THE EFFECTS OF TEMPERATURE AND SHORT-TERM EXPOSURE TO 17α-ETHYNYLESTRADIOL ON THE GROWTH AND PHYSIOLOGY OF JUVENILE RAINBOW TROUT (ONCORHYNCHUS MYKISS)

A Report of a Senior Thesis

by

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ABSTRACT

Exposure to natural and synthetic estrogens and androgens from the environment has been shown to cause cellular, developmental, behavioral, and physiological changes in fish. This experiment examined the combined effect that exposure to two different temperatures, 9°C and 19°C, and a synthetic estrogen, 17α- ethynylestradiol, had on behavior, tissues, and weight gain in juvenile rainbow trout (*Oncorhynchus mykiss*). Forty juvenile *O. mykiss* were exposed to either 50ng/L of 17α- ethynylestradiol (EE_2) or no EE_2 for 21 days, and each of these groups were housed at either 9°C or 19°C. Four groups (10 trout per group) were exposed to the following conditions: (1) 1750ng/tank EE_2, 9°C, (2) 1750ng/tank EE_2, 19°C, (3) no EE_2, 9°C, and (4) no EE_2, 19°C. The fish were monitored over the exposure period for changes in aggression, and analyzed for hematocrit, hepatosomatic indices, gonad histology, and weight gain after euthanasia. Several hypotheses were made. First, it was hypothesized that fish exposed to EE_2 would have increased levels of aggression, as compared to those in the control group, but statistical analyses showed

that neither temperature nor EE_2 had a significant effect on the number of approaches (p = 0.8560, p = 0.5869, respectively) and number of attacks (p = 0.5472, p = 0.2322, respectively). Second, it was hypothesized that fish exposed to EE_2 would have lower levels of hematocrit, but fish exposed to EE_2 had significantly higher levels of hematocrit (p = 0.0483). Only temperature (19°C) had a significant increasing effect on the hepatosomatic indices (p = 0.0002), contrary to the third hypothesis that exposure to both 19°C and EE_2 would cause increased hepatosomatic indices. No conclusions could be made as to the effect of temperature and treatment with EE_2 on gonad morphology. Finally, in support of the hypothesis that fish at a colder temperature would grow at a faster rate, fish exposed to 9°C showed significantly greater weight gain over those housed at 19°C (p = 0.0217).

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CHAPTER I

INTRODUCTION

Mechanisms of Sex Determination

The manner in which sex is determined in vertebrate species has evolved over millions of years, resulting in an extreme diversity of sex determining mechanisms expressed in phylum chordata (Figure 1). It is possible that all vertebrates began with the same type of gender determination mechanism and developed modifications to better survive in their environments. Sex is determined in mammals, birds, amphibians, and some fish by genetic mechanisms, whereas environmental factors play a primary role in sex determination in some species of amphibians, reptiles, and fish.

Mechanisms of Sex Determination in Mammals, Birds, Amphibians, and Reptiles

Sex determination in mammals results primarily from the sex chromosomes, but also from the some genes located on autosomes. In mammals, the sex chromosomes in females are homogametic (XX) and in males are heterogametic (XY). Gender is determined by the

presence of *Sry* gene (sex-determining region of the Y chromosome), which encodes the testis-determining factor on the Y chromosome (Morrish & Sinclair, 2002). There are rare or exceptional cases in which homogametic (XX) phenotypic males and heterogametic (XY) phenotypic females occur, which is often a result of the translocations of the *Sry* gene from the Y chromosome to the X chromosome (Jameson, 1988). Such translocations occur in the seventh week of development when a recombination results in the shifting of the *Sry* gene from the Y chromosome to the X chromosome (Hartwell et al., 2000). If an XX egg containing the *Sry* gene is fertilized, a phenotypic male XX develops. The H-Y antigen on the Y chromosome plays an important role in the phenotype of a male by interacting with the undifferentiated gonad tissue during embryologic development and causing testicular organogenesis. Though an organism may have the XY sex chromosome set, without the H-Y antigen or cells that code for the H-Y antigen receptors, the organism will not undergo testicular organogenesis or express primary male sexual characteristics. Species can have one genotype, but functionally be the other gender. There are specific symbols for these unconventional sex chromosomes, each of which are different from the typical XX/XY or ZZ/WZ determination (van Tienhoven, 1983).

In contrast to mammals, birds and snakes express female heterogamety (WZ) and male homogamety (ZZ). Graves and Shetty (2001) show that the XX/XY and WZ/ZZ sex chromosome systems evolved independently of each other and contain different genes. By mapping 17 genes of the chicken Z chromosome, it was found that none of the 17 are found on the mammalian X. In addition, none of the six human genes mapped in chicken were found on the chicken Z chromosome. This shows that there is a complete lack of homology of the bird Z and mammalian X chromosome, thus illustrating the lack of ancestral commonality in the sex determining mechanism.

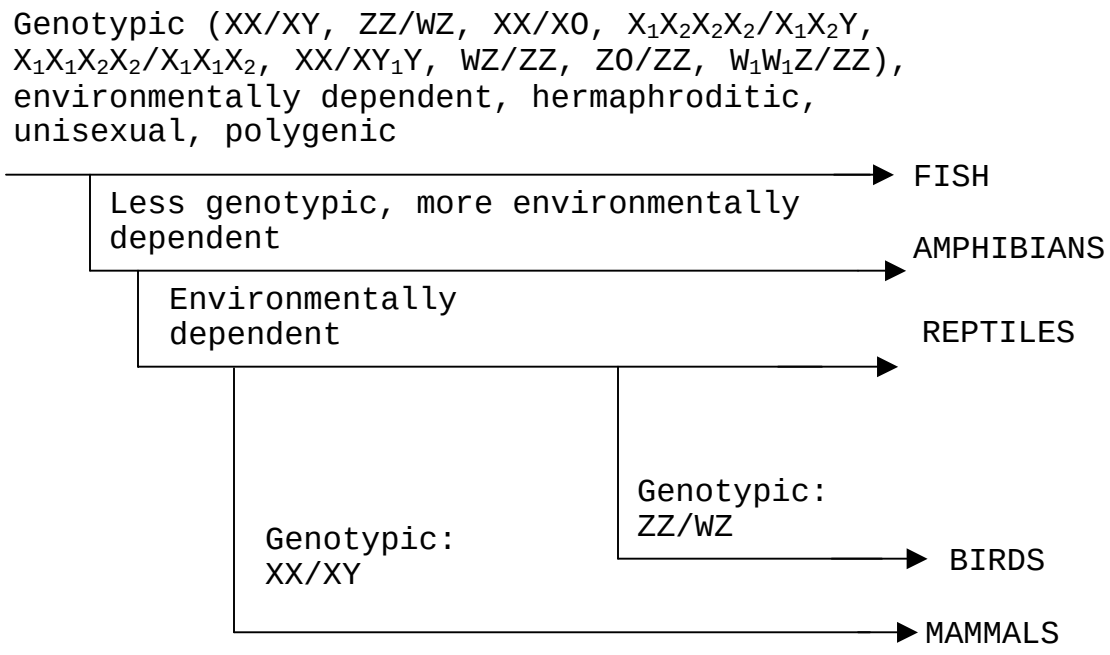

Figure 1. Phylogenetic tree of sex determination mechanisms in fish, amphibians, reptiles, birds, and mammals (based on Price, 1984; Graves & Shetty, 2001; Sparling, Linder, & Bishop, 2000; Lang & Andrews, 1994).

