ORGANOCHLORINE PESTICIDE REPRODUCTIVE EFFECTS IN FATHEAD MINNOWS (*Pimephales promelas*): COMPARISON OF EMBRYO AND MATERNAL EXPOSURE

By

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Previous studies from this laboratory have documented decreased reproductive efficiency and altered hormone concentrations in wild populations of American alligators (*Alligator mississippiensis*) and largemouth bass (*Micropterus salmoides*) from sites contaminated with organochlorine pesticides (OCPs). These bioaccumulate significantly in fish. Our purpose was to determine whether adverse effects to reproductive fitness are caused by OCP-induced effects on maternal reproductive physiology.

We investigated maternal transfer of the OCPs dichlorodiphenyldichloroethylene (DDE), dieldrin, chlordane, and toxaphene from adult fathead minnows (*Pimephales promelas*) to egg and larvae. Adult female fathead minnows were dosed at rates of high, middle, and low. The high dose was a complex mixture comparable to concentrations found in blue tilapia (*Oreochromis aurea*) from a historically contaminated lake in central Florida. The middle dose was 50% of the high dose, and the low dose was 25% of the high dose. We recorded the frequency of spawns, the number of eggs laid per

spawn, the number of eggs hatched, and the number of larvae surviving to Day 14. At the end of the dosing period, we made histology slides of male and female gonads to determine stage of sexual maturation. We calculated GSI on male and female gonads. We used GC-MS analysis to analyze concentration levels of OCPs in fathead minnow eggs, male and female gonads, and whole body burdens. Statistical analysis was completed by using a One-way ANOVA to compare treatment groups.

Significant differences among control and treatment groups were detected when examining egg counts, the number of eggs hatched, clutch viability percentage, survivorship percentage to Day 7, and survivorship percentage to Day 14. No significant differences were detected between treatment groups when examining GSI. In conclusion, laboratory populations of fathead minnows maternally exposed to OCPs spawn less frequently, have a decreased hatch rate, and have decreased survivorship.

CHAPTER 1
INTRODUCTION

Background

The group of chemicals known as organochlorine pesticides (OCPs) consists of structurally diverse compounds used to control pests that have potential to damage agricultural crops, and to serve as vectors for human diseases. Because of the chemical stability, lipophilicity, and low water solubility of OCPs, these compounds and their metabolites persist in the environment. The OCPs can bioaccumulate in vertebrate species, causing negative effects in developmental and reproductive potential. OCPs alter enzyme activities (such as $Ca2^{+}$-ATPase and phosphokinase), and alter electrophysical properties (such as K^{+}, Na^{+} ion exchange of nerve cell membranes), thus affecting neural transmission. Because of their environmental persistence and their ability to biomagnify in food webs, the U.S. Environmental Protection Agency (USEPA) began to restrict (or in some cases, ban) the use of OCPs on agricultural lands between 1978 and 1983.

Growing concern has arisen that many environmental pollutants have the capability to interfere with normal function of human and animal endocrine systems. This type of pollutant is known as an endocrine disruptor. The endocrine system is composed of numerous types of tissues and can briefly be described as any tissues or cells that release chemical messengers or hormones that signal or trigger a physiological response from a target tissue (Thomas and Thomas, 2001). The endocrine system is integrated with many other biological functions and is widely dispersed throughout entire organisms. Because

of this, a wide range of toxicological responses may develop after exposure to endocrine disrupting compounds (EDCs) (Newman and Unger, 2003). Responses may include sexual developments, sexual differentiation, and success rates of reproduction. Reproductive processes in fish and other species of wildlife are largely controlled by complex hormonal pathways, thus giving endocrine disruptors the potential to target reproductive organs.

The USEPA developed a tiered testing paradigm, and paralleled assays to identify potential EDCs such as OCPs (USEPA, 1998). Some OCPs alter endocrine pathways controlled by thyroid hormones, estrogens, and androgens. Recommendations for the initial (Tier 1) screening assays include 3 assays using male or female rats at different life stages, using the amphibian (*Xenopus laevis*) as metamorphosis test, and a short-term reproduction test with the fathead minnow (*Pimephales promelas*) (USEPA, 1994). In general, Tier 1 screening assays include exposing fathead minnows to the chemical of concern for up to 21 days. Post exposure, the survival, behavior, fecundity, and secondary sexual characteristics are assessed. Fertility and early development of the F1 generation may also be evaluated. At the end of the test, plasma concentrations of sex steroids (β-estradiol, testosterone, 11-ketotestosterone [11-KT]) and vitellogenin (Vtg) are measured. Gonadal status is also assessed using a gonadosomatic index (GSI) and histopathology.

Although a general reduction in use of OCPs has been observed, several field studies suggest that OCPs adversely affect endocrine function in fish. Thus indicating aquatic and semi-aquatic organisms are continuously being exposed to levels of toxicants capable of altering reproductive parameters. Marburger *et al*. (2002) tested OCP levels in

soil from the Emeralda Marsh Conservation Area (on the north-east shore of Lake Griffin, Florida, United States), a historically contaminated lake. The lake contained concentrations of *p,p'*-DDE, dieldrin, and toxaphene over 3,000, 500, and 40,000 ng/g, respectively. The same study showed concentrations of OCPs in largemouth bass (*Micropterus salmoides*) ovaries and fat reached levels of 4,000 and 17,000 ng/g respectively, for total DDT derivatives, over 100 and 700 ng/g for dieldrin, and over 4,000 and 20,000 ng/g for toxaphene.

Selection of Animal Model

Numerous reasons exist for selecting the fathead minnow as a model species for EDC screening (Ankley, 2000). The fathead minnow is a member of the Cyprinidae family. Cyprinidae represent the largest fish family in the world. Over 2,000 species of Cyprinids or true minnows make up 25% of all freshwater fish and 9% of all fish species (Nelson, 1984). The Cyprinids are distributed in the fresh waters of North America, Europe, Asia, Africa, and Australia. They exist in virtually every freshwater habitat including swamps, sloughs, springs, ponds, lakes, large rivers, and tiny creeks. Although considered a freshwater species, some Cyprinids have been known to frequent tidal fresh and brackish water (Jenkins and Burkhead, 1994). North America contains approximately 295 described species of Cyprinids, 9 being exotic (Miller, 1996; Robins *et al*., 1991). Although most minnows are commonly considered small, the Cyprinidae family contains the smallest American minnow, the Mexican shiner (*Notropis saladonis*), reaching 150 mm total length (TL), as well as the endangered Colorado squawfish (*Ptychocheilus lucius*) which is known to reach a TL of 1800 mm and weigh as much as 45 kg (Smith, 1945).

Specifically, the fathead minnow represents an excellent model for the recognition of contaminant accumulation for numerous reasons. Adult fathead minnows are omnivores. Different populations have shown that one ate primarily insects, another only algae, another entirely detritus (Cahn 1927; Coyle, 1930 in Scott and Crossman, 1973; Starrett 1950) and yet another consumed microcrustaceans, insects, algae, and detritus (Pearse, 1918). Because of the fathead minnow's range in diet, they possess a moderately coiled gut which is an intermediate between the long and coiled gut of detritivores and herbivores and the S-shaped gut found primarily in carnivores. Their wide variety in diet insures they have the potential to eat, digest, and possibly bioaccumulate environmental contaminants located in various levels of the food web.

Approximately 11,080 developmental and survival tests in support of regulatory programs in North America and Europe (USEPA 1982, 1989, 1991, and 1994) have been established, giving an extensive background in sub-lethal and lethal effects on fathead minnows caused by agents and metabolites in numerous classes of environmental toxicants.

Fathead Minnow Reproductive Cycle

Because the fathead minnow is widely distributed across the United States and North America, specific times of maturation and spawning temperatures are difficult to pinpoint. The fathead minnow is native to the central portion of North America (Scott and Crossman, 1973). However, numerous populations exist in California, Arizona, Texas, and into the New England states; all of which possess different maturation times and spawning temperatures.

Generally, spawning occurs in water temperatures of 15 to 32 ºC (Markus, 1934). Because the population distribution of the fathead minnow ranges from southern Texas to

northern Minnesota, actual months of the year that the fathead minnow spawns will vary tremendously. When photoperiod and temperature reach acceptable limits, males develop dark vertical bands, turn a rusty brown, and develop white breeding tubercles that are prominent on the tip of the snout and top of the head. Once a dominant male is selected, he establishes a territory which he defends against other subordinate males in the local vicinity. He will then clean the nesting site which may be the underside of a flat rock or submerged vegetation, stones, logs or other acceptable substrates. Spawning typically takes place between dawn and 10:00am. After engaging in courtship, the female will deposit eggs onto the prepared surface of the selected substrate. Gale and Gerard (1982) showed in a study that female fathead minnows laid between 9 and 1,136 eggs per session (clutch). They also showed that between May 22 and August 22 five pairs of fish produced 16 to 26 clutches of eggs. Post fertilization, the male will assume responsibility of nest guarding. Again, depending on water temperature, the eggs will hatch into larvae in approximately six days and remain in the nesting area for several more days until their yolk material is absorbed.

Along with environmental cues such as photoperiod and temperature, teleost fish reproductive cycles are also regulated by endogenous hormonal cues (Gross *et al*., 2002). A combination of these factors stimulate the hypothalamus of the fish to release gonadotropin-releasing hormone (GnRH), norepinephrine (NE), as well as other neuropeptides to stimulate the pituitary gland which releases the primary teleost gonadotropins GTH-I and GTH-II (Van Der Kraak *et al*., 1998). These two gonadotropins are similar to the mammalian lutenizing hormone (LH) and follicle stimulating hormone (FSH) (Redding and Patino, 1993). GTH-I is the gonadotropin

associated with stimulation of events leading to vitellogenesis and spermiogenesis as well as early gonadal development. GTH-II is involved in the stimulation of events that will eventually lead to the final oocyte maturation and ovulation in females, as well as spermiation in males. The primary sex steroids involved in regulation of gametogenesis in the majority of male and female teleost fish are 11-KT and 17β-Estradiol. An increase in plasma concentrations of these hormones has been shown to be associated with the onset of seasonal reproductivity (Gross *et al*., 2002).

In females, an increase in estrogen (E_2) levels within the blood stimulates the liver to produce vitellogenin, a phosphoglycolipoprotein that serves as a precursor to yolk production in oviparous vertebrates (Wahli *et al*., 1981). Vitellogenin is then released into circulation where it travels to the gonad and is used as a nutrient source for developing oocytes. Within the promoter region of the vitellogenin gene lies an estrogen responsive element which is transcribed in response to an estrogen receptor (ER) complex (Wahli *et al*., 1981). The surface of the oocyte contains vitellogenin receptors, which once these receptors are occupied, the oocyte is cleaved into smaller yolk proteins. The yolk proteins are embodied into yolk granules which will in turn constitute majority of the mature oocytes. The yolk granules are stored and will serve as the nutrient source for developing embryos (Wahli *et al*., 1981). Once oocytes have reached their properly developed size, vitellogenesis stops, and the oocytes complete maturation. During this maturation phase, follicles increase in size due to hydration, as well as collect additional vital proteins. At the time of germinal vesicle breakdown, protein uptake ceases. Due to hydration, the follicle volume continues to increase. It is at this time the cellular envelope that surrounds an egg in preparation for ovulation known as the chorion, begins to

develop. The time of ovulation is species specific and will take place when follicles reach a specific size (Wallace and Selman, 1981).

