# Behavioral Effects of Exposure to the Endocrine Disrupting Chemical, Ethinyl Estradiol, on the Fourspine Stickleback (*Apeltes quadracus*)

by

Kristine K. Kuroiwa

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy,
The City University of New York

2009

© 2009
KRISTINE K. KUROIWA
All Rights Reserved

Approval page

This manuscript has been read and accepted for the
Graduate Faculty in Biology in satisfaction of the dissertation
requirement for the degree of Doctor of Philosophy.

Dr. Jennifer Basil

__________ ______________________________

Date Chair of Examining Committee

Dr. Laurel A. Eckhardt

__________ ______________________________

Date Executive Officer

Dr. Peter Moller

____________________

Dr. Robert Rockwell

____________________

Dr. Martin Schreibman

____________________

Dr. Judith Weis

____________________

THE CITY UNIVERSITY OF NEW YORK

# Abstract


The Behavioral Effects of Exposure to the Endocrine Disrupting Chemical, Ethinyl Estradiol ($EE_2$), on the Fourspine Stickleback (*Apeltes quadracus*)



By

Kristine K. Kuroiwa

Adviser: Professor Jennifer Basil



There is growing evidence that endocrine disrupting chemicals polluting the environment have the potential to affect animal health and behavior. Ethinyl estradiol ($EE_2$), the active ingredient in contraceptive pills and hormone-replacement therapies, is a particularly potent endocrine disrupting chemical found in our waterways. In this study, adult male and female fish were used to investigate the effects of a 60 day $EE_2$ exposure on 1) ecologically relevant behaviors, 2) gonadal state, and 3) male reproductive coloration. Exposure to levels of $EE_2$ documented in the environment (10, 70 and 100 ng/L) detrimentally altered fish behavior. Fish exposed to $EE_2$ were less active, more aggressive, and avoided an artificial predator less often. 100 days in clean water reversed some but not all of these behavioral changes. In both males and females, gonads in exposed groups differed from controls, and included the presence of ovotestes found only in males exposed to $EE_2$. Reproductive coloration in males was not affected by exposure. This is the first report of long-lasting behavioral aberrations caused by $EE_2$ exposure in adult fish.

# Acknowledgements

The encouragement and support of many people have contributed to this dissertation. First and foremost, I would like to extend thanks and appreciation to my mentor, Dr. Jennifer Basil for her guidance, support, and for giving me the opportunity to work in a very stimulating multi-disciplinary project, which broadened my knowledge of animal behavior and helped me mature as a scientist. I also owe special thanks to my doctoral committee: Drs. Peter Moller, Robert Rockwell, Martin Schreibman and Judith Weis for sharing their expertise and time over the years with me.

I would also like to express my gratitude to my dear colleagues and friends, Dr. Christian Soucier and Dr. Robyn Crook, who both helped me tremendously during these past few years. My warmest thanks to Martin Zartarian, Doug Laing, Rob Dickie and all of the undergraduate students from the Basil lab, whose help made my research possible and my years at Brooklyn College particularly enjoyable.

Furthermore, I would like to thank my family, especially my parents, William & Karlyn Pearl, and Ken & Pat Kuroiwa, and my brother Jeffrey Kuroiwa, whose encouragement and inspiration have been central in getting me to this point.

Lastly, I am especially grateful to my husband Giorgio, for his patience and unconditional support of all my undertakings.

## Table of Contents

Abstract iv
Acknowledgements v
Table of Contents vi
List of Tables viii
List of Figures ix

**Chapter 1: Introduction 1**

1.0 Introduction ....1
1.1 Endocrine Disrupting Chemicals and their Presence in the Environment ....3
1.2 Ethinyl Estradiol as an Endocrine Disrupting Chemical in Fish ....5
1.3 Stickleback Natural History ....9
1.4 Sticklebacks as Indicator Species of EDC Toxicity ....11

**Chapter 2: General Methods 16**

2.0 General Methods & $EE_2$ Treatment ....16
2.1 $EE_2$ Water Testing ....24

**Chapter 3: Histology & Coloration 26**

3.0 Introduction: Gonad Histology ....26
3.1 Methods: Gonad Histology ....27
3.2 Results: Gonad Histology ....29
3.3 Discussion: Gonad Histology ....40
3.4 Introduction: Red Coloration in Males ....45
3.5 Methods: Red Coloration in Males ....46
3.6 Results: Red Coloration in Males ....46

3.7 Discussion: Red Coloration in Males ....47

**Chapter 4: Activity Experiment** **49**

4.0 Introduction: Activity Experiment ....49

4.1 Methods: Activity Experiment ....50

4.2 Results: Activity Experiment. ....52

4.3 Discussion: Activity Experiment. ....57

**Chapter 5: Aggression Experiment** **61**

5.0 Introduction: Aggression Experiment ....61

5.1 Methods: Aggression Experiment ....63

5.2 Results: Aggression Experiment. ....69

5.3 Discussion: Aggression Experiment. ....74

**Chapter 6: Predator Avoidance Experiment** **76**

6.0 Introduction: Predator Avoidance Experiment ....76

6.1 Methods: Predator Avoidance Experiment ....77

6.2 Results: Predator Avoidance Experiment. ....81

6.3 Discussion: Predator Avoidance Experiment. ....90

**Chapter 7: General Conclusions** **94**

7.0 General Conclusions ....94

**Literature Cited ....99**

## List of Tables

Table 1.1 Levels of $EE_2$ measured in sewage, rivers, streams and potable water from the USA, UK and Germany ........................................6

Table 2.1 Tank Spiking Regime for $EE_2$ Series 1 ......................................................19

Table 2.2 Tank Spiking Regime for $EE_2$ Series 2 ......................................................20

Table 3.1 Male Gonad Categories: Post-Exposure . ...................................................30

Table 3.2 Male Gonad Categories: Post-Depuration. .................................................33

Table 3.3 Female Gonad Categories: Post-Exposure .................................................36

Table 3.4 Female Gonad Categories: Post-Depuration ...............................................38

Table 4.1 Activity Test: Number of Subjects in Each Testing Period ........................51

Table 5.1 Aggression Test: Number of Subjects in Each Testing Period ...................63

Table 5.2 Aggression Test: Total Number of Male or Female Subjects .....................63

Table 6.1 Predator Avoidance Test: Total Number of Successful Trials/Attempted Trials by $EE_2$ Level and Sex............................................77

Table 6.2 Predator Avoidance Test: Number of Subjects with Data for Both Testing Periods . ..............................................................................77

Table 6.3 Predator Avoidance Test: Number of Fish that Approached the Food Cup Post-Exposure..........................................................................................81

Table 6.4 Predator Avoidance Test: Number of Fish that Approached the Food Cup Post-Depuration.........................................................................................82

Table 7.1 Summary of Results.........................................................................................98

## List of Figures

Figure 1.1 Illustration of *Apeltes quadracus* .......................................10

Figure 1.2 Approximate Distribution of *A. quadracus* . ..............................11

Figure 2.1 Map of *A. quadracus* Collection Site ..........................................16

Figure 2.2 Plastic Mesh Basket Used to Separate Fish . ..............................17

Figure 3.1 Control Male, Post Exposure, Representative Category 1 Testis . ..........................................................30

Figure 3.2 Control Male, Post Exposure, Representative Category 3 Testis . ..........................................................31

Figure 3.3 10ng/L Male with Ovotestis, Post Exposure, Category 3 with Close-up of Oogonia in Testis ...........................31

Figure 3.4 100 ng/L Male with ovotestis, Post Exposure, Category 2 with Close-up of Oogonia in Testis ...........................32

Figure 3.5 10ng/L Male with ovotestis, Post Exposure, Category 3 with Close-up of Oogonia . ........................................32

Figure 3.6 Control Male, Post Exposure, Representative Category 1 Testis ..........................................................33

Figure 3.7 10ng/L Male, Post Depuration, Representative Category 2 Testis . ..........................................................34

Figure 3.8 70ng/L Male with Ovotestis, Post Depuration, Category 2 with Close-up of Oogonia in Testis ...........................34

Figure 3.9 100 ng/L Male, Post Depuration Representative Category 3 Testis . ..........................................................35

Figure 3.10 Control Female, Post Exposure Representative
Category 4 Ovary ....................................................................37

Figure 3.11 100ng/L Female, Post Exposure, Representative
Category 2 Ovary ....................................................................37

Figure 3.12 Control Female, Post Depuration, Representative
Category 2 Ovary ....................................................................39

Figure 3.13 70ng/L Female, Post Depuration, Representative
Category 4 Ovary ....................................................................39

Figure 4.1 Activity Experiment: Set Up....................................................50

Figure 4.2 Activity Experiment: Average Effects of Testing Period. ............54

Figure 4.3 Activity Experiment: Mean % Time Fish Were Active,
Post-Exposure .........................................................................55

Figure 4.4 Activity Experiment: Mean % Time Fish Were Active,
Post-Exposure .........................................................................56

Figure 5.1 Aggression Experiment: Photograph of a Male Intruder. ............64

Figure 5.2 Aggression Experiment: Photograph of a Female Intruder. .........64

Figure 5.3 Aggression Experiment: Set Up ...............................................65

Figure 5.4 Aggression Experiment: Diagram of Tank Sections. ..................66

Figure 5.5 Aggression Experiment: Average Effects of Testing
Period on Approaches to Intruder. ............................................70

Figure 5.6 Aggression Experiment: Mean % Time Hiding...........................71

Figure 5.7 Aggression Experiment: Mean % Time Swimming……………72

Figure 6.1 Predator Avoidance Experiment: Set-Up....................................78

Figure 6.2 Predator Avoidance Experiment: Fish that Approached the Food Cup.......................................................................82

Figure 6.3 Predator Avoidance Experiment: Number of Bites at Food, Post-Exposure. ....................................................................83

Figure 6.4 Predator Avoidance Experiment: Time Spent Swimming, Post-Exposure. ...................................................................84

Figure 6.5 Predator Avoidance Experiment: Latency to Approach the Food Cup, Post-Depuration.....................................................85

Figure 6.6 Predator Avoidance Experiment: Time Spent Within One Body Length of the Food Cup, Post-Depuration...................86

Figure 6.7 Predator Avoidance Experiment: Average Effects of Testing Period for Bites at Food...............................................87

Figure 6.8 Predator Avoidance Experiment: Average Effects of Testing Period for Approaches to Food Cup. ............................88

Figure 6.9 Predator Avoidance Experiment: Average Effects of Testing Period for Swimming...................................................89

## 1.0: Introduction

Environmental exposure to pharmaceuticals, pesticides, metals, and a variety of other substances present serious risks for wildlife. Some of these pollutants are part of a group called endocrine disrupting chemicals (EDCs). Since the 1990s, the term endocrine disrupting chemical has been used to describe a broad range of natural and synthetic compounds that affect the endocrine system of animals (Colborn, vom Saal, & Soto, 1993). EDCs can cause deleterious effects by acting as mimics, agonists, or antagonists of endogenous hormones (Colborn et al.; Crisp et al., 1998; Crews, 1992). Consequently, EDCs disrupt the normal functioning of the endocrine system by interfering with the manufacture, release, transport, metabolism, binding action or elimination of endogenous hormones (Kavlock et al., 1996). Although the preponderance of EDC research focuses on steroidogenic or anti-steroidogenic compounds, EDCs can also interfere with thyroid hormones (in teleosts: Picard-Aitken, Fournier, Pariseau, Marcogliese & Cyr, 2007; Zhou, John-Alder; review, Propper, 2005).

Hormonal activity plays an important role in the fitness-related processes of living organisms including development, growth, and behavior. Any disruption in these functions could cause detrimental effects on wildlife populations as a whole (Bayley, Neilsen, & Baatrup, 1999; Halldin, Berg, Brandt, & Brunstrom, 1999; Van der Kraak, Hewitt, Lister, McMaster, & Munkittrick, 2001). In fact, EDCs were deemed dangerous enough by the U. S. government that Congress passed the Food Quality and Protection Act and to amend the Safe Drinking Water Act in 1996. Both of these laws require the government to test chemicals and pesticides for endocrine disrupting effects. In addition, the amendment called for the formation of an advisory committee by the EPA (Endocrine

Disrupter Screening and Testing Advisory Committee) to oversee the compliance of these laws.

Historically, the acceptable limits for dangerous compounds in the environment were based on acute toxicity tests, although this practice is losing favor among researchers (Fossi & Leonzio, 1994). The majority of acute toxicity tests use a method developed by J. W. Trevan in 1927: the $LD_{50}$. The $LD_{50}$ is the lethal dose that kills half of the test population. In EDC research today, the majority of scientists have focused on interruptions of the development and physiology of organisms as indicators for toxicity (Zala & Penn, 2004). EDCs and their effects on organisms have been studied at all levels of organization, from the biochemical to the cellular, and to a lesser extent, at the individual and population levels. The manner in which the components interact with one another, however, is difficult to measure and is not well understood.

More recently, the study of sublethal effects such as alterations in behavior have become of interest. Behavior is a logical choice to study the impact of pollutants on wildlife. In vertebrates, gonadal steroids direct reproductive and territorial behaviors (Bass, 1998). Changes in gonadal steroid levels caused by EDCs may affect hormone levels along the hypothalamus-pituitary-gonadal axis, also resulting in behavioral changes. This is important because the behavior of an organism is a reflection, in part, of its biochemical state and is the culmination of interactions between the physiological state (internal) of the organism and its environment (external). Also, behavior is sensitive to small changes in hormone levels which can be altered by EDCs (vom Saal, Nagel et al., 1995) and neurotoxic chemicals (Beauvais, Jones, Parris, Brewer, & Little, 2001; NRC, 1992). Under the assumption that particular behaviors have adaptive value, and

that departures from 'normal' behavior may result in a reduction in fitness, EDCs that affect behaviors can have deleterious consequences (Adkins-Regan & Weber, 2002). Given that natural selection acts on outcomes, in this case an individual's behavior, these changes in behavior can also affect responses at the population and community levels (Weis, Smith, Zhou, Santiago-Bass, & Weis, 2001). Practically speaking, behavioral studies are also favored because they are inexpensive to conduct and noninvasive in nature (Clotfelter, Bell, & Levering, 2004; Kavlock et al., 1996).

Despite recent interest in the sublethal effects of EDCs on wildlife, some research gaps still exist, such as how EDCs affect males and females differently and the effects of EDCs on native species. Researchers argue that more consideration should be given to incorporating the use of males and females in studies and using native species to determine effects on wildlife populations (Burger, Fossi, McClellan-Green, & Orlando, 2007; Orlando & Guillette, 2007).

## 1.1: Endocrine Disrupting Chemicals and their Presence in the Environment

Many substances have been found to disrupt the endocrine system, but due to their variable characteristics, EDCs can not be classified by a single chemical or physical property. Over 100 compounds with reported EDC properties are introduced into the environment from agricultural, manufacturing, and pharmaceutical industry sources (review in Crisp et al., 1998). Although a number of these compounds have lethal effects in high doses, they can also cause detrimental effects on wildlife at sublethal doses. Pharmaceuticals such as natural and synthetic hormones are some of the most potent

EDCs causing sublethal effects in fish at low doses (Larsson et al., 1999; Maunder, Matthiessen, Sumpter, & Pottinger, 2007; Purdom et al., 1994; Scholz & Gutzeit, 2000).

Traditionally, toxicology researchers have relied on the dose-response model based on the assumption that toxic thresholds exist. Two major problems with the threshold dose-response model are that 1) most exposures fall below the threshold and 2) below the threshold level the model predicts random responses that fall close to the control value (Calabrese, 2008). In 2002, Calabrese & Baldwin provided evidence that another model, the hormesis model, predicted effects below the threshold level with greater accuracy than the dose-response model. Hormesis is the phenomenon of low-dose stimulation and high-dose inhibition with specific quantitative and temporal characteristics (Calabrese). Proponents of the hormesis model contend that using the dose-response model has led to faulty hazard assessment protocols and risk assessment practices and that some toxins at low doses may have beneficial effects (Calabrese, 2008). The disagreement over the opposing models continues today. Despite the debate regarding modeling the effects of EDCs on organisms there is no doubt that EDCs are entering the environment and affecting the wildlife that lives there.