The determination of gender in amphibians and reptiles is a result of both genetic and environmental factors. Amphibians do not utilize one set pattern of genetic sex determination (Sparling et al., 2000). Some species exhibit male heterogamety, whereas others exhibit male homogamety. In addition, gonadal development in anurans may depend on hormone concentrations of estrogen and testosterone (Sparling

et al., 2000). Many reptilian species, such as the American alligator (*Alligator mississipiensis*), have temperature dependent sex determination. Lang and Andrews (1994) completed a study examining the evidence of temperature-dependent sex determination in 11 crocodilian species and found evidence supporting temperature-dependent sex determination in *A. mississippiensis* and *Caiman crocodiles*. Eggs incubated at lower temperatures and higher temperatures, they discovered, result in female offspring, whereas eggs incubated at intermediate temperatures resulted in males. This thermosensitive period for temperature-dependent sex determination in *A. mississipiensis* occurs during stages 21 to 24 (days 30-45), during which gonadal differentiation occurs (Lang & Andrews, 1994). Unlike mammals, reptiles, amphibians, and birds, however, fish do not utilize one primary mechanism of sex determination.

Mechanisms of Sex Determination in Fishes

Fish express an extreme variety in both sexuality and sex determination methods, including hemaphroditism, polygenic sex determination, the mammalian XX/XY mechanism, avian WZ/ZZ mechanism, environmental sex determination, and modifications of these mechanisms. The sexuality of fish ranges from hermaphroditism, unisexuality, and bisexuality. Hemaphroditic fishes can be either synchronus or

asynchronous. In synchronous fish species, the egg and sperm mature at the same time (leading to self-fertilization); asynchronus fishes, however, function as only female or male at one time. It has been proposed that hemaphroditism arose several times independently, since it occurs in several orders of fish. Unisexual fishes are less common than hermaphroditic species; these fish reproduce through either gynogenesis or hybridogenesis. There are several genotypes of male heterogamety in fish: XX/XY, XX/XO, $X_1X_2X_2X_2/X_1X_2Y$, $X_1X_1X_2X_2/X_1X_1X_2$, and XX/XY_1Y_2 (Price, 1984). Female heterogamety is of the types: WZ/ZZ, ZO/ZZ, and W_1W_1Z/ZZ (Price, 1984). Along with hermaphroditism and polygenic sex determination, sex is determined in bisexual species of fish using the XX/XY and WZ/ZZ systems. *Poecilia reticulata* possess the XX/XY sex chromosome system, whereas *Gasterosteus aculeatus* possess the WZ/ZZ system (van Tienhoven, 1983). Similar to many species of the salmon family, rainbow trout (*Oncorhynchus mykiss*) express genetic sex determination as female homogamety (XX) and male heterogamety (XY) (Quillet, Aubard, & Queau, 2002). Bisexual fish compose the majority of fish species. In some bisexual fish, sex is determined not by the sex chromosomes alone, but by the minor genes throughout the chromosomes (polygenic sex determination).

Polygenic sex-determination relies on the autosomes of the epistatic genes. The Y chromosome has epistatic M genes and the X chromosome has epistatic F genes (Yamamoto, 1969). If the number of autosomes is in balance, as it usually is, gender determination is based primarily on the XX or XY sex chromosomes. If the number of X and Y autosomes are not in balance, however, the fish utilizes the polygenic sex determination mechanism, in which gender is determined by the sum of the F autosomes or the sum of the M autosomes. There are cases in which the sex chromosomes specify XY, but the sum of the female autosomes is greater than that of the male autosomes.

Similar to the mechanism through which gender is determined in reptiles and amphibians, environmental factors play a key role in the sex determination of some fish species. Temperature, photoperiod, rainfall, and salinity influence sex determination in many species of fish (Jobling, 1995). In the Atlantic silverside (*Menidia menidia*), sex determination is under genetic and environmental control. Conover and Kynard (1981) found that a larger population of females developed at colder temperatures and more males developed at slightly higher temperatures (as cited in Jameson, 1988).

Sexual Characteristics in Fishes

Sexual dimorphism is expressed in the males and females of class Osteichthyes through secondary sexual characteristics. Primary characteristics relate directly to the reproductive process, whereas secondary sexual characteristics relate to the accessories to reproduction, such as body size, fin shape, head ornamentation, and body coloration (Lagler, Bardach, & Miller, 1962). The primary sexual characteristics that differentiate male and female fish are the presence of testes and ducts or ovaries and accompanying ducts (Lagler et al., 1962). Marks of sexual distinction for secondary sexual characteristics relate to the additional physical characteristics of the individual.

Many physical characteristics are varied in male and female fishes. There is an overall size difference between genders in most species of fishes (Nikolsky, 1963). Mature females are generally larger than males because of the reproductive organs they contain in their bodies. Fin morphology also differs between the sexes. Males generally have longer, larger fins than those of females, a modification that often serves both a physical and adaptive function in reproduction (Nikolsky, 1963). Male fishes of the order Cyprinodoniformes, such as *Poecilia reticulata*, have a modified anal fin called the gonopod (Lagler et

al., 1962). Landsman, David, and Drew (1987) found that the development the gonopod is under androgen control, as female *P. reticulata* fed 17α–methyltestosterone developed gonopodia (as cited in Bayley, Junge, and Baatrup 2002). The gonopod serves to guide the spermatophores from the male into the female during copulation, thus resulting in more successful egg fertilization. Female fishes may also possess an accessory reproductive structure, called the ovipostior. The ovipositor, found in *Rhodeus amarus* and *Caraproctus*, was derived from the genital papilla and is used to deposit eggs (Lagler et al., 1962).

Male fishes generally have brighter, more intense coloration of the body than that of a female. This sexual dichromatism is thought to have arisen to attract females to males during mating (sexual selection). Baatrup and Junge (2001) show that male *P. reticulata* lose their orange coloration with hormonal and chemical changes; it is possible that this change in coloration would cause a reduction in female receptivity during mating. Brooks and Endler (2000) found a clear positive correlation between attractiveness (coloration) and successful mating of *P. reticulata*, thus showing the important link between secondary sexual characteristics and mating success. Many species of male fish develop head ornamentation

during the breeding season that females do not possess. For example, breeding males of the salmon and trout (Salmoninae) possess knobby hooks on their upper and lower jaws, whereas male chimaeras (Chimaeridae) develop a spiny retractile knob in the upper part of the head (Lagler et al., 1962).