Although male and female fish contain the vitellogenin gene, concentrations of E_2 normally only found in females are needed to produce measurable levels of vitellogenin (Wallace, 1970; Ryffel, 1978; Hori *et al*., 1979). Presence of vitellogenin in males can then be used as an indicator of estrogenic compound exposure (Pelissero *et al*., 1993; Sumpter and Jobling, 1995; Shelby *et al*., 1996; Okoumassoun *et al*., 2002).

OCP Characteristics

OCPs are synthetic compounds that contain certain amounts of chlorine substituted for hydrogen on a hydrocarbon backbone, also known as chlorinated hydrocarbons. However, also included in this group are a few compounds such as dieldrin and methoxychlor that have oxygen incorporated in their structure. Most OCPs do not resemble naturally occurring organic compounds in their structure, hence, they degrade slowly within their environment (Newman and Unger, 2003). Because OCPs have high molecular weights and are non-polar, they have low water solubilities and are soluble in lipids that are found within living organisms. The lipophilicity of OCPs causes bioaccumulation and possibly biomagnification.

One of the first widely used OCPs was dichloro-diphenyl-trichloroethane (DDT). DDT became a popular pesticide for numerous reasons. DDT has the capability to control or kill a wide range of insect pests, it is persistent in the environment, and it has low mammalian toxicity. DDT was used during World War II to control fleas, lice, flies, and mosquitoes which are vectors for malaria and typhus transfer to servicemen and civilian populations. Based on the success of DDT during World War II, DDT became the pesticide of choice for agricultural and commercial use. Gildersleeve *et al*. (1985)

and Bryan *et al*. (1989) were able to demonstrate through laboratory studies that in ovo treatment with DDT to Japanese quail resulted in female progeny that lay eggs without shells on a regular basis, as well as, have reproductive tract malformations. These, along with numerous other studies on exposure of DDT to wildlife led to the banning of DDT by the Environmental Protection Agency (EPA) in the United States at the end of 1972. DDT undergoes degradation in the environment to the metabolites DDD and DDE. Both of these metabolites have a half life on the order of years. Most samples taken in the environment today contain predominately the DDE and DDD isomers.

Another class of insecticides commonly found in the environment that act similar to DDT are the Cyclodienes. Included in the Cyclodiene class are toxaphene, dieldrin, and chlordane. One complex mixture of polychlorobornanes and camphenes is known as toxaphene. The chlorination of technical camphene or α-pinase can consist of over 300 congeners (Di Giulio and Tillitt, 1997). Between 1972-1975, toxaphene was widely used in the United States on cotton crops. Toxaphene was banned in the United States in 1982, however, each structurally different compound in the mixture will have a specific set of chemical properties, and thus predicting its environmental fate proves difficult.

Dieldrin is a common name of an insecticide that was routinely used in the 1950's to the early 1970's. It was used in agriculture for soil and seed treatment and in public health to control disease vectors such as mosquitoes and tsetse flies. It was also used as a sheep dip and has been used in the treatment of wood and woolen products. Most uses of dieldrin were banned in 1975 and currently it is not produced or imported by the United States. Studies have shown that oral uptake, as well as, exposure through fish gills can

adversely affect fish nitrogen content in the liver and reduce swimming speed at which fish can maintain themselves (Mayer, 1971).

Chlordane is another insecticide within the Cyclodiene class that was used in the United States between 1948 and 1988. Chlordane is not a single chemical, but is a mixture of many related chemicals of which approximately 10 are major components including trans-chlordane, cis-chlordane, heptachlor, and trans-nonachlor. Prior to 1978, chlordane was used as a pesticide on agricultural crops. From 1983-1988, chlordane was only approved to be used as a pesticide to control termites in homes. Because of concerns over cancer risk and adverse affects in wildlife, the EPA cancelled the use of chlordane on food crops and phased out other above-ground uses.

OCP Exposure Responses in Fish

Numerous studies have linked disturbances in reproductive function to exposure of different classes of toxic chemicals (Jobling *et al*., 1998). However, when looking specifically at OCPs, much research has been concentrated on endocrine disruption (Rauschenberger *et al.*, 2004; Ree and Payne, 1997; Russell *et al.* 1999; Gleason and Nacci, 2001). It is thought that one area of reproductive success that is affected by OCP exposure is circulating sex steroids. Studies have shown that abnormalities exist in concentration levels of circulating sex steroids in fish exposed to OCPs. Johnson (2005) found that Florida largemouth bass (*Micropterus salmoides floridanus*), exposed to *p,p'*-DDE and dieldrin for a 120-day period, led to depressed concentrations of plasma E_2 for female largemouth bass, increases in 11-KT in female largemouth bass and a lack of consistent increases and/or depressions of male largemouth bass E_2 and 11-KT concentrations. Female largemouth bass in another study (Muller, 2003) also showed higher circulating E_2 concentrations when exposed to 2.5 mg of DDE compared to

control groups. Male largemouth bass when exposed to 5.0 mg of DDE also consistently showed higher circulating levels of E_2. Not only have laboratory studies shown altered sex steroid hormones, but also fish captured from wild populations. Largemouth bass collected from the Emeralda Marsh Conservation Area (EMCA) located on the north-east shore of Lake Griffin, Florida, USA, demonstrated depressed levels of sex steroid hormones 17β-estradiol and 11-KT concentrations that are possibly a result of a disruption in the hypothalamus-pituitary-gonad axis which is responsible for stimulating synthesis and secretion of sex steroid hormones (Gross *et al*., 2003).

Another example of altered circulating sex steroids was demonstrated by (Kelce *et al*., 1995). They demonstrated that *p,p'*-DDE acts as a potent anti-androgen in rats by binding to the androgen receptor (AR), preventing testosterone synthesis, and causing demasculinization. This phenomenon has also been demonstrated in several fish species including white sturgeon (*Acipenser transmontanus*) (Foster *et al*., 2001) and goldfish (*Carassius auratus*) (testes only), (Wells and Van Der Kraak, 2000).

Numerous mechanisms of action exist for EDCs and may interrupt multiple pathways along the hypothalamic-pituitary-target-tissue axis. These interruptions may disturb the transport, binding, release, biotransformation, elimination or normal synthesis of natural hormones. EDCs may alter the hypothalamic-pituitary axis which could have a cascading affect on the endocrine system downstream of the hypothalamus. EDCs may interfere with neurotransmitters that control GnRH secretion resulting in decreased levels of GnRH production, as well as a reduction in gonad size, and may be responsible for the alterations in the concentrations of circulating sex steroids (Jansen *et al*., 1993). Specific endocrine tissues synthesize hormones that are secreted into the bloodstream where they

are bound to proteins and transported to target tissues where they interact with receptors, bring about responses, or may be metabolized or degraded. EDCs may block or enhance the function of these hormones by interfering with one or several of these steps. EDCs may cause synthesis failure of certain hormones or limit the uptake of critical precursors to produce hormones. Additionally, EDCs may alter the rate at which hormones are metabolized as is the case with the super family of enzymes, CYP 450 which are critical in the synthesis and metabolism of steroid hormones. EDCs may also induce hormone-like effects due to the alternating rates of degradation. EDCs may interfere with hormones binding to transport proteins thus preventing delivery to target tissues (Darnerud *et al*., 1996). Some EDCs have the ability to mimic estrogens or androgens. These EDCs may bind to globulin proteins, thus displacing and possibly increasing the elimination of endogenous circulating sex steroids (Rosner, 1990). EDCs may also have the potential to bind to hormone receptors and either activate, (agonize), (Flouriot *et al.*, 1995) or inhibit (antagonize) (Danzo, 1997) receptor function. Particular research interest has been focused on EDCs and their ability to bind to estrogen receptors (ER). Most ER are located in the nucleus of target cells. ER-DNA complexes interact with chromosomal proteins and transcription factors in order to induce or inhibit transcriptions of specific genes, thus enabling endocrine specific responses. It is possible for EDCs to enhance or block the function of a hormone or endocrine target tissue by interfering with one or several critical steps in the transcription process (Hoffman *et al*., 2003). Unlike ER that have a specific E_2 response element, EDCs that have androgenic activities may exert broader effects than those attributed to a simple androgen mimic. Endocrine-disrupting effects may also occur due to direct or indirect toxicities on specific target

tissues. Lipophilic EDCs such as OCPs will accumulate primarily in fatty tissues (Raushenberger, 2004) such as gonads and the liver, potentially interfering with the mobilization and synthesis of lipids, thus inhibiting specific endocrine related functions such as vitellogenesis.

Significance of this Study

Numerous oviparous vertebrates become subjects for studies because they are sensitive to adverse effects of chemical contaminants released into the environment, highly visible by the public, or obtain interest by organizations because they are viewed as limited natural resources. However, a species that attracts attention by the public or is federally listed may not be considered appropriate for laboratory studies. It is crucial when assessing risks to wildlife populations that not only must one understand environmental exposures, but also dose-response relationships using measured endpoints. Endpoints may be measured as lethal (mortality) or as sub-lethal effects such as adverse changes in reproduction or development.

In many instances use of a surrogate species may be appropriate to assess possible risks involved in exposure to chemical contaminants. Biological endpoints can be used to help express potential for chemicals to cause negative ecological effects. Surrogate species are often used to in laboratory tests to complete acute or chronic toxic effects for specific chemicals (Zeeman and Gifford, 1997). Most surrogate species are selected because they are easily cared for and cultured under laboratory settings and provide an inexpensive and reliable assessment of acute and chronic toxicity of potential chemical contaminants. Identifying potential ecological and physiological characteristics related to the susceptibility to effects of contaminants, along with their exposure routes is important in managing wildlife populations for the optimal use by humans (Rauschenberger, 2004).

The ability to develop the fathead minnow into a biological model would provide wildlife managers a standard by which to measure ecological impacts brought about by anthropogenic chemicals dispersed throughout aquatic environments.

The objectives of this study were:

1) to determine if OCPs are maternally transferred from the adult female fathead minnow to her offspring;

2) to determine if maternal OCP exposure adversely affects hatch rate and larval mortality; and

3) to determine if oral exposure of OCPs cause masculinization in female fathead minnow gonads and/or feminization in the gonads of male fathead minnows.

Previous studies have shown that OCPs may act as endocrine system modulators affecting reproductive success in numerous animal species (Gallager *et al.*, 2001; Gross *et al*., 1994; Mills *et al.*, 2001; Muller *et al*., 2004). We hypothesized that oral exposure to fathead minnows by a mixture of *p,p'*-DDE, dieldrin, toxaphene, and chlordane at increasing concentrations would bioaccumulate in muscle tissue and in the gonads of both male and female fathead minnows due in part to the highly lipophilic nature of these compounds, and thus be maternally transferred to fathead minnow eggs. Exposure to the OCP mixture would also demonstrate an enzyme inducing and/or estrogenic properties that would directly or indirectly increase larval mortality, adversely affect clutch size and frequency of spawns per fathead minnow pair, and would cause feminization or masculinization of fathead minnow gonads.