EDCs enter the aquatic environment directly as sewage treatment plant effluent (point source) and from agricultural and industrial run off (non-point source) (Desbrow, Routledge, Brighty, Sumpter, & Waldcock, 1998; Gomes, Scrimshaw, & Lester, 2003; Halling-Serensen et al., 1997; Routledge et al., 1998; Ternes et al., 1999). EDCs have been detected in U. S. streams, rivers, lakes, groundwater, and oceans around the world (Atkinson, Atkinson, & Tarrant, 2003; Baronti et al., 2000; Fowler, 1990; Hohenblum, Gans, Moche, Scharf, & Lorbeer 2004; Kolpin et al., 2002; Rodriguez-Moza, Lopez de

Alda, & Barcelo, 2004; Shore, Gurevitz, & Shemesh, 1993; Stumpf, Ternes, Haberer, & Baumann, 1996). In fact, the EPA reported in their National Water Quality Inventory to Congress in 1988 that 35% of the rivers and streams in the U. S. were impaired by pollutants (USEPA, 2000). In light of this phenomenon, it is no surprise that organisms that live in and/or feed from these aquatic environments are exposed to high levels of EDCs via bioaccumulation in their surroundings and biomagnification in their diets (Ikonomou, Rayne, & Addison, 2002). For this reason, EDC research is dominated by, but not limited to, work focusing on organisms living in aquatic environments (Sumpter & Johnson, 2005).

## 1.2: Ethinyl Estradiol and its Impact as an EDC in Fishes

One of the most prescribed types of pharmaceutical EDCs in the United States is synthetic estrogen, ethinyl estradiol ($EE_2$), in the form of birth control pills and hormone replacement therapy (Zoeller, 1998). In 2002, The Center for Disease Control survey reported that 82.3% of sexually active women have used birth control pills (Chandra, Martinez, Mosher, Abma, & Jones, 2005). The use of birth control is certainly not limited to the United States. A 2007 report from the United Nations' Department of Economic and Social Affairs: Population Division estimated that worldwide over 96 million married women use birth control pills (United Nations, 2007). Due to the extensive use of birth control throughout the world, it is not surprising that $EE_2$ is found in concentrations of 5-200 ng/L in raw sewage, sewage effluent, potable water, rivers, and streams in Europe and North America (Aherne & Briggs, 1989; Desbrow, Routledge, Brighty, Sumpter, & Waldcock, 1998; Kolpin et al., 2002; Kuch & Ballschmiter, 2000;

Snyder et al., 1999; Stumpf, Ternes, Haberer, & Baumann, 1996; Ternes et al., 1999; Tabak, Bloomhuff, & Bunch, 1981) (Table 1.1).

| Source | Location | $EE_2$ Level (ng/L) | Reference |
|---|---|---|---|
| raw sewage | USA | average =1210 | Tabak, Bloomhuff, & Bunch, 1981 |
| treated sewage | USA | average=810 | Tabak et al., 1981 |
| treated sewage | UK | maximum=62 | Stumpf, Ternes, Haberer, & Baumann, 1996 |
| treated sewage | UK | range=1-7 | Aherne & Briggs, 1989 |
| treated sewage | UK | range=0-7 | Desbrow, Routledge, Brighty, Sumpter, & Waldcock, 1998 |
| treated sewage | Germany | median 1-9 | Ternes et al., 1999 |
| river | UK | range 2-15 | Aherne & Briggs, 1989 |
| river | UK | average= 5 | Stumpf et al., 1996 |
| stream | USA | median=73,max=831 | Kolpin et al., 2002 |
| potable water | UK | <1-4 | Aherne & Briggs, 1989 |

Table 1.1**:** Levels of $EE_2$ measured in sewage, rivers, streams and potable water from the USA, UK and Germany

In rivers and streams, levels of $EE_2$ ranging from 2-15 ng/L (UK) (Aherne & Briggs, 1989) to a median level of 73 ng/L (USA) have been detected. High consumption of birth control is not the only reason for the presence of $EE_2$ in the environment. The chemical properties of $EE_2$ largely contribute to its ubiquity as well.

The underlying actions of natural hormones like estradiol are essentially the same across vertebrates (vom Saal, 1995). Given that $EE_2$ was designed as a mimic for the natural estrogen, estradiol, it is not surprising that $EE_2$ is an active hormone mimic in fish. $EE_2$ is a lipophilic compound and is synthesized to bind to estrogen receptors at levels of equal (Kasper & Wintzel, 1985) or greater affinity (Blair et al., 2000) than estradiol. $EE_2$ and natural estrogen are excreted in urine and feces which then pollute the aquatic environment via sewage effluent (Denison, Chambers, & Yarbrough, 1981). In comparison to natural estrogens, $EE_2$ is quite stable and has a much longer plasma half-

life; it therefore remains active in sewage effluent longer than estradiol (Tabak, Bloomhuff, & Bunch, 1981). In addition, the pharmacokinetics of $EE_2$ increases its potency. Schultz, Orner, Merdink, and Skillman (2001) found that enterohepatic recirculation of $EE_2$ in rainbow trout resulted in a re-dosing effect. Researchers have accordingly warned of potential immediate negative effects on aquatic life and also of future problems due to the accumulation of synthetic steroids in our waters (e.g., Tabak et al., 1981).

In light of this information, researchers began the process of documenting the dangerous effects on aquatic life exposed to estrogens at environmentally relevant concentrations. One of the first documented occurrences of EDC-related abnormalities in fish was the discovery of hermaphroditic fish in sewage treatment plant lagoons by fishermen in the United Kingdom (unpublished observation cited in Purdom et al., 1994). To investigate the cause of this phenomenon, Purdom et al. placed cages filled with rainbow trout downstream from the sewage-treatment plant in question. After finding that both the male and female fish caged downstream of the effluent had significant increases in vitellogenin, the researchers determined that some component of the sewage effluent must be estrogenic. Vitellogenin is an egg yolk precursor protein that is stimulated by estrogen and is usually only produced by females. Although they could not identify the estrogenic component conclusively, further laboratory studies suggested $EE_2$ as the likely culprit. These studies further revealed that $EE_2$ at levels as low as 1 to 10 ng/L produced effects similar to those found in fish exposed to sewage effluent. Subsequent studies established the NOEC (no observed effect concentration) for $EE_2$ on sexual behavior and secondary sexual characteristics in fathead minnows at 4 n/L (Lange

et al., 2001) and at 1 ng/L for vitellogenin production and changes in liver and testes (Pawlowski, Aerle, Tyler, & Braunbeck, 2004). These levels are much lower than the critical levels established for fish survival, 200 ng/L, and the LOEC (lowest observed effect concentration), 400 ng/L (sheepshead minnow (*Cyprinodon variegatus*), Zillioux et al., 2001).

EDC studies using toxicological parameters like reproduction, development, and physiology to identify the effects of exposure to exogenous estrogens have uncovered a wealth of information. Reproductive anomalies like atypical sex ratios (guppies (*Poecilia reticulate)*, Toft & Baatrup, 2003; fathead minnows (*Pimephales promelas*), Lange et al., 2001) and decreased fecundity (Japanense medaka *(Oryzias latipes),* Oshima et al., 2003) were discovered to be useful parameters to identify exposure to estrogens. Developmental effects such as the occurrence of intersex individuals in threespine stickleback (Hahlbeck, Griffiths & Bengtsson, 2004) and the absence of secondary sexual characteristics in fathead minnows (Lange et al., 2001) have also been used as indicators. Lastly, physiological changes like vitellogenin production in males (fathead minnows, Panter, Thompson, & Sumpter, 1998), kidney hypertrophy (threespine stickleback, Hahlbeck et al., 2004) and altered levels of spiggin production (the glue-like substance produced by males to construct their nests) in threespine stickleback (Hahlbeck et al., 2004; Katsiadaski & Scott, 2002) have successfully been used as parameters in toxicology studies testing estrogen exposure.

The use of behavioral measures to examine EDC toxicity is relatively new (reviews in Clotfelter, Bell, & Levering, 2004; Zala & Penn, 2004). Animals display a multitude of behavioral aberrations after exposure to natural and synthetic estrogens. In

teleosts, exposure can alter social, reproductive, and predator-prey behaviors. Estrogen exposure increases non-reproductive social behaviors such as aggression (threespine stickleback; Bell, 2001) and causes aberrant grouping behavior (goldfish (*Carassius auratus*), Saglio & Trijasse, 1998). Not surprisingly, reproductive behaviors have been widely studied. Researchers have found that estrogen exposure induces irregular courtship behavior in threespine stickleback (Bayley, Neilsen, & Baatrup, 1999; Bell, 2001), guppies (Toft & Baatrup, 2003), western mosquito fish (*Gambusia affinis*, Toft & Guillette, 2005), Japanese medaka (Oshima et al., 2003) and goldfish (Bjerselius, Lundstedt-Enkel, Olsen, Mayer, & Dimberg, 2001), and delays in threespine stickleback nest building (Wibe, Rosenqvist, & Jenssen, 2002). In terms of predator-prey interactions, environmental pollutants have been shown to increase risk taking (threespine stickleback, Bell, 2004) and to reduce foraging ability (Japanese medaka, Gormley & Teather, 2003). Significant changes in any of these behaviors may ultimately affect the reproductive success and survivorship of fishes (Zala & Penn, 2004).

## 1.3: Stickleback Natural History

*Apeltes quadracus* (fourspine stickleback) is a member of the stickleback family Gasterosteidae (Wooton, 1984). Species in the Gasterosteidae family are widely studied in behavioral and evolution research---making the fourspine stickleback an ideal choice for this project. As its name indicates, the fourspine stickleback has naked skin (*Apeltes*) -- no plates covering its body -- and it has four dorsal spines (*quadracus*) (Figure 1.1).

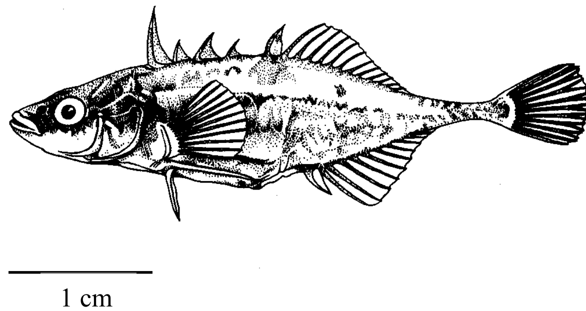


Figure 1.1: Illustration of a female *Apeltes quadracus* from St. Lawrence Estuary, Canada. (Wooton, 1984).

The fourspine stickleback is found predominantly on the Atlantic coast of North America, areas that are densely populated by humans (Figure 1.2). They inhabit estuaries and lagoons characterized by calm waters and subaquatic vegetation (Wooton, 1984). Fourspine sticklebacks have a brown-olive dorsal surface with black patches, a silvery-white ventral surface and they grow to a maximum of 6 cm. They become sexually mature at about 1 year and then breed annually between late March and early August. Generally, the lifespan of a stickleback ranges from 1 to 3 years (Wooton, 1984). Fourspine stickleback are sexually dimorphic in size (males are smaller) and in coloration (Bond, 1968), with male reproductive coloration limited to red pelvic spines. Male coloration and female egg production are modulated by androgens (Borg, 1981) and estrogen respectively, in concert with temperature and photoperiod (Wooton, 1984). The endocrine system of fourspine sticklebacks fits the basic vertebrate structure with the pituitary organ at the core of the system. Males produce androgens in the testes and females produce estrogen and progesterone in their ovaries (Wooton, 1984).

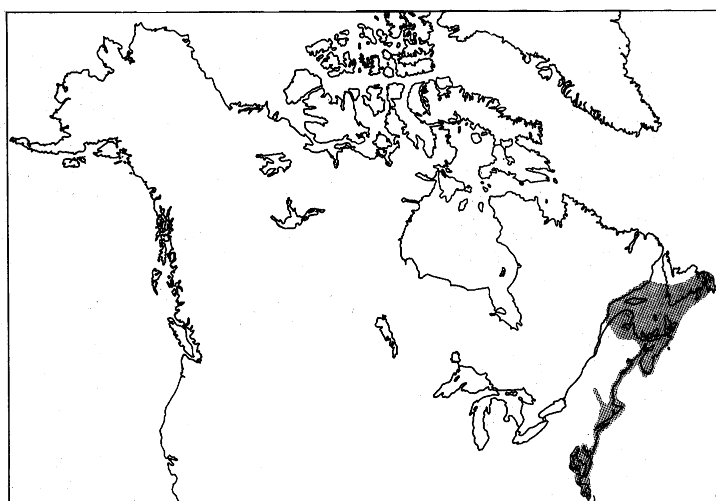

Figure 1.2: Approximate distribution (shaded area) of *Apeltes quadracus* (Wooton, 1984).

Sticklebacks are visual predators (Beukema, 1968) and they forage in loose schools in search of brachiopods and ostracods (Wooton, 1984). Locomotion is produced by a rapid rowing movement of the pectoral fins (Lindsey, 1978). Sticklebacks are a major food source for a number of predators, making them a critical part of the food web in coastal regions (Wooton). Their predators include fish-eating birds (i.e., herons, gulls, terns, kingfishers and ducks) and many species of fishes (i.e., cod, trout and char) (Wooton).

## 1.4: Sticklebacks as Indicator Species of EDC Toxicity

Fourspine sticklebacks are an ideal choice as indicator species to evaluate the effect of EDCs for a number of reasons. In sticklebacks, testosterone and estrogen are responsible for the expression and development of secondary sexual characteristics and behavior of males and females, respectively. As a member of the NYC estuarine community, this species presents the potential to understand the impact of $EE_2$ exposure

on species in an urban coastal area. Moreover, as the endocrine systems of vertebrates are similar, these findings can be used to make informed decisions regarding vertebrates in general (Crews, Willingham, & Skipper, 2000).

Another member of the stickleback family (threespine stickleback) is already in use as a model system in toxicology (Bell, 2001, 2004; Hahlbeck, Griffiths, & Bengtsson, 2004; Hahlbeck & Katsiadaki et al., 2004; Holm, Lundstrom, Andersson, & Norrgren, 1994; Katsiadaki & Scott, 2002; Katsiadaki, Scott, & Mayer, 2002; Rouse, Coppenger, & Barnes, 1977; Wibe, Rosenqvist, & Jenssen 2002). Sticklebacks are an ecologically diverse and therefore a relevant choice as indicator species, serving as a reference point for EDC toxicity in a large part of the northern hemisphere. Much is known about sticklebacks in terms of their morphology and evolution (Bell & Foster, 1994; Wooton, 1984) and their ease of care in the laboratory makes them a desirable choice (Wooton).

Assessing the risks that wildlife populations experience due to exposure to $EE_2$ requires understanding not only physiological and morphological consequences of exposure in males and females but also behavioral changes. Organizational effects of hormones on behavior occur during early development and are permanent while activational effects are temporary and can occur throughout adulthood (Cooke, Hegstrom, Villeneuve, & Breedlove, 1998; Phoenix, Goy, Gerall, & Young, 1959). By using adult fish we were able to assess activational effects that may have resulted in differences in behavior after exposure to $EE_2$. Based on the idea that activational effects of $EE_2$ can directly impact behavior without necessarily changing gonadal state, we investigated if exposure to $EE_2$ could result in behavioral changes without being accompanied by corresponding gonadal abnormalities.

Three experiments were conducted on fourspine sticklebacks to assess how levels of $EE_2$ that may occur in their natural environment (10 ng/L, 70 ng/L and 100 ng/L) affect behavior. Males and females were used in all three behavioral experiments to explore possible sex differences in the behavioral effects of exposure to $EE_2$ (Burger, Fossi, McClellan-Green, & Orlando, 2007). These experiments were designed to investigate if exposure to $EE_2$ alters activity, aggression, and predator avoidance behaviors and, if behavioral changes occurred, if they persisted after depuration in clean water. This is of particular importance since little is known about the recovery from $EE_2$ exposure in adult wildlife. To determine if behavioral changes were long lasting, the fish were tested in each behavioral experiment at two time points: 1) immediately after a 60-day exposure (Toft & Baatrup, 2001) to one of 3 concentrations of $EE_2$ and then again 2) after a 100 day depuration in clean water. This testing regime represents the kind of variability in $EE_2$ exposure a fish may experience due to natural variation in the temporal and spatial distribution of effluent plumes in their environment (Martinovic, Hogarth, Jones, & Sorensen, 2007).