Effects of Environmental Contaminants on Fish Behavior, Sex Characteristics, and Physiology

Natural and anthropogenic compounds released into the environment can have detrimental effects on the organisms to which they are exposed. Chemicals, including those that are endocrine disruptors, are released daily into waterways from agricultural sources and municipal sources such as factories, sewage treatment plants, and power plants. Organisms that live in aqueous environments, including fish, can easily bioaccumulate these chemicals in their bodies because of continued exposure. Studies have shown that thermal and metabolic factors can affect the bioaccumulation of certain chemicals in fish. Tarja, Kirsti, Marja, and Kari (2003) exposed *O. mykiss* to triazine herbicides terbutryn and terbuthylazine at water temperatures of 4°C, 10°C, and 17°C. They found that temperatures had a significant effect on the bioaccumulation of terbutryn, but not terbuthylazine. Such chemicals may interfere with the endocrine system of organisms by changing naturally occurring hormone

concentrations or mimicking naturally occurring hormones, thus altering reproductive capacities, behavior, and normal physiology. By acting as either agonists or antagonists, endocrine disrupting chemicals can binding to hormone receptors or blocking hormone receptors to alter the normal chemical pathways.

Not all species of fish, however, are equally susceptible to environmental contaminants because of the different mechanisms in which gender is determined. The endocrine system functions such that hormones are secreted from specific hormonal glands into the bloodstream and influence cell functions at other specific locations in the body. Steroid hormones are secreted by the adrenal cortex, testes, ovaries, and placenta and transported bound to plasma proteins (Porterfield, 1997). Secretion of such steroids in humans occurs in concentrations of one picogram per milliliter of blood, and rates of secretion are typically measured in micrograms or milligrams per day (Guyton & Hall, 2000).

Some of the first studies of environmental endocrine disruption in fish included the examination of estrogenic effect caused by natural and synthetic estrogens. Researchers have completed many experiments in which species of fish are exposed to a chemical and examined for resulting effects on

behavior, sexual characteristics, or physiology. Not all species of fish, or members of the same species, it has been found, react identically to exposure to the same chemical or concentration of a chemical. For example, only some of *O. mykiss* exposed to a series of dilutions of wastewater effluents showed an estrogenic response to the exposure (Pickering & Sumpter, 2003). This lack of intraspecific variability may be the result of additional factors playing a role in the disruption of the endocrine system in members of the same fish species. In various fish species, however, the differing effects may be a result of the effects of different mechanisms of sex determination, resulting in a varying level of susceptibility. Though fish of different species may not experience the same effects of a contaminant, most exposed species exhibit some sort of changes in behavior, secondary sexual characteristics, and physiology (see Table 1).

Behavior Endpoints	Species	Chemical exposure	Effect	Reference
Sigmoidal swimming	*Poecilia reticulata*	Vinclozolin[1], flutamide[2]	Altered swimming pattern in males, resulting in suppressed courtship behavior	Bayley et al., 2002
Phenotypic Endpoints				
Gonopodium development	*Poecilia reticulata*	Vinclozolin[1], flutamide[2], *p,p'-DDE*[3]	Inhibited development	Bayley et al., 2002
Body coloration	*Poecilia reticulata*	Vinclozolin[1]	Reduced orange display coloration in area and intensity in males	Baatrup & Junge, 2001
Testis size	*Oncorhynchus mykiss*	17 α-ethynylestradiol	Inhibited development	Schultz et al., 2003
Sperm count	*Poecilia reticulata*	Vinclozolin[1], *p,p'-DDE*[3]	Highly significant reduction in number of sperm cells	Bayley et al., 2002
Gonad development	*Oncorhynchus mykiss*	Estradiol-17β	Males developed female gonadal structures	Krisfalusi & Cloud, 1999
Gonad development	*Oncorhynchus mykiss*	non-aromatizable androgen 17α-methyldihydrotestosterone	Females developed testes	Krisfalusi & Cloud, 1999
Physiological Endpoints				
Change in testosterone concentrations	*Cyprinus carpio*	municipal sewage effluent	Depressed serum testosterone concentration	Folmar et al., 1996
Vitellogenin concentration	*Oncorhynchus mykiss*	Nonyphenol	Males synthesized vitellogenin	Schwaiger et al., 2002

Effects of Environmental Endocrine Disruptors on Courtship Behavior in Fish

The hormones of the endocrine system dictate fish courtship behavior, so when exposed to environmental endocrine disruptors, changes may occur in such behaviors. Many species of fish perform elaborate courtship ceremonies to attract the opposite sex for copulation. Displays performed by many males include weaving, prodding, rubbing or bunting the female (Lagler et al., 1962). Courtship by males of *P. reticulata* involves the male placing himself in front of the female and performing a sigmoid display. During this display, the male's body moves in the shape of a C or S, and vibrates while displaying his orange-yellow coloration (Bayley et al., 2002). Such actions can occur at a rate of one per minute, and can last for several minutes until the female submits. Certain acts of courtship induce hormonal changes in fish and prepare them for copulation. From this, one can see the important relationship of the endocrine system and courtship behavior in fishes through hormonal concentrations.

In males, androgen concentration directly affects courtship behavior. Fish exposed to antiandrogens often experience a loss of typical courtship display behavior. When male mosquitofish (*Gambusia holbrook*) from contaminated Lake Apopka, Florida, were examined

for sigmoid displays, a correlation between mean testosterone concentration and corresponding sexual behavior was established (Toft, Edwards, Baatrup, & Guillette, 2003). Bayley et al. (2002) exposed juvenile *P. reticulata* to three antiandrogens, vinclozolin, *p.p'*-DDE, and flutamide, for two weeks, after which frequency of sigmoid displays and other variables were observed and measured. To measure sigmoid displays, each male was placed with a non-receptive female and the sigmoid displays were recorded and analyzed using computer aided DISPLAY vision system software. Parameters of measurement included: position of the females relative to the male, position of the males relative to the female, the distance between the two fish, male swimming speed, male lateral (angular) displacement, male lateral swimming speed, and male curvature (Bayley et al., 2002). Bayley et al. (2002) found that the treatment of flutamide and vinclozolin had a significant affect on both number and duration of sigmoid displays. A similar experiment using adult *P. reticulata* exposed to the same antiandrogens resulted in a reduction in orientation by the male towards the female in courtship (when exposed to vinclozolin) and a reduction in sideways swimming action (when exposed to *p,p'*-DDE) (Baatrup & Junge, 2001). This impairment of courtship behavior by environmental endocrine

disruptors would affect a male's success in mating, as several studies have shown that female guppies are attracted to males with a high sigmoid display rate (as cited in Bayley et al., 2002). Because courtship behavior is linked with the endocrine system and hormone concentrations, the effects of such chemicals on this delicate balance could lead to detrimental effects on successful fish reproduction and overall population numbers.