CHAPTER 2
MATERIALS AND METHODS

Experimental Animals

Male and female fathead minnows of 5 to 7 months of age were obtained from Aquatic Bio Systems Inc. in March 2005. The fish were transported to the USGS-FISC laboratory where they were maintained in 10-gallon aquariums with a flow-through water system supplied by on-sight well water and aeration. Upon arrival, all fish looked healthy, disease free, and weighed between 2.20 and 4.13 grams (g). Fish were fed a diet of Ziegler "Prime Tropical 45-9 Flake Feed" *ad libitum* once per day. The fish were allowed two weeks to acclimate to their new environment prior to dosing.

Water quality parameters measured included temperature, pH, and dissolved oxygen. Water quality parameters remained within acceptable ranges for the duration of the experiment. Temperature ranges were from 21.30 to 22.30 ºC. Dissolved oxygen ranges were from 5.30mg/L to 6.35mg/L. pH levels were from 7.90 to 8.70.

Chemicals and Dosing

The organochlorine pesticide 1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4,5,8-dimethanonaphthalene (dieldrin, Lot#77H3578) was obtained from Sigma (St. Louis, MO). The organochlorine pesticide 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene (*p,p'*-DDE, Lot#0902KU) was obtained from Aldrich Chemical Co. (Milwaukee, WI). The organochlorines chlordane (Lot#303-16B) and toxaphene (Lot#302-125B) were purchased from Chem Service (West Chester, PA). The chemicals were added to 1690.0 ml of Yelkin oil provided by Ziegler Bros., Inc. at the

following concentrations: dieldrin 5.0 mg, *p,p'*-DDE 12.0 mg, chlordane 10.0 mg, toxaphene 95.0mg. The contaminated Yelkin oil was then mixed with 25.0 pounds (lbs.) of "Prime Tropical Flake Feed" by Ziegler Bros., Inc.

Pilot Study

Prior to the conduction of the experiment, a pilot study was undertaken to establish body burdens and possible lethal doses of the experimental fish, as well as contaminant concentration amounts present in the treated and control feeds. The control and treated feed was analyzed by the Center for Environmental and Human Toxicology, University of Florida, Gainesville, Florida. Screen assays were performed to establish limits of detection, as well as limits of quantification (Table 1).

When determining the amount of contaminants to add to the flake feed, an attempt was made to achieve target parts per million (ppm) values comparable to what is seen in blue tilapia (*Oreochromis aureus*) stocked at the north shore restoration area in Lake Apopka, Florida, a historically hypereutrophic and contaminated lake (Pollman *et al*., 1988). A biomagnification factor of 15 was used based on previous studies performed at the USGS-FISC, Gainesville, Florida, laboratory.

Post feed analysis, the control and treated feed was mixed to establish a high, middle, and low dose. One hundred twenty grams of control feed was added to a 64.0 ounce (oz.) clean plastic container. One hundred twenty grams of the high dose feed was also added to a 64.0 oz. clean plastic container. The medium dose was established by mixing 60.0 g of control feed with 60.0 g of treated feed in a 64oz. clean plastic container. The low dose was established by mixing 90.0 g of control feed with 30.0 g of treated feed in a 64.0 oz. clean plastic container. The middle and low doses were then shook to ensure thorough mixing and stored in a refrigerator at 5 ºC.

Five female fish of each treatment group and the control were placed in separate 10-gallon aquariums. Fish were then fed approximately 2.5% of their body weight once per day for 30 consecutive days. At the end of 30 days, all fish were separately wrapped in foil, frozen, and delivered to the Center for Environmental and Human Toxicology, University of Florida, for chemical analysis. During the analysis, 2 fish from each treatment group were individually assayed, and the remaining 3 were analyzed via a composite assay with the exception of the low dose treatment group and control group where each sustained one mortality. In this case, 2 individual fish from the low dose treatment group and control group were analyzed and the remaining 2 fish from each group were analyzed via a composite assay. The results of the individual and composite assays are located in Table 2.

Nanoinjection Experiment

A nanoinjection experiment was performed that involved four replicate trials. Each trial contained five treatments. Eggs were injected at a low, middle, and high dose rate at 5, 10, and 20 ppm of *p,p'*-DDE at a quantity of 83.5-116.9 micrograms. A control that was not injected, as well as a vehicle control that was injected with the same quantity of triolein was used. Eggs were injected with a Kanetec MB-B manipulator and pre-made needles comprised of aluminosilicate 0.68-mm capillary tubes. Eggs were held in place for injection by agar placed on microscope slides acting as a substrate. Once the eggs were injected, they were placed in a flow-through incubator for hatching. The number of hatched larvae, as well as the number of surviving larvae were monitored to Day 11.

Maternal Transfer Experiment

Ten female fathead minnows per treatment group and control were randomly selected and placed in separate 10-gallon aquariums supplied by flow-through, on-sight

well water and aeration. Water quality parameters were measured, and remained within acceptable levels throughout the experiment. Photoperiod was gradually increased from an 8 light/16 dark hour day to a 12 light/12 dark hour day. Temperature was gradually increased from 22 ºC (± 1 ºC) to 25 C (± 1 ºC). The fish were dosed with the same feed mixture as in the pilot study. Based upon the amount of food consumed by the fish during the pilot study, a reduction was made in the amount of food provided to the fish to approximately 2% of the fish's body weight. The fish were then dosed for a consecutive 30 days.

At the end of the dosing period, male fish were separated from each other, and randomly placed in 10-gallon aquariums. Female fish were then randomly paired with the male fish. Each treatment and control groups contained 10 pairs of male and female fish. Water quality was monitored throughout the experiment and aquariums were siphoned as needed. Control fish were fed .18± .02 g per pair of Ziegler "Prime Tropical 45-9 Flake Feed" once per day; while the 3 treatment groups remained on their diet of the previously mixed treated feed at .18± .02 g per pair once per day.

Spawning substrates consisted of PVC pipe with a 4-inch diameter cut in half to 4 inches long. The substrates were inspected for the presence of eggs daily at approximately 10:30am. Substrates that contained eggs were removed, and the eggs counted under a bench magnifying glass. The substrates with eggs were then placed into a 64 oz. plastic container with 1000 micron mesh attached to the sides in order to allow water flow-through. The container was then placed into a flow-through water bath at 22.2±1 ºC with a constant drip consisting of aerated well water. A 2-inch airstone

supplied heavy aeration into the container to prevent fungus from attaching to the eggs. Egg substrates were replaced to aquaria.

Larvae began hatching 7 to 11 days after spawning. Hatched larvae were transferred to a clean 64 oz. container and individually counted. This process was repeated on Days 7 and 14 after hatch. A composite assay containing 0.2 g to 0.4 g of eggs with all four treatment groups was performed to confirm that contaminants were being maternally transferred. This assay was performed by the Center for Environmental and Human Toxicology, University of Florida, Gainesville, Florida. The trial was repeated dosing 40 female fathead minnows 7-9 months of age for 30 consecutive days. At the end of the 30-day dosing period, female fathead minnows were paired with male fathead minnows 7 to 9 months of age into individual 10-gallon aquariums with the same flow-through water system. Each pair of fish was fed 0.4 g of control and treated feed once daily for an additional 30 consecutive days. A spawning substrate constructed of a 4-inch long, 4-inch diameter PVC pipe cut in half was placed in the aquarium. Spawning substrates were checked daily for the presence of eggs. Any eggs present were individually counted and recorded. A composite sample containing 0.2 g to 0.4 g of the control and 3 treatment group eggs were collected at the beginning, middle, and end of the 30-day trial. The Center for Environmental and Human Toxicology, University of Florida, Gainesville, Florida, performed an OCP screen on the eggs to affirm the presence of contaminants.

Analysis of Fathead Minnow Tissues and Eggs for OCPs

Analysis was conducted by the Center for Environmental and Human Toxicology, University of Florida. Briefly, the OCPs in a weighed, homogenized portion of sample 2.0 g were extracted into ethyl acetate. Sample clean-up included use of C18 and NH2

solid-phase extraction cartridges prior to analysis by GC-MS. Each analyte was quantified against a standard curve having at least five points and a correlation coefficient $\geq$0.995. For the flake feed analysis, the Est LOD-LOQ range was 0.3-7.5 (ng/g) for chlordane, 0.1-7.5 (ng/g) for DDE (metabolites), 0.8-1.5 (ng/g) for dieldrin, and 63-1500 (ng/g) for toxaphene. Fathead minnow body burdens had an Est LOD-LOQ range of 0.3-1.5 (ng/g) for chlordane, 0.1-1.5 (ng/g) for DDE (metabolites), 0.5-1.5 (ng/g) for dieldrin, and 41-1500 (ng/g) for toxaphene. The fathead minnow gonads had an Est LOD-LOQ range of 1.2-7.5 (ng/g) for chlordane, 0.1-7.5 (ng/g) for DDE (metabolites), 0.4-1.5 (ng/g) for dieldrin, and 42-1500 (ng/g) for toxaphene. Fathead minnow eggs had an Est LOD-LOQ range of 0.2-1.5 (ng/g) for chlordane, 0.1-1.5 (ng/g) for DDE (metabolites), 0.8-1.5 (ng/g) for dieldrin, and (ng/g) 42-1500 for toxaphene.

Gonad Histology

Tissue staining with hematoxylin and eosin (H&E), sectioning and slide mounting was performed by Histology Tech Services (Gainesville, Florida). Ovaries were observed under a light microscope at 40X and stage of sexual maturation was assigned (Sepúlveda, 2000).

Briefly, stage 1 ovaries are undeveloped with mostly primary phase follicles. Stage 2 ovaries are pre-vitellogenic with primary and secondary phase follicles, but have no vitellogenic follicles. Stage 3 ovaries are early vitellogenic with some vitelline granules in follicles of varying size and no fully developed eggs. Stage 4 ovaries are late vitellogenic with a majority of follicles containing numerous vitelline granules and fully developed eggs are present (Muller, 2003).

Stage 1 testes are non-spermatogenic, with an extremely thin germinal epithelium and no sperm present. Stage 2 testes show low spermatogenic activity, with a thin

epithelium that contains scattered proliferation and maturation of spermatozoa. Stage 3 testes show moderate spermatogenic activity, thick germinal epithelium and diffuse to moderate proliferation and maturation of sperm. Stage 4 testes show thick germinal epithelium, high proliferation and maturation of sperm.

Statistical Analysis

All statistical analyses were performed using Statistical Analysis system (SAS) Enterprise Reporter software version 2.6. The experimental design required statistical analysis comparing each treatment group by a One-Way ANOVA. ANOVAs were performed and significance was set at alpha = 0.05. Results are represented as means ± standard error.

A Tukeys Multiple Comparison test ($P \leq 0.05$) was used to compare treatment groups and the stage of the gonad in both male and female fathead minnows.