We predicted that fish exposed to $EE_2$ would demonstrate: 1) higher overall activity, 2) impaired predation avoidance, and 3) less aggression after exposure to $EE_2$. These predictions were based on evidence that exposure to $EE_2$ increases activity and decreases predator avoidance in adult fish exposed to $EE_2$ from fertilization to adulthood (Bell, 2004). Exposure to $EE_2$ may increase activity levels and predator avoidance indirectly due to the positive effect of gonadal steroids on fish growth rates (Donaldson & Hunter, 1981). Fish with higher growth rates would presumably have higher food requirements making it necessary for the fish to spend more time actively searching for

food. Likewise, the higher food requirements may outweigh the risk involved in searching for food resulting in a decrease in predator avoidance. Research also indicates that exposure to $EE_2$ decreases aggression in male fish (Bell, 2001). $EE_2$ exposure could cause down regulation of androgen production or impair androgen function resulting in decreased frequency of androgen related behaviors like aggression. In addition, we predicted that depuration would eliminate the behavioral changes caused by $EE_2$ exposure.

To describe the relationships between behavior and underlying physiological changes, males were evaluated for reproductive coloration, and gonads from both males and females were described histologically. We predicted that males exposed to $EE_2$ would express lower levels of red reproductive coloration. Given that reproductive coloration is modulated by androgens, any interruptions in androgen function mediated by $EE_2$ at the physiological level could ultimately alter coloration (Wai & Hoar, 1963). Two predictions regarding the effect of $EE_2$ on the gonads of fish were generated based on two hypotheses. First, these adult fish were past the organizational phase of development, when permanent anatomical changes from EDC exposure can occur (Phoenix, Goy, Gerall, & Young, 1959). Exposure during adulthood when the endocrine system is mature may mitigate any detrimental effects of EDC exposure (Damstra, Barlow, Bergman, Kavlock, & Van der Kraak, 2002). Second, the fish were kept at a temperature and photoperiod representative of the reproductive season (summer). In females, summer photoperiod and temperatures stimulate the ripening of ovaries but have the opposite effect on sperm production which is signaled by winter photoperiod and temperature. As a result, we predicted that: 1) adult male gonads would be resistant to

anatomical changes mediated by exposure to $EE_2$ (they would not have ovotestes), and 2) male gonads would be quiescent and female gonads would be ripe. These anatomical measures would also help to inform any behavioral changes resulting from exposure and from depuration. Since the fish were exposed as adults we would expect that the behavioral effects would be activational rather than organizational and that the fish would recover normal behaviors and gonads after depuration. Activationally induced factors are temporary and reversible changes caused by exposures that occur after critical periods of development. Organizationally induced factors are permanent changes caused by exposure during critical periods of development. Any long-lasting changes in behavior and gonad state would indicate that even adults can experience permanent physiological and anatomical changes in response to EDCs.

## 2.0: General Methods & $EE_2$ Treatments

Animal Care:

Fourspine sticklebacks, *A. quadracus,* were caught with seine nets in May of 2005 and 2006 from Moriches Bay in eastern Long Island, NY (Figure 2.1). They were held in 37.9 L home tanks (50.8 x 25.4 x 30.5 cm), 15 fish per tank, until they were separated and $EE_2$ exposures began. Tanks were filled with 35 liters of artificial sea water (Instant Ocean™), fitted with sponge filters and gravel, and kept at 28 ppt salinity at a temperature of 20±2 °C. Fish were fed daily with frozen *Artemia.* Once a week, water and waste were aspirated from the substrate and tanks were replenished with fresh artificial sea water.

Figure 2.1: Map of area *A. quadracus* collection site (Long Island, NY). Circle indicates actual collection site.

Ten days before experimental trials began, each treatment tank was fitted with a housing unit with individual dividers to separate and identify each fish. The housing

units were constructed with black plastic mesh (75% open area) and fastened with cable ties (Figure 2.2). Each fish was randomly numbered and assigned to a compartment in the basket, minimizing any bias during data analysis since fish number and level of $EE_2$ exposure were not related.

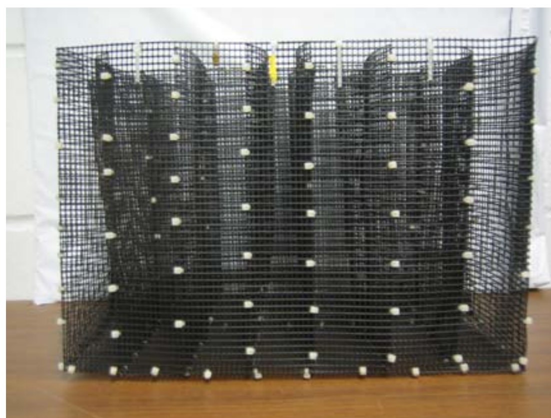

Figure 2.2: Compartmentalized housing unit used to separate and identify fish

Environmental Parameters:

Fish were housed in a Brooklyn College laboratory and maintained on a summer photoperiod (16L/8D) at a temperature of 20 ± 2 °C. These conditions are similar to those used in previous studies with threespine sticklebacks (Bell 2001, 2004). Environmental parameters remained the same as above during 1) $EE_2$ exposure and 2) depuration in clean water.

Ethinyl Estradiol Stock Solution:

A stock solution of $EE_2$ (17α-ethinyl estradiol) was prepared by combining 2 mg of a powdered form of $EE_2$ (Fisher Scientific ™) and 200 ml of 100% ethanol and mixing it with a magnetic stirrer for three days, following the methods of Bell (2001). This produced a concentration of 10 ng of $EE_2$ for every µL of stock solution. *Note:* Bell

(2004) found that sponge filters did not remove $EE_2$ from the water and that the concentrations of $EE_2$ changed only very slightly over time

Project Timeline:

Due to constraints on the restrictions of obtaining wild-caught fish and the time-intensive behavioral measurements, we conducted the experiments in two phases. Series 1 of this project was a pilot study and started in May of 2005. Series 2 started in May of 2006. Series 1 included behavioral data for activity and aggression at post-depuration only (pilot). Series 2 included behavioral data for activity, aggression and predator avoidance at both testing periods, post-exposure and post-depuration. The total number of trials for each behavioral measure is as follows: 177 activity trials, 158 aggression trials and 132 predator avoidance trials. All trials totaled to 621 hours of video for behavioral analysis.

$EE_2$ Treatment Groups:

In this experiment we used 2 controls and 5 different concentrations of $EE_2$: 1) a control (0 ng/L $EE_2$), 2) a solvent control (0 ng/L $EE_2$), 3) a 10 ng/L concentration of $EE_2$, 4) a 70 ng/L concentration of $EE_2$, and 5) a 100 ng/L concentration of $EE_2$. These concentrations of $EE_2$ were selected to reflect the levels of $EE_2$ that are found in the environment (see Chapter 1.2 for details). The solvent control tank was spiked with 100% ethanol, the solvent used to dissolve $EE_2$. This was done to distinguish between any changes in the fish's behavior due to exposure to the solvent or to the $EE_2$. To produce the necessary concentrations of $EE_2$ in each experimental treatment tank, the appropriate volumes of $EE_2$ stock solution, solvent and/or salt water were added to the tanks filled with 35 L (Series 1) or 10 L (Series 2) of artificial seawater (see Tables 2.1

and 2.2 for details). To restore the necessary concentrations of $EE_2$ after removing $H_2O$ for cleaning purposes, the appropriate amount of $EE_2$ stock solution, solvent, or sea water was replenished. Water samples were collected, frozen and tested with an $EE_2$ ELISA kit (Ecologiena™, Abraxis) to verify the concentration of $EE_2$ in all treatment tanks (see Chapter 5 for details).

Experimental note: The 70 and 100 ng/L concentrations of $EE_2$ may be at the high end of the scale for levels found in the environment. However, these levels may be representative of the total estrogenic load (ethinyl estradiol, estriol, 17β-estradiol, estrone, and estranol) that sewage treatment plants discharge into waterways (Kolpin et al., 2002). In addition, it was anticipated that the nominal concentrations would be lower.

| $EE_2$ Exposure Level | $EE_2$ Stock Solution Initial Amount | $EE_2$ Stock Solution Replenish Amount | Solvent[1] Salt Water[2] Initial Amount | Solvent[1] Salt Water[2] Replenish Amount |
|---|---|---|---|---|
| 0 ng/L | ----- | ----- | 350 µL[1] | 100 µL[1] |
| 0 ng/L (solvent control) | ----- | ----- | 350 µL[2] | 100 µL[2] |
| 10 ng/L | 35 µL | 10 µL | ----- | ----- |
| 70 ng/L | 245 µL | 70 µL | ----- | ----- |
| 100 ng/L | 350 µL | 100 µL | ----- | ----- |

Table 2.1: Tank spiking regime for $EE_2$ Series 1(35 L Tanks). The replenish amount was based on removing 10 L of water for cleaning purposes. [1]= solvent, [2]=salt water.

| $EE_2$ Exposure Level | $EE_2$ Stock Solution Initial Amount | $EE_2$ Stock Solution Replenish Amount | Solvent[1] Salt Water[2] Initial Amount | Solvent[1] Salt Water[2] Replenish Amount |
|---|---|---|---|---|
| 0 ng/L | ----- | ----- | 150 µL[1] | 42.5 µL[1] |
| 0 ng/L (solvent control) | ----- | ----- | 150 µL[2] | 42.5 µL[2] |
| 10 ng/L | 15 µL | 4.25 µL | ----- | ----- |
| 70 ng/L | 105 µL | 29.75 µL | ----- | ----- |
| 100 ng/L | 150 µL | 42.5 µL | ----- | ----- |

Table 2.2: Tank spiking regime for $EE_2$ Series 2 (15 L Tanks). The replenish amount was based on removing 4.25 L of water for cleaning purposes. [1]= solvent, [2]=salt water.

Baseline "wild fish" Behavior:

To compare potential differences in behavior between fish kept under laboratory conditions and behaviors in wild fish, a group of wild-caught fish (N=19) was tested soon after capture on all three behavioral measures: activity, aggression and predator avoidance. These data served as a baseline of what would be expected from fish that had not been held in captivity for a prolonged period of time.

Replicate Tanks:

All experiments in Series 2 of this project were conducted using replicate tanks to determine if there were any effects specific to a particular tank. Fish in each of the five treatment groups were kept in two exposure tanks, with 6-10 fish in each replicate tank. Replicates of each exposure-level tank were used to reduce possible pseudoreplication and to strengthen the likelihood that differences found between treatments were not related to the conditions of a particular tank other than the level of $EE_2$.

Depuration:

Depuration occurred after the 60-day exposure to $EE_2$ and after post-exposure behaviors were measured. Depuration was carried out by moving fish from the $EE_2$ exposure tanks to clean tanks of the same size equipped with sponge filters and filled with artificial sea water. No $EE_2$ was introduced into the tanks during the depuration period of 100 days. Animal care, feeding, lighting regime, water changes, and other environmental parameters remained the same as during the exposure period.

Behavioral Experiment Room and Testing Tanks:

All of the experiments were conducted in a small, windowless experiment room in a Brooklyn College laboratory. The room was equipped with 4-18.9 L behavioral testing tanks (40.6 x 20.3 x 25.4 cm) that were filled with 15 L of artificial sea water and outfitted with sponge filters and gravel. Water quality in these tanks was identical to the home tanks but did not contain $EE_2$ or solvent. The salinity and temperature remained the same in home and testing tanks.

General Behavioral Testing Protocol:

Each fish was tested for all behavioral measures (activity [Chapter 4], aggression [Chapter 5], and predator avoidance [Chapter 6]) at two points in time, right after 60-d $EE_2$ exposure (post-exposure), and immediately following the 100-d depuration (post-depuration) – a mixed design (both within-subject and between-group factors). Behavioral testing protocols for each behavior were identical for both testing periods. Figures of testing arenas are illustrated in the corresponding chapters. Testing order of the animals was random and included all treatment groups.

All behavioral experiments were recorded with Sony digital cameras (DCR-VX1000) positioned 50 cm in front of each of two tanks to enable accurate off-line analysis of the target behaviors. Two fish were tested in the separate tanks, at the same time, to accommodate time constraints. Data were analyzed frame by frame with a digital video recorder (Sony-DHR 1000) and monitor (Sony-HR Trinitron). Inter-observer reliability was confirmed using two observers who were blind to treatment.

Experiment Timeline:

At 5 PM the day before behavioral testing started, 4 fish were removed from their home tanks and each placed into one of the 4 experimental tanks (A, B, C, and D) allowing the fish to adjust to the experiment tank overnight. The next morning, Day 1 of the experiments, activity tests were performed with fish in tanks A and B from 10 am to 1 pm. Subsequently, aggression tests (with either the male or female intruder) were performed on the fish in all four tanks (A-D) from 1-2 pm. On Day 2, the same timeline was followed for the activity tests for fish in tanks C and D, and the aggression test (with either the male or female intruder) for fish in tanks A-D. The sex of the intruder fish was alternated so that each fish was tested against both a male and a female intruder. After all fish were tested for activity and aggression on days 1 and 2, all fish were tested for predator avoidance on Day 3. At the end of Day 3, all fish were returned to their home tanks. All of the experimental tanks were cleaned and new artificial salt water was added.

Fish Mortality:

Mortality occurred in all fish groups probably due to non-treatment factors such as age and undetermined disease. $EE_2$ treatment level did not appear to be a factor in

mortality as it was not significantly different among the $EE_2$ concentration levels. The number of fish that died in each $EE_2$ treatment level was as follows: control=2, 10 ng/L $EE_2$=2, 70 ng/L $EE_2$=2, 100 ng/L $EE_2$=1. The difference in the number of fish in each $EE_2$ exposure level was due to two factors. First, to compensate for potential loss at the highest level of $EE_2$, there were greater numbers of fish in the 100 ng/L $EE_2$ group compared to intermediate levels. Second, the need to include 2 control groups (regular control and solvent control) boosted the total number of control fish. With similar numbers of non-treatment mortalities across all animals, the 10 and 70 ng/L groups were disproportionately affected. The within-subject design of this study with set exposure and depuration periods and timed behavioral tests prevented fish replacement after each mortality event occurred.

Statistical Analysis:

Data were entered into SPSS (version 11.5) for statistical analysis. Gonad categories were analyzed with Kruskall-Wallis and Mann-Whiney U since they were ranked. Aggression and activity data were analyzed using parametric tests (Pearson's Coefficient, and mixed ANOVAs). For predator avoidance data, non-parametric Mann-Whitney U Tests and Wilcoxon Signed Ranks Test were used to analyze the responses to attack while Fisher's Exact Probability Test was used to track the probability of approaching a food cup in the presence of a predator. The remaining behaviors in the predator avoidance experiment were analyzed using parametric paired t-tests and independent t-tests. An alpha level of 0.05 was used for all statistical tests.

## 2.1: $EE_2$ Water Testing

To quantify the actual amount of $EE_2$ in the treatment tanks, water samples were collected from each tank once a week for testing. The samples were collected before the weekly water change during the 60-day exposures for Series 1 and 2. A water sample from the fish-collection site in the wild was also obtained. Samples were labeled and frozen until the end of the exposure periods and tested with an $EE_2$ ELISA (Ecologiena®, Japan EnviroChemicals, Ltd., Abraxis).

As expected, actual $EE_2$ concentrations were lower than the nominal concentrations. The ELISA had a lower detection limit of 50 ng/L. The level of $EE_2$ in both of the control tanks, the 10 ng/L $EE_2$, tanks and the water sample from the collection site were undetectable. The average actual concentration of samples from the 70 ng/L $EE_2$ tanks was 35 ng/L of $EE_2$ (range = 26.29-64.77, $SE$ = 3.17), 50% of its nominal concentration. The average actual levels of $EE_2$ in the 100 ng/L $EE_2$ tanks was 46.5 ng/L (range = 38.42-75.065, $SE$ = 2.82), 46.5% of its nominal concentration.