Effects of Environmental Endocrine Disruptors on Phenotypic Sexual Characteristics of Fish

Steroid hormones secreted from the endocrine system have secondary effects on phenotypic characteristics of an organism. Such hormones travel to specific target sites throughout the organism and cause changes at sites that initiate a cascade of events that control specific elements in overall development. Body coloration and gonad development in male fish, for example, is under androgenic control in *P. reticulata* (as cited in Bayley et al., 2002). Changes in normal steroid secretion, therefore, could inhibit normal gonadal development or display coloration. Though all species of fish do not respond uniformly to exposure to endocrine disruptors, there are examples in which acute and chronic exposure have caused changes in phenotypic sexual characteristics of fish.

Head ornamentation, fin morphology, and body coloration are phenotypic characteristics that develop under the control of estrogenic or androgenic hormones. Breeding males of the salmon and trout (Salmoninae) possess knobby hooks on their upper and lower jaws, whereas male chimaeras (Chimaeridae) develop a spiny retractile knob in the upper part of the head (Lagler et al., 1962). Since these sexually dependent accessories are present only during the breeding period, the appearance of such characteristics implies that they are dependent on specific hormone concentrations. Normal fin morphology can also be inhibited through exposure to endocrine disrupting chemicals. Bayley et al. (2002) exposed juvenile *P. reticulata* to the fungicide vinclozolin (3-(3,5-dichlorophenyl)-5-methyl-5-vinyloxazolidine-2,4-dione), the insecticide *p,p'-DDE* (*p,p'*-1,1-dichloro-2,2-*bis*(*p*-chlorophenyl) ethylene), or the commercial antiandrogen flutamide (4'-nitro-3'-trifluromethyllisobutyranilide) until the fish reached adulthood, at which time the degree of gonopodium development was observed. It was found that males exposed for 30 days to flutamide (0.01 or 1.0 μg/mg fodder; used as a positive control), DDE (0.1 μg/mg fodder), and vinclozolin (0.1 μg/mg fodder) had significantly shorter gonopodium than those in the control groups. Such changes were not observed,

however, in male *P. reticulata* that were exposed to the same chemicals at similar concentrations as adults (Baatrup & Junge, 2001). This lack of comparable response by the same species to the same compound at similar concentrations suggests that endocrine disruptors may play a significant role in the developmental stages, and that adults are insensitive to gonadal morphological changes after completed gonadal development.

Studies have shown that exposure to certain compounds can cause changes in the body coloration of particular species of fish, the most studied of which is *P. reticulata*. Juvenile *P. reticulata* exposed 30 days to fungicide vinclozolin (3-(3,5-dichlorophenyl)-5-methyl-5-vinyloxazolidine-2,4-dione) showed a significant reduction in area and color intensity of orange coloration (Baatrup & Junge, 2001). In a different study, female *P. reticulata* exposed 42 days to a diluted effluent from a Swedish kraft pulp mill showed a greater intensity in body coloration over time than that of the control group (Larsson et al., 2002). Changes in male and female body coloration after exposure to chemicals is a clear indication that those particular chemicals are endocrine disruptors.

Physiological Effects on Fish by Environmental Endocrine Disruptors

Many researches have focused on examining the effects of environmental endocrine disruptors on fish physiology. Researches have used different fish species and exposed them to various concentrations of various compounds to see how reproduction and normal physiology is altered through exposure. Though not all fish react with the same physiological changes, evidence has shown that natural and synthetic steroid hormones and other compounds can alter natural hormone concentrations in fish and promote functions in male fish that normally occur in females, such as vitellogenin synthesis.

Endocrine disrupting chemicals are compounds that interact with components of an organism's endocrine system and interfere with some step of the regulated system. Such changes in the system may include changes in hormone synthesis, clearance, and receptor-mediated hormone actions (Pickering & Sumpter, 2003). Many of the compounds used in experiments are natural and synthetic sources of steroids, including estrogen and testosterone. Natural estrogens include 17β-estradiol (E_2) and estrone (E_1), whereas 17β-ethinylestradiol (EE_2) is a synthetic estrogen. Changes in natural hormone levels caused by environmental contaminants can stimulate

vitellogenesis in males and secretion of other hormones. Schultz, Skillman, Nicolas, Cyr, and Nagler (2003) studied the effects on reproductive capacities of *O. mykiss* with short-term exposure to 17 α-ethynylestradiol. After a 62-day exposure prior to spawning, several endpoints were considered, including circulating plasma levels of the sex steroids 17 α, 20 β-dihydroxyprogesterone (17,20-DHP) and 11-ketotestosterone (11-KT). They found a two-fold increase of 17,20-DHP in exposed fish, whereas levels of 11-KT were significantly reduced in fish exposed to 100ng/L of EE_2. Such results show that male *O. mykiss* are susceptible to hormonal concentration changes if exposed to relevant levels of EE_2 (10-100ng/L). Other steroids may not cause hormonal changes in *O. mykiss*. When *O. mykiss* are exposed at the embryonic stage to external sources of steroids, they may not experience treatment-dependent changes in hormone concentrations. For example, embryonic *O. mykiss* exposed by microinjection showed no morphological changes in testis formation or changes in sex-ratio six months later (Carlson, Curtis, & Williams, 2000).

The naturally occurring 17β-estradiol (E_2) has also been shown to affect the hormones of the endocrine system of *O. mykiss*. Vizziano, Le Gac, and Fostier (1996) exposed *O. mykiss* to three testicular steroids and measured the effect the steroids had on

the secretion of 17,20 β-dihydroxy-4-pregnen-3-one (17,20 β-OHP), a primary hormone in spermatogenesis. Pieces of testes were incubated in one of the steroids of 24 to 48 hours, then examined for the level of secretion of 17,20 β-OHP. It was found that only E_2 had a significant effect on the secretion of 17,20 β-OHP at concentrations higher than 50 ng/ml. Thus, E_2 changes the secretion of a hormone important to the process of spermatogenesis. From this, one can conclude that exposure to certain steroid hormones, whether they are natural or synthetic in nature, can affect an organism in both acute and chronic reproductive capacities.