CHAPTER 3
RESULTS

Maternal Exposure

Maternal exposure to contaminant mixture led to a decrease in the percent of spawning pairs in all three treatment groups when compared to the control. The percent of females spawning per treatment group resulted in the following: Control group 80%, Low treatment group 60%, Middle treatment group 40%, and High treatment group 50%. Significant differences were observed between the control and the middle treatment group and the control and the high treatment group when using a One-way ANOVA ($P<.05$). The total number of potential spawning pairs for the control and treatment groups were 10 pairs over a 30-day period.

Exposure to the contaminant mixture led to an increase in the mean number of eggs laid per spawn in the low treatment group 350 vs. 220, and in the high treatment group 270 vs. 220, but a decrease in the number of eggs laid per spawn in the middle 190 vs 220. The mean and standard error of eggs laid for each treatment group is represented in Figure 2. The mean number of eggs laid for the control group ± standard error was 220.04 ± 32.36. The mean number of eggs laid for the low treatment group was 351.67 ± 41.16. The mean number of eggs laid for the middle treatment group was 188.73 ± 34.41. The mean number of eggs laid for the high treatment group was 271.68 ± 29.41. The controls laid the most total number of eggs at 5501, the low treatment group laid the second highest amount of eggs at 5275, the high treatment group laid the third most

number of eggs at 4890, and the middle treatment group laid the fewest number of total eggs at 2831.

Exposure to the contaminant mixture led to an increase in the mean number of eggs hatched in the low treatment group, but a decrease in the mean number of eggs hatched in the middle and high treatment groups when compared to the control. This is the same phenomenon witnessed in the mean number of eggs laid. The mean and standard error number of hatched eggs for each treatment group is represented in Figure 3. The mean number of hatched eggs for the control group and ± standard error was 144.64 ± 25.77. The mean number of eggs hatched for the low treatment group was 265.60 ± 31.98. The mean number of eggs hatched for the middle treatment group was 69.67 ± 21.66. The mean number of eggs hatched for the high treatment group was 82.95 ± 28.06. Significant differences were detected among the low vs. mid treatment groups, low vs. control treatment groups, and low vs. high treatment groups.

Exposure to the contaminant mixture also led to an increase in clutch viability for the low treatment group and a decrease in clutch viability for the middle and high treatment groups when compared to the control. This is the same phenomenon in witnessed in the mean number of eggs laid and hatched. The mean and standard error of clutch viability percentage is represented in Figure 3. The mean number of clutch viability percentage for the control group was 64.98 ± 6.19. The mean number of clutch viability percentage for the low treatment group was 76.39 ± 3.72. The mean number of clutch viability percentage for the middle treatment group was 30.64 ± 9.71. The mean number of clutch viability percentage for the high treatment group was 28.57 ± 8.35. Significant differences were detected among the low vs. middle treatment groups, low vs.

high treatment groups, control vs. middle treatment groups, and control vs. high treatment groups.

Exposure to the contaminant mixture led to a decrease in the percent of larvae surviving to Day 7 in all three treatment groups when compared to the control. The mean and standard error of surviving larvae to Day 7 percentage is represented in Figure 4. The mean and standard error of surviving larvae to Day 7 percentage in the control treatment group was 43.59 ± 6.98. The mean number of surviving larvae to Day 7 percentage in the low treatment group was 31.35 ± 5.98. The mean number of surviving larvae to Day 7 percentage in the middle treatment group was 8.35 ± 4.91. The mean number of surviving larvae to Day 7 percentage in the high treatment group was 15.71 ± 7.09. Significant differences were detected in the control vs. middle treatment groups and the control vs. high treatment groups.

Exposure to the contaminant mixture led to a decrease in the percent of larvae surviving to Day 14 in all three treatment groups when compared to the control. The mean and standard error of surviving larvae to Day 14 percentage is represented in Figure 5. The mean and standard error of surviving larvae to Day 14 percentage in the control treatment group was 32.52 ± 8.14. The mean number of surviving larvae to Day 14 percentage in the low treatment group was 39.67 ± 9.62. The mean number of surviving larvae to Day 14 percentage in the middle treatment group was 7.60 ± 4.57. The mean number of surviving larvae to Day 14 percentage in the high treatment group was 7.06 ± 4.03. Significant differences were detected between the control vs. middle treatment groups.

With the exception of the high treatment group in regards to female GSI, the mean GSI for the low and middle remained approximately the same when compared to the control, while the high treatment group increased. The mean and standard error for GSI in the female control groups was 10.44 ± 5.57. The mean and standard error for GSI in the female low treatment group was 9.44 ± 1.79. The mean and standard error for GSI in the female middle treatment group was 9.50 ± 1.99. The mean and standard for GSI in the female high treatment group was 14.12 ± 1.63. Mean GSI for male and female fathead minnows showed no effect.

With the exception of the middle treatment group in regards to male GSI, the mean GSI for the low and high groups remained approximately the same when compared to the control, while the middle treatment group decreased. The mean and standard error for GSI in the male control treatment group was 1.36 ± 0.79. The mean and standard error for GSI in the male low treatment group was 1.33 ± 0.15. The mean and standard error for GSI in the male middle treatment group was 0.66 ± 0.13. The mean and standard error for GSI in the male high treatment group was 1.36 ± 0.23. No significant differences were observed among treatment groups or sex in regards to GSI.

Nanoinjection Experiment

Nanoinjection trials indicated that fathead minnow eggs injected with *p,p'*-DDE were adversely affected by the chemical. Trials 1, 2, and 3 showed eggs injected with 20, 10, and 5 ppm of *p,p'*-DDE had lower hatch and survivorship when compared to control eggs. Trial 4 showed hatch and survivorship to be higher in the *p,p'*-DDE injected eggs than the controls. Overall, hatch and survivorship rates during this trial were lower than previous trials.

The percent of hatched eggs for the control group was 81 with a standard error of ± 10. The triolein group showed a percent of hatched eggs at 67 ± 7. The treatment group at 5 ppm of *p,p'*-DDE had a percent of hatched eggs at 49 ± 17, the treatment group at 10 ppm had a percent of hatched eggs at 56 ± 18, and the treatment group at 20 ppm had a percent of hatched eggs at 54 ± 2. The percent of hatched eggs when comparing treatment groups to the controls showed no significant differences.

The percent of fry surviving to pre-swim-up stage for the control group was 68 with a standard error of ± 11. The triolein group showed the percent of survived fry to pre-swim up at 83 ± 8. The treatment group at 5 ppm of *p,p'*-DDE had a percent of survived fry to pre-swim up stage at 60 ± 9, the treatment group at 10 ppm had a percent of survived fry to pre-swim up stage at 56 ± 18, and the treatment group at 20 ppm had a percent of survived fry to pre-swim up stage at 54 ± 2.

The percent of fry surviving to post-swim-up stage for the control group was 93 with a standard error of ± 4. The triolein group showed the percent of survived fry to post-swim up at 93 ± 4. The treatment group at 5 ppm of *p,p'*-DDE had a percent of survived fry to post-swim up stage at 90 ± 6, the treatment group at 10 ppm had a percent of survived fry to post-swim up stage at 88 ± 6, and the treatment group at 20 ppm had a percent of survived fry to post-swim up stage at 97 ± 3.

The percent of survivorship to Day 30 for the control group was 51 with a standard error of ± 11. The triolein group showed the percent of survivorship to Day 30 at 51 ± 6. The treatment group at 5 ppm of *p,p'*-DDE had a percent of survivorship at 26 ± 7, the treatment group at 10 ppm had a percent of survivorship to Day 30 at 28 ± 10, and the treatment group at 20 ppm had a percent of survivorship at 31 ± 9. Although I observed a

lower percentage of hatched larvae in fathead minnow eggs injected with *p,p'*-DDE, there was not a decrease in survivorship.

Use of a Tukeys Multiple Comparison Test showed that there were no significant differences in male or female fathead minnow gonads when comparing the stage of the ovary and/or testis to the treatment group when the *p* value was declared at equal or lower than 0.05. Only 1 sample collection was performed for both male and female fathead minnows at the end of the 30-day exposure.

Control flake feed did exhibit some levels of OCP contaminants however, this is expected due to the fact that the flake feed is comprised of wild-caught fish and shellfish that naturally contain some levels of contaminants. The levels of OCPs in the contaminated feed were within the selected range while attempting to achieve ppm values comparable to blue tilapia (*Oreochromis aureus*) located at the north shore of Lake Apopka, Florida, USA, with a biomagnification factor of 15 (Table 1).

Again, the controls of body burdens exhibited minimal levels of OCP contaminants, while the treatment groups displayed a dose-response relationship in all contaminants in the mixture (Table 2). When examining the levels of OCPs maternally transferred to the eggs in treatment groups, it was demonstrated that individual contaminants either did not transfer from the female to the egg or did not show a dose-response pattern. In some cases, the amount of contaminant transferred to the control group was indeed higher than those transferred to the treatment group (Table 3). Because these patterns were unexpected, a second round of maternal transfer from dosed female fathead minnows to eggs was performed to confirm our findings (Table 4). In early clutches (Days 1-5), again, the controls showed a higher amount of contaminant was

transferred in the controls than in the treatment groups. In the middle clutches (Days 13-17), there were no dose-response patterns observed with some contaminants being higher in the controls than the treatment groups, some contaminants being higher in the medium treatment groups, and some contaminants being the mid-range of the high treatment groups. The late clutches (Days 25-30), displayed no obvious dose-response relationship throughout all contaminants. While the chlordane did show a dose-response in the control and treatment groups, *p,p'*-DDE, dieldrin, and toxaphene did not.

Gonad OCP concentrations displayed a dose-response relationship across all contaminants with the only exception being the low treatment group of chlordane and the medium treatment group of chlordane (Table 5).

Table 1. Day 30 GC-MS results of flake feed OCP concentrations (ng/g). N.D. is defined as not detected.

	Control	Treated
Chlordanes	5.57	105.74
DDE (metabolites)	44.61	528.67
Dieldrin	N.D.	200.0
Toxaphene	3850.37	6447.13

Table 2. Day 30 GC-MS results of female whole body burden OCP concentrations (ng/g). N.D. is defined as not detected.

	Control	Low	Middle	High
Chlordane	3.54	8.5	21.07	40.07
DDE (metabolites)	7.07	93.44	102.42	206.57
Dieldrin	N.D.	6.8	15.06	33.92
Toxaphene	N.D.	N.D.	N.D.	N.D.

Table 3. Day 30 GC-MS results of Egg OCP concentrations (ng/g). N.D. is defined as not detected.

	Control	Low	Middle	High
Chlordane	N.D.	N.D.	N.D.	2.79
DDE (metabolites)	298.11	117.19	79.3	103.26
Dieldrin	3.77	N.D.	N.D.	2.79
Toxaphene	N.D.	N.D.	N.D.	N.D.

Table 4. Day 30 GC-MS results of OCP concentrations in eggs (ng/g) taken during Days 1-4 (early clutches), 13-16 (mid clutches) and 27-30 (late clutches). N.D. is defined as not detected.

Early Clutches

	Control	Low	Middle	High
Chlordanes	N.D.	14.46	11.62	.88
DDE (metabolites)	157.9	108.43	75.58	4.44
Dieldrin	52.63	28.92	17.44	.89
Toxaphene	N.D.	N.D.	N.D.	N.D.