The decrease in $EE_2$ nominal concentrations to measured concentrations is universal in toxicant studies using fish. Researchers using a variety of techniques to verify $EE_2$ concentrations report levels that range from 14-15% (gas chromatography-mass spectrometry, Hahlbeck et al., 2004), 10-20% (liquid chromatography/mass spectrometry, Peters, Courtenay, Cagampan, Hewitt, & MacLatchy, 2007), 58-84% (radioimmune assay/liquid scintillation counting data, Lange et al., 2001) and 82-86% (liquid chromatography/mass spectrometry, Andersson, Katsiadaki, Lundstedt-Enkel, & Orberg, 2007) of the nominal $EE_2$ concentrations used in their work. The measured levels of 50% and 46.5% in this study are in accordance with the research in this field.

The general decline in $EE_2$ levels can perhaps be attributed to the uptake of $EE_2$ by the fish, adsorption to the tank, and biological degradation throughout the exposure period.

## 3.0 Introduction: Gonad Histology

Fish used in this study were considered sexually mature based on their size (females) and the presence of red pelvic fins (males) (Wooton, 1976). Histological examinations of both male and female gonads were conducted to determine: 1) if $EE_2$ exposure caused gonadal anomalies, 2) if these effects were dose dependent (different $EE_2$ exposure levels), and 3) if the gonadal changes persisted after depuration. Fish were sacrificed from each treatment group at two points in time, right after 60 days exposure to $EE_2$, and after 100 days of depuration (these time periods reflect the behavioral testing periods). Acquiring samples from the two testing periods made it possible to determine if the gonads can 'recover' from the $EE_2$ exposure if, indeed, there is an exposure effect.

In male sticklebacks, there is a negative correlation between spermatogenesis and high levels of circulating androgens (Andersson, Mayer, & Borg, 1988; Borg, 1981). Spermatogenesis is quiescent during the breeding season. Once spermatogenesis is complete, the testes contain only spermatozoa (Borg & Mayer, 1995; Craig-Bennett, 1931). During the breeding season androgen levels are much higher than in the non-breeding season (Andersson, Mayer, & Borg). This pattern is in contrast to the mechanisms by which androgens affect spermatogenesis in many other fish species (see review in Schulz & Miura, 2002). Under long photoperiods, representative of the breeding season, testes in threespine stickleback lack spermatocytes and spermatids (Bornestaf, Antonopoulou, Mayer & Borg, 1997). If $EE_2$ inhibits androgen function in fourspine sticklebacks, fish exposed to $EE_2$ will have show signs of active spermatogenesis (an abundance of spermatids/spermatozoa but few

spermatocytes/spermatogonia) and controls should possess quiescent testis (the converse of above -- predominantly spermatogonia).

Oogenesis in female sticklebacks follows the general teleost pattern (Scott, 1979). Female fecundity is a function of body size which is in turn related to food availability (Wooton, 1976). Several clutches of eggs are released during each breeding season (spring - summer) (Wooton). Oocytes develop in synchrony -- with batches of oocytes developing together to form clutches of eggs (Wallace & Selman, 1979; Wooton, 1984). However, different stages of oogenesis can be found everywhere in the ovary and are not restricted to particular areas (Wallace & Selman). Previous work on the effects of $EE_2$ on oogenesis in teleosts revealed that gonadal somatic index (GSI, ratio of gonad weight and whole body weight) and number of spawned eggs is reduced at exposure levels of 10-100 ng/L (medaka, Scholz & Gutzeit, 2000; mummichogs (*Fundulus heteroclitus*), Peters, Courtenay, Cagampan, Hewitt, & MacLatchy, 2007). If $EE_2$ inhibits the maturation of eggs at 10-100 ng/L of $EE_2$, the histological analysis of gonads here should reveal mature eggs in the controls and only oogonia and oocytes in the $EE_2$ treatment groups (10, 70 and 100 ng/L).

## 3. 1 Methods: Gonad Histology

Males and females from each $EE_2$ exposure level and testing period were sampled for histological examination. Since our animal numbers were restricted, we chose to sample more animals immediately post-exposure to discover any abnormalities directly resulting from $EE_2$ exposure, as that has not been described in this animal to date. Males examined from the post-exposure testing period included 3 controls, 2 10ng/L, 1 70ng/L

and 4 100/ng/L. Males examined from the post-depuration testing period included 2 controls, 2 70ng/L, 2 10ng/L and 2 100ng/L. Females examined from the post-exposure testing period included 4 controls, 4 10 ng/L, 4 70 ng/L and 4 100ng/L. Females examined at the post-depuration testing period included 2 controls, 2 10 ng/L, 2 70 ng/L, and 2 100 ng/L. To reduce the number of fish sacrificed, the limited number of fish that were used for histology was skewed to highlight the extreme levels of $EE_2$ (control and 100 ng/L of $EE_2$) used in this study. Two groups of fish (N=32; N=11) were exposed and sacrificed for histology (May 2007 and July 2008). Both groups of fish were used in behavioral experiments as controls.

Total length and weight of each fish was measured and then they were sacrificed by decapitation. Gonads and livers were excised and weighed to calculate the GSI and hepatosomatic index (HSI, ratio of liver weight and whole body weight), respectively. After weighing, gonads were fixed for 24 hours in Bouin's fluid and then transferred to 70% ethanol.

Gonads were prepared for histological sectioning with a stepwise method of dehydration in ethanol and butanol and then they were embedded in PolyFin paraffin (Polysciences, Inc.) (Pressnell & Schreibman, 1997). Eight-µm longitudinal sections of testes and ovaries were cut using a microtome and every fifth section was stained with hematoxylin and eosin. All stained sections of ovaries and testes were microscopically examined and graded using the categories described below. Observers blind to treatment also inspected 10% of the gonad sections to check for inter-observer reliability using an Olympus DP71 microscope digital camera. 100% of randomly sampled sections that were scored by unbiased observers concurred with original measurements by K.K.

Testes were evaluated using the following criteria (Sheahan et al., 1994):

Category: 1= spermatogonia predominate

2= spermatocytes and spermatids predominate

3= spermatids and spermatozoa predominate

Ovaries were evaluated using the following criteria (adapted from Sheahan et al., 1994):

Category: 1=small oogonia predominant

2=oocytes without oil droplets predominant

3=oocytes with oil droplets predominant

4=mature ova, yolky oocytes, present

## 3. 2 Results: Gonad Histology

Photos of gonadal sections representing the various testis and ovary categories found in both testing periods are depicted in Figures 3.1-3.13. The features of the gonads from the two groups sampled for histology were not different from each other.

Males:

Post-Exposure: Fish sacrificed post-exposure did not exhibit significantly different stages of spermatogenesis among controls and $EE_2$ treatment groups (Kruskall-Wallis, $H$ (3, $N$ = 10) = 3.57, $p$ = 0.31) (Table 3.1).

| $EE_2$ Exposure | Category | Ovotestis |
|---|---|---|
| 0 ng/L | 1 | no |
| 0 ng/L | 1 | no |
| 0 ng/L | 3 | no |
| 10 ng/L | 3 | yes |
| 10 ng/L | 3 | no |
| 70 ng/L | * | - |
| 70 ng/L | 3 | no |
| 100 ng/L | 1 | yes |
| 100 ng/L | 2 | yes |
| 100 ng/L | 2 | no |
| 100 ng/L | 3 | no |

Table 3.1: Male Gonad Categories - Post-Exposure. *70 ng/L fish died before the sampling date and was not included in histological examination. Control N=3, 10 ng/L N=2, 70 ng/L N=1, 100 ng/L N=4, total N = 10.

Two controls scored a category 1 (spermatogonia predominate) (Figure 3.1) and the third fish scored a category 3 (spermatids and spermatozoa predominate) (Figure 3.2).

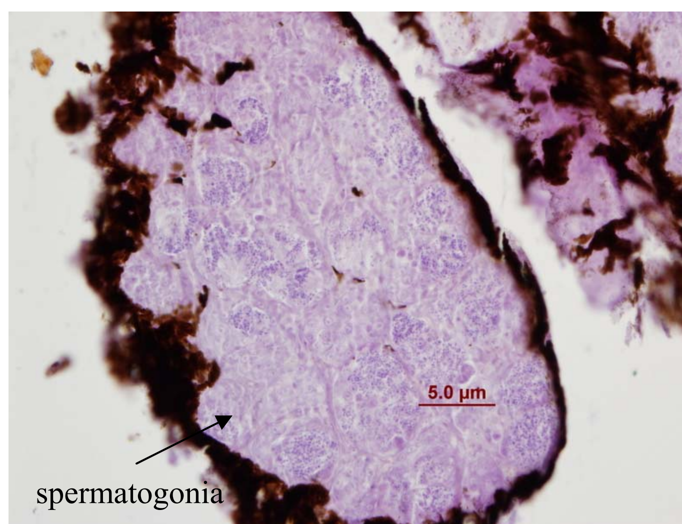


Figure 3.1: Control Male, Post-Exposure, representative category 1 testis, magnification 400x.

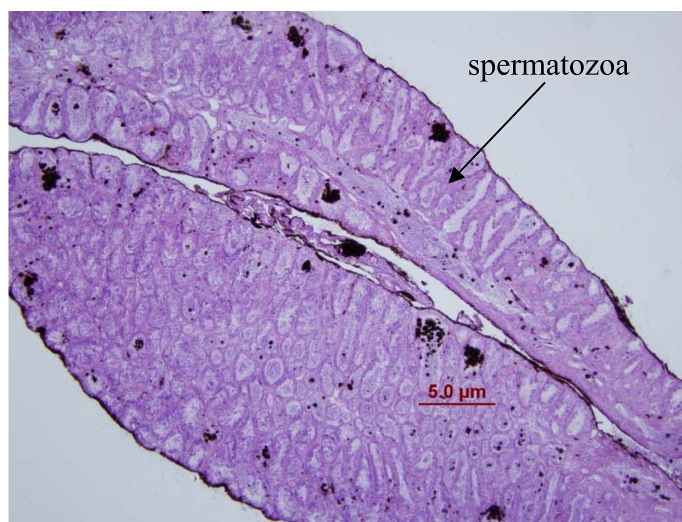


Figure 3.2: Control Male, Post-Exposure, representative category 3 testis. magnification 400x.

Both 10 ng/L treated fish (Figure 3.3) and the 70 ng/L treated fish scored a category 3. One of the 100 ng/L treated fish scored a category 1, 2 scored a category 2 and one scored a 3. Three fish sampled post-exposure developed ovotestis -- one was exposed to 10 ng/L (Figure 3.3) and the two others were exposed to 100 ng/L of $EE_2$ (Figures 3.4, 3.5).

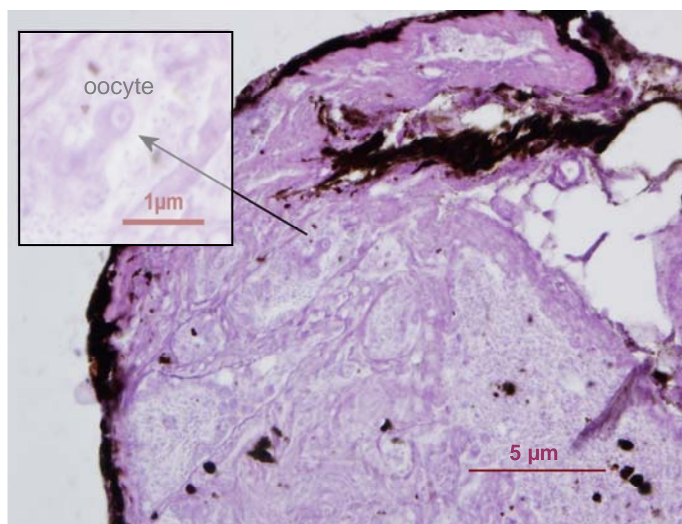


Figure 3.3: 10ng/L Male with ovotestis, Post-Exposure, category 3 with close-up of oogonia in testis, magnification 400x.

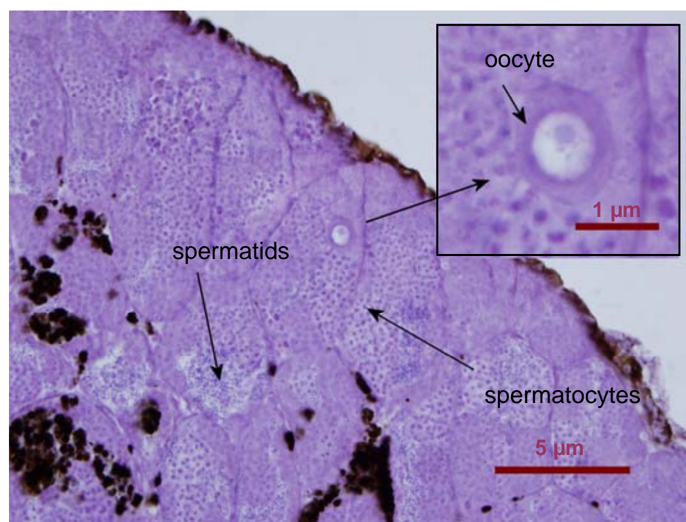


Figure 3.4: 100 ng/L Male with ovotestis, Post-Exposure, category 2 with close-up of oogonia in testis, magnification 400x.

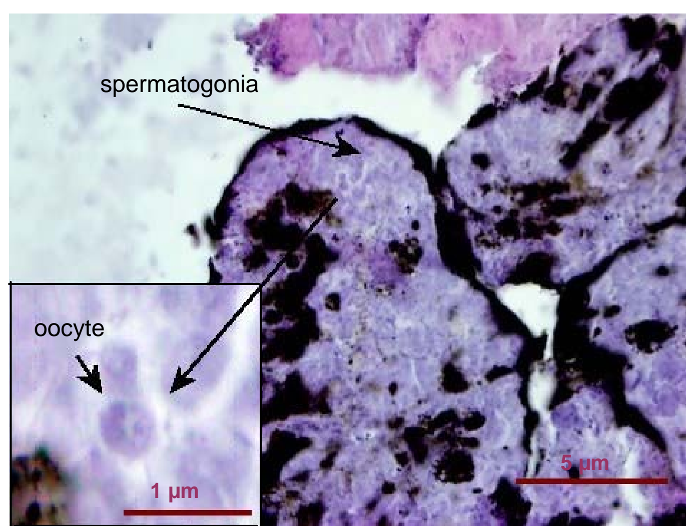


Figure 3.5: 100 ng/L Male with ovotestis, Post-Exposure, category 1 with close-up of oogonia in testis, magnification 400x.

Neither GSI (one-way ANOVA, $F(3, 9) = 1.33$, $p = 0.35$) nor HSI (one-way ANOVA, $F(3, 9) = 0.40$, $p = 0.76$) were significantly different among $EE_2$ levels.

Post-Depuration: Fish sacrificed post-depuration did not exhibit significantly different stages of spermatogenesis among controls and $EE_2$ treatment groups (Kruskall-Wallis, $H$ (3, $N$ = 8), $p$ = 0.11) (Table 3.2).

| $EE_2$ Exposure | Category | Ovotestis |
|---|---|---|
| 0 ng/L | 1 | no |
| 0 ng/L | 1 | no |
| 10 ng/L | 2 | no |
| 10 ng/L | 2 | no |
| 70 ng/L | 2 | yes |
| 70 ng/L | 2 | no |
| 100 ng/L | 2 | no |
| 100 ng/L | 3 | no |

Table 3.2: Male Gonad Categories - Post-Depuration. Control N=2, 10 ng/L N=2, 70 ng/L N=2, 100 ng/L N=2, total N = 8.

Both of the controls scored a category 1 (Figure 3.6) and all of the 10 (Figure 3.7) and 70 ng/L males scored a category 2.

