) putative atretogenic factors, [dimethylbenz[a]anthracene (DMBA; 100 μM), testosterone (Tes; 3 μM), or cadmium chloride ($CdCl_2$; 100 μM), and a combination of forskolin and isobutyl-methylxanthine and cadmium chloride 100 μM (For/Cd100).


A
3'-End Labeling (nmol/ug DNA)
10
8
6
4
2
0
Control
For/IBMX
E2
EGF
PMA
n = 7
n = 5
n = 5
n = 6
n = 6
*
*
*

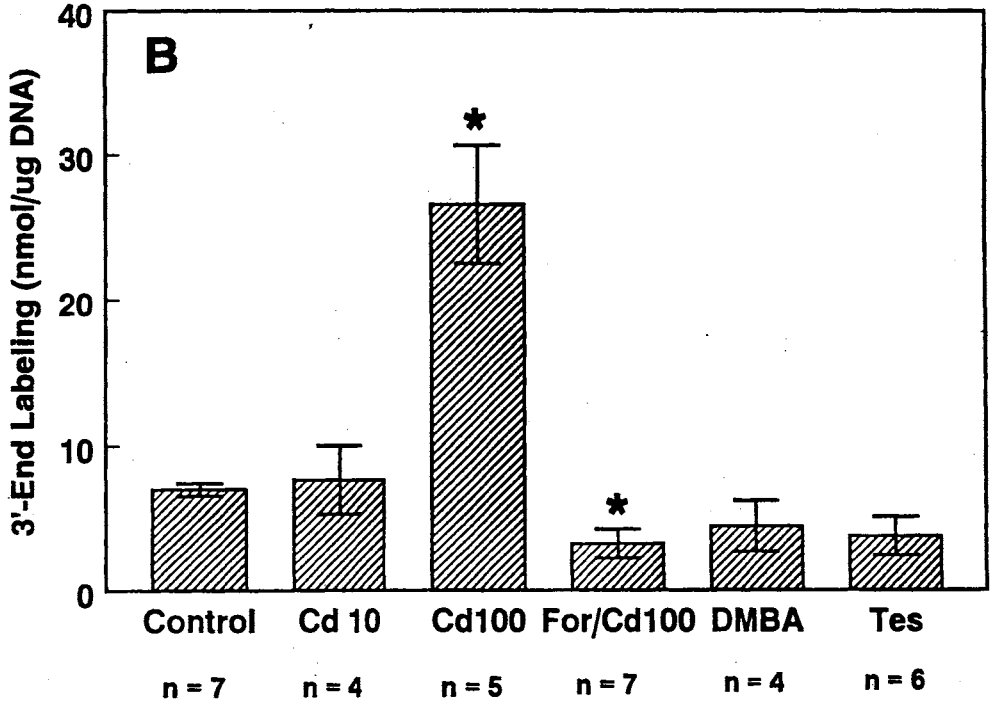
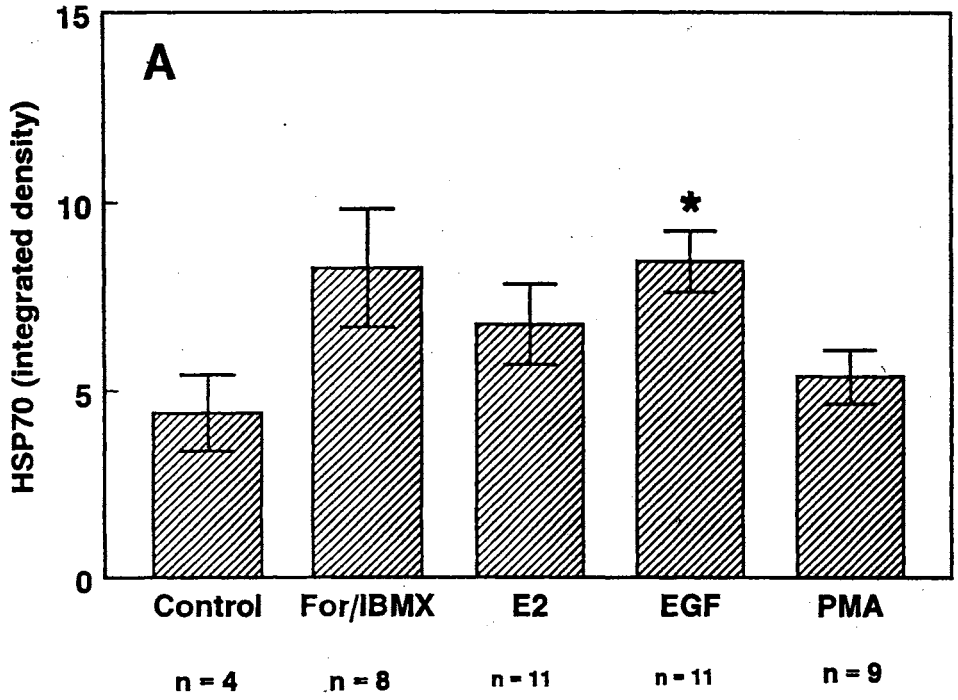

B
3'-End Labeling (nmol/ug DNA)
40
30
20
10
0
Control
Cd 10
Cd100
For/Cd100
DMBA
Tes
n = 7
n = 4
n = 5
n = 7
n = 4
n = 6
*
*

FIGURE 8. Expression of HSP70 protein in preovulatory fathead minnow ovarian follicles as determined by Western blotting. (A) Densitometric estimation of HSP70 bands in follicles cultured with putative survival factors (For / IBMX, EGF, PMA, E_2), or (B) putative atretogenic factors, (DMBA, testosterone, or $CdCl_2$), as well as For/ IBMX and Cd Cl_2. Data are expressed as means ± SEM (n = 5-14). NIH Image software was used to assess band density.


A
HSP70 (integrated density)
15
10
5
0
*
Control
For/IBMX
E2
EGF
PMA
n = 4
n = 8
n = 11
n = 11
n = 9

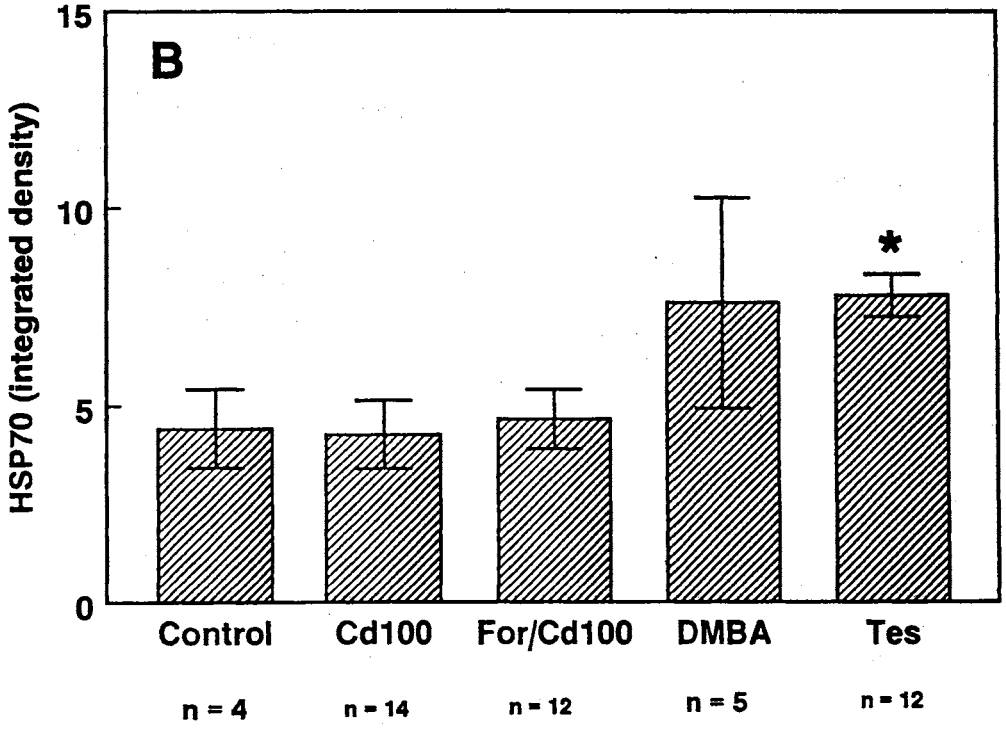
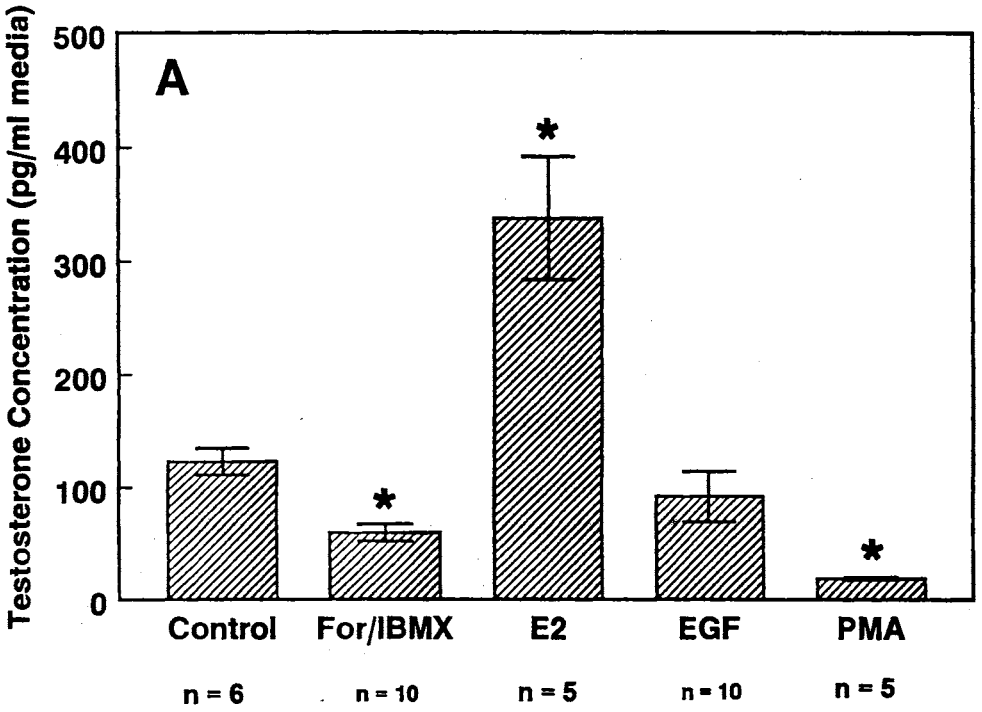

B
HSP70 (integrated density)
15
10
5
0
*
Control
Cd100
For/Cd100
DMBA
Tes
n = 4
n = 14
n = 12
n = 5
n = 12

FIGURE 9. In vitro testosterone production by fathead minnow follicles cultured for 48 hours in serum-free medium (control) or in presence of chemical treatments as described in Figure 2. Testosterone levels were determined using an enzyme-linked immunosorbent assay (ELISA). Data were expressed as means ± SEM (n = 4-10). An sterisk indicates significantly different from control.