Mid Clutches

	Control	Low	Middle	High
Chlordanes	N.D.	N.D.	N.D.	21.36
DDE (metabolites)	119.41	73.17	245.9	106.76
Dieldrin	29.85	14.63	98.36	32.03
Toxaphene	N.D.	N.D.	N.D.	N.D.

Late Clutches

	Control	Low	Middle	High
Chlordanes	N.D.	N.D.	5.22	10.06
DDE (metabolites)	42.61	57.84	46.96	110.56
Dieldrin	17.05	14.46	10.43	30.15
Toxaphene	N.D.	N.D.	N.D.	N.D.

Table 5. Day 30 GC-MS results of OCP concentrations in male and female gonads (ng/g). N.D. is defined as not detected.

	control		low		medium		high	
	male	female	male	female	male	female	male	female
Chlordanes	17.54	7.0	28.16	26.0	126.58	23.0	132.35	70.77
DDE (metabolites)	61.4	20.0	126.76	182.18	746.84	224.22	779.41	549.91
Dieldrin	8.77	7.0	14.08	15.0	113.92	31.0	117.65	65.34
Toxaphene	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

CHAPTER 4
DISCUSSION

The results of this study suggested that 30-day exposure to diets containing the OCPs chlordane, *p,p*'-DDE, dieldrin, and toxaphene at varying doses can result in internal carcass and gonad accumulation. The accumulation of OCPs is then maternally transferred to the eggs of fathead minnows at various concentrations. These results are consistent with other studies that suggest maternal OCP transfer (Rauschenberger, *et al*. 2004; Johnson, 2005). The value of our study was that OCP concentrations in maternal tissues and egg yolks appear to be strongly correlated with one another. Our data suggests maternal exposure to OCPs adversely affects reproduction by lowering the percentage of spawning pairs, lowering the survival of larvae up to Day 14, decreases the number of eggs produced over time, and lowers clutch viability over time.

In analyzing fathead minnow whole body tissue, gonadal tissue, and eggs, (Tables 1 through 3) we can see that numerous OCP analytes were detected in the flake feed, whole body tissues, gonads, and eggs. This suggests that when making predictions about possible effects to wildlife populations or human risk assessment, the composition of the toxicant is a crucial factor. One reason for this is that different xenobiotic compounds may inhibit or induce specific biotransformation enzymes (Rauschenberger, 2004). Because genetic variability exists among populations, it is possible that certain individuals or populations may lack the genetic ability to produce a biotransformation enzyme that is required for the detoxification of specific OCP analytes. Examination of Table 2 shows that a dose-response relationship was established within whole body

burdens of fathead minnows. While examining gonadal tissues, we also saw a dose-response relationship (Table 5) with the one exception of low and medium treatment groups of chlordane. Rauschenberger (2005) showed that in alligators, an adipose-to-yolk concentration ratio was close to 1. This would suggest that OCPs reach equilibrium within adipose tissue, and that lipids and OCPs are mobilized and subsequently incorporated into developing yolks. However, as seen in Table 3 and Table 4, there was no evidence to suggest that a consistent amount of chlordane, *p,p'*-DDE, dieldrin, or toxaphene was transferred to mature eggs. For this reason, the hypothesis that direct toxicity occurs to fathead minnow eggs in the natural environment and adversely affects fertilized egg development and/or survivorship of larvae was abandoned, and the nanoinjection portion of this thesis was terminated. Table 4 shows that over a 30-day period, while females were continually dosed, with the exception of toxaphene which was not detected in any samples except the flake and dieldrin which remained relatively constant from the early sampling period to the late sampling period, chlordane and *p,p'*-DDE either decreased to a non-detectable amount or decreased to nearly 50% of the initial concentration. Xie and Klerks (2002) showed that least killifish (*Heterandria Formosa*) exposed to cadmium developed a resistance to toxicity within a six generation timeframe. It is possible that the fathead minnows are able to adapt, possibly by increasing activity or production of enzymes that transform OCPs from hydrophobic xenobiotics to hydrophilic metabolites that are excreted, thus decreasing the potential for adverse affects.

When examining Figure 1, we can observe that there were significant differences detected among the control vs. high and middle treatments and although not significantly

different, a 20% decrease between the control and low treatment groups. Previous studies have demonstrated that exposure to certain contaminants adversely affects mating behavior in cyprinids. Jones and Reynolds (1997), found that exposure to various contaminants affected mating behavior in such ways as: increases or decreases in mating displays, increased courtship duration, performance of male-like behavior of masculinized females, decreased nest-building ability, decreased offspring defense, or changes in division of parental care between sexes. It is also known that cyprinids secrete steroid hormones that act as pheromones during courtship. It is possible that OCPs may disrupt the activity of these pheromones causing a disruption in courtship behavior. Cyprinidae are known to have complex mating behaviors, thus the possibility exists that exposure to OCPs may adversely affect mating behavior and consequently decrease the ability for fathead minnow pairs to successfully spawn.

Figures 3 and 4 show that the mean number of eggs hatched and clutch viability both show similar trends in the controls and treatment groups. While the low treatment group displayed an increased number of eggs hatched, and a higher clutch viability when compared to the control, the middle and high treatment groups showed a decrease in the number of eggs hatched, as well as clutch viability. Because OCP levels in the eggs of the fathead minnow were either non-detectable or inconsistently transferred from the adult female to the egg; conclusions can be drawn that there is not a correlation between maternally transferred OCPs to eggs and reduced mean number of eggs hatched and reduced clutch viability. In an attempt to explain the significant decreases in the middle and high treatment groups, it should be noted that the intermediate and highly exposed females continued to lay eggs that were not significantly different than the mean number

of control eggs. The middle and high treatment females continually produce and deposit eggs, however, the ova appear to be unable to sequester the nutrients required to produce healthy and viable eggs. A lack of nutrients results in a decreased amount of energy, as well as, structural supplies that are available for developing embryos and possibly yolk sacs that larvae use as a source of nutrition for the first several days after hatching. This is an observation that has been speculated in other studies (Rauschenberger, 2004; Johnson, 2005) however, until the completion of this study, little evidence has been able to support this hypothesis.

Although circulating sex steroids were not analyzed in this experiment, effects that these hormones have on reproductive success should be addressed. Gross *et al.* (2002) found that circulating concentrations of E_2 in female largemouth bass and 11-KT in male largemouth bass exposed to OCPs were on average1,500 pg/mL less than what was reported for pond-reared largemouth bass sampled in the same calendar year. The ability for any exogenous compound to bind a sex steroid hormone receptor and agonize and/or antagonize the action of an endogenous hormone can severely affect normal endocrine function. The reasoning for this is that normal estrogen or testosterone concentrations and actions are critical for development of gonads in both male and female fish (Johnson, 2005). It is possible that fathead minnows exposed to OCPs may alter the concentrations of these two circulating sex steroids thus reducing reproductive success.

Vitellogenin is a protein synthesized by the liver and its uptake by growing oocytes and its storage as yolk serves as a nutrient source by developing embryos. During this period, extraovarian proteins are gathered, processed, and packaged into oocytes. Consequently, this period is of particular importance when considering maternal transfer

by lipophilic proteins of OCPs to developing oocytes (Di Giulio and Tillitt, 1999). The possibility exists that because studies have shown that circulating vitellogenin acts as an important transport protein that binds lipophilic hormones, OCPs may bind to vitellogenin and agonize and/or antagonize vitellogenin receptors on gonads, thus impeding the deposition of yolk reserves, and therefore oocyte growth. Again, this is one explanation as to why decreased reproductive success is observed in my experiment while direct embryo toxicity is not.

Favorable water temperature, salinity, food availability, as well as egg quality are critical to the survivorship, growth, and the metamorphosis to the juvenile stage of larval fish. Fast growth to fish larvae has been correlated to the presence of high plankton concentrations where larvae have adequate opportunities to capture prey. Other environmental factors that influence larval development include dissolved oxygen, turbidity, nutrients, water movement, and meteorological events. The first stage of larval development is the yolk sac phase. The yolk sac contains nutrition used by larval fish while it adapts to its new aquatic environment. Larvae absorb the yolk and will continue to grow and develop while they begin to look for their first prey, plankton. As larvae continue to develop, they begin to take on the appearance of juveniles and further develop the ability to swim and capture prey. Typically, fathead minnows can not swim for long periods of time as larvae, they can however, swim long enough to seek appropriate resting habitat. During the course of our experiment, every effort was made to eliminate any possibility of larvae mortality due to water quality parameters or insufficient food availability. At no time during the course of the experiment were hatched larvae exposed to harmful levels of dissolved oxygen, salinity, or temperature.

Hatched larvae were also fed *ad libitum* adequate supplies of artemia. Because all obvious growth and developmental parameters were satisfied, it leads us to believe that the significant differences in the percent of larvae survived to Day 7 and Day 14 (Figures 5 and 6) were solely due to OCP exposure. While a dose-response relationship was not established for either Day 7 or Day 14 larvae survivorship, all three treatment groups for the percent of larvae survived to Day 7 were either lower or significantly lower when compared to the control, and all three treatment groups of the percent of larvae survive to Day 14 were significantly lower when compared to the control.

While no teratological or morphometrical measurements were taken during the course of this experiment, further discussion should be made as to the specifics of the high mortality observed in this experiment. Most teratogenic contaminants are believed to be non-specific (Newman and Unger, 2003). According to Karnofsky's law, any agent will be teratogenic if it is present at concentrations or intensities that produce cell toxicity (Bantle, 1995). Teratogens act by disrupting mitosis, interfering with transcription and translation, disturbing metabolism, and producing nutritional deficits (Weis and Weis, 1987). Consequences of these disruptions may include abnormal cell interactions, excessive growth, or cell death.

Retardation of growth, adverse effects on the skeletal, musculature, circulatory, and optical system are the most common effects seen in larval fish exposed to toxicants. Skeletal problems in fish may include lateral curvature of the spine (scoliosis) or the extreme forward curvature of the spine (lordosis). Wies and Wies (1989), showed that the mummichog, (*Fundulus heteroclitus*) when exposed to 10 mg l^{-1} of Pb^{2+} failed to uncurl it's tail post hatch, essentially leaving the larval fish to certain death. Sepúlveda *et*

al. (2000) found that largemouth bass larvae exposed to *p,p'*-DDE and dieldrin showed yolk sac edema, and eye deformities including opaque corneas. Studies of largemouth bass larvae exposed to paper mill effluent also showed deformities including: increased head abnormalities, a decrease in length and weight, and a shortened vertebral column (Sepúlveda *et al.*, 2003). Any or all of these abnormalities may have led to the decreased survival rates observed in my experiment.