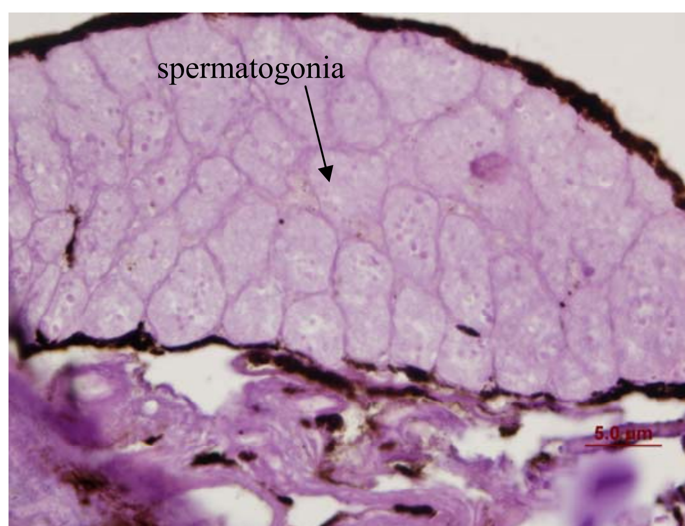


Figure 3.6: Control Male, Post Depuration, representative category 1 testis, magnification 600x.

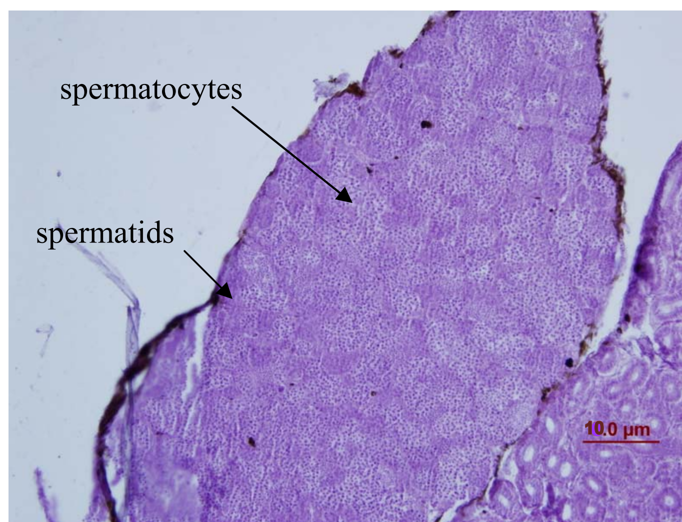


Figure 3.7: 10ng/L Male, Post Depuration, representative category 2 testis, magnification 400x.

Ovotestis was evident in one fish, a 70 ng/L male, at the post-depuration testing period (Figure 3.8).

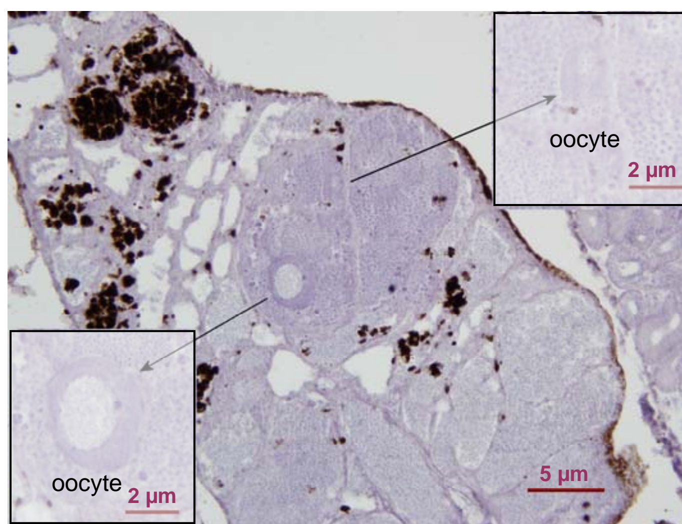


Figure 3.8: 70ng/L Male with ovotestis, Post Depuration, category 2 with close-up of oogonia in testis, magnification 400x.

One of the 100 ng/L treated fish was categorized as 2 and the other 3 (Figure 3.9).

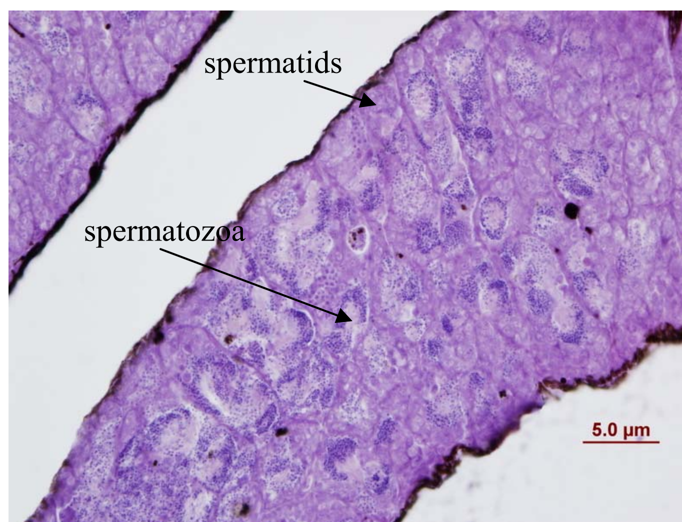


Figure 3.9: 100 ng/L Male, Post Depuration representative category 3 testis, magnification 400x.

GSI was significantly different among $EE_2$ levels (one-way ANOVA, $F(3,7) = 11.80$, $p = 0.02$). Fish exposed to 100 ng/L of $EE_2$ ($M = 0.0019$) had a GSI significantly smaller than the control ($M = 0.0040$, $p = 0.03$), 10ng/L ($M = 0.0038$, $p = 0.04$) and 70 ng/L ($M = 0.0041$, $p = 0.02$) of $EE_2$ levels (post hoc Tukey). HSI was not significantly different among controls and other $EE_2$ levels $F(3,7) = 0.12$, $p = 0.90$.

Females:

Post-Exposure: Fish sacrificed post-exposure exhibited significantly different stages of oogenesis among the control and $EE_2$ exposure groups (Kruskall-Wallis, $H$ (3, $N$ = 16 (4 in each $EE_2$ level)) = 8.80, $p = 0.03$) (Table 3.3).

| $EE_2$ Exposure | Category |
|---|---|
| 0 ng/L | 4 |
| 0 ng/L | 4 |
| 0 ng/L | 4 |
| 0 ng/L | 4 |
| 10 ng/L | 4 |
| 10 ng/L | 4 |
| 10 ng/L | 4 |
| 10 ng/L | 4 |
| 70 ng/L | 2 |
| 70 ng/L | 2 |
| 70 ng/L | 3 |
| 70 ng/L | 4 |
| 100 ng/L | 2 |
| 100 ng/L | 2 |
| 100 ng/L | 2 |
| 100 ng/L | 4 |

Table 3.3: Female Gonad Categories - Post-Exposure. Control N=4, 10 ng/L N=4, 70 ng/L N=4, 100 ng/L N=4

The gonadal states of the 70 ng/L treated fish had ranks that were significantly different than the control ($p = 0.046$) and the 10 ng/L treated fish ($p = 0.046$). Gonads of the 100 ng/L treated fish had ranks that were significantly different from the control ($p = 0.04$) and the 10 ng/L treated fish ($p = 0.04$). The control, the 10 ng/L, and one of the 70 ng/L $EE_2$ exposed fish all had mature eggs, category 4 (Figure 3.10).

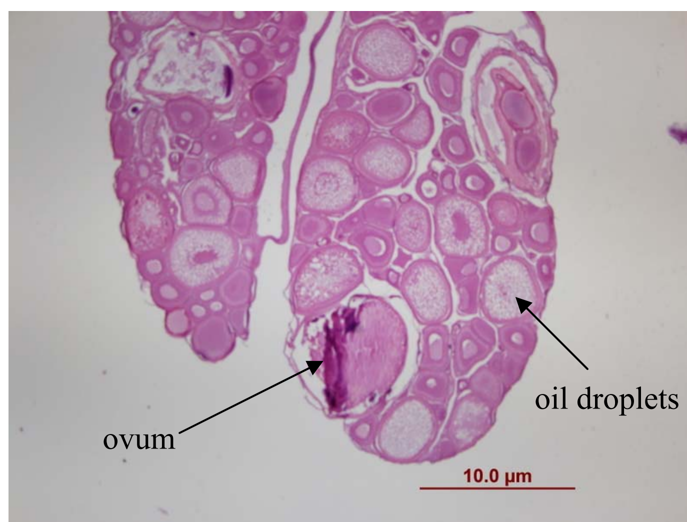


Figure 3.10: Control Female, Post Exposure representative category 4 ovary, magnification 60x.

The remaining 70 ng/l fish and both of the 100 ng/L females had oocytes without oil droplets, category 2 (Figure 3.11).

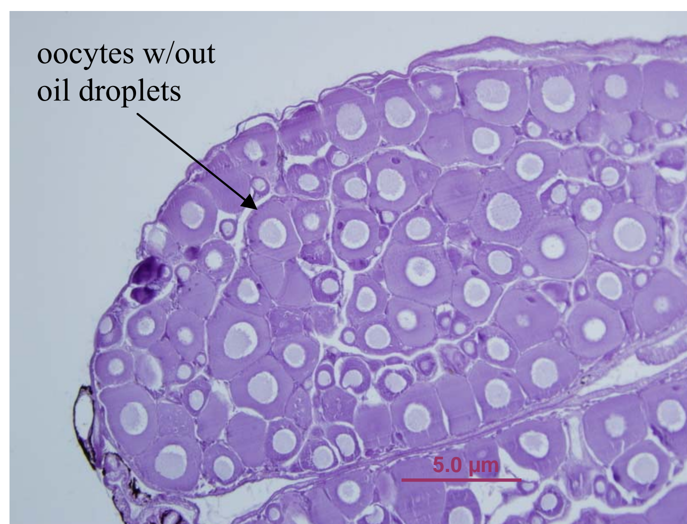


Figure 3.11: 100ng/L Female, Post Exposure, representative category 2 ovary, magnification 100x.

GSI was significantly different among the control and $EE_2$ treatment groups (one-way ANOVA $F(3, 15) = 3.69$, $p = 0.04$). The 10 ng/L treated fish ($M = 0.11$) had a higher GSI than the 100 ng/L treated fish ($M = 0.01$) (post-hoc Tukey, $p = 0.04$). The other treatment groups were not significantly different from each other. HSI was not significantly different among $EE_2$ levels (one-way ANOVA $F(3, 15) = 2.70$, $p = 0.09$).

Post-Depuration: Fish sacrificed post-depuration did not exhibit significantly different stages of oogenesis among controls and $EE_2$ treatment groups (Kruskall-Wallis, $H$ (3), $p = 0.07$) (Table 3.4).

| **$EE_2$ Exposure** | **Category** |
|---|---|
| 0 ng/L | 2 |
| 0 ng/L | 2 |
| 10 ng/L | 2 |
| 10 ng/L | 2 |
| 70 ng/L | 4 |
| 70 ng/L | 4 |
| 100 ng/L | 2 |
| 100 ng/L | 2 |

Table 3.4: Female Gonad Categories - Post-Depuration. Control N=2, 10 ng/L N=2, 70 ng/L N=2, 100 ng/L N=2

The controls and the fish exposed to 10 ng/L and 100 ng/L of $EE_2$ sampled post-depuration lacked mature ova. The ovaries contained only oocytes without oil droplets, category 2 (Figure 3.12).

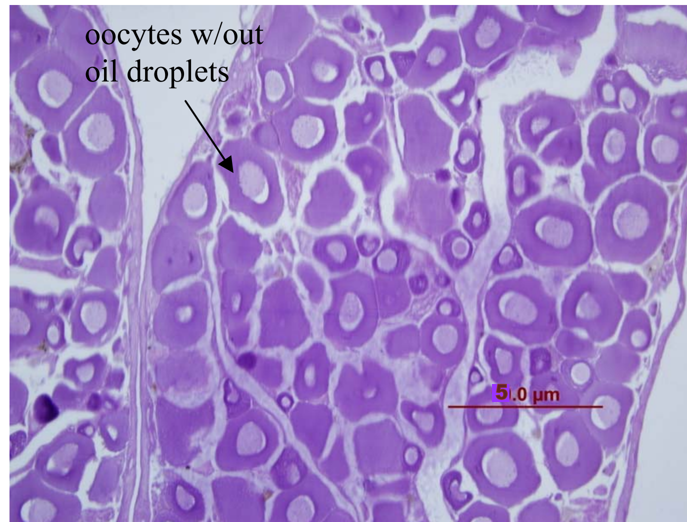


Figure 3.12: Control Female, Post Depuration, representative category 2 ovary, magnification 100x.

Only the fish exposed to 70 ng/L had mature ova, category 4 (Figure 3.13).

Figure 3.13: 70ng/L Female, Post Depuration, representative category 4 ovary, magnification 40x.

Neither GSI (one-way ANOVA $F(3, 7) = .92$, $p = 0.51$) nor HSI (one-way ANOVA $F(3, 7) = .81$, $p = 0.55$) were significantly different in these animals.

### 3.3 Discussion: Gonad Histology

These data do not support the prediction that adult sticklebacks are resistant to gonadal changes that $EE_2$ exposure can cause in developing animals. More mature stages of spermatogenesis were represented in males exposed to lower levels of $EE_2$ and earlier stages of spermatogenesis were represented in males exposed to higher levels of $EE_2$. Nielson and Baatrup (2006) also reported this result after exposing guppies to similar concentrations of estradiol and $EE_2$. In EDC research, this occurrence of low-dose stimulation and high-dose inhibition with specific quantitative and temporal characteristics is termed hormesis (Calabrese & Baldwin, 2002). Hormesis is also used to describe does-response relationships (biphasic, non-monotonic, U-shaped, and inverted U-shape; Southam & Erhlich, 1943).

In males, 4 of the 5 controls (testing periods combined) possessed testis with only spermatogonia (category 1), indicative of the quiescent stage of spermatogenesis in sticklebacks. None of the fish exposed to $EE_2$ at either testing period had quiescent testes without ovotestes. All fish exposed to $EE_2$ developed spermatocytes, spermatids or spermatozoa (categories 2 and 3) except for one of the fish exposed to 100 ng/L. Given that high levels of testosterone inhibit spermatogenesis, this indicates that $EE_2$ may have been inhibiting androgen function. Likewise, since low levels of testosterone are necessary for spermatogenesis to occur, it can be assumed that the controls had higher testosterone levels than the $EE_2$ exposed fish (Andersson, Mayer & Borg, 1988; Borg,

1981). One possible mechanism for this could be that $EE_2$ acts antagonistically and blocks or reduces androgen production or function. While it is unknown if this occurs in fish, in rats 17b-estradiol will bind to androgen receptors but with a much lower affinity than testosterone (Michel & Baulieu, 1980).

After 100 days of depuration, all males originally treated with 10 and 70 ng/L of $EE_2$ and one of the fish treated with 100 ng/L contained secondary spermatocytes and spermatotids (category = 2) in their testes. This difference in gonad category when compared to exposed fish can be interpreted as a partial recovery from $EE_2$ exposure. A similar study on adult zebra fish exposed to $EE_2$ reported complete gonad recovery after depuration (Van den Belt, Wester, Van der Ven, Verheyen, & Witters, 2002). However, gonad recovery after depuration does not occur in every case. Adult guppies exposed to 500 ng/L $EE_2$ did not recover after depuration but this could be due to the extremely high level of estradiol used in the study (Toft & Baatrup, 2001).

Ovotestes were found in 4 out of 20 males in the different $EE_2$ treatment groups and testing periods. In fish sampled immediately after exposure, one 10 ng/L and two 100 ng/L treated fish exhibited ovotestes. At post-depuration, only one fish from the 70 ng/L group exhibited ovotestis. Ovotestes have been found in threespine stickleback exposed to 27.7 ng/L of $EE_2$ during the critical time of sexual differentiation (post-hatch to 4 weeks) and then raised in clean water to adulthood (Maunder, Matthiessen, Sumpter, & Pottinger, 2007). Other studies on fish exposed to EDCs resulting in intersex gonads demonstrated that the fish also have different steroid profiles and reduced fertility (Jobling et al., 2002). It is interesting to note that, in our study, fish with ovotestes that were exposed to higher levels of $EE_2$ (70 ng/L and 100 ng/L) did not have spermatozoa

while fish without ovotestes that were exposed to the same levels of $EE_{2,}$ did have spermatozoa. Perhaps the mechanism that triggers the development of ovotestes at higher levels of $EE_2$ exposure also depresses the maturation process of sperm. It was unexpected to find ovotestis in the present study given that fish were exposed as adults only and not during sexual differentiation. No ovotestes were found in any of the controls indicating that it was likely the exposure to $EE_2$ in adulthood that caused the gonadal changes.