Exposure to steroid hormones has also been shown to cause synthesis of vitellogenin in male fish. Vitellogenin is a major yolk protein precursor produced in the liver of sexually maturing female oviparous vertebrates. When vitellogenin undergoes enzymatic cleavage, it produces oocyte yolk proteins necessary for the viability of developing embryos (Kwon, Prat, Randall, & Tyler, 2001). Under normal conditions, male fish synthesize small amounts of estradiol, the hormone that regulates the synthesis and secretion of vitellogenin and thus, males have no circulating vitellogenin concentrations (Jobling, Sheahan, Osborne, Matthiessen, & Sumpter, 1996). When males or females are exposed to excess concentrations

of estrogens, vitellogenin is synthesized and secreted from the liver. Thus, male vitellogenin concentration is used as a biological indicator of exposure to estrogenic compounds. Excess production and accumulation of vitellogenin can cause death through kidney failure.

Schwaiger et al. (2002) exposed sexually maturing *O. mykiss* to 1μg/L and 10μg/L of the high volume industrial chemical nonylphenol four months before spawning. After the four months, all fish showed an increase in levels of plasma vitellogenin, and at concentrations of 10μg/L, reproduction was impaired. The progeny were also examined for altered levels of vitellogenin; there was no effect on males, but females had a slightly higher level than that of the control progeny. Though vitellogenin levels were not altered in male offspring, they were in the parental generation, showing a negative affect of nonylphenol on *O. mykiss*.

Purpose and Outline of Study

The objective of this study is to examine the responsiveness of *O. mykiss* exposed to 17α-ethynylestradiol at two temperatures. This study is important as it is becoming increasingly vital to understand the effects that chemicals released into the environment have on organisms, such as fish. It is necessary to investigate and take measures to

control any possible negative effects such chemicals may have in order to maintain the natural structure of the aquatic environment.

No apparent studies have been identified that use sexually undifferentiated *O. mykiss* to test for affects of temperature and EE_2 on gonadal development, hematocrit, hepatosomatice index, or behavioral changes. Five hypotheses were made in this experiment. Because hormones affect behavior, it was hypothesized that fish exposed to EE_2 would have increased levels of aggression, as compared to those in the control group. Second, it was hypothesized that fish treated with EE_2 would have lower levels of hematocrit, as estrogens have an inhibiting effect on erythropoiesis-stimulating factor. Because exposure to toxic conditions can cause changes in the liver, it was hypothesized that both EE_2 and a water temperature of 19°C would result in higher hepatosomatic indices in those fish groups. Gonad morphology was hypothesized to be significantly different in those fish exposed to EE_2 and those of the control groups, in terms of development. Finally, fish exposed to colder temperatures (9°C) were hypothesized to have a greater overall weight gain than the fish housed at warmer temperatures (19°C).

CHAPTER II

MATERIALS AND METHODS

Fish

Forty juvenile *O. mykiss* were obtained from Tuckaleechee Trout Farm and Restaurant in Townsend, Tennessee, on 10 Jan 2004. Wet weight was recorded for each individual and each was placed in one of four 227L Rubbermaid Roughneck® tubs that were acclimated to the appropriate temperature (19°C or 9°C). The fish remained in the 227L tubs 4 days prior to initiation of the experiment.

Experimental Design

Eight 35L glass aquaria were filled with dechlorinated Maryville City water (Tetra Aqua) and acclimated to either 19°C (4 tanks) or 9°C (4 tanks) 24 hours prior to the introduction of the fish. Each tank contained two airstones weighed down by a granite rock, an Aqua-Tech 5-15 power filter, and a new Aqua EZ-change filter cartridge. Each tank contained five *O. mykiss* of similar sizes (ANOVA of weight by tank $p = 0.71952$). At each temperature, two tanks were controls, dosed with 175µL dechlorinated water, and two

were dosed with 175 μL of 50ng/L 17α-ethynylestradiol (EE_2)(SIGMA, 103k1230), resulting in a final concentration of 1750ng/tank. The tanks were dosed after the fish had been acclimated for 4 days in the glass aquaria, initiating day one of the experiment. A photoperiod of 11:13 daylight/night was set throughout the experiment. Fish were fed 0.35g of Zeigler finfish starter #2 crumble (Zeigler) twice a day until the night before euthanization. The carbon filters were rinsed out when excess of food and feces was observed. Dechlorinated water was added as needed to maintain the original volume of the tank (35L).

Behavioral Analysis

Temperature was recorded for approximately 30 seconds twice daily using a digital thermometer. Aggressive behavior shown toward the thermometer by the fish was noted five days after exposure. To measure aggression, the thermometer was held in the front center of the tank for 30 seconds and a count of approaches and attacks was made by the observer. Approaches were defined as deliberate turn towards and bodily contact made by the fish on the thermometer, excluding oral contact. Attacks were defined as an approach in which the fish makes oral contact with the thermometer tip.

Growth Measurements and Blood Sampling

After 20 days of exposure, wet weights were recorded of fish at 19°C. Wet weights were recorded of fish at 9°C after 21 days exposure. The fish were individually anesthetized in 50mg/L of ethyl aminobenzoate (SIGMA, lot 092K1175), and removed from the anesthesia tank following cessation of opercular movement. A sharp single-edged razor was used to cut through the caudal peduncle to collect blood for analysis. Blood was collected immediately using a labeled 75mm heparinized capillary tube (Fisherbrand red-tips) by touching the capillary tube to the dorsal aorta. The capillary tubes were sealed with Crit-o-seal, chilled, centrifuged, and scored for immediate determination of hematocrit. The plasma was suctioned out and put into a bullet tube using a pipette. Plasma samples were stored at -75°C.

Following collection of blood, the fish were euthanized by decapitation. The gonads and liver were removed and each was placed in a 10% formaldehyde solution (FisherChemicals, lot no.012507). Liver weight was recorded to determine the hepatosomatic indices.

Gonadal Histology

Following four days of fixation, the gonads were placed in cassettes and cleared using 70% ethanol. Histotechnique was used following *Humason's Animal*

Tissue Techniques (1997). Prior to embedding the tissues in wax blocks, the tissues were dehydrated using a graded percentage of alcohol. The tissues were exposed to 80% ethanol for 120 minutes, 95% ethanol for 90 minutes, and twice at 100% ethanol for 60 minutes. The tissues were then placed in Citrasolv (Fisherbrand) twice for 60 minutes. The tissues were infiltrated with paraffin wax under a vacuum (FisherScientific, Isotemp vacuum oven model 280A), for 60 minutes at 12psi for wax I, 60 minutes at -15psi for wax II, 60 minutes at -21psi for wax III, and 60 minutes at -25psi for wax IV. After wax IV, the tissues were placed in a wax pot and embedded in a wax block. The gonads were oriented such that they were flush with the bottom of the block.