Gonadosomatic index (GSI) is defined as the ratio of the weight of the gonad to the weight of whole body. Although GSI is not specific to a particular mechanism of toxicant action, it is an endpoint commonly used in toxicological studies, and is a good predictor of reproductive success that may be linked to population level responses to contaminants. However, caution must be applied when using GSI to make a hypothesis concerning contaminant effects to individuals. Dependent upon the species of fish and the time of the year, an increase in GSI may be a result of responses to reproductive and/or environmental cues. Gross *et al.* (2000) found that gonads in largemouth bass began to mature in October and reached a considerable size (approximately 5% of fish body weight) by January, and continued to peak until February. Depending on the month or season of the year, GSI may not be an accurate endpoint for contaminant exposure. Also, fathead minnows are batch spawning fish. Consequently, their gonads undergo rapid cyclical changes over short periods of time (every few days) as successive batches of eggs or sperm are produced, and thus means the size of the gonads in breeding adults can vary considerably between individuals at any point in time (Harries *et al.*, 2000). In my study, neither male nor female displayed significant differences in mean GSI (Figures 7 and 8). This is consistent with a study that exposed largemouth bass to 10.0 mg/pellet

of *p,p'*-DDE and 1.0 mg/pellet of dieldrin (Muller, 2003). My results are however, not consistent with a study of male guppies (*Poecilia reticulata*) who when fed a diet of 150ug of *p,p'*-DDE for 30 consecutive days showed a decrease in GSI. Sepúlveda (2000) showed that there can be differences in GSI not only between various spawning seasons, but also between different habitats within the same area. Again, these inconsistencies with GSI should throw caution when using this tool as an endpoint to measure reproductive success or toxicant exposure.

There were significant differences in the percent of females spawning and the number of spawns (Figure 9). Over a 30-day period, the percent of females spawning in the control went from 80% to 10% after the fifth spawn. The low treatment group went from 70% of females spawning to only 30% after the third spawn. Although the middle treatment group spawned the most in a 30-day period, six times, the percent of females spawning started at 40% and remained relatively constant throughout the 30-day period at 20%. The high treatment group started at 50% and by the fifth spawn dropped to only 10%. The frequency of spawnings in our control fish were slightly lower than what has been reported in other studies. Gale and Buynak (1982) reported that the mean number of spawns in their experiment was every 3.9 days. In contrast, in my study, the low treatment group spawned approximately every 8 days, the middle treatment group spawned approximately every 5 days, and the high treatment group spawned approximately every 6 days. Although the frequency of spawning was reduced in the control and treatment groups, two critical observations should be made. The first observation is that as previously mentioned, not only did treatment groups have reduced frequency in spawning, so did the control. Our data shows that over a 30-day period the

percent of control females spawning went from 80% to 0%. This critical observation is in that numerous state, federal, and private institutions use the fathead minnow as a model in aquatic toxicity tests. Protocols should be established that limit the frequency a specific breeding pair of fathead minnows is used without adequate recovery time before employing them in continuous toxicity tests. The second observation that should be made is that as seen in Figure 10, altered frequency of spawns is also correlated with the mean number of eggs laid. This would suggest that in reference to a population of fathead minnows in a particular ecosystem, altered frequency of spawning would not be offset by an increase in fecundity. This is in contrast to a study where fathead minnows exposed to weak active endocrine mimics had a decrease in the number of spawnings, however, there was a reciprocal increase in the size of the egg batch (Harries *et al.*, 2000).

The mean number of eggs laid per spawn showed a lower number of eggs produced for treated versus controls (Figure 10). This figure also showed altered patterns across treatments. Although the control started with a low mean number of eggs laid, by the second spawn, the mean number of eggs laid per spawn decreased, and fish stopped spawning after the forth spawn within a 30-day period. The low treatment group showed a similar pattern with the most eggs laid during the first spawn, the mean of eggs laid tapering off until the third spawn when no more eggs were laid. Although the middle treatment group displayed a similar pattern, having the greatest number of eggs during the first spawn, then gradually tapering off, there was a lower mean number of eggs laid on the first spawn, however, this treatment group spawned the most times, six, out of all treatment groups. The fact that the middle treatment group continued to spawn for a

longer period of time than other treatment groups, may indicate the fathead minnow has adapted to mid-exposure and although fewer eggs are being produced, the frequency of spawning is significantly longer. The high treatment group continued to show the same pattern of the most eggs being spawned early, then gradually tapering off until no further spawning took place. Although OCPs may affect the frequency of spawning as well as the mean number of eggs laid per spawn, it could also be possible that the differences observed in mean number of eggs is in part due to differences in the sizes of the fish. It is apparent however, that the largest clutch sizes take place early and gradually taper off in the control and treatment groups possibly due to the availability of mature oocytes.

Control clutch viability varied from 55% to 90% throughout the number of times spawned within the 30 trial period. There appeared to be no distinguishable pattern within the percent ranges (Figure 11). The low treatment group did however, show a higher clutch viability toward the early stages of the experiment than the later stages. From this, one might assume that bioaccumulation of OCPs are decreasing clutch viability, however, the middle and high treatment groups show an increase and then decrease in clutch viability over the number of times spawned as well as the 30-day period. Although no obvious patterns across treatments were observed, all three treatment groups had significantly lower clutch viability percentages when compared to the control. This observation is similar to alligator eggs exposed to OCPs which also showed decreased clutch viability (Rauschenberg, 2004). This indicates decreased clutch viability is due to decreased egg quality associated with senescence.

Recently, numerous studies have clearly established that various man-made and natural chemicals exist within aquatic environment that have the potential to mimic

androgens, estrogens, or their antagonists. A number of EDCs bioaccumulate and/or result from environmental degradation or metabolism from their parent compound. In order to establish a cause-effect relationship, between exposure to EDCs and reproductive success and survivorship, it is necessary to conduct whole animal studies. Given the large number of chemicals that have the potential to be EDCs, as well as are regularly found within the aquatic environment, there is a need to develop a biological model that is practical to work with in a laboratory setting that is capable of demonstrating quantifiable reproductive parameters as well as other endocrine disruptive biomarkers such as vitellogenin induction. Other studies have demonstrated documented bioaccumulation of OCPs in the carcass and gonads of largemouth bass (Johnson, 2005), and endocrine modulation caused by OCPs (Muller, 2004). Sepúlveda *et al*. (2004) found alterations in endocrine function and increased developmental mortality in largemouth bass inhabiting the Emeralda Marsh. In another study, relative contributions of losses during in ovo development in alligators at impacted sites in Florida are lower clutch viability, higher rates of damaged eggs, higher rates of early embryo mortality, and higher rates of late embryo mortality, all of these were due to exposure to OCPs (Rauschenberger, 2004). While all of these studies displayed affects caused by exposure to OCPs, these were all captive studies that were both labor intensive and financially demanding. Hence, the need to develop a series of short-term, economical, laboratory tests using a biological model that shows effects of exposure to EDCs including fecundity, GSI, induction of vitellogenin and other circulating sex steroids (E_2 and 11-KT), and survivorship is needed to predict and assess potential impacts of EDCs either on larger individuals or on populations within ecosystems.

Further research on the reproductive effects of *p,p'*-DDE, dieldrin, chlordane, and toxaphene on fathead minnows could focus on the measurement of additional reproductive endpoints. For example, estradiol-17β, 11-KT and vitellogenin would potentially add additional information concerning the development stages of oocytes and address the size and condition of gonads. Although male gonads were staged, assessing gamete viability in males would eliminate the possibility that EDCs are disrupting male reproductive potential. Nakayama *et al*. (2005) found that when Japanese medaka (*Oryzias latipes*) were exposed to tributyltin (TBT), the number of eggs laid remained relatively constant, however, the number of fertilized eggs decreased. Another study showed that as levels of TBT increased the percent of sperm that lacked flagellum or had a decrease in the volume of milt also increased (McAllister and Kime, 2003). Another factor that may affect OCP toxicity that was not addressed in my experiment is in that low temperatures have been associated with increased DDT toxicity in fish (Rattner and Heath, 2003). Fluctuation in water temperature, as well as fluctuations in OCP levels, mimicking "hotspots" may also provide invaluable insight into the correlation between OCP exposure and reduction in reproductive success.

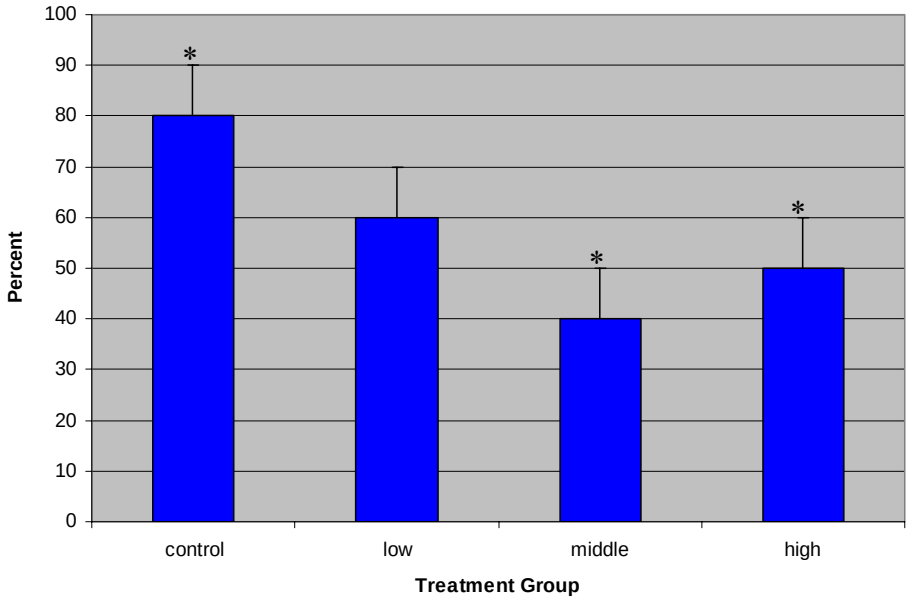

Figure 1. The percent of spawning pairs among treatment groups. Control, low, middle, and high treatment groups had 10 potential spawning fathead minnow pairs. Mean ± standard error results are shown. Significant differences between control middle and high treatment groups using One-way ANOVA (P<.05). Asterisks indicate differences in relation to controls.

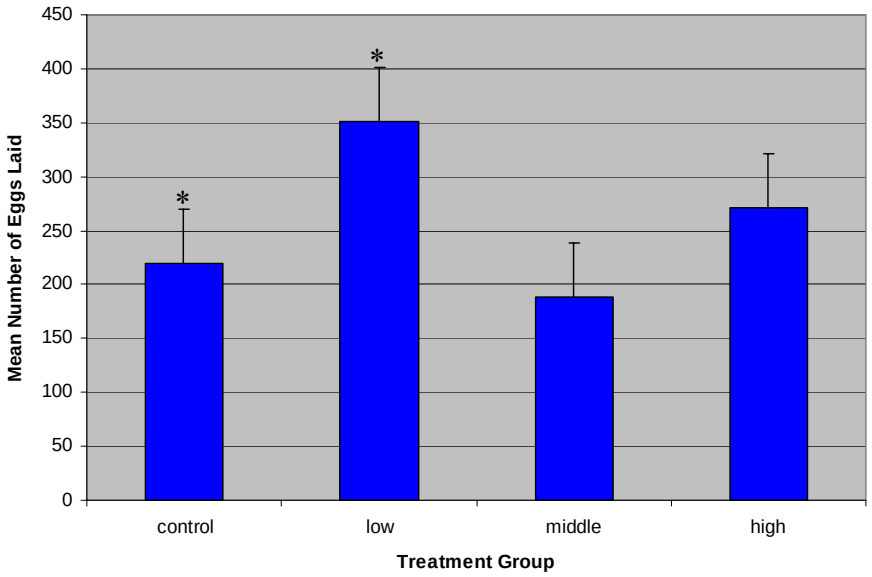

Figure 2. The mean number of eggs laid per spawn among treatment groups. Control, low, middle, and high treatment groups had 10 potential spawning fathead minnow pairs. Mean ± standard error results are shown. Significant differences between low and control treatment groups, low and middle treatment groups using One-Way ANOVA (P<.05). Asterisks indicate differences in relation to controls.