A significant increase in GSI is often an indicator of $EE_2$ exposure. Adult male mummichogs exposed to 100 ng/L of $EE_2$ for 28 days showed a significant increase in GSI (Peters, Courtenay, Cagampan, Hewitt, & MacLatchy, 2007). Here, post-exposure GSI was not significantly different among $EE_2$ exposure levels but this was not the case post-depuration. Post-depuration, 100 ng/L males had a significantly smaller GSI compared to all other $EE_2$ exposure levels. The difference in GSI indices between testing periods could be due to the difficulty of excising the very small testis. If the excised tissue included other tissue in addition to the testis or only part of the testis, this would have resulted in a miscalculation of the gonad weights.

In females, $EE_2$ affected oogenesis at the higher levels of $EE_2$ used in this study, which was contrary to our predictions. Controls and fish exposed to the lowest level of $EE_2$, 10 ng/L, had mature eggs. In contrast, one of the fish exposed to 70 ng/L and both of the 100 ng/L fish had oocytes without oil droplets. Similar results were found in fathead minnows exposed for 3 weeks to comparable levels of $EE_2$ (Pawlowski, Aerle, Tyler, & Braunbeck, 2004), medaka after 2 months of exposure to 10 and 100 ng/l of $EE_2$ and after 6 weeks of depuration (Scholtz & Gutzeit, 2000), and mummichogs exposed to

100 ng/L for 4 weeks (Peters, Courtenay, Cagampan, Hewitt, & MacLatchy, 2007). The results here also support the notion that $EE_2$ may inhibit the maturation of fish eggs. One possible explanation is that $EE_2$ has an inhibitory effect on gonadotropin secretion. In fish, estradiol has a negative feedback relationship with gonadotropins, GTHI (Saligaut et al., 1998) and GTHII (Khan, Hawkins, & Thomas, 1999). GTHI is important for vitellogenesis and early ovary development and GHTII stimulates oocyte maturation.

Females examined post-depuration exhibited differences in ovaries in the control and 10 ng/L $EE_2$ groups compared with females exposed to the two higher levels of $EE_2$. Post-exposure, the control and the 10 ng/L treated fish had ovaries containing mature eggs but post-depuration the ovaries lacked mature oocytes and oocytes with oil-droplets. Fish exposed to the higher levels of $EE_2$, 70 ng/L and 100 ng/L, generally remain in the same state from post exposure to post depuration. This outcome implies that perhaps higher levels of $EE_2$ have longer lasting effects, regardless of the nature of the effect (Maunder, Matthiessen, Sumpter, & Pottinger, 2007).

In females, none of the $EE_2$ exposure groups had a significantly different GSI compared to the control. One notable difference post-exposure was the larger GSI for females exposed to 10 ng/L compared to the 100 ng/L. Maunder, Matthiessen, Sumpter, and Pottinger (2007) reported that female threespine stickleback exposed to 27.7 ng/L of $EE_2$ had a significantly higher GSI compared to controls. Though the results here do not conform to Maunder et al., they do point to the possibility of another hormetic relationship between $EE_2$ exposure level and GSI. In this study, females exposed to the lowest level of $EE_2$, 10 ng/L, developed much larger ovaries in relation to their body weight -- indicating stimulation of ovary growth. In contrast, fish exposed to 100 ng/L

had smaller ovaries in relation to their body weight suggesting inhibition of ovary growth. This was not the case for either group post-depuration.

Differences in gonadal state across sampling periods in both males and females was not surprising, given that depuration may allow fish to recover (Gray, Niimi, & Metcalfe, 1999) and that different fish were sampled in each testing period. Studies assessing the reproductive effects of life-long exposure to $EE_2$ on zebrafish (*Danio rerio*) show that depuration does not always reverse the physiological effects of $EE_2$ (Nash et al., 2004; Van den Belt, Verheyen, & Witters, 2003). After a 5 month depuration, zebrafish exposed to 5 ng/L of $EE_2$ from fertilization to 75 days post fertilization had 24% nonviable eggs compared to 7.8% in the controls (Nash et al.). $EE_2$ caused some changes immediately after exposure, but it seems that some of the effects may take time to become evident. By examining animals in the two sampling periods, it was possible to investigate the post-exposure and post-depurational effects of $EE_2$ exposure, though obviously different fish had to be used for each period.

It is not surprising that the trends for spermatogenesis and oogenesis were different from one another given that estrogen is the primary female sex hormone in teleosts. The mechanism at work is not clear except that it most certainly can function on the activational level since exposure (and changes) occurred in adults. For this reason, the incidence of ovotestes in a few of the males exposed to $EE_2$ is all the more surprising. Despite the small number of fish sampled for these descriptions, these data indicate that short-term exposure to $EE_2$ can affect gonadal state in adult male and female sticklebacks. Further targeted studies examining a greater number of fish from known age groups would provide insights into the manner in which $EE_2$ affects spermatogenesis

and oogenesis in these animals. Here these gonadal descriptions served primarily for confirmation of gonadal state and to determine if an overall increase in the incidence of intersex animals arose from exposure to $EE_2$. The data presented here support that these changes are indeed likely after exposure to $EE_2$.

### 3.4 Introduction: Reproductive Coloration in Males

In sticklebacks, reproductive coloration of males is thought to be an honest signal of energy investment in body condition (Frischknecht, 1993) and is positively correlated with male status (Bakker & Sevenster, 1983). These characteristics have a positive relationship with foraging ability and the quality of one's territory (Rowe, Baube, Loew, & Phillips, 2004). For these reasons, females find colorful males more attractive (Bakker, 1993). The consequence of having poor male reproductive coloration can include low success in acquiring the best territories and mates, and ultimately lead to low fitness.

Reproductive coloration is important to evaluate because the secondary sexual characteristics of male sticklebacks and many other fish is modulated by androgens (Wai & Hoar, 1963). It is possible that exogenous estrogen could cause a down-regulation of endogenous androgen production resulting in the reduction of androgen dependent sexual characteristics like reproductive coloration (Bell, 2001). To this researcher's knowledge there aren't any reports of work on the reproductive coloration of sticklebacks after exposure to $EE_2$ as adults.

### 3.5 Methods: Reproductive Coloration

The reproductive coloration of males (Series 2 = post-exposure and post-depuration) was confirmed by photographing the ventral side of each fish with a digital camera (Canon SD200). Twenty five fish were photographed: five control, four 10 ng/L, four 70 ng/L and twelve 100 ng/L $EE_2$ treated fish. Each fish was placed in a Petri dish that was filled with salt water and set in a frame that allowed photography from the underside of the dish. All fish were photographed under the same lighting conditions (fluorescent lighting in a windowless room, 400 lx). The photos were downloaded to a computer and analyzed for red intensity using Adobe Photoshop 7.0. The values for red, blue and green from 0-255 (RBG) (Toft & Baatrup, 2001) were recorded for two points on the pelvic fins of each fish (proximal and middle). [Adobe Photoshop has an 'Info Palette' with an option that allows one to acquire RBG levels by placing the cursor over an image.] After calculating the average of the RBG values for the pelvic fins, an index was calculated using the following formula after Frischknecht (1993):

$$\text{Red index} = 1 - \frac{R}{(R+G+B)}$$

### 3.6 Results: Reproductive Coloration

The red index of the pelvic fins of the males did not differ among the four $EE_2$ exposure levels from post-exposure to post-depuration (two-way mixed ANOVA, $F$ (3, 22) = 0.279, $p$ = 0.84). The testing period ($F$(1, 22) = 0.59, $p$ = 0.45) nor the interaction between $EE_2$ exposure level and testing period were significant ($F$ (3, 22) = 0.33, $p$ = 0.80).

### 3.7 Discussion: Reproductive Coloration

The reproductive coloration of males did not change among $EE_2$ exposure groups from post-exposure to post-depuration. There are several reasons that might account for this finding. The most obvious possible explanation is that exogenous estrogen, or the levels that were used in this study, had no affect on the reproductive coloration in adult male fourspine sticklebacks. In fact, changes in the reproductive coloration in sticklebacks after adult exposure to estrogens have not been previously reported. It is possible that this androgen-dependent secondary sexual characteristic would only be affected if $EE_2$ exposure occurred during the sensitive period of sexual differentiation which is the first two weeks post-hatch (threespine stickleback, Hahlbeck, Griffiths & Bengtsson, 2004). Notably, the red coloration in male pelvic spines is evident throughout the year (Bond, 1968) and consequently may not be affected by seasonal fluctuations of androgens. Reduction in reproductive coloration does occur in threespine sticklebacks exposed as juveniles and in other species as adults, however.

Reductions of the red index have been reported in adult threespine sticklebacks that were exposed as fry to 27.7 ng/L of $EE_2$ (exposure: post-hatch to 4 weeks) (Maunder, Matthiessen, Sumpter, & Pottinger, 2007). In this case, exposure as juveniles had a long lasting effect on reproductive coloration even though the exposure was short-term and the measurements were taken when the fish reached adulthood. Similar reductions in reproductive coloration were obtained in juvenile guppies exposed to 500 ng/L 17b-estradiol for 90 days (Toft & Baatrup, 2003). It is acknowledged that guppies have different reproductive strategy from sticklebacks; however, as in sticklebacks, reproductive coloration in guppies is androgen dependent (Pandey, 1969) and can be

compared, in general terms, to sticklebacks. A comparable study using adult guppies exposed to 30 ng/L of estradiol (60 day exposure) reported a reduction in the reproductive coloration index (Toft & Baatrup, 2001). These results suggest that perhaps higher exposure concentrations would diminish the red index in male sticklebacks or that they are not as labile as other fish when exposed as adults.

Another possibility is that the fish did not have the proper nutrients to synthesize the reproductive coloration. This is not a likely explanation as the fish in this study were fed a diet of brine shrimp (*Artemia*). Brine shrimp provided canthaxanthin (Nelis et al., 1988) which is essential for the development of the reproductive coloration in sticklebacks (Czeczuga, 1980). Lastly, the method used to obtain the color index may have been too insensitive (Toft & Baatrup, 2001).

## 4.0 Introduction: Activity Experiment

Activity is a widely studied behavior that is used as a measure of toxicant exposure effects (Zhou & Weis, 1998). Exposure to chemicals ranging from pharmaceuticals like DES, diethylstilbestrol, a drug once used to prevent miscarriages, and pesticides like DDT result in increased activity levels in rats (Kubo et al., 2003) and goldfish (Weis & Weis, 1974), respectively. Swimming is affected by changes in toxicants at levels that are 0.1 to 5.0 % of the concentration that kills half of the exposed organisms ($LC_{50}$) (Little, 2002). The frequent use of swimming behavior in behavioral toxicology is based on the observation that changes in swimming behavior, even at sublethal levels of exposure, can cause impairments in bodily functions that are vital for living organisms (Little). Swimming activity, in particular, is one of the most reliable measures in toxicity tests when comparing different chemicals and diverse species (Little, Archeski, Flerov, & Kozlovskaya, 1990). Locomotor activity can be modulated by many variables including hunger (Milinski & Heller, 1978) and, important here, steroids (Bell, 2004).

The ability to move around is essential to forage for food, learn home ranges, find mates, and for successful breeding. The swimming activity of an organism can therefore affect its growth and survivorship (Werner & Anholt, 1993). As is the case for most behaviors that are necessary for survival, they must be balanced with the dangers of predation. Foraging requires a high level of activity yet it is an act that leaves prey more vulnerable to predators. As a result, individuals with high activity rates in the presence of predators may have reduced fitness (Werner & Anholt).

## 4.1: Methods: Activity Experiment

The objective of this experiment was to establish if exposure to $EE_2$ alters the overall activity of sticklebacks. Activity rate was documented by recording the activity of a solitary fish (N=66, number of male and female fish tested at both testing periods at all levels of treatment) for 2 seconds every minute, for a period of three hours. The behavioral arena for the experiment is depicted in Figure 4.1; see the General Methods section for details of $EE_2$ exposure treatments.

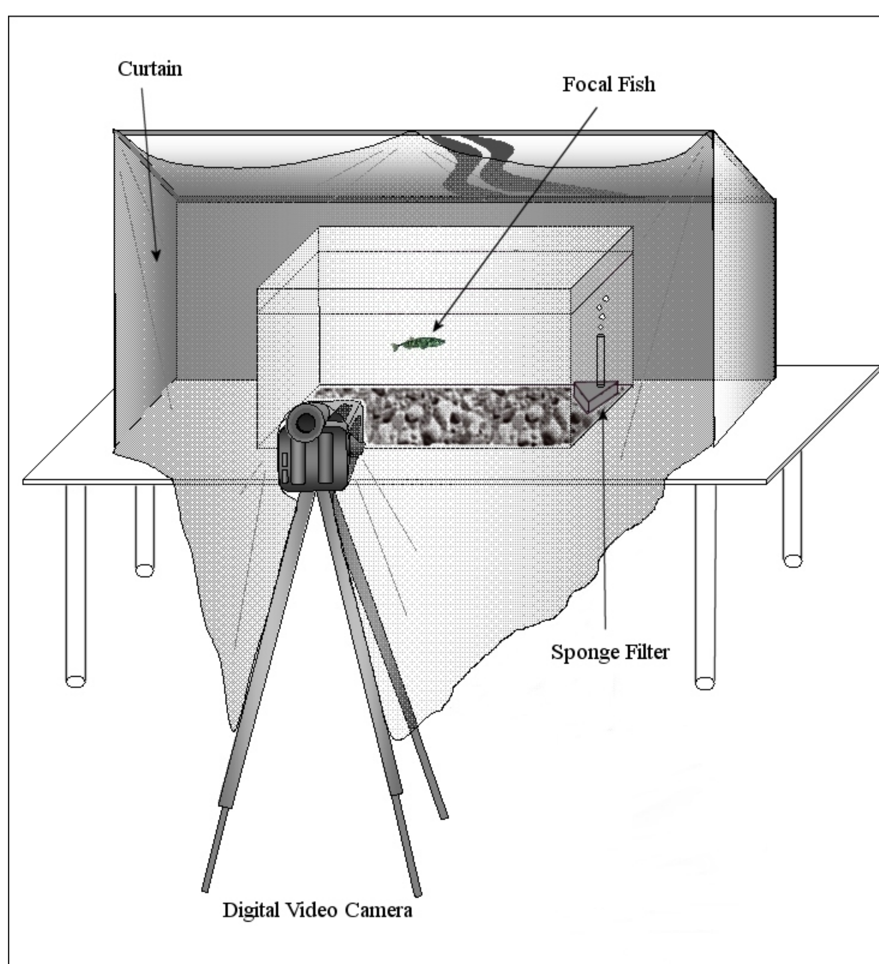


Figure 4.1: Activity Experiment (figure adapted from Crook, 2004). Tank dimensions = 40.6 x 20.3 x 25.4 cm (not to scale), camera positioned 50 cm from the front of the tank.

Table 4.1 lists the number of fish, sex, and level of $EE_2$ exposure that were tested post-exposure and post-depuration in this experiment.

| | Post-Exposure | | | | | Post-Depuration | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| $EE_2$ ng/L | 0 | 10 | 70 | 100 | Total | 0 | 10 | 70 | 100 | Total |
| Males | 10 | 3 | 3 | 10 | **26** | 10 | 3 | 3 | 10 | **26** |
| Females | 18 | 4 | 3 | 15 | **40** | 18 | 4 | 3 | 15 | **40** |
| Total | 28 | 7 | 6 | 25 | **66** | 28 | 7 | 6 | 25 | **66** |

Table 4.1: Activity Test: Number of subjects in each testing period, $EE_2$ concentration, and sex. A total of 66 fish were tested in the Activity Experiment.