A
Testosterone Concentration (pg/ml media)
500
400
300
200
100
0
Control
For/IBMX
E2
EGF
PMA
n = 6
n = 10
n = 5
n = 10
n = 5

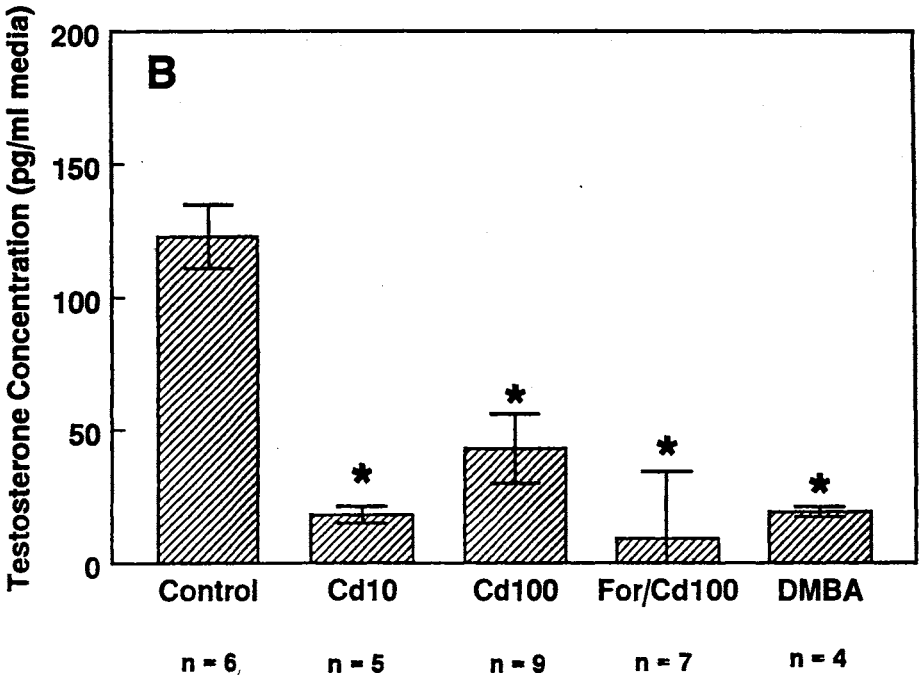
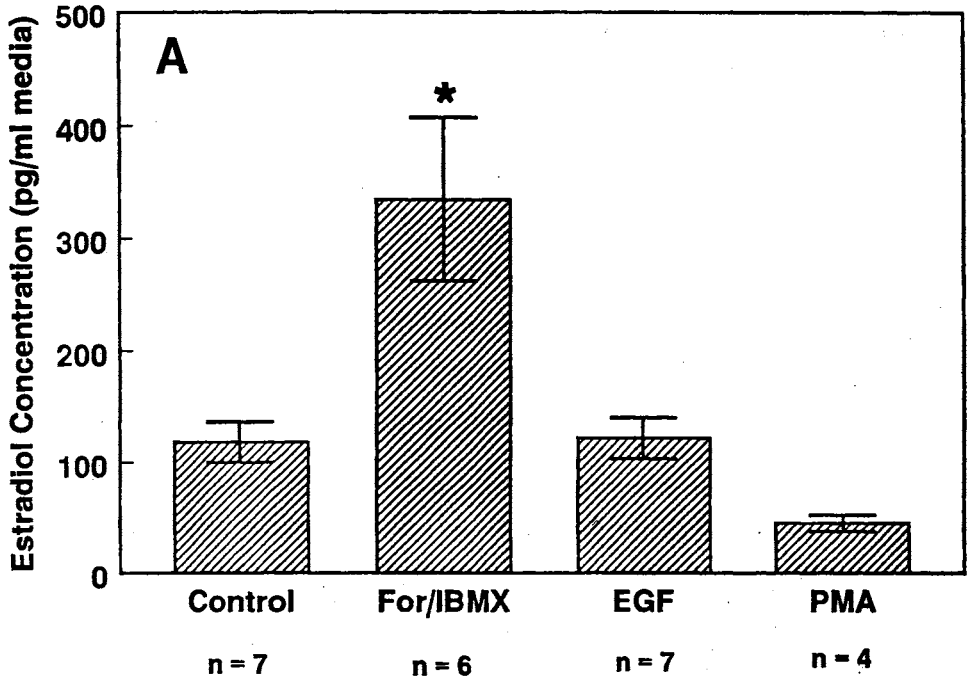

B
Testosterone Concentration (pg/ml media)
200
150
100
50
0
Control
Cd10
Cd100
For/Cd100
DMBA
n = 6
n = 5
n = 9
n = 7
n = 4

FIGURE 10. In vitro 17β-estradiol production by fathead minnow follicles was measured by an enzyme-linked immunosorbent assays (ELISA). (A) In presence of survival factors, and (B) atretogens. Values are mean ± SEM of 4 to 7 samples per treatment.


A
Estradiol Concentration (pg/ml media)
500
400
300
200
100
0
*
Control
For/IBMX
EGF
PMA
n = 7
n = 6
n = 7
n = 4

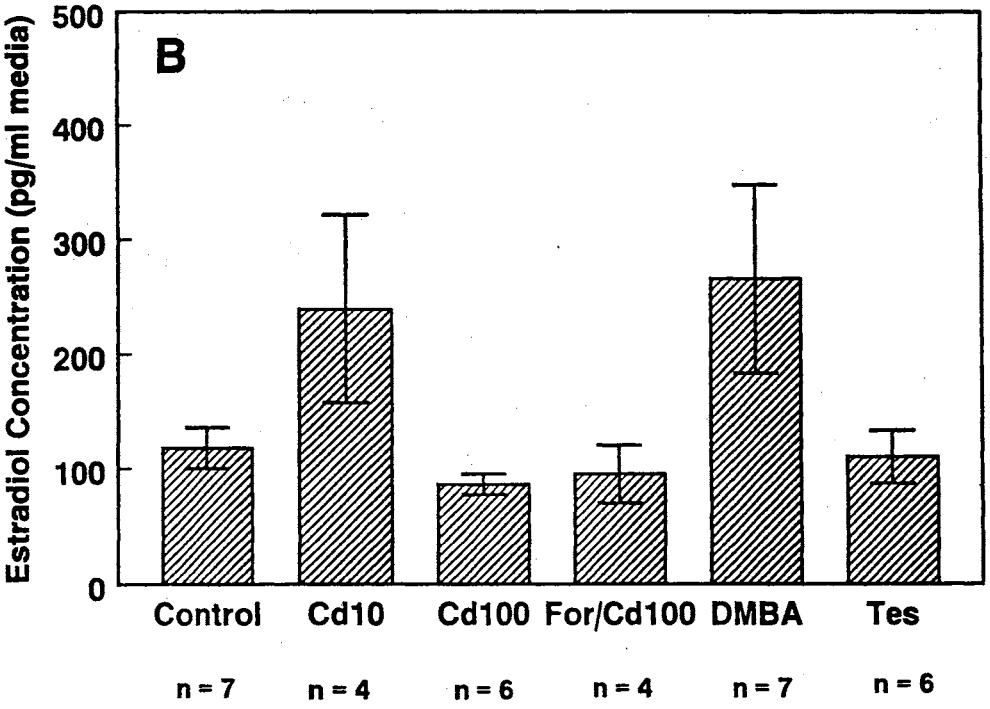
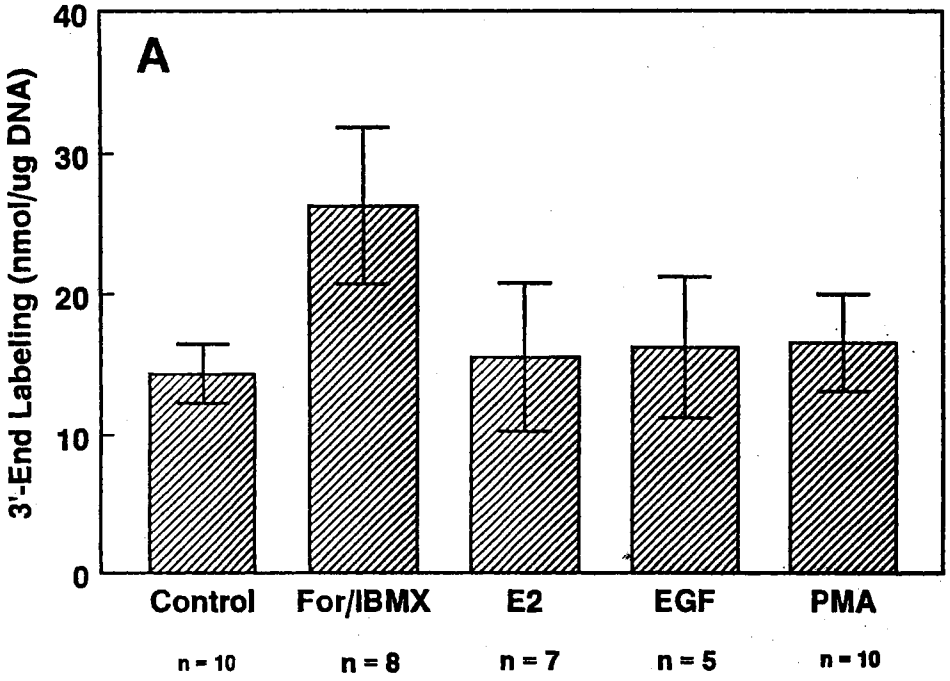