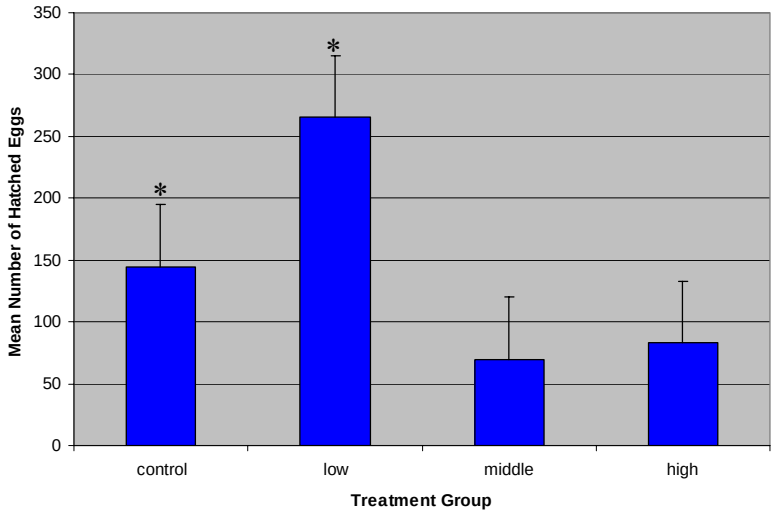

Figure 3. The mean number of eggs hatched among treatment groups. Control, low, middle, and high treatment groups had 10 potential spawning fathead minnow pairs. Mean ± standard error results are shown. Significant differences between low and control treatment groups, low and high treatment groups, and low and middle treatment groups using One-Way ANOVA (P<.05). Asterisks indicate differences in relation to controls.

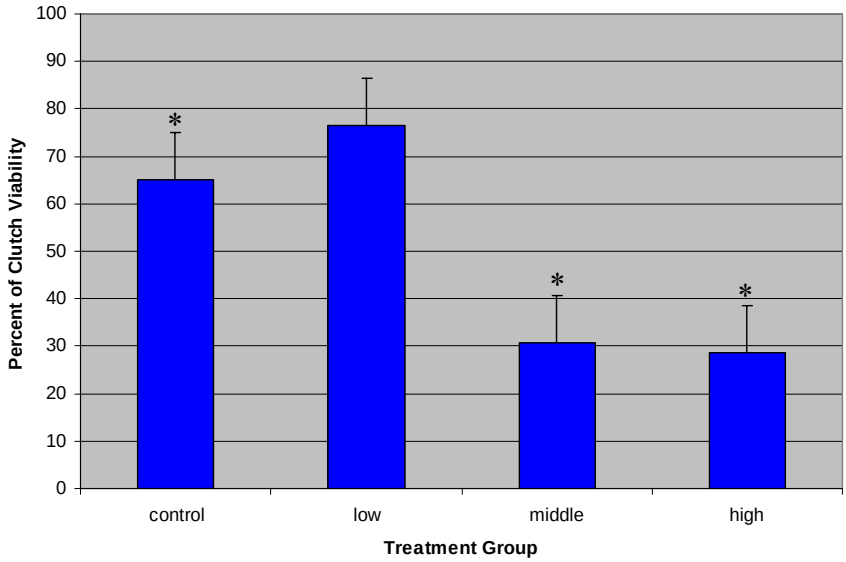

Figure 4. The percent of clutch viability among treatment groups. Control, low, middle, and high treatment groups had 10 potential spawning fathead minnow pairs. Mean ± standard error results are shown. Clutch viability = No. of eggs yielding a live hatchling / Fecundity x 100. Significant differences between low and middle treatment groups, low and high treatment groups, control and middle treatment groups, control and high treatment groups, and middle and low treatment groups using One-Way ANOVA (P<.05). Asterisks indicate differences in relation to controls.

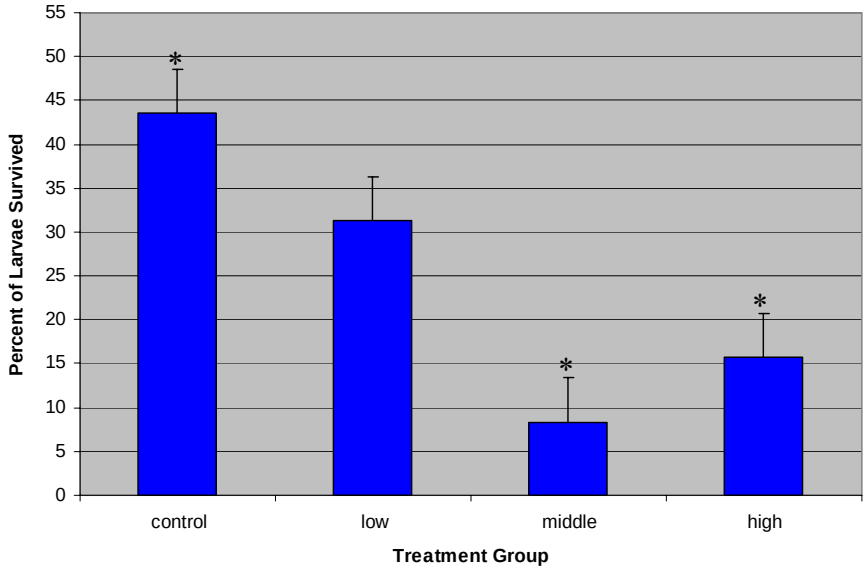

Figure 5. The percent of larvae survived to Day 7 among treatment groups. Control, low, middle, and high treatment groups had 10 potential spawning fathead minnow pairs. Mean ± standard error results are shown. Significant differences between control and high treatment groups and control and middle treatment groups using One-Way ANOVA (P<.05). Asterisks indicate differences in relation to controls.

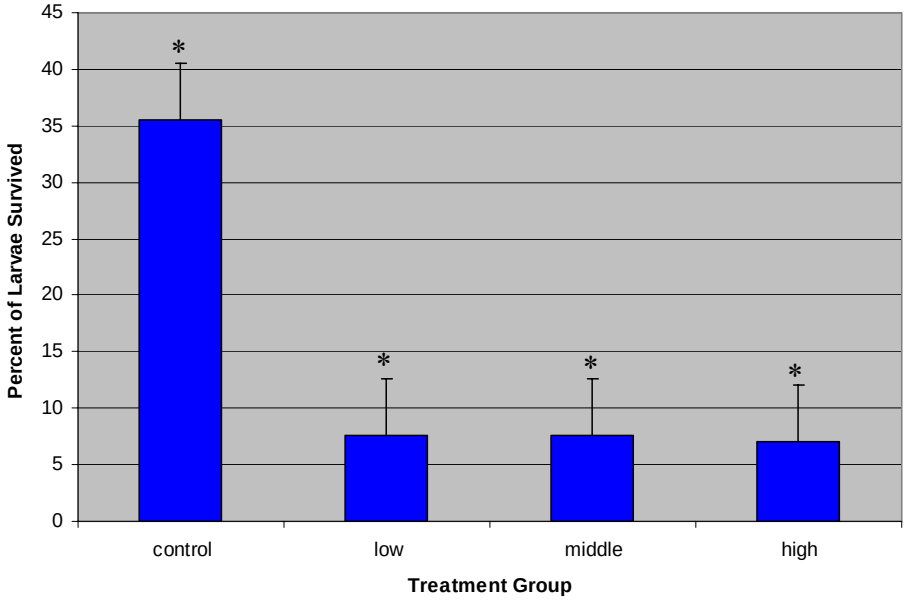

Figure 6. The percent of larvae survived to Day 14 among treatment groups. Control, low, middle, and high treatment groups had 10 potential spawning fathead minnow pairs. Mean ± standard error results are shown. Significant difference between control and middle treatment groups using One-Way ANOVA (P<.05). Asterisks indicate differences in relation to controls.

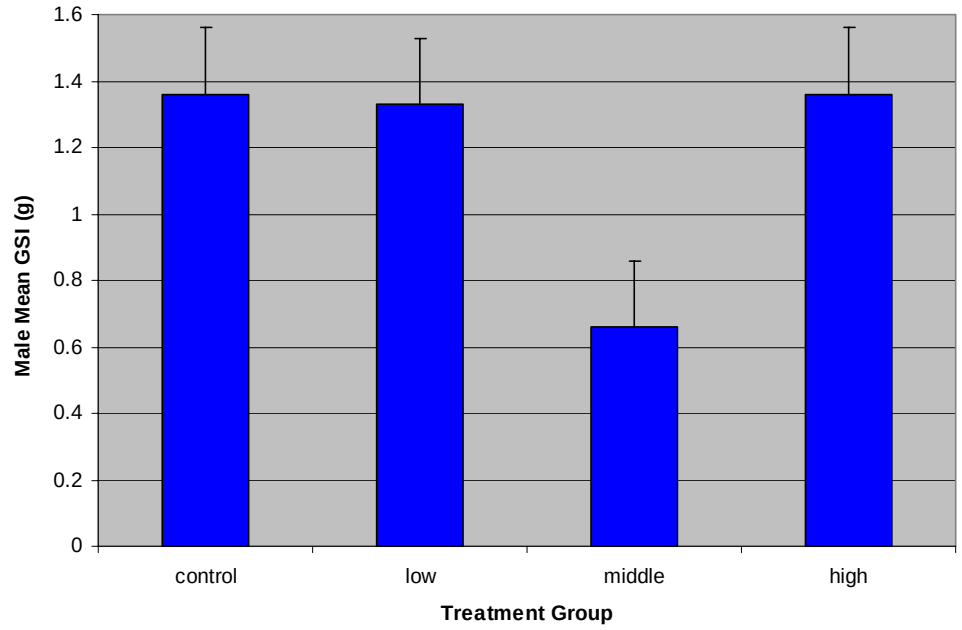

Figure 7. Mean GSI among male treatment groups. Control, low, middle, and high treatment groups had 10 potential spawning fathead minnow pairs. Mean ± standard error results are shown. No significant differences using One-Way ANOVA (P<.05).