Activity testing trials took place from 10 a.m. to 1 p.m. The time-interval settings on a Sony digital camera were programmed to record for two seconds every minute (a “bin”*)*, for three hours (total bins = 180/fish). Each two-second bin was scored by observers blind to treatment using the standardized system described below.

During the initial laboratory observations for this project, sticklebacks placed in individual aquaria displayed three basic behaviors: 1) swimming around the tank, 2) hiding motionless behind the sponge filter, and 3) lying motionless on the substrate. Based on these observations, fish behavior was scored in the following manner: if the fish was not visible, the score was recorded as 0, if the fish was visible but not moving the score was recorded as 1, if the fish moved less than a body length the score was recorded as 2, and if the fish moved more than a body length the score was recorded as 3.

The time-lapse recordings were scored by viewing the tapes frame-by-frame offline, by K.K. and observers blind to treatment (177 hours of tape). Inter-observer

reliability indicated that there was a high level of agreement between observers (Pearson's coefficient, $r = 0.90$, $p < 0.0005$). Scores 0, 1, and 2 were collapsed into one group representing no activity (score =0). Percentage bins in which the animal was considered "active" was defined as the proportion of the bins in which a fish moved greater than one body length (bins with a score of 3) divided by 180. The data were analyzed with SPSS.

The proportionate data (p) were transformed (arcsine ($p^{1/2}$)) to stabilize variances. They were evaluated on both transformed and untransformed scales. As transformed and untransformed scales were concordant, only untransformed scale results are presented here. Since this was a repeated-measures design, 2-way mixed ANOVAs were used to analyze the data (with $EE_2$ level and sex as between-subject factors and testing period as the within-subject factor).

<u>Control Data</u>

Since behavioral data from both of the control groups, the solvent control and no-solvent control, were not significantly different they were combined and treated as the single control for subsequent data analyses.

## 4.2: Results: Activity Experiment

Animals exposed to different levels of $EE_2$ expressed different activity levels from one another (mixed ANOVA, $F(3, 58) = 3.95$, $p = 0.01$). Control animals were significantly more active than fish exposed to 100 ng/L of $EE_2$ (post hoc Tukey, $p = 0.03$). The effect of fish sex and the interaction between $EE_2$ level and sex on activity were not significant. The interaction between testing period and $EE_2$ exposure on activity

was significantly different indicating that the exposure effects differed as a function of testing period ($F(3, 58) = 9.33$, $p = < 0.0005$). Figure 4.2 depicts the calculated effect of testing periods for each $EE_2$ exposure level (Mellor & Rockwell, 2006). Note that the confidence intervals did not overlap 0 for the control and 100 ng/L $EE_2$ meaning that each group's activity is significantly different from post-exposure to post-depuration. Control animals decreased their activity from post-exposure to post-depuration ($M = 0.60$ to $M = 0.44$), whereas fish exposed to 100 ng/L of $EE_2$ increased activity levels from post-exposure to post-depuration ($M = 0.13$ to $M = 0.50$). Testing period, the interactions between testing period and sex, and the interactions between testing period, $EE_2$ level and sex were not significant.

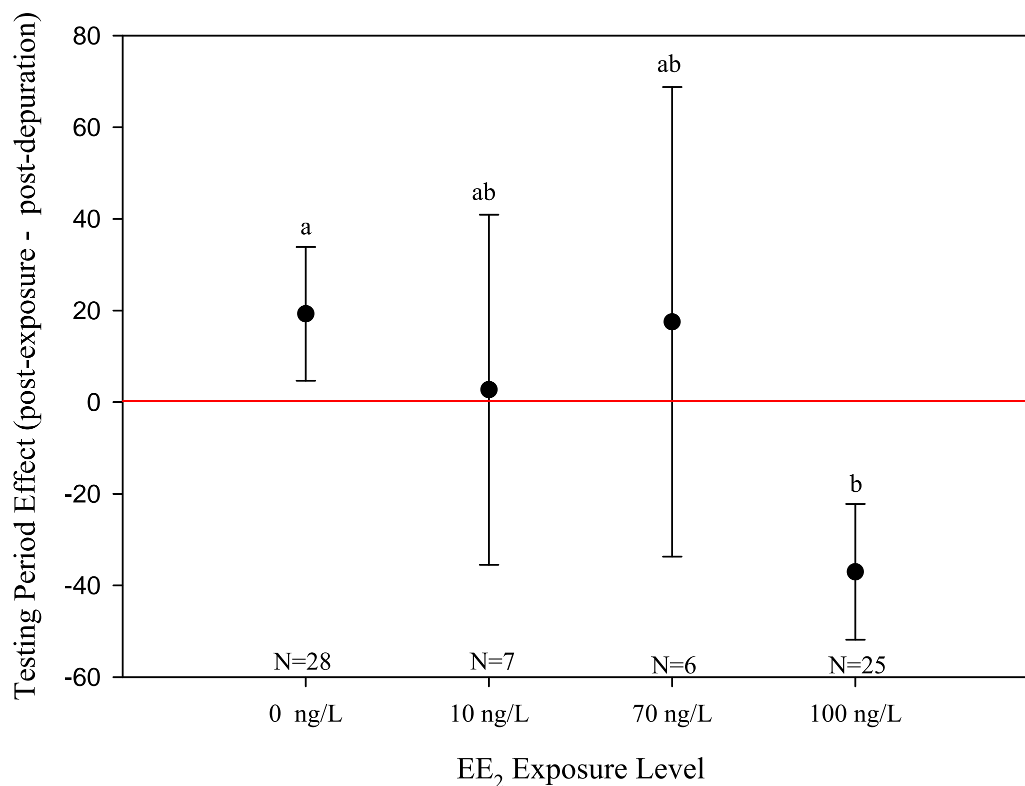


Figure 4.2: Average effects of testing period (males and females pooled), mean % time active post-exposure – mean % time active post-depuration for all $EE_2$ exposure levels. Error bars represent 95% confidence limits. Different letters, a and b, indicate a significant difference in means ($p < 0.05$). Paired letters indicate overlap in confidence intervals.

Since exposure effects differed as a function of testing period, two-way ANOVAs were used to determine how these differences manifested. Post-exposure, the behavior of animals treated with different levels of $EE_2$ differed significantly $F(3, 58) = 11.84$, $p < 0.0005$. Specifically, the control and 100 ng/L fish expressed different levels of activity

(post hoc Tukey, $p < 0.0005$; Figure 4.3). The effects of sex and the interaction between sex and $EE_2$ level on activity were not significantly different among animals.

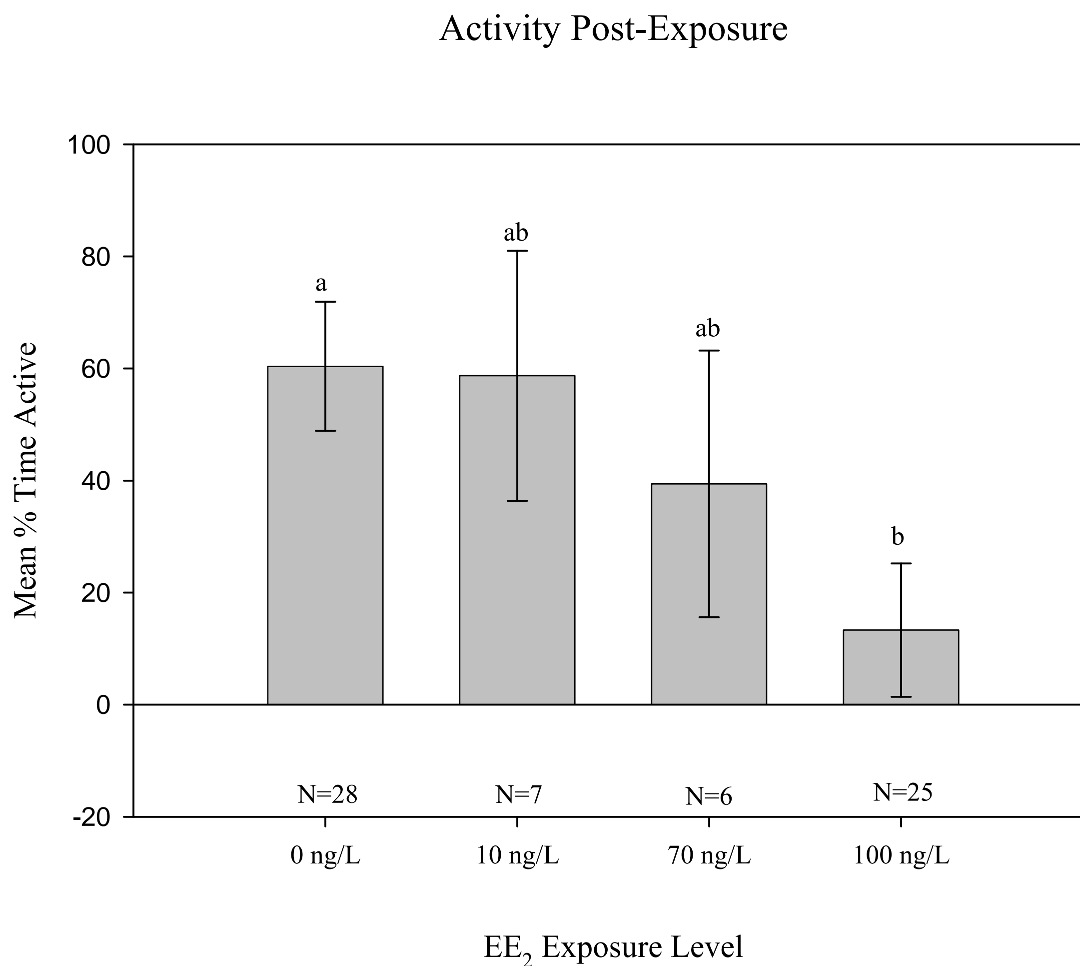


Figure 4.3: Mean % time fish were active (males and females pooled) post-exposure. Error bars represent 95% confidence limits. Different letters, a and b, indicate differences in means of $p < 0.05$.

Post-depuration, activity levels as a function of sex, $EE_2$ level, or the interaction between sex and $EE_2$ level were not significantly different (Figure 4.4).

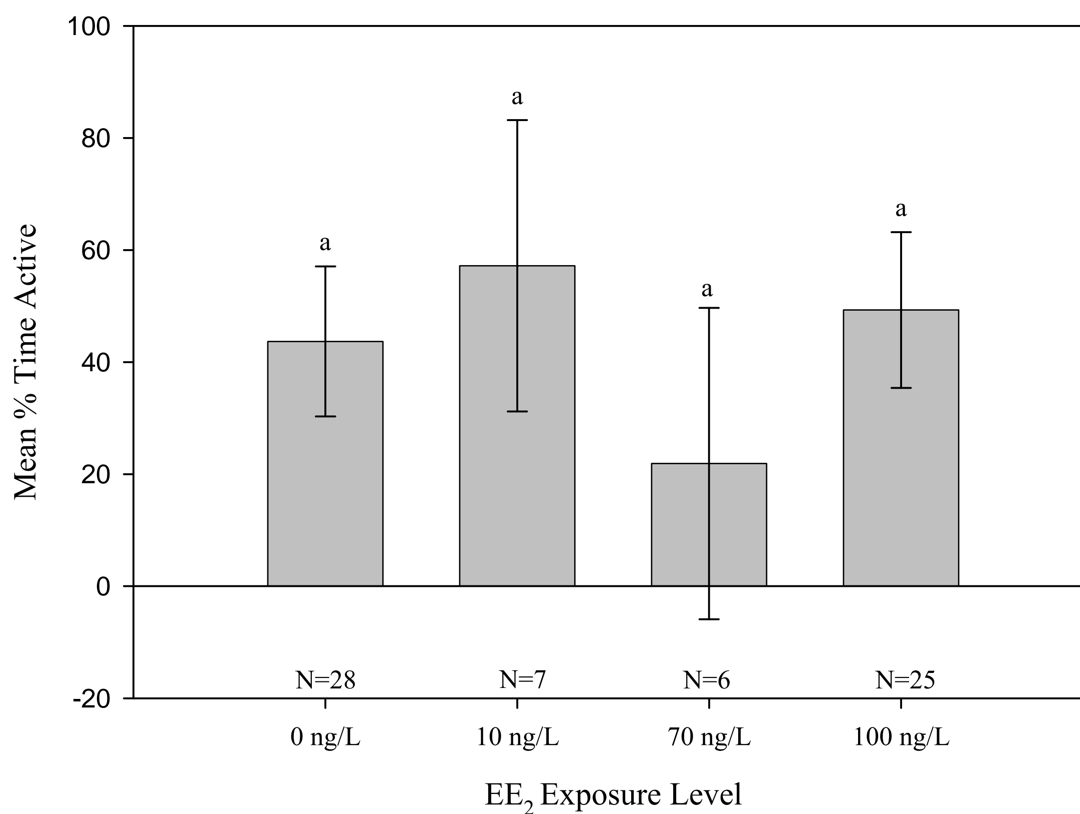


Figure 4.4: Mean % time fish were active (males and females pooled) post-depuration. Error bars represent 95% confidence limits. Different letters indicate $p < 0.05$.

The Effect of Captivity on Activity Levels

Activity levels of "wild" female fish tested immediately after capture ($M = 0.802$, $SE = 0.063$) decreased significantly after 60 days in captivity ($M = 0.52$, $SE = 0.08$) (paired samples t-test, $t(12) = 4.75$, $p < 0.0005$). The males' (after capture, $M = 0.91$, $SE = 0.06$ to after 60 days in captivity, $M = 0.62$, SD = 0.16) activity levels were not significantly different ($t(5) = 1.49$, $p = 0.19$).

## 4.3 Discussion: Activity Experiment

The key finding from this experiment is that exposure to a level of $EE_2$ found in the environment, 100 ng/L (measured concentration 46.5 ng/L), resulted in the decline of activity in adult male and female fourspine sticklebacks. Based on a few previous studies using similar levels of $EE_2$, we expected that activity would increase in the fish exposed to $EE_2$ (Bell, 2004). Bell found that exposure to $EE_2$ increased activity in threespine stickleback; however, differences in methodology may account for the discrepancy. Bell used a much longer exposure time (211 days) that spanned critical points in development, from fertilization to juvenile stage, when organizational changes of the endocrine system occur. Assuming that the fish tested in this study were living in a pristine environment, these fish were exposed as adults, not during critical points in their early development. It may be that organizational changes due to $EE_2$ exposure during development alter activity differently in adults than, perhaps, activational effects off $EE_2$ exposure which occur after development is complete (Breedlove & Hampson, 2002; Cooke, Hegstrom, Villeneuve, & Breedlove, 1998).

Another difference in our study was the duration of the trials in which activity was measured (3 minutes in Bell; 3 hours here). One of the aims for this project was to acquire data that were a true representation of a fish's activity. This was done by evaluating activity over a long period of time. The rationale for assessing activity for 3 hours was to avoid only measuring exploration of a novel environment in the first few minutes of exposure, which is naturally a time when animals, including fish, show marked increases in activity (e.g. Basil & Sandeman, 2000; Teyke, 1989). This trend alone could possibly account for the increase in activity that was reported by Bell (2004).

The variable that Bell used to measure activity, the number of times the fish moved, may have been an indicator of the intensity of exploratory behavior and not the general activity level of a fish.

Although we did not expect this negative relationship between $EE_2$ exposure and fish activity, decreases in activity from exposure to pollutants is not uncommon. Fish exposed to lead (mummichog, Weis & Weis, 1998), organophosphorus defoliant (rainbow trout, Little, Archeski, Flerov, & Kozlovskaya, 1990), and aluminum sulfate (brook trout (*Salvelinus fontinalis*), Cleveland, Little, Ingersoll, Wiedmeyer, & Hunn, 1991) exhibited decreased levels of swimming activity. While the end result is similar, the mechanism by which these pollutants decrease activity may differ. Due to their diverse chemical properties, mimicking, and chemical structures, it is probable that they operate in different modes. The mechanism behind the changes in activity levels due to $EE_2$ exposure is not known. One hypothesis for this decrease in activity is that $EE_2$ increases the production of vitellogenin that is released into the bloodstream (Hansen et al., 1998) which dilutes oxygen levels in the blood resulting in lower activity levels.