B
Estradiol Concentration (pg/ml media)
500
400
300
200
100
0
Control
Cd10
Cd100
For/Cd100
DMBA
Tes
n = 7
n = 4
n = 6
n = 4
n = 7
n = 6

FIGURE 11. Apoptotic DNA fragmentation in preovulatory channel catfish follicles cultured for 48 hours in serum-free medium (control), in presence of (A) putative survival factors forskolin (10 μM) and isobutyl-methylxanthine (0.1 mM) (For / IBMX treatment), epidermal growth factor (EGF; 0.1 μg/ml), phorbol myracetate (PMA; 0.4 μM), or 17β-estradiol (E_2; 4μM), and (B) putative atretogenic factors, [dimethylbenz[a]anthracene (DMBA; 100 μM), testosterone (Tes; 3 μM), or cadmium chloride ($CdCl_2$; 100 μM), and a combination of forskolin and isobutyl-methylxanthine and cadmium chloride 100 μM (For/Cd100). DNA was radiolabeled and fractionated on agarose gel by electrophoresis. Low-molecular-weight DNA (<15kb) was excised from the gel and incorporated radioactivity was quantified using Cerenkov counting. Data are expressed as means ± SEM (n = 4-10). An asterisk indicates significantly different from control.


A
3'-End Labeling (nmol/ug DNA)
40
30
20
10
0
Control
For/IBMX
E2
EGF
PMA
n = 10
n = 8
n = 7
n = 5
n = 10

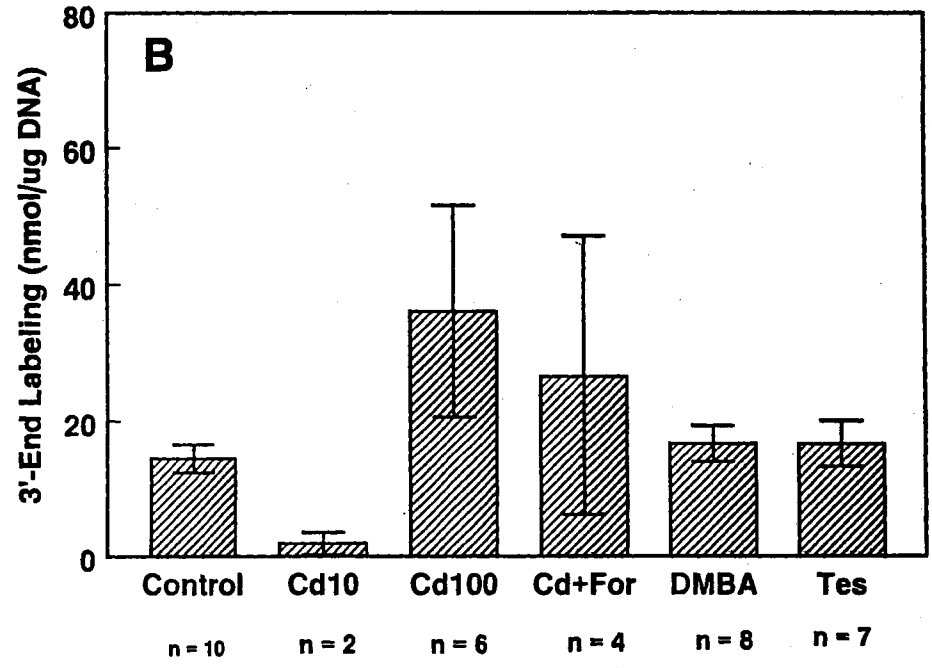
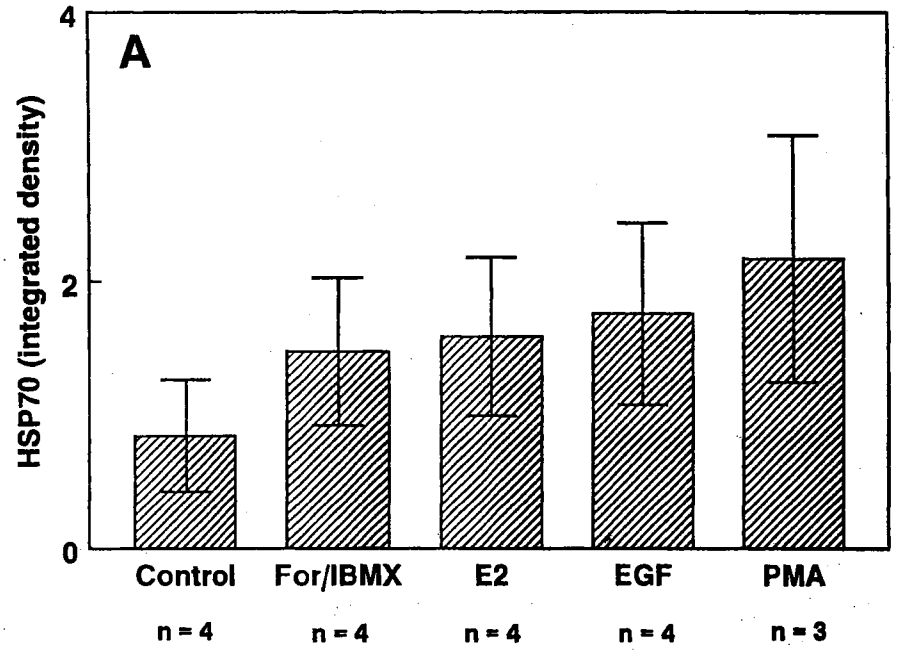

B
3'-End Labeling (nmol/ug DNA)
80
60
40
20
0
Control
Cd10
Cd100
Cd+For
DMBA
Tes
n = 10
n = 2
n = 6
n = 4
n = 8
n = 7

FIGURE 12. Expression of HSP70 protein in preovulatory channel catfish ovarian follicles as determined by Western blotting. (A) Densitometric estimation of HSP70 bands in follicles cultured with putative survival factors (For / IBMX, EGF, PMA, E_2), or (B) putative atretogenic factors, (DMBA, testosterone, or Cd Cl_2), as well as For/ IBMX and $CdCl_2$. Data are expressed as means ± SEM (n = 2-10). NIH Image software was used to assess band density. An asterisk indicates significantly different from control.


A
HSP70 (integrated density)
4
2
0
Control
For/IBMX
E2
EGF
PMA
n = 4
n = 4
n = 4
n = 4
n = 3

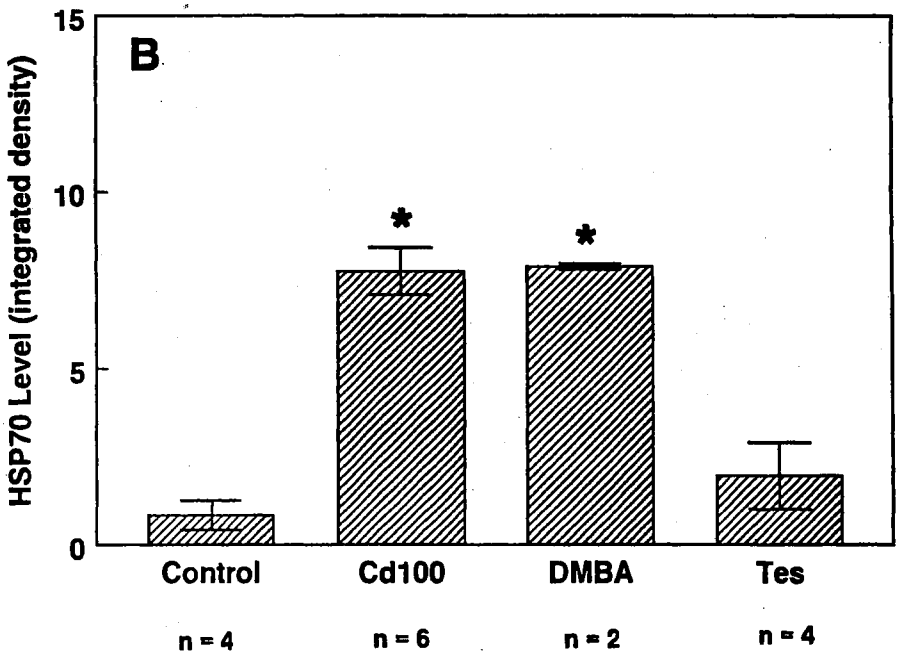
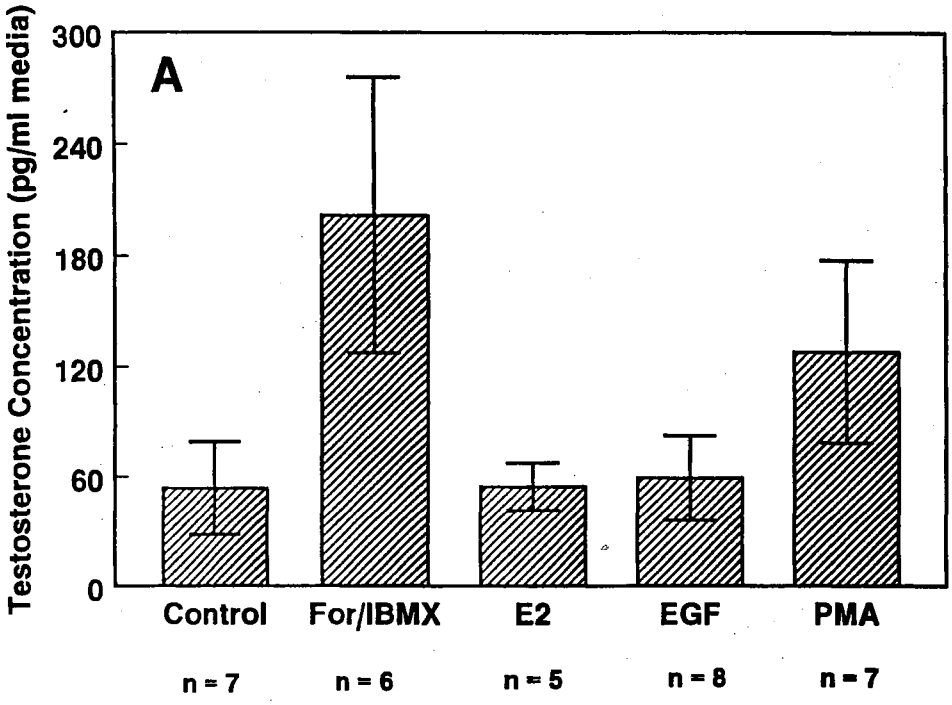

B
HSP70 Level (integrated density)
15
10
5
0
*
*
Control
Cd100
DMBA
Tes
n = 4
n = 6
n = 2
n = 4

FIGURE 13. In vitro testosterone production by channel catfish follicles cultured for 48 hours in serum-free medium (control) or in presence of chemical treatments as described in Figure 9. Testosterone levels were determined using an enzyme-linked immunosorbent assay (ELISA)(A) in presence of putative survival factors, and (B) putative atretogenic compounds. Data are expressed as means ± SEM (n = 5-8). An asterisk indicates significantly different from control.