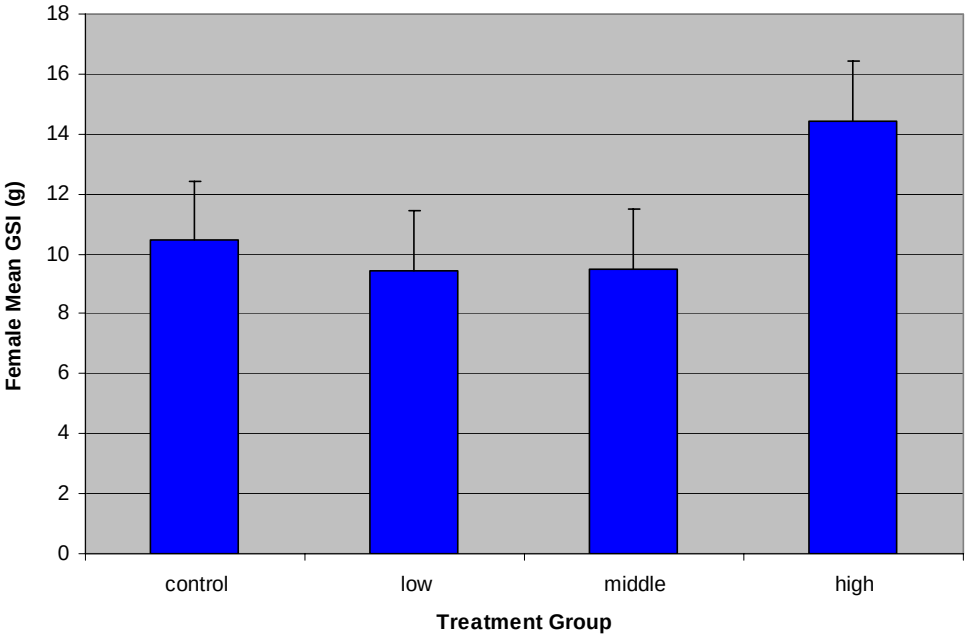

Figure 8. Mean GSI among female treatment groups. Control, low, middle, and high treatment groups had 10 potential spawning fathead minnow pairs. Mean ± standard error results are shown. No significant differences using One-Way ANOVA (P<.05).

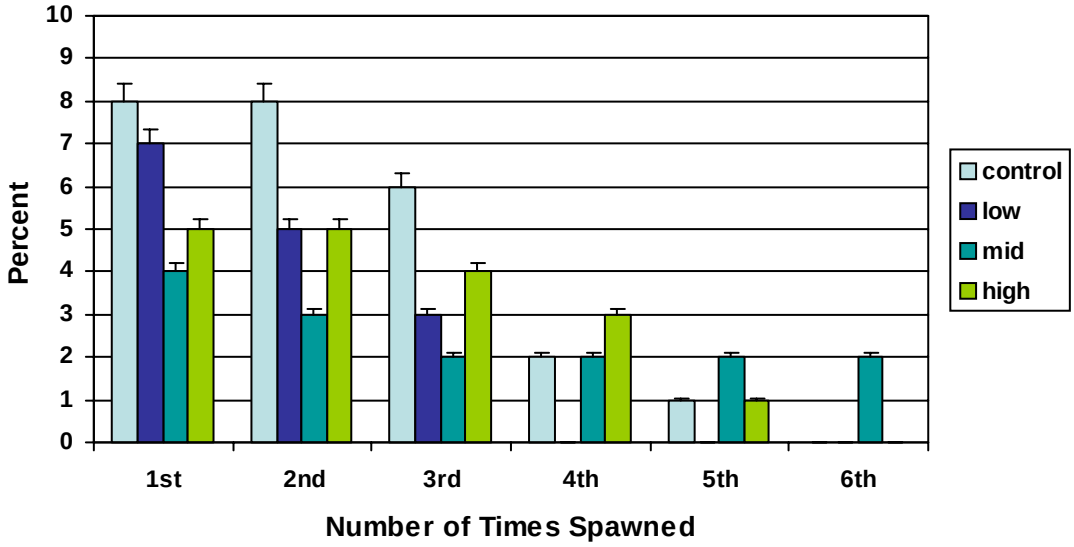

Figure 9. Percent of Females Spawning vs. Number of Spawns. Control, low, middle, and high treatment groups had 10 potential spawning fathead minnow pairs. Mean ± standard error results are shown. Note: Decreased number of spawning females for treated versus controls and altered patterns across treatments.

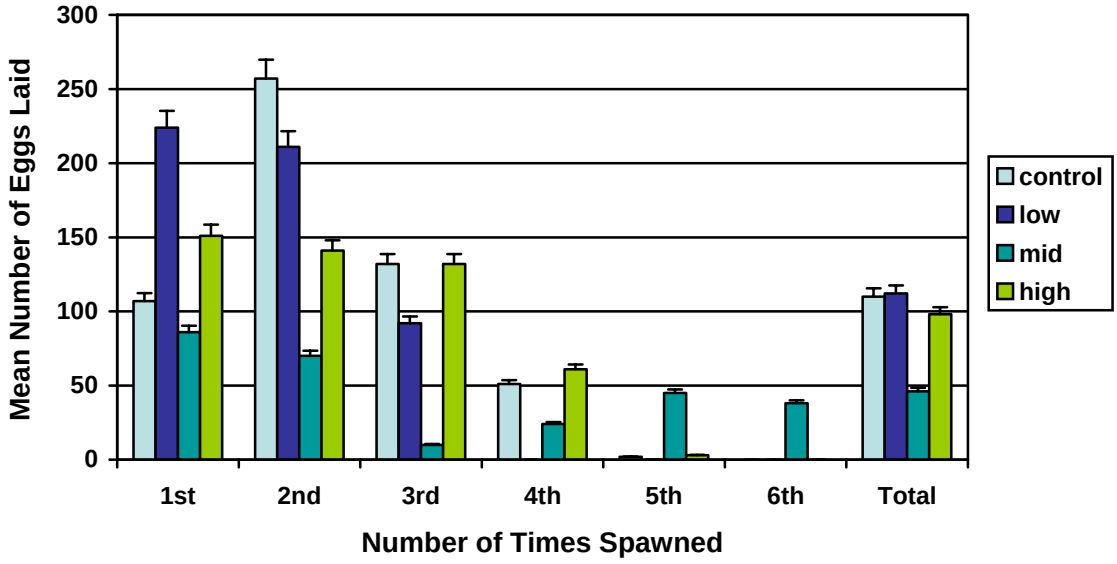

Figure 10. Mean Number of Eggs Laid vs. Number of Times Spawned. Control, low, middle, and high treatment groups had 10 potential spawning fathead minnow pairs. Mean ± standard error results are shown. Note: Lower number of eggs produced for treated versus controls and altered patterns across treatments.

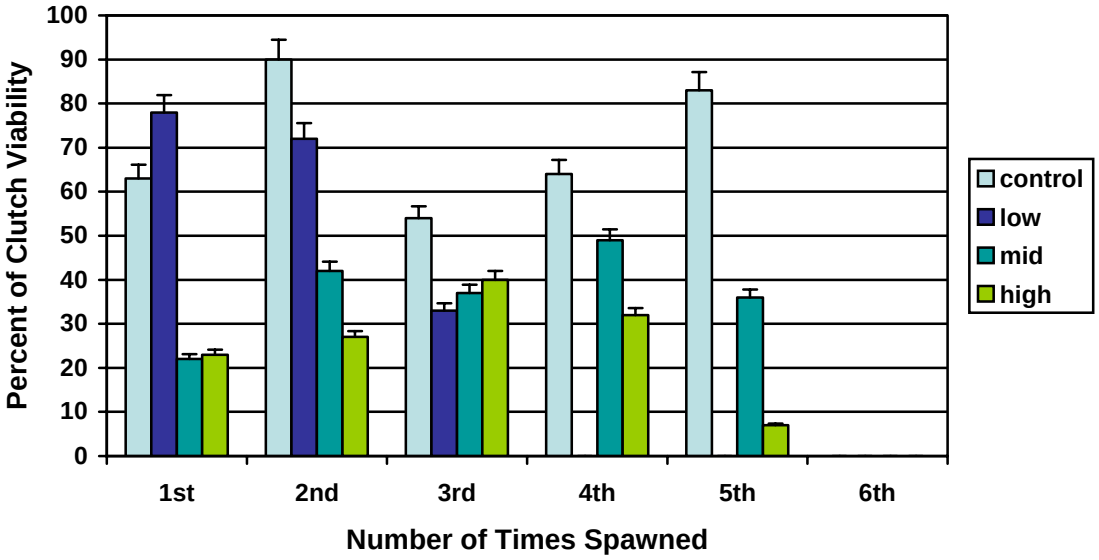

Figure 11. Percent of Clutch Viability vs. Number of Times Spawned. Control, low, middle, and high treatment groups had 10 potential spawning fathead minnow pairs. Mean ± standard error results are shown. Note: Lower clutch viability produced for treated versus controls and altered patterns across treatments.

CHAPTER 5
CONCLUSION

Research from this study provides evidence that dietary exposure to a mixture of *p,p*'-DDE, dieldrin, toxaphene, and chlordane bioaccumulate in maternal tissues and at inconsistent rates are transferred to developing eggs. Our data shows that maternal exposure to OCPs indicates endocrine disruption, and adversely affects reproduction by lowering the percentage of spawning pairs, lowering the survival of larvae up to Day 14, decreases the number of eggs produced over time, and lowers clutch viability over time.

The fathead minnow could potentially be used as a biological model to assess effects of various contaminant exposures including pharmaceuticals, sewage effluent, paper mill effluent, and numerous heavy metals. Adult fathead minnows are omnivores, consuming a variety of resources as food, hence giving the fish an opportunity to accumulate numerous toxicants. My study has shown OCPs bioaccumulate in fathead minnow adipose and gonadal tissue and is maternally transferred at various concentrations to their eggs. Fathead minnows in my study responded similarly to largemouth bass and alligators that were orally exposed to various concentrations of OCPs, making the fathead minnow an eco-relevant model. However, unlike largemouth bass, fathead minnows are not seasonal spawners, multi-generation tests can easily be performed, and costs of performing these tests are greatly reduced in comparison with largemouth bass. The ease of laboratory handling, the low financial burden, as well as the ability of fathead minnows to bioaccumulate contaminants makes it a good model to

examine potential reproductive effects, thus giving researchers the ability to extrapolate cause-effect relationships in higher order organisms.

My data also concluded that although fathead minnows exposed to OCPs continued to produce and deposit eggs, the ova appear unable to sequester the proper nutrients required to produce healthy eggs. The conclusion can be made that bioaccumulation of OCPs in the female ovaries may cause lower egg quality prior to the induction of vitellogenesis, however, total protein or lipid analysis would further support this conclusion. Inconsistent amounts of OCPs were transferred to fathead minnow eggs, however, the relatively same endpoints were observed whether high quantities of OCPs were transferred or low quantities were transferred. This would suggest that the mechanism of action is maternal.

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BIOGRAPHICAL SKETCH

Dane H. Huge was born August 29, 1970. He attended Fredericktown High School in Fredericktown, Ohio, and graduated in 1989. Dane then served 5 years in the U.S. Navy as a photographer. Dane graduated from the University of Florida in 1999. Post graduation, Dane worked as a biological technician at the United States Geological Survey working on spawning behaviors of invasive and listed species of minnows. Dane then enrolled as a graduate student in spring 2004 under the guidance of Dr. Timothy S. Gross. He focused his work on reproductive effects of organochlorine pesticides on fathead minnows. Dane received his Master of Science degree from the Department of Physiological Sciences, College of Veterinary Medicine, at the University of Florida in May 2006.