After the wax hardened, the paraffin block was trimmed around the tissue. The block was mounted on a microtome and sectioned at 12µm thickness. The ribbons were floated on a warm water bath containing a small amount of gelatin and mounted on slides. The slides dried overnight on a slide warmer prior to staining. Hematoxylin and Eosin were used to stain the slides. The slides were stained according to the following procedure: Citrisolv for 10 minutes, 100% ethanol for 1 minute, 95% ethanol for 1 minute, 70% ethanol for 1 minute, running water 4 minutes, Hematoxylin 4 minutes, running water 4 minutes, Scott solution 2

minutes, running water 4 minutes, Eosin 3 minutes, 70% ethanol 2 dips, 95% ethanol 2 dips, 100% ethanol 2 minutes, 100% ethanol 2 minutes, Citrisolv 4 minutes, and Citrisolv 4 minutes. After staining, a coverslip was added to the slides using Permount.

Statistical Analysis

A one-way analysis of variance (ANOVA) was used to test the effects of treatment (exposure to EE_2) and temperature on behavior, tissues, and weight gain in the 40 juvenile *O. mykiss*. Statistical analyses were conducted using StatView. Exposure levels and temperature levels were compared to identify significant differences in aggression, hematocrit, hepatosomatic index (HSI; liver weight x 100/total body weight), and overall weight gain.

CHAPTER III

RESULTS

Effects of treatment (exposure to 17α-ethynylestradiol (EE_2)) and temperature on behavior, tissues, and weight gain in 40 juvenile *O. mykiss* were examined. The effects of temperature and exposure were examined through measuring and comparing aggression, overall weight gain, hematocrit, hepatosomatic index (HSI), and gonadal histology.

Behavior Analysis

There were no significant differences in aggression of fish due to treatment or temperature. In terms of temperature and treatment, both the number of approaches (p = 0.8560, p = 0.5869, respectively) and number of attacks (p = 0.5472, p = 0.2322, respectively) did not differ significantly between the groups. Table 2 shows the mean approaches and attacks for the four groups.

Tissue Analyses

Percent Hematocrit

Treatment had a significant effect on hematocrit in *O. mykiss* (p = 0.0483), whereas temperature did not

(p = 0.2738). *Oncorhynchus mykiss* exposed to EE_2 had higher hematocrit percentages than did those in the control group, as shown in Table 2. The number of hematocrit samples, however, differed between the treatment groups: the EE_2 group had 17 samples and the control group had 11 samples.

Hepatosomatic Indices

Temperature had a significant effect on the hepatosomatic index (HIS) of *O. mykiss* (p = 0.0002), irrespective of treatment. Fish housed at 19°C had greater HSI than fish housed at 9°C. Table 2 shows the values for mean ratio of HSI.

Gonadal histology

Analysis of histology was performed on the undifferentiated gonads of juvenile *O. mykiss*. Figure 2 shows gonadal tissue from a juvenile *O. mykiss*.

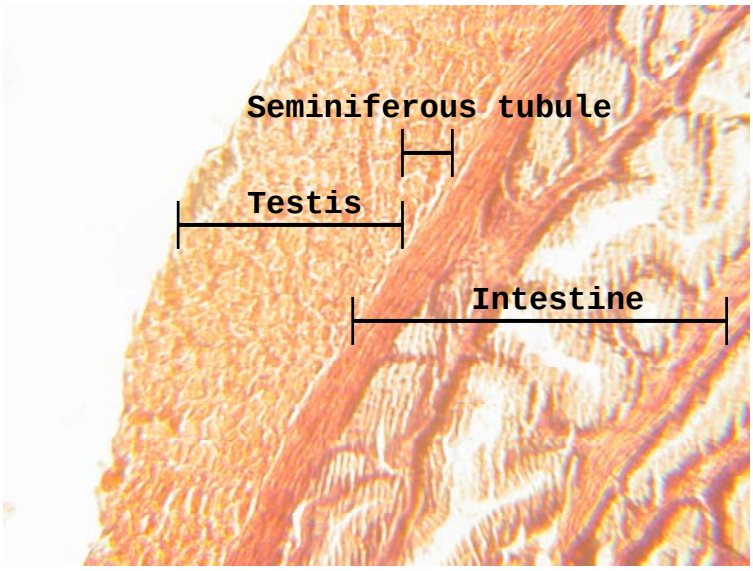

Figure 2. Immature gonadal tissue from *Oncorhynchus mykiss* (78.75x)

Complete analysis of gonadal histology was not possible due to incompletion of gonadal histology.

Whole Body Analysis

Although there was no significant initial difference in body masses of the 40 *O. mykiss* as shown by the interaction of temperature and treatment (p = 0.4393), temperature had a significant effect on weight gain after 21d in two of the four groups of fish. Fish housed at 9°C in both the control and EE_2 group had a significant weight gain after 21d compared to those housed at 19°C (p = 0.0217). Table 2 shows the mean initial weights, final weights, and overall

weight gain for each group of fish with standard error.

As compared with fish housed at 9°C, the fish at 19°C displayed greater signs of loss of equilibrium, darker body coloration, and sites of inflammation or fungal infection on the body.

Figure 3 shows the overall weight gain of the control and EE_2 groups exposed to two temperatures.

Table 2. Results (+1SE) for analyzed aggression, percent hematocrit, hepatosomataic indices, and mean weights in *Oncorhynchus mykiss*. (* donotes significance at $p < .05$)

	9°C, Control	9°C, EE_2	19°C, Control	19°C, EE_2
Aggression				
Mean approaches (±SE)	1.6 (0.846)	1.7 (0.907)	1.4 (0.872)	2.2 (0.629)
Mean attacks	0.20 (0.200)	0.90 (0.547)	0.30 (0.213)	0.40 (0.221)
*Percent Hematocrit (±SE)**	39.5 (5.01)	38.2 (1.18)	28.6 (1.89)	36.7 (2.31)
Mean liver:final body mass (±SE)	1.567 (0.0650)	1.592 (0.137)	1.984 (0.131)	2.143 (0.116)
*Mean weights (g)**				
Mean initial weight	3.507	4.062	4.086	4.002
Mean final weight	5.851	5.624	4.545	4.255
Overall mean weight gain (±SE)	2.344 (0.505)	1.562 (0.505)	0.459 (0.578)	0.253 (0.631)