A
Testosterone Concentration (pg/ml media)
300
240
180
120
60
0
Control
For/IBMX
E2
EGF
PMA
n = 7
n = 6
n = 5
n = 8
n = 7

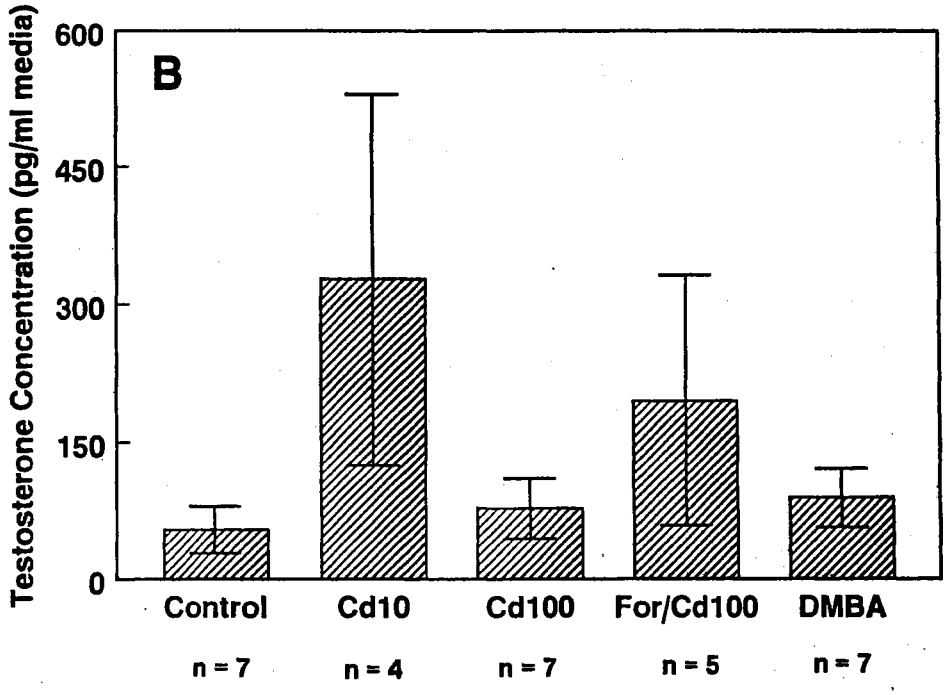
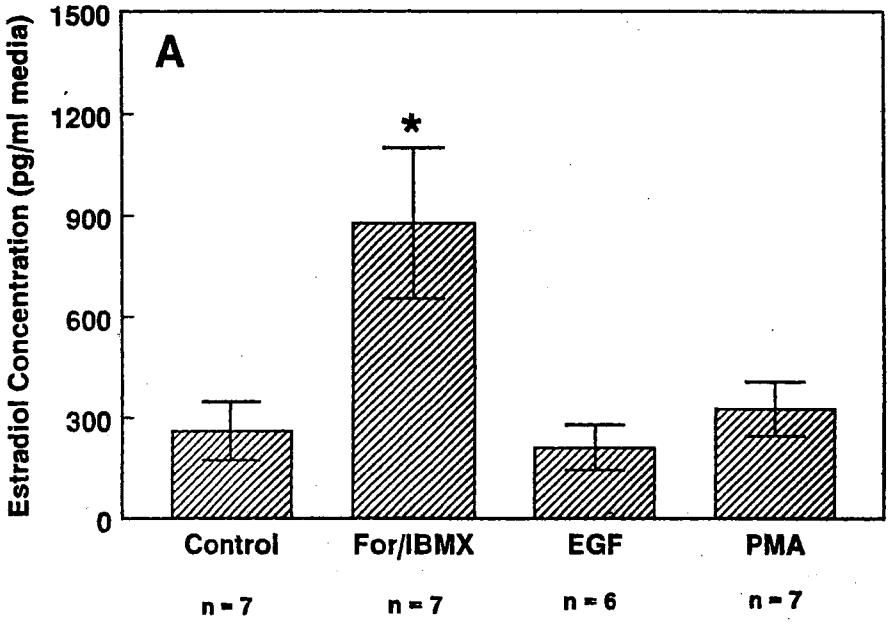

B
Testosterone Concentration (pg/ml media)
600
450
300
150
0
Control
Cd10
Cd100
For/Cd100
DMBA
n = 7
n = 4
n = 7
n = 5
n = 7

FIGURE 14. In vitro 17β-estradiol production by channel catfish follicles was measured by an enzyme-linked immunosorbent assays (ELISA). Values are mean ± SEM of 6 to 8 samples per treatment. An asterisk indicates significantly different from control.


A
Estradiol Concentration (pg/ml media)
1500
1200
900
600
300
0
*
Control
For/IBMX
EGF
PMA
n = 7
n = 7
n = 6
n = 7

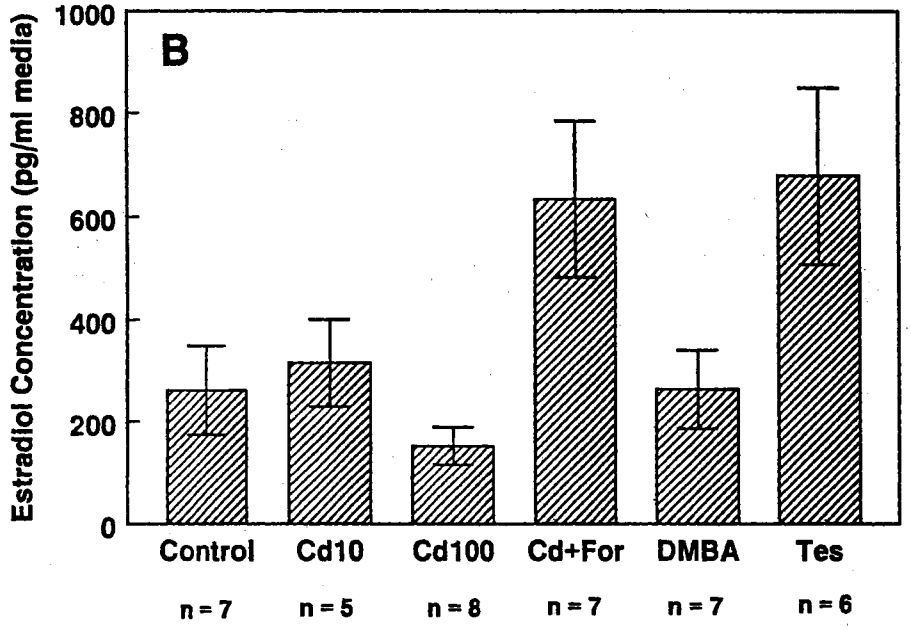
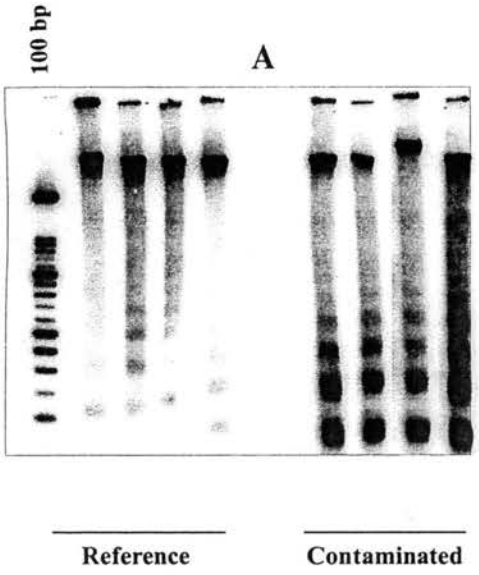
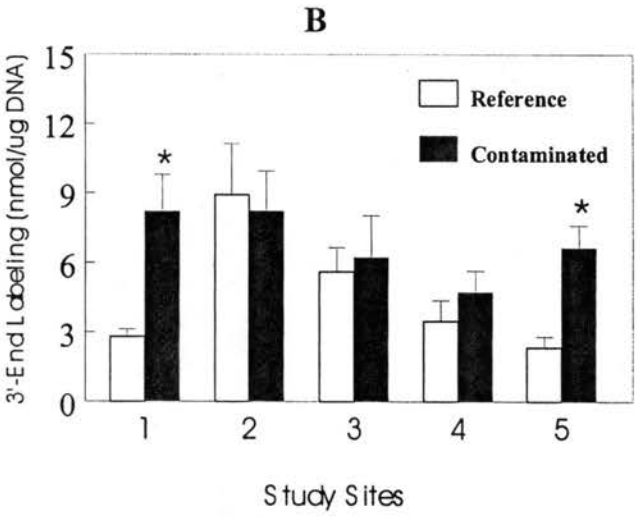
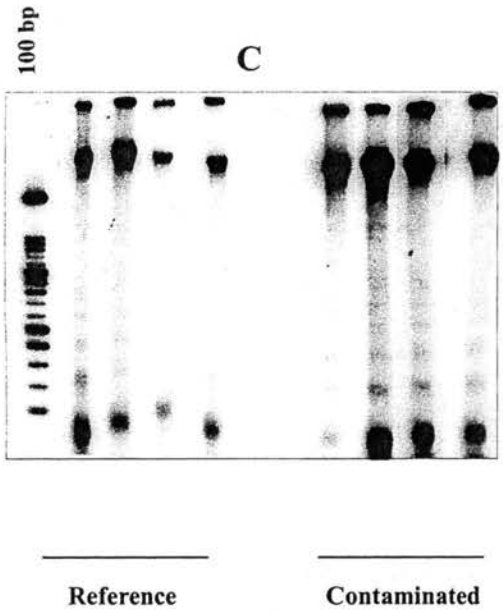

B
Estradiol Concentration (pg/ml media)
1000
800
600
400
200
0
Control
Cd10
Cd100
Cd+For
DMBA
Tes
n = 7
n = 5
n = 8
n = 7
n = 7
n = 6

FIGURE 15. Apoptotic DNA fragmentation in ovaries and thymuses collected from wild female cotton rats inhabiting replicated petrochemical-contaminated and reference sites in Oklahoma. A and C, autoradiograms illustrating representative DNA integrity in ovarian cells and thymocytes, respectively. B and D, quantitative estimation of low molecular weight (<15 kb) DNA fragments from individual sites. Data were analyzed by Student t-test ($p < 0.05$). An asterisk indicates significantly different from reference site by ANOVA ($p<0.05$).


100 bp
A
Reference
Contaminated


B
3'-End Labeling (nmol/ug DNA)
15
12
9
6
3
0
Reference
Contaminated
*
*
1
2
3
4
5
Study Sites


100 bp
C
Reference
Contaminated

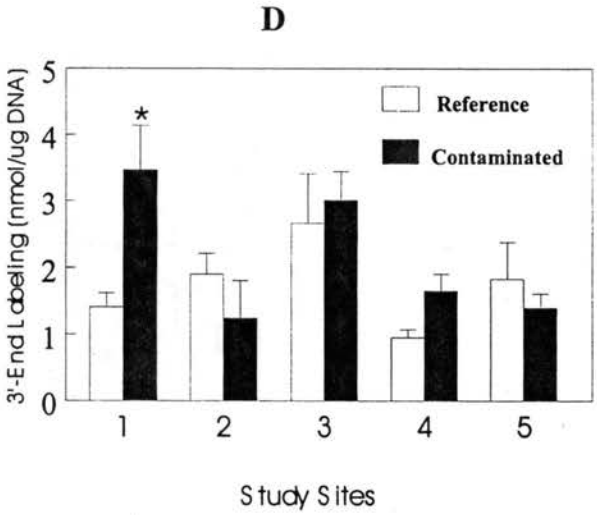
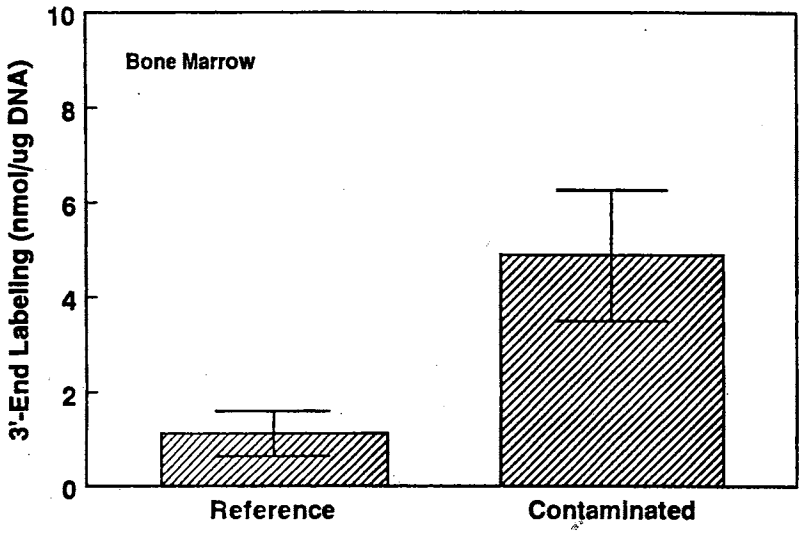
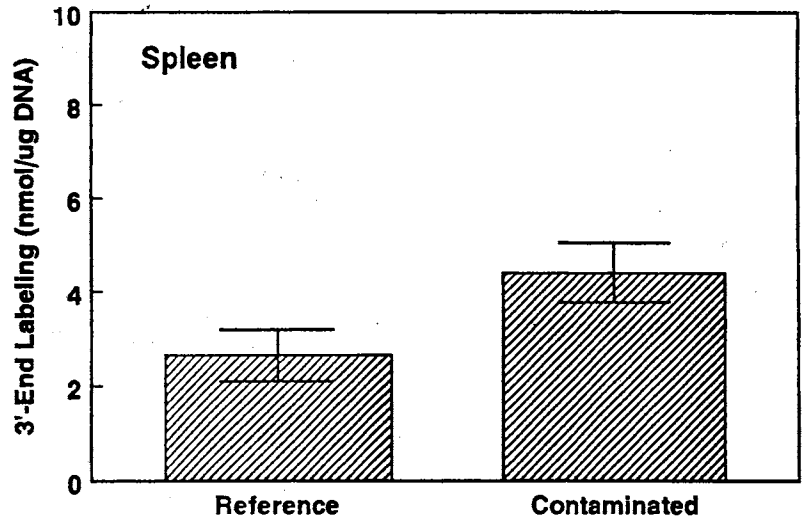

D
3'-End Labeling (nmol/ug DNA)
5
4
3
2
1
0
Reference
Contaminated
*
1
2
3
4
5
Study Sites

FIGURE 16. Apoptotic DNA fragmentation in bone marrow and splenocytes of wild female cotton rats collected from petrochemical-contaminated (n = 6 and 4) and reference (n = 6 and 6) sites in Oklahoma. Quantitative estimate of low molecular weight (<15 kb) DNA fragments from individual site and its replicated reference site. Data were analyzed by Student t-test ($p < 0.05$).


Bone Marrow
3'-End Labeling (nmol/ug DNA)
10
8
6
4
2
0
Reference
Contaminated


Spleen
3'-End Labeling (nmol/ug DNA)
10
8
6
4
2
0
Reference
Contaminated

Discussion

The overall goal of this dissertation was to investigate effects of DMBA and cadmium on three highly conserved molecular processes (steroidogenesis, heat shock protein (HSP70) induction, and apoptosis) that maintain ovarian function and homeostasis and are adversely affected by exposure to environmental contaminants. We used the ovarian follicle culture method to determine the effects of hormones and toxicants on these processes.

Organization of the discussion:

First I will contrast synchronous and asynchronous ovaries in fish and go on to discuss the relevance of a time course study for apoptosis, HSP70 expression and steroidogenic capacity in each species. I will then continue with the discussion of the actions of hormones/toxicants in the microenvironment of the ovary in fathead minnow and catfish and finally discuss our data on apoptosis in cotton rat ovary, thymus, bone marrow, and spleen.

Contrasting models of ovarian development in fish; synchronous (channel catfish) and asynchronous (fathead minnow) teleosts

Oogenesis is a hormonally controlled process during which ovarian follicles grow, mature, and are finally ovulated (Tyler and Sumpter, 1996; Dye et al., 1986;). We chose to study two fish species with contrasting reproductive strategies (synchronous and asynchronous) to further the current understanding of endocrine regulation of oogenesis in the teleost ovary and to investigate effects of environmental contaminants on ovarian development and function.

In fish, an asynchronous ovary contains oocytes at different stages of follicular development throughout the year. In an asynchronous ovary, complex endocrine mechanisms maintain different follicles at various stages of development and simultaneously remove follicles, unfit for ovulation, from the ovarian tissue (Janz and Van Der Kraak 1997). Such fish are able to spawn several times during a breeding season. In contrast, synchronized ovaries contain follicles that are generally at the same stage of follicular development. In synchronous species of fish, ovulation is often an annual event (Dye et al.,

1986). We were interested in similarities and differences between these two modes of ovarian development and function in terms of three highly conserved processes; steroidogenesis, heat shock protein induction, and apoptosis, because they are involved in maintenance of ovarian follicle growth and development in all classes of vertebrates (Steller, 1995; Hsueh et al., 1996; Nagahama et al., 1994).

Additionally, there is ample scientific evidence, in diverse species and across many geographic locations, to suggest that exposure to environmental contaminants can alter the processes of steroidogenesis, heat shock protein induction, and apoptosis (Kime, 1996, 1998, 1999). Moreover, such perturbations have been shown to cause reproductive impairment in exposed wildlife species (Kime, 1998; Hose et al., 1981, 1989; Spies et al., 1985; Johnson et al., 1988; Singh, 1989; Thomas 1988; Thomas and Khan, 1997; Trent and Thomas, 1989).

Time course of ovarian apoptosis, HSP70 expression, and steroidogenic capacity; a comparison between synchronous and asynchronous ovaries of fish

Apoptosis

The phenomenon of spontaneous apoptosis has been reported in cultured pre-ovulatory follicles of rats (Chun and Hsueh, 1994) and in cultured granulosa cells from rat as well as chicken ovaries (Tilly et al., 1991, 1992). In mammals, where physiological cell death has been most studied, apoptosis occurs during all stages of ovarian development and survival or demise of a follicle depends on many growth and hormonal factors (Hsueh et al., 1994). In the absence of appropriate hormones and growth factors follicles rapidly undergo apoptosis *in vivo* and are removed from ovarian tissue (Hsueh et al., 1994).