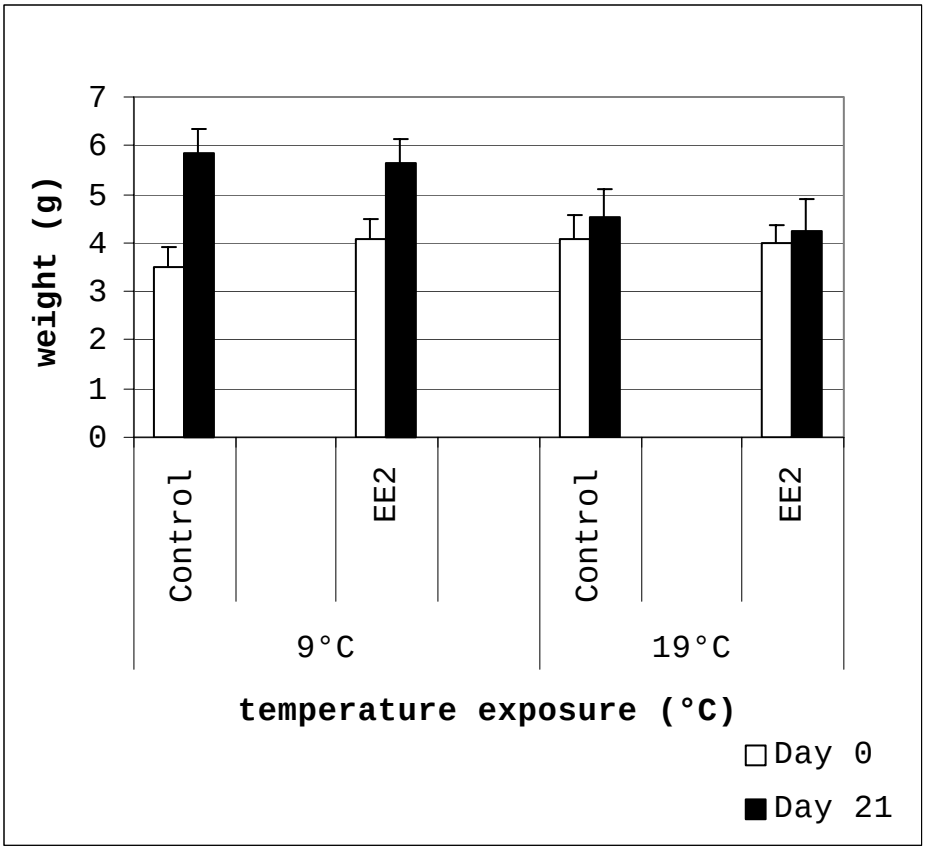

Figure 3. Initial and final weight (+1SE) in the four groups of *Oncorhynchus mykiss* (N= 40).

CHAPTER IV

DISCUSSION AND CONCLUSIONS

The results of this study suggest that temperature and treatment with 17α-ethynylestradiol (EE_2) independently have an effect on certain developmental and tissue characteristics of juvenile *O. mykiss*. Irrespective of temperature, exposure to EE_2 had a significant effect on hematocrit. Temperature, irrespective of EE_2 treatment, had a significant effect on the hepatosomatic index (HSI) and mean weight gain. Neither temperature nor treatment had a significant effect on the aggressive behavior of *O. mykiss*.

Behavior

Few studies have examined the influence of estrogens on the behavior of fish. The effects of various antiandrogens on fish behavior, however, have been well studied. Baatrup and Junge (2001) and Bayley, Junge, and Baatrup (2002) found that antiandrogens alter behavior in other teleosts by causing suppression of courtship behavior. Thus, it was expected that fish treated with EE_2 would show a

significant difference in behavior, as compared to the control groups. It did appear that EE_2 exposure increased aggression, but the results were not significant. It is highly probable that significant results would be attained if the experiment were run longer.

Tissue

Percent Hematocrit

Houston and Koss (1984) found that other than at 17°C and 25°C, increasing temperatures from 10°C and 26.1°C did not significantly influence hematocrit or hemoglobin in rats. The results of the present experiment confirm this finding in fish, as temperature did not have a significant effect on hematocrit. However, it was found that fish treated with EE_2 had significantly higher hematocrit.

Based on previous research, it was hypothesized that treatment with EE_2 would inhibit erthyropoiesis, resulting in lower hematocrit values for fish exposed to EE_2. As previous research has shown, estrogen inhibits erythropoiesis-stimulating factor. For instance, Mirand and Gordon (1966) showed that estrogens inhibit erythropoiesis by blocking the erythropoiesis-stimulating factors. In the present experiment, exposure to EE_2 caused a slightly significant increase in hematocrit. Because EE_2 should have an inhibitory effect on erythropoiesis and thus

result in lower hematocrit, another factor must have caused the fish exposed to EE_2 to have higher hematocrit.

One possibility is that androgens influenced hematocrit, as androgens have been shown to stimulate erythropoiesis in mice (as cited in Mirand & Gordon, 1966). Though the juvenile *O. mykiss* in this experiment had undifferentiated gonads, circulating testosterone in male fish would have resulted in the males having higher levels of erythropoiesis than those of females. It is possible that the proportion of male fish to female fish was skewed towards males; this proportion could have caused the average hematocrit value for treated fish to be high enough to have significance.

Another possible explanation for the unexpected levels of hematocrit is that estrogens were converted to androgens, or that the EE_2 was increasing testosterone production. A recent study showed that 4-tert-octylphenol, which has weak estrogenic activity, caused an increase in testosterone production in the Leydig cells of male rats (Murono & Derk, 2002). This reasoning would explain why the groups exposed to EE_2 had significantly higher percent hematocrit when significantly lower values were expected. It is possible that given a long enough exposure, however, the EE_2 would have had a more

significant inhibitory effect on erythropoiesis, causing the hematocrit to be lower in treated fish, as was hypothesized. Future studies should examine the mechanism through which EE_2 alters hematocrit.

Hepatosomatic Indices

Oncorhynchus mykiss housed at 19°C had greater hepatosomatic indices, yet gained less weight overall. The livers of the fish in these groups could have been enlarged due to hypertrophy or allometric growth. Mackay and Lazier (1993) exposed juvenile *O. mykiss* to estradiol (E_2) at 9°C and 15°C and found that fish at 15°C had a significantly greater liver-somatic index (LSI, calculated as liver weight/body weight) than fish housed at 9°C. Similar results were found in the present study. It is possible that the fish housed in the less ideal environment (19°C) experienced greater levels of internal stress and thus greater levels of hypertrophy, specifically in the liver.

Whole Body Analysis

The significant weight gain shown by the fish housed at 9°C can be explained in several ways. The fish housed at 9°C did have one additional day of growth that the fish at 19°C did not have. This additional day could have allowed for a small increase in body weight, but this is not expected to be the major reason for weight gain. A more likely explanation for this weight gain is the increased

health of the fish and their increased appetite at 9°C. Because *O. mykiss* survive best at water temperatures between 10°C and 18°C, and generally thrive at the lower end of the range, the fish housed at 9°C were in a more ideal environment. From this, one can conclude that the fish housed at 9°C fish would thus thrive over those housed at the warmer temperature. A healthier fish and thus greater appetite would result in an overall greater mean weight gain.

Different metabolic rates could have resulted in different mean weight gains. Temperature does affect certain physiological and biochemical processes in many ectotherms, such as fish (Houlihan, Mathers, & Foster, 1993). With an increase of 10°C, a significant change can be observed in physiological processes, such as the rate of oxygen consumption and protein synthesis (Houlihan, Mathers, & Foster, 1993). The 10-degree difference water temperature in the present experiment could have affected physiological processes of the *O. mykiss* at 19°C, such had they had lower mean weight gain as compared to those housed at 9°C.