Fish follicles also undergo spontaneous apoptosis *in vitro* (Janz and Van Der Kraak, 1997) and we found that ovarian follicles of fathead minnow and catfish are similarly prone to apoptosis when deprived of serum factors that promote life of a follicle (Fig. 1 and 4). Fish ovarian follicles also responded similarly to exposure to toxicants (Janz et al., 1997)

The asynchronous fathead minnow ovarian follicles proved to be highly resistant to apoptotic cell death and this finding is in agreement with results from a preliminary study in goldfish (Janz unpublished data), which is also an asynchronous fish. Levels of intra-ovarian apoptosis in synchronous follicles (channel catfish) were twice that of asynchronous fathead minnow and it is entirely plausible that resistance to apoptosis, which protracts the life span of a follicle in an asynchronous ovary, would evolutionarily be advantageous in terms of maximizing the probability of ovulation and fertilization, which would enhance species fitness.

In contrast, synchronous follicles of catfish were more susceptible to apoptosis most likely because ovulation takes place at once and there is no real advantage in maintaining life of a follicle long after the short period of spawning. After 12 hours of incubation in serum free medium apoptosis was 4-fold higher in catfish follicles when compared to fathead minnow ovarian follicles (Fig. 4). At 24 hour of incubation there was a decline in fathead minnow follicular apoptosis but apoptosis continued to increase in catfish ovaries. At this time, 24 hour past incubation in serum free media, catfish follicles exhibited a 6.5-fold higher level of apoptosis in comparison to

fathead follicles. The trend for higher susceptibility to apoptosis in catfish follicles continued but at a smaller magnitude as time passed such that at 48 hours of incubation apoptosis was reduced to only 4-fold higher in catfish follicles when compared to fathead minnow follicles. A similar magnitude of spontaneous apoptosis has also been reported in another synchronous species, the rainbow trout. Incubation of rainbow trout pre-ovulatory follicles in serum free media causes a five-fold increase in follicular cells apoptosis after 24 hours of incubation as reported by Janz and Van Der Kraak (1997).

It is apparent that most cell types undergo apoptosis when deprived of survival factors that originate from neighboring cells and/or extra-cellular matrix. Based on our findings we suggest that the ovarian follicle culture method is a sensitive and reliable model for examining the effects of different survival and atretogenic factors on apoptosis in the ovary (Hsueh et al., 1994).

Heat shock proteins

Heat shock proteins are cellular chaperones that participate in protein biosynthesis, transport, and repair and are also expressed in response to a wide range of biotic and abiotic stressors (Feder and Hofmann, 1999;

Welch, 1993). It has been suggested that regulation of HSP expression is based on the ability of a stressor to denature native proteins (proteotoxiciy). Proteotoxicity results in denatured proteins that can act as potent inducers of HSP and that all stressors at some point act by damaging cellular proteins (Morimoto et al., 1994). We were interested in levels of HSP70 in follicles during our time course study and in response to toxicant/hormone treatments, because HSP70 is a major sensor molecule that mediates crucial cellular events and eventually sets off production of additional HSP70 in the cells (Morimoto et al., 1994; Voellmy, 1996; Welch, 1993).

There are a number of other signal transduction pathways for cellular stress sensors that initiate and/or regulate HSP70 production. Redox changes in the cell have been suggested to affect HSP biosynthesis (Voellmy, 1996). Various stressors that initiate a stress response in a cell also cause an increase in the intra-cellular calcium concentrations and activate enzymes such as phospholipases, which are important messengers in heat shock response (Calderwood et al., 1993; Kiang and McClain, 1993). These events and processes can then affect phosphorylation of heat shock protein factors that are crucial in regulation of HSP production. In addition, stress can cause changes

in membrane fluidity that may be involved in heat shock response by cells (Bensaude et al., 1996).

In fish, HSPs are induced by a variety of stressors including disease, severe temperature fluctuations, and/or exposure to environmental contaminants (Iwama et al., 1998). Environmental contaminants such as heavy metals, bleached kraft pulp mill effluents (BKME), PAH, industrial waste (i.e. solvents, paints, and lubricants), and herbicides have all been shown to induce HSP70 levels in fish tissue (Chen et al., 1988; Cho et al., 1997; Forsythe et al., 1997; Gedamu et al., 1983; Janz et al., 1997, 2001; Kobanet al., 1987; Koban et al., 1991; Luft et al., 1996; Mazur, 1996; Weber and Janz, 2001; Hassanein et al., 1999; Schroder et al., 2000).

In our time course study, HSP70 was decreased in follicular cells of fathead minnow during the first 12 hours of incubation in serum-free media (Fig. 2). HSP70 levels initially diminished in follicular cells perhaps in response to stress induced by lack of essential serum proteins and growth factors. The decline in HSP70 levels continued in fathead follicles for 24 hours but its production resumed and its levels built up in the cells at 48 hours of incubation. This indicates the ability of

fathead follicles to up-regulate HSP70 production at a time of stress, possibly to protect them from undergoing apoptosis.

Interestingly, intra-ovarian levels of HSP70 in fathead minnow follicles were twice as high as that in catfish ovaries. This suggests that fathead follicles are by nature better protected against various stressors. In catfish follicles HSP70 declined more rapidly during the time course study such that at 24 hours its levels had decreased to less than one third of the initial (0-hour) levels (FIG. 5). Lower constitutive levels of HSP70 in catfish follicles may provide an explanation for a more rapid utilization of this protein in the event of an insult.

Moreover, catfish follicles higher susceptibility to apoptosis may be explained by their inherent tendency to maintain low levels of HSP70 in their cells and their inability to increase HSP70 production after an insult. Conversely, resistance of fathead minnow follicles to apoptosis can be attributed to their inherent ability to normally maintain much higher levels of HSP70 within the cell and their ability to up-regulate HSP70 biosynthesis after an insult by an stressor.

Steroidogenesis

Gametogenesis and steroidogenesis in the vertebrate ovary are regulated primarily by actions of pituitary gonadotropins, and follicular atresia is a rapid response to reduction in gonadotropin levels in the ovary (Hirshfield, 1991). In mammals androgens have also been shown to act as atretogenic factors and to induce atresia in ovarian follicles (Billing et al., 1993).

In the time course study, testosterone biosynthesis by fathead minnow and catfish follicles increased steadily and peaked at 48 and 24 hours of incubation respectively (Fig. 3A). However, the two species differed in steroidogenic capacity (i.e. fathead follicles had a higher capacity for testosterone production). The significant increase in testosterone production in both models also coincided with elevated apoptosis, which supports the hypothesis that similar to mammals; testosterone acts as an atretogenic factor in the teleost ovary.

We have documented clear differences in the ability of asynchronous and synchronous follicles to produce sex steroid hormones. Asynchronous fathead minnow follicles can more effectively increase testosterone biosynthesis while synchronous catfish follicles are more prone to induce 17β-

estradiol productions in serum free media. A comparative time course study of synchronous and asynchronous teleosts suggests that fathead minnow follicles with slightly higher levels of constitutive HSP70 are better equipped to increase HSP70 levels shortly after environmental perturbation or insult. This ability of fathead minnow follicles coincided with follicular preparedness to rapidly increase testosterone biosynthesis. In contrast, catfish follicles have lower levels of constitutive HSP70 and after an insult, do not up-regulate production of this protein as efficiently as fathead follicles. Moreover, catfish follicles tend to increase production of 17β-estradiol during stressful conditions.

Similar to testosterone biosynthesis, 17β-estradiol production by fathead and catfish ovarian follicles increased during the time course study and peaked at 48 and 24 hour(s) respectively (Fig. 3B). Interestingly, at the peak of follicular steroidogenic activity the ratio of testosterone/estradiol was 3-fold higher in fathead follicles when compared to catfish. The presence of high levels of testosterone in follicular cells of fathead minnow contradicts their high resistance to apoptosis.

Perhaps testosterone is a much less potent androgen and 17β-estradiol a much more effective estrogen in teleost model.

Actions of selected factors (hormone/toxicants) in the microenvironment of the ovary

Effects of putative survival and atretogenic factors on cultured intact ovarian follicles were studied to investigate possible linkages between apoptosis, HSP70 expression, and steroidogenesis in the teleost ovary. We used chemicals/factors to stimulate four main cellular signal transduction pathways: (1) cAMP-dependent protein kinase (For/IBMX), (2) nuclear hormone receptor (17β-estradiol and testosterone), (3) tyrosine kinase-coupled receptor (EGF), and (4) protein kinase C activation (PMA) to determine their effects on apoptosis in the two teleosts of contrasting reproductive strategies. We examined fathead minnow as representative of an asynchronous teleost and channel catfish as a synchronous spawner.

Fathead minnow follicle treatments

We present that 17β-estradiol, EGF, and PMA protect preovulatory fathead minnow follicular cells from undergoing apoptosis (Fig. 7A). A combination of forskolin (activator of adenylyl cyclase) and IBMX (a

phosphodiesterase inhibitor) was used to stimulate cAMP-dependent protein kinase and mimic the mechanism of action of pituitary gonadotropins.

It is well established that gonadotropins control germ cell development and production of steroid hormones in vertebrates (Hirshfield, 1991). Gonadotropins are also the primary hormones involved in follicular survival (Hsueh et al., 1994). Without sufficient gonadotropin stimulation, ovarian follicles of mammals and birds undergo the rapid apoptotic process of atresia. In addition, exogenous gonadotropins as well as a number of growth factors (i.e. EGF) have been shown to inhibit ovarian follicle apoptosis both in mammals and in fish (Kaipia and Hsueh, 1997; Janz and Van Der Kraak, 1997).

1-Apoptosis

Treatment of fathead minnow preovulatory follicles with forskolin / IBMX, a gonadotropin pathway activator, suppressed the onset of DNA fragmentation by 32 % although this reduction in cell death was not statistically significant compared to control follicles (Fig. 7A). Data from studies in mammals and fish also indicate suppression of DNA fragmentation as a result of activation of gonadotropin pathway. Similar to rainbow trout, 17β-

estradiol suppressed apoptosis in fathead minnow follicles (82 %) but appeared to be a much more effective survival factor in the fathead minnow when compared to rainbow trout in which apoptotic DNA fragmentation was suppressed only by 26 % in the presence of the same concentration of 17β-estradiol. The higher efficiency of 17β-estradiol, as a survival factor, in the asynchronous ovary of fathead minnow may be explained by the fact that these follicles are naturally more resistant to apoptosis. In addition, high efficiency of 17β-estradiol in fathead model may explain why testosterone at its high levels does not induce apoptosis in this model. It appears that anti-apoptotic survival properties of 17β-estradiol dominates in fathead ovary overriding atretogenic effects of testosterone.

Similar to rainbow trout (Janz and Van Der Kraak, 1997), EGF caused a decrease in DNA fragmentation of follicular cells. Treatment of follicles with EGF did not affect production of 17β-estradiol (Fig. 10A) or testosterone (Fig. 9A) suggesting that the ability of EGF to suppress apoptosis is unrelated to steroidogenesis (Janz and Van Der Kraak, 1997).

PMA is a tumor promoter and recent evidence suggests that tumor growth *in vivo* depend on avoidance of normal

cellular control mechanisms that induce apoptosis (Wright et al., 1994, 1996). In agreement with this data, PMA in our study suppressed apoptotic cell death in follicular cells of fathead minnow (Fig. 7A). Wright et al (1994) have demonstrated that over 10 tumor-promoting factors block apoptosis and DNA fragmentation in a variety of carcinoma cell lines. In this realm, the presence of exogenous environmental toxicants that can suppress apoptosis may be important in the development of disorders that are initiated by environmental contaminants, such as cancer caused by environmental factors that suppress apoptosis.

Of all apoptogens investigated in this study Cd was the only one to induce apoptosis in fathead minnow follicular cells (Fig. 7B). Cadmium toxicity may be due to its ability to interfere with calcium homeostasis in the cell. Calcium is an important messenger (i.e. second messenger) that is involved in many cellular transduction pathways and can independently or after converging with one another initiate apoptosis in cells (Hajnoczky et al., 2000; Putney and Riberio, 2000). Another proposed mechanism of cadmium toxicity is its ability to generate hydroxyl radicals that can damage mitochondria and/or depress the level of endogenous antioxidants thereby

increasing oxidative stress in the cell. Forskolin/IBMX treatment of follicles suppressed cadmium-induced apoptosis and rescued cells from programmed cell death confirming the activation of gonadotropin pathway in follicular cells.

In vitro exposure to DMBA, which is a widely used in experimental trials and much data is available on its reproductive effects in fish and mammalian systems, in our study, did not cause DNA fragmentation in either of the two species we tested (Fig. 7B and 11B). However, *in vivo* exposure to DMBA has been shown to cause massive apoptosis in juvenile catfish ovary (Weber and Janz, 2001) suggesting that metabolic activation of DMBA may be necessary for its action in the ovary. Addition of activated microsomes to the wells may have provided the necessary biotransformathon processes for DMBA and enabled us to see effects of DMBA on follicular cells.

In addition, developmental differences may be responsible for differing effects of DMBA on follicular cells. Weber and Janz (2001) reported that follicular cells in earlier stages of development were more susceptible to DMBA exposure compared to fully reproductively developed cells of pre-ovulatory follicles. Similarly in our study mature pre-ovulatory ovarian

increasing oxidative stress in the cell. Forskolin/IBMX treatment of follicles suppressed cadmium-induced apoptosis and rescued cells from programmed cell death confirming the activation of gonadotropin pathway in follicular cells.

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lead to a significant negative relationship between expression of HSP70 and apoptosis (Weber and Janz, 2001). Similar to our finding, Weber and Janz (2001) reported no effects of DMBA on more reproductively mature catfish in their study.

Collectively, these observations suggest developmental differences in follicle susceptibility to exposure to DMBA with more mature follicles exhibiting resistance to DMBA toxicity. The significant increase we observed in HSP70 levels as a result of follicle treatment with testosterone appears to be unrelated to apoptosis in the fathead ovary because testosterone treatment did not alter the rate of apoptotic fragmentation in follicular cells of fathead minnows.

3-Steroidogenesis

It is well established that sex steroid hormones affect apoptosis in the ovary with estrogens inhibiting and androgens enhancing ovarian cell apoptosis in mammals. These hormones have been suggested to exert their regulatory effect by acting via a Ca^{2+}/Mg^{2+}-dependent endonuclease and by influencing steroid-dependent gene expression and/or synthesis of growth factors such as EGF/TGFα (transforming growth factor-alpha) (Billing et

al., 1993; Hsueh et al., 1994). Lund et al. (1999) has recently proposed a more immediate cytoprotective role for 17β-estradiol. These investigators suggest an oxygen-scavenging mode for 17β-estradiol actions in the ovary. Removal of oxygen radicals from the ovarian tissue is suggested to relieve cells from apoptosis induced by oxidative-stress.

Testosterone

Forskolin/IBMX treatment did not inhibit apoptosis in ovarian cells of fathead minnow (Fig. 7A) but suppressed testosterone production by these cells (Fig. 9A). Since forskolin/IBMX treatment did not inhibited testosterone biosynthesis while it induced 17β-estradiol productions in follicular cells, it may be that this gonadotropin analog protected cells by enhancing aromatase activity in follicular cells. Aromatase of mammals, birds, and fish converts testosterone to 17β-estradiol.

17β-estradiol treated follicles exhibited elevated testosterone levels which can be attributed to suppressed aromatase activity in response to excess 17β-estradiol in the immediate surrounding of the cells. In fathead minnow follicles EGF, in agreement with available data (Srivastava and Van Der Kraak, 1994) had no effect on either sex

steroid production. Therefore, inhibition of apoptosis by EGF cannot be attributed to its effects on steroidogenesis.

Cadmium chloride (CdCl10, CdCl100), DMBA, and PMA, all suppressed testosterone production in follicular cells of fathead minnow. This consistency in suppression of testosterone biosynthesis in toxicant exposed cells is in agreement with field studies that demonstrate similar effect in fish exposed to environmental ovotoxicants (Janz et al., 1997; Spies et al., 1985; Johnson et al., 1988; Singh, 1989; Thomas 1988, 1990; Thomas and Khan, 1997). Although testosterone synthesis was inhibited by all atretogens, we saw no effect on 17β-estradiol biosynthesis in follicular cells of fathead minnow. In catfish ovarian follicles however, biosynthesis of testosterone and 17β-estradiol was not altered by atretogens (Fig. 13B and 14B). This suggests that there was no effect on aromatase activity in follicular cells of either species.

17β-estradiol

Forskolin/IBMX treatment inhibited apoptosis in ovarian cells of fathead minnow (Fig. 7A) while it induced 17β-estradiol production (Fig. 10A). This observation agrees with mammalian studies in which 17β-estradiol inhibits apoptosis of ovarian cells. In agreement with

studies in goldfish and trout preovulatory follicles (Srivastava and Van Der Kraak, 1996) EGF in our study had no effect on 17β-estradiol in fathead minnow follicular cells.

17β-estradiol biosynthesis was virtually unaffected by toxicant treatments suggesting that its biosynthesis is unaffected by exposure to toxicants (Fig. 10B and 14B). This is not consistent with the studies that report altered 17β-estradiol levels in feral fish exposed to environmental contaminants (Janz et al., 1997; McMaster et al., 1996).

In summary, the fathead minnow experiments provide evidence that the teleost model is an appropriate system in which to assess effects of toxicants on ovarian function and homeostasis. The study of linkages between highly sensitive biochemical pathways (i.e. apoptosis, steroidogenesis, and HSP induction) and their response to ubiquitous environmental toxicants is possible in this simple and inexpensive model. Fine-tuning of a teleost ovarian model can assist in studies of signal transduction pathways that are sensitive to exposure to toxicants and adversely effect reproduction in aquatic organisms.

Channel catfish follicle treatments

1-Apoptosis

The catfish model proved to be less responsive than the fathead minnow model to treatment with apoptogens and/or survival factors. Wild catfish used in this study were overall more genetically heterogeneous than the laboratory raised fathead minnows and the high degree of variability in catfish data may have obscured our results and subsequently our ability to see clear trends in catfish apoptosis data. Catfish follicles treated with survival factors did not exhibit a detectable trend of reduced apoptosis.

Similar to fathead minnow follicles catfish ovarian cells were unaffected by testosterone or DMBA exposure (Fig. 11B). Testosterone effects on apoptosis may have been negligible since it appears to be a less potent atretogen in the teleost ovary when compared to mammalian model. Furthermore, DMBA's rate of biotransformation by follicular cells may have been too low to cause a significant build up of apoptogenic DMBA metabolites in follicular cells and cause an induction in apoptotic cell death in DMBA treated catfish preovulatory follicles.

2-HSP70 expression

Variability in this data set does not allow for further speculation with regards to the role of HSP70 in

catfish ovary at this time. The increase in HSP70 levels we observed in cadmium-treated catfish follicles was not similar to our finding in fathead minnow model where cadmium had no effect on levels of HSP70 (Fig. 12B). Therefore, at this time there is no evidence to suggest that HSP70 protects against cadmium-induced apoptosis in synchronous or asynchronous teleost ovarian follicle culture model.

HSP70 levels have been shown to increase as a result of exposure to DMBA in juvenile catfish ovaries (Weber and Janz, 2001). In agreement with this *in vivo* study, preovulatory catfish follicles exposed to DMBA exhibited a trend for elevated HSP70 levels. However, as it was discussed earlier, DMBA had no effect on HSP70 levels in fathead ovarian follicles. These differences in DMBA effects on follicular HSP70 levels may be due to species differences (catfish Vs fathead minnow) or the necessity for metabolic activation of DMBA before manifestation of its toxic effects on follicular cells.

Recent studies in mammals as well as juvenile catfish have shown that low HSP70 precedes cellular apoptosis and may be the cause rather than the consequence of apoptosis (Samali et al., 1999; Mallouk et al., 1999; Weber and Janz,

2001); however our results do not indicate such a relationship between HSP70 induction and apoptosis *in vitro*. Testosterone treatment, which had induced HSP70 expression in fathead minnow follicles, remained ineffective in catfish model. This is perhaps because of differing efficacy of 17β-estradiol in the two species as well as the role it plays in maintenance of follicular homeostasis in our test species.