Conclusions

From this experiment, it has been shown that temperature and short-term treatment with EE_2 do not have a significant collective effect on behavior, tissues, or weight gain in juvenile *O. mykiss*.

Independently, however, increased temperature does increase hepatosomatic index and EE_2 exposure does increase hematocrit. The conclusions drawn from the analysis and interpretation of the results lead to the possibility of further research that can be done in this field.

If it were found that estrogens could in fact be converted into androgens or stimulate androgen production in fish, a long-term experiment would allow researchers to determine the collective effect that this process and exposure to different concentration of estrogens has on fish, specifically *O. mykiss*. In addition, it would be beneficial to expose *O. mykiss* to environmentally significant water temperatures and estrogen concentrations so one could extrapolate conclusions made from the experiment to real-world situations.

APPENDIX

MARYVILLE COLLEGE
HUMAN AND ANIMAL PARTICIPANTS REVIEW COMMITTEE
ANIMAL STUDY APPLICATION FORM

1. Student Name: Lauren Butz

2. Date: 24 November 2003

3. Senior Thesis Advisor: Drew Crain

4. Pain or Distress Category: B (See listing of Pain or Distress Categories below)

For categories C,D, or E, USDA regulations require that the investigator consider alternative procedures. Please provide a narrative (for instance the end of Chapter 1) describing the methods and sources used to determine that alternatives are not available. If a computer assisted literature search was conducted, provide the names of the database(s) and date(s) of the search.

PAIN OR DISTRESS CATEGORIES

A. ACUTE STUDIES
Studies performed under anesthesia from which the animals are not permitted to regain consciousness, or performed on excised animal tissues collected under anesthesia or following euthanasia.

B. PAIN OR DISTRESS - NONE OR MINOR
Chronic studies that DO NOT involve survival surgery, induction of painful or stressful disease conditions, or pain or distress in excess of that associated with routine injections or blood collection. Included are induction or transplantation of tumors in animals (so long as the tumors do not cause pain and the animals are terminated prior to becoming seriously ill), administration of mildly toxic substances or drugs that cause no significant disease or distress, and antibody production as long as significant disease does not result and antigen booster doses do not include Complete Freund's Adjuvant (CFA).

C. PAINFUL PROCEDURES WITH ANESTHESIA/ANALGESIA
a. Survival surgical procedures.
b. Painful or potentially painful non-surgical procedures; e.g. bone marrow taps, injections into particularly sensitive areas such as foot pads, cardiac punctures, or traumatic procedures such as burns (burns may be category D, depending on severity).

D. MODERATE DISTRESS OR PAIN GENERALLY WITHOUT ANESTHESIA/ ANALGESIA/ TRANQUILIZERS
Induction of moderately distressful or painful disease conditions (examples: arthritis, administration of toxic chemicals, infectious challenges, immunosuppression resulting in infectious disease, peritonitis, severe inflammation, especially of weight bearing surfaces or resulting in external sores), whole body irradiation, stress models, septic shock, hypotensive shock, moderate painful stimuli (examples: low level electrical shock or heat), survival surgical procedures that have the potential to result in long term distressful illness (organ transplants, for example), induction of cardiac ischemia, booster immunizations with CFA, tumor induction or animal cultures that cause significant distress or pain, sight deprivation, restraint for periods longer than 12 hours.

E. INTENSE SUSTAINED OR REPEATED PAIN WITHOUT ANESTHESIA/ANALGESIA
Direct stimulation of CNS pain tracts, nociceptor stimulation by physical or chemical means that causes severe pain (e.g., corneal abrasions), or any category C (see above) procedure if performed without chemical relief of pain.

5. Species to be used *Oncorhynchus mykiss*

6. Age of animals juveniles

7. Number of animals in study ~30

8. Duration of study 21 day exposure (during January 2004)

9. Location of animals during the study (building and room) Sutton Science Center Rm. 102

10. List personnel to call if problems with animals develop:

Name	Daytime Phone	Nighttime Phone	Emergency No.
Lauren Butz	981-8582	981-8582	981-8582
Drew Crain	981-8238		
Ben Cash	981-8009		

Investigator Assurance

The information provided in this protocol form accurately reflects the intended use of animals for this research activity. Significant changes in procedures will not be undertaken without prior notification and approval of the Human and Animal Subjects Review Committee.

All persons involved in the use of animals on this protocol have been informed of the experimental objectives and methods. Each has received training in the execution of animal-related procedures he/she will perform prior to participation in the protocol, and will participate in any educational or training programs deemed appropriate or necessary by the Human and Animal Subjects Review Committee.

I agree to follow the provisions of the Animal Welfare Act and the guidelines of the National Institutes of Health on the care and use of laboratory animals.

I agree to use anesthesia, analgesia and tranquilization to relieve pain or distress whenever use of these agents will not jeopardize the scientific validity of the data. I have specifically consulted with the Human and Animal Subjects Review Committee regarding any experiments that are classified in pain/distress categories C, D, or E.

I will take appropriate steps to avoid exposure of persons working with these animals to any biohazardous agents used in the study.

State the reasons if you cannot attest to the accuracy of any of these statements:

11. HUSBANDRY REQUIREMENTS: Is anything other than routine care and equipment required?
YES_x_ No __ If "YES", please list below.
Chemicals for experiment: ethyl-4-aminobenzoate, 17α-ethynylestradiol, and one additional chemical

12. Is it likely that pain/discomfort will be experienced by animals in this protocol?
YES___ NO_x_ If "YES", describe:

13. What will happen to the animals at the end of the study? If euthanasia is required, state the methods.

The fish will be euthanized using >250mg/L of ethyl-4-aminobenzoate (benzocaine hydrochloride) (SIGMA) after the 21 day exposure period. The following method for euthanasia was adapted from the "2000 Report of the AVMA Panel on Euthanasia*." Benzocaine hydrochloride is water soluble and can be used directly for anesthesia or euthanasia. For euthanasia, the chemical can be put in a bath or recirculation system. A concentration of >250 mg/L should be used for euthanasia. Fish should be left in the solution for at least 10 minutes following cessation of opercular movement. Death occurs though hypoxia attributed to depression of vital centers and occurs rapidly when using the correct concentration of the chemical. There is no harm presented to the experimenter using ethyl-4-aminobenzoate.

14. Briefly describe your proposed research project (or attach a research proposal). Be sure to include a justification for the species and number.

See attached

* American Veterinary Medical Association (2000). 2000 Report of the AVMA Panel on Euthanasia. Journal of American Veterinary Medical Association, 218, 669-696.

This project has been reviewed by the Maryville College Human and Animal Use Committee.

Nov 26, 2003

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