3-Steroidogenesis

Testosterone

Testosterone biosynthesis was unaltered by survival factors we used in our study suggesting that none of the survival pathways we examined had any effect on testosterone production in catfish (Fig. 13A). In contrast to our major finding that all apoptogens significantly suppressed follicular synthesis of testosterone in fathead follicles, testosterone biosynthesis was unaffected by apoptogen treatments in the catfish model (Fig. 13B). Our inability to detect a similar trend in catfish ovary may be attributable to high variability in the data. The catfish we used in the present study were collected in late April, May, and early weeks of June at a time when water temperatures ranged from 23-27°C. Subtle, but significant,

developmental differences may have existed between experimental animals and may have been overlooked because of the similarity in follicle size and condition. In addition, greater genetic diversity of wild fish may be responsible for our inability to distinguish altered patterns of steroid hormone biosynthesis in catfish.

17β-estradiol

Forskolin/IBMX treatment in catfish induced 17β-estradiol production by follicular cells (Fig. 14A). When used in combination with Cd100, For/IBMX increased 17β-estradiol productions and this event coincided with lower apoptotic cell death in follicular cells strengthening the hypothesis that 17β -estradiol acts as a survival factor in teleost ovarian tissue.

Chronic exposure to environmental chemicals and elevated apoptosis in ovary and thymus of small mammals

Combinations of gonadal toxicants that are being released into the aquatic and terrestrial environments threaten gonadal function (Kime, 1998; Tilly, 1998). In mammals, females of the species are especially at risk of gonadal damage since, unlike males, females are born with a

finite and irreplaceable stock of germ cells in their ovaries. The mammalian ovary plays a crucial role in the regulation of reproduction because (1) ovaries are the site for development and maturation of female gametes and (2) ovaries produce and release sex steroids that maintain and regulate ovarian cyclicity (Hoyer and Sipes, 1996).

Exposure to environmental chemicals can affect ovarian development and function by acting at a variety of sites in the reproductive axis (i.e. hypothalamus, pituitary, or ovary) (Hoyer et al., 2001). However, this discussion will only be concerned with ovotoxicants that act by inducing apoptosis in the ovary. Toxicant-induced oocyte destruction is not well understood but it may be due to one or several mechanisms. Oocyte destruction may be the result of a direct effect of the toxicant on the ovarian follicles or toxicity may occur by one of several indirect routes.

The ovary contains biotransformation enzymes (i.e. epoxide hydrolase, glutathione-S-transferase, and cytochrome P450 monooxygenases) that are responsible for detoxification of many known ovarian toxicants (Bengtsson et al., 1983, 1992). Indirect ovotoxicity may occur as a result of biotransformation of toxicants in selected

regions within the ovary that cause specific exposure of certain class of oocyte to toxic effects of the compound. In addition, since granulosa cells encircle oocytes at all stages of follicular development, ovotoxicity may be due to the somatic cells losing ability to maintain the oocyte (Buccione et al., 1990).

The hypothesis that many environmental contaminants induce apoptosis in the oocyte or the supporting somatic cells and cause increased atresia and loss of viable ovarian tissue in a variety of organisms is strongly supported by an increasing number of investigations (Tilly, 1998; Springer et al., 1996; Kao et al., 1999; Borman et al., 2000; Hoyer and Sipes, 1996; Hoyer et al., 2001; Heimler et al., 1998). Environmental toxicants that are produced during the manufacture of many industrial and agricultural chemicals induce apoptosis in the ovary of rat and mice and possibly humans (Hoyer et al., 2001; Tilly, 1998).

For instance, PAHs (i.e. benzo[a]pyrene, 3-methyl-cholanthrene, DMBA) destroy oocytes in small primordial follicles of rats and mice (Mattison and Thorgeirsson, 1979). Another environmental chemical, 4-vinylcyclohexene, which is produced during manufacturing of tires,

insecticides, and plasticizers, as well as dioxins, have also been shown to cause atresia in ovarian follicles of rats (Hoyer et al., 2001; Heimler et al., 1998).

In addition to studies in mammals that demonstrate increased gonadal apoptosis as a result of exposure to environmental toxicants, elevated gonadal cell apoptosis has also been reported in larval pink salmon (*Oncorhynchus gorbuscha*) exposed to crude oil containing a complex mixture of PAHs (Marty et al., 1997). Complex chemical mixtures such as pulp mill effluents have also been shown to induce apoptosis in feral fish ovaries (Janz et al., 1997; 2001). Based on field and laboratory studies that associate exposure to environmental ovotoxicants with increased ovarian apoptosis we hypothesized that the rate of apoptosis will be higher in ovaries of cotton rat inhabitants of toxic waste sites.

Here we report the first evidence of elevated rates of ovarian and thymic apoptosis in wild mammals chronically exposed to environmental toxicants (Fig. 15A and B). The increase in ovarian apoptosis of rats from contaminated sites also coincided with fewer numbers of uterine scars (Fig. 15C and D). The number of uterine scars is a measure of previous successful pregnancies and provides evidence

that exposed females may have had lower reproductive fitness than unexposed females from non-contaminated sites.

Thus, it appears that chronic exposure to complex mixtures of environmental toxicants, such as those present in pulp mill effluents and petrochemical-contaminated soils, are able to stimulate apoptotic cell death in ovarian cells of aquatic and terrestrial vertebrates. In summary, data suggest that chronic exposure to certain components of petrochemical-contaminated soil stimulate the rate of ovarian cell and thymocyte apoptosis in feral cotton rats.

Elevated apoptosis in immune and hematopoetic cells of small mammals chronically exposed to environmental contaminants

Immune cells are the most common cell types to normally undergo apoptosis in adult mammals (Kelley et al., 1998; Raff, 1992; Cohen and Eisenburg, 1991). In hematopoietic tissue, apoptosis is an active process that is initiated by outside stimuli or loss of growth factors as well as some cytokines (Fairbairn et al., 1993; Koury and Bondurant 1990; Kalf et al., 1996).

Recent *in vitro* studies have demonstrated induction of apoptosis in immune and hematopoietic cell lines of humans, mice, and fish in response to exposure to environmental chemicals such as PAHs, organochlorine pesticides, and benzene metabolites (Kamath et al., 1997; Mann et al., 2001; Hinoshita et al., 1992; Kannan et al., 2000; Moran et al., 1996; Kiraku and Kawanishi, 1996; Ross et al., 1996; Kalf et al., 1996; Yoon et al., 2001; Bratton et al., 2000).

Many metals are ubiquitous environmental contaminant and are frequently found on petrochemical contaminated sites. Metals such as cadmium have been shown to induces apoptosis in human immune cell lines and cultures of mouse thymocytes (Tsangaris and Tzortzatou-Stathopoulou, 1998; Moran et al., 1996; Fujimaki et al., 2000). In addition, *in vivo* exposure to environmental toxicants such as PAHs and benzene cause a decrease in cellularity of spleen, bone marrow and thymus of mice and an increase in apoptosis of thymocytes and hemopoetic cells of humans, mice, and fish (Holladay and Smith, 1995; Vojdani et al., 1997; Farris et al., 1997; Holladay et al., 1998; Gogal et al., 2000).

Hemopoietic progenitor cells of the bone marrow and stromal cells are important control points for regulation

of hematopoiesis (Ross et al., 1996). Perturbations of homeostasis in immune and hematopoietic cell lines may have grave consequences for the individual's immune competence and has been the subject of many current studies. These investigations have proposed a number of mechanisms for immunotoxic effects of benzene, a highly volatile and widespread aromatic hydrocarbon.

The present study documents a four-fold increase in bone marrow cell apoptosis, and a two-fold increase in splenocyte cell death of cotton rat inhabitants of petrochemical-contaminated sites (Fig. 16). Although statistically not significant, such increases in the rates of cellular apoptosis may be biologically significant and hinder maintenance of animal's immune and hematopoietic system integrity and competence which has long been reported in these mammals (Lochmiller et al., 1999).

The long reported immunosuppression observed in small mammals living on contaminated sited might be the result of this loss of immune and hematopoietic cell types to apoptosis and has the potential to threaten individual survival in the wild (McMurry et al., 1999; Rafferty et al., 2001; Kim et al., 2001; Propst et al., 1999). It is therefore possible that environmental chemicals initiate

apoptotic signaling in bone marrow and spleen cells of cotton rat inhabitants of contaminated sites.

In summary, our data suggest that chronic exposure to certain components of petrochemical-contaminated soils stimulates the rates of ovarian, thymic, bone marrow, and splenic apoptosis in feral cotton rats. Wildlife species face a variety of natural stressors including nutritional deficiencies, disease, climatic extremes, reproduction and intraspecific interactions. These factors, in combination with the genetic heterogeneity of feral populations, create difficulties when attempting to evaluate toxicological risks to wildlife exposed chronically to environmental toxicants. Overall, the results of this study indicated that rates of apoptotic cell death in reproductive and immune organs represent an early cellular indicator of toxicity in wildlife exposed to complex mixtures of environmental contaminants.

Concluding Remarks

A rising number of hormonally active compounds (pesticides, herbicides, bactericidal agents, and synthetic hormones) are released into both aquatic and terrestrial environments. These compounds are often widespread, highly

persistent, bioavailable, and most importantly present at physiologically relevant concentrations in the environment. Many aquatic and terrestrial populations have exhibited signs of distress or have already been severely impacted by these compounds. Apoptosis is a well-conserved hormonally controlled physiological pathway that is highly sensitive to exposure to environmental pollutants and xenobiotics. Endocrine active compounds can cause perturbations in hormonal control of apoptosis and alter development and/or maintenance of reproductive tissue in growing or reproductively mature vertebrates. Such alterations can lead to reduced reproductive capacity of the exposed individual.

Here we present the first evidence that environmental exposure to a host of petrochemical contaminants (PAHs and metals) indeed induce apoptosis in highly proliferating reproductive (ovary), hematopoetic, and immune (thymus, bone marrow, and spleen) tissues of small mammals living on contaminated sites.

We also present evidence that the teleost ovarian follicle culture method which uses intact follicles, preserving the essential interconnections between granulosa and thecal cells, is a sensitive and reliable model in

which to assess effects of environmental toxicants on vertebrate ovarian homeostasis and function.

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