These data support the prediction that changes in activity levels would not persist after depuration. This was the case for both males and females and suggests that deleterious effects on activity levels from a 60 day exposure to $EE_2$ are reversible in both male and female adult fish. It is to be expected that the timing of the exposure is a critical factor in the reversibility of these changes. As the fish in this experiment were exposed as adults only, it is reasonable to assume that these results would be different from studies using a full-life cycle exposure or exposures at critical times of development. Currently outside of this study, there are no other behavioral experiments

with adult $EE_2$ exposure that incorporate a depuration period in the experimental protocol for comparison. The finding that depuration allows fish to recover from $EE_2$ exposure is promising although it does not guarantee that this would be the case for all wildlife or for organisms exposed during different life stages.

Even if depuration facilitates recovery, abnormally low levels of activity for any period of time can have devastating effects on fitness by disrupting normal feeding patterns (Steele, 1983). A likely consequence of a change in feeding pattern is a deleterious cycle of reduced food consumption resulting in less energy to expend for locomotion. Less energy, in turn, could slow down an organism resulting in an increase in vulnerability to predation (Weis, Smith, Zhu, Santiago-Bass, & Weis, 2001). Predatory organisms with low activity levels will also have reduced search areas and encounters with prey (Laurence, 1972). However, a decline in activity levels does have the benefit of a decreased likelihood of detection by predators.

Although sex-specific differences were not found in activity levels, the importance of evaluating sex as a factor is paramount in toxicology research to determine if males and females respond differently to EDC exposure. The effect of hormones on sexual differentiation and development underscores this importance (Devlin & Nagahama, 2002). Today, many EDC studies use exclusively one sex, or use both sexes but do not include sex as a factor in the statistical analysis (see reviews: Burger, 2007; Orlando & Guillette, 2007). To determine risk assessment and develop monitoring programs for EDCs, including sex as a factor in these studies is essential.

In light of the decrease in activity in fish exposed to $EE_2$, one must consider that living in captivity may have an effect on the activity levels of fish. Testing the wild

caught fish immediately after capture served as a proxy for activity levels of fish in the wild. The data presented here revealed that male ‘wild’ fish did not have decreased levels of activity after 60 days in captivity. In contrast, females did exhibit a decrease in activity indicating that perhaps females are more sensitive to living in laboratory conditions. No differences in activity levels between males and females were evident in the remaining data.

The results from this study demonstrate that exposure to levels of $EE_2$ found in the environment, much below those in acute toxicity tests, result in significant changes in an important behavior and that, once animals are removed from $EE_2$. the changes are reversible. This study also illustrates the need for changes in the way environmental pollutants and their effects on wildlife are assessed to determine toxicity. Namely, terms of the levels of pollutants tested, the indicator responses used, sex, and life stage of study organisms.

## 5.0 Introduction: Aggression Experiment

There is general acceptance among researchers that a relationship exists between gonadal sex steroids and aggressive behavior in a wide array of vertebrates (Svare, 1983). Sticklebacks are no exception; androgens modulate the full expression of aggressive behavior in males (Hoar, 1962; Wooton, 1984). Castrated sticklebacks do not defend territories or court females, and androgen (11-ketoandrostenedione) treatment will restore both (Borg & Mayer, 1995). Researchers have exploited this relationship between sex steroids and aggression to study the effects of EDCs on important behaviors like aggression (Bell, 2001; Porter, Jaeger, & Carlson, 1999).

Aggressive behavior can also be modulated by variables other than hormones including the presence of a nest (Van Iersel, 1958), and visual signals (red color) (Pelkwijk & Tinbergen, 1937) in sticklebacks. Male threespine sticklebacks show aggression in the context of acquiring territories, mates, and food or to protect their offspring (Wooton, 1976). Higher levels of fitness are associated with success at these tasks. The best territories for nest building are large and associated with resources (Wooton, 1976) and males will actively fight over such habitats. Aggression, however, is not limited to males in reproductive condition (Murckensturm, 1965); males will exhibit aggression outside of the breeding season. Importantly, females will also display aggressive behavior toward conspecifics although male aggression is described in the literature most frequently (Wooton, 1984).

In this experiment, a number of behaviors were examined immediately after exposure to $EE_2$, and also after depuration to determine if aggression can be modulated by $EE_2$ exposure in fourspine stickleback adults. Measured variables included: 1) time

spent within one body length of an intruder, 2) time spent hiding, 3) time spent within one body length of a shelter, 4) time spent swimming, 5) number of bites directed toward an intruder, 6) areas of the tank used, and 7) number of approaches toward an intruder (Bell, 2001; Huntingford, 1976b). We also wanted to see if $EE_2$ exposure affected the time that it took to recover from exposure to a conspecific. Recovery from the presence of an intruder in this context was measured as the proportion of time fish spent: 1) hiding, 2) within one body length of a shelter, 3) swimming, and 4) the number of areas of the tank used after the intruder was removed from the tank. It is important to note that several of these behaviors are mutually exclusive (time spent hiding and swimming; time spent near the shelter and near the intruder) and some occur together (time spent hiding and near the shelter).

These behaviors were selected since the duration or frequency at which animals display them indicate heightened levels of aggression. Specifically, 1) increased time spent near the intruder, and swimming, and 2) increased numbers of approaches toward the intruder, bites, and areas of the tank used would be expected in aggressive fish. Low levels of the same set of behaviors would indicate lower levels of aggression. Aggressive fish would be expected to spend less time spent hiding, and near the shelter. Fish with higher aggression levels might also be expected to recover from the presence of an intruder more readily than a less aggressive fish. Recovery would be characterized by more time spent swimming in more areas of the tank and less time spent hiding and staying near the shelter compared to the aggression test. The opposite scenario would be predicted for fish with low aggression levels.

## 5.1 Methods: Aggression Experiment

The overall objective of this experiment was to establish if $EE_2$ exposure in adult sticklebacks alters aggression toward conspecifics. Individual sticklebacks (N=66 for both testing periods), male and female, were removed from their home tanks and placed singly into experimental tanks (refer to General Methods Section for details) at two time points, post-exposure and post-depuration. See Tables 5.1 & 5.2 for the number of males and females in each treatment that were tested. Aggression levels were assessed by introducing a "conspecific intruder" (a fish model) into the experimental tank after the resident had established a territory (17 hours after placement into the experimental tank). Behavioral studies have revealed that sticklebacks react to simple models (Tinbergen, 1951); these "intruders" were made from fishing lures (WildEye® Swim Shad, Figures 5.1 & 5.2). The sizes of the models were altered to match the lengths of the live fish and also modified with nontoxic paint to simulate reproductive coloring. Pelvic fins of the male intruders were painted red (reproductive coloration) to illicit a more robust response (Tinbergen, 1951). Also, the models were positioned in the tank in a threatening "head-down" pose (Hall, 1956; Van Iersal, 1958).

| | Post-Exposure | | | | Post-Depuration | | | |
|---|---|---|---|---|---|---|---|---|
| $EE_2$ ng/L | 0 | 10 | 70 | 100 | 0 | 10 | 70 | 100 |
| Males | 10 | 3 | 3 | 10 | 10 | 3 | 3 | 10 |
| Females | 18 | 4 | 3 | 15 | 18 | 4 | 3 | 15 |
| Total | **28** | **7** | **6** | **25** | **28** | **7** | **6** | **25** |

Table 5.1: Aggression Test: Number of subjects in each testing period, separated by $EE_2$ concentration, and sex.

| | |
|---|---|
| Total Males | 26 |
| Total Females | 40 |
| Overall Total | 66 |

Table 5.2: Aggression Test: Overall total number of male or female subjects.

Figure 5.1: Photograph of a male intruder used in the aggression experiment.

Figure 5.2: Photograph of a female intruder used in the aggression experiment.

Each resident fish was tested against one male and one female “intruder” of similar size (within 5 mm) for a 5 minute aggression test. The sex of the “intruder” was random for the first aggression test and balanced thereafter. The intruder was introduced into the center of the tank suspended by fishing line 4 cm above the substrate and then video recording began. The fishing line attached to the intruder was secured to a guide that fit over the tank to maintain a consistent position for all subjects (Figure 5.3).

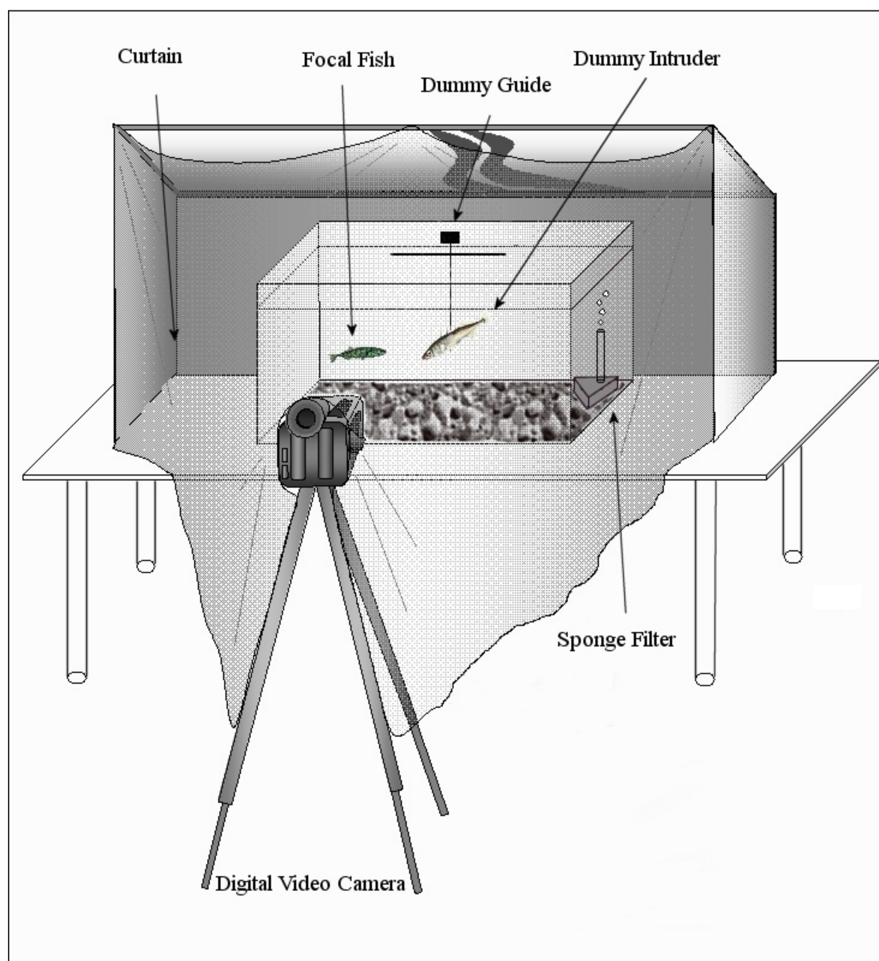


Figure 5.3: Aggression Test Experiment Set-Up (figure adapted from Crook, 2004). Tank dimensions = 40.6 x 20.3 x 25.4 cm (not to scale), camera positioned 50 cm from the front of the tank.

To determine the spatial areas of the tank that the fish used, a template to separate the areas of the tank was drawn directly on the video monitor (Figure 5.4) during video analysis. As the video was analyzed the area of the tank that the fish occupied was documented by recording the letter that corresponded to the particular area.

**Fish Tank: front view**

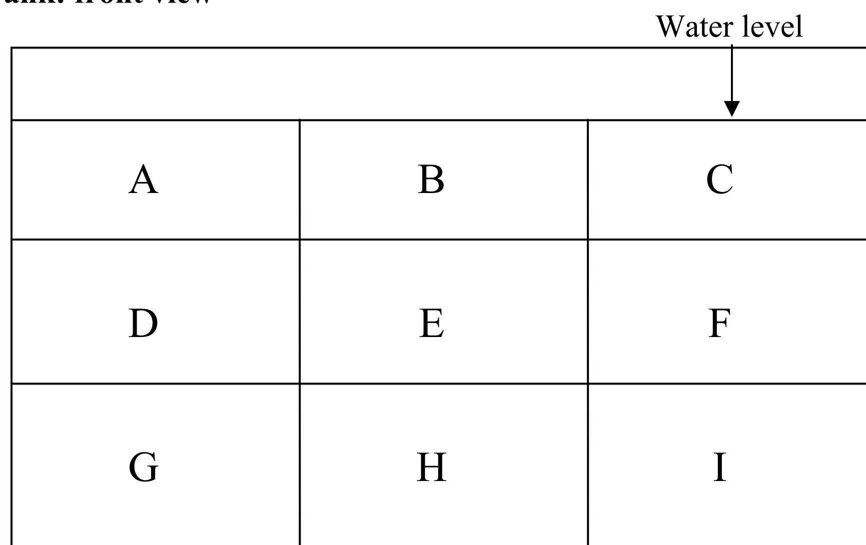


Figure 5.4: Diagram of tank sections to determine spatial areas used by fish. Tank dimensions = 40.6 x 20.3 x 25.4 cm. Each area (A-I) was 13.53 x 8.46 cm. Tank areas were marked on the video monitor with a dry-erase marker. Each time the fish changed position in the tank, the spatial area was recorded.

The model remained in the tank for 5 minutes after which it was removed. To assess recovery behaviors, video recording continued for 5 minutes after the removal of the intruder. Recovery time was included to test for any long-lasting effects of an aggressive bout on the subject. The following behavioral measures were recorded offline, by frame-by-frame analysis, and collected in 1-minute bins, for both the aggression tests and the recovery tests:

1) time spent within one body length of the intruder (horizontally and vertically)
2) the number of approaches to the intruder (an approach was defined as directional movement towards the intruder, subsequent approaches were counted if the fish turned away and approached again)
3) the number of bites directed toward the intruder (bites were characterized as physical contact between the fish's mouth and the intruder )
4) time spent hiding behind the sponge filter

5) time spent within one body length of the shelter (sponge filter provides a hiding spot)
6) time spent swimming
7) number of areas of the tank used

(As in Bell, 2001 (#1); Huntingford, 1976b (#3), 1976a)

The recovery test observations included only behavioral measures 4-7.

## Data Collection and Analysis for Aggression Experiment

The aggression test recordings were scored by viewing the tapes frame-by-frame offline, by K. K. and observers blind to the treatment (a total number of 158 hours of video was analyzed for this experiment). The proportional behavioral measures [1) time spent within one body length of the intruder; 2) time spent hiding; 3) time spent within one body length of the sponge filter; and 4) time spent swimming] were defined as the average number of seconds fish spent engaged in each behavior in the 5 min trial and was calculated as follows:

Let $t_1$ = time (s) engaged in behavior for the first minute, $t_2$ = time (s) engaged in behavior for the second minute, etc.

$$\text{Trial average} = \frac{t_1 + t_2 + t_3 + t_4 + t_5}{300s}$$

The number of two dimensional areas used was determined by recording each of the areas of the tank that the fish used during the trial. The "average area covered" was calculated by taking the sum of the number of areas that the fish occupied during each one-minute bin and dividing by 5 for the average number of areas occupied for the whole trial. For the remaining behavioral measures (1) the number of bites directed toward the intruder; and 2) the number of approaches to the intruder) the sums over the entire 5

minute aggression test were used for data analysis. The data for the recovery test were calculated in the same manner.

Based on evidence that males and females react differently physiologically and behaviorally to toxic substances, statistical analyses were conducted to determine if differences in behavior existed between males and females (Johnston, Fisk, Whittle, & Muir, 2002; Kvarnemo, Forsgren, & Magnhagen, 1985; Orlando & Guillette, 2007). Since the behavior of the animals was measured at post-exposure and post-depuration, a mixed 2-way ANOVA was conducted to track any differences in behavior as a function of time, $EE_2$ exposure level, and fish's sex. The advantage of comparing the behavior of each fish after exposure to $EE_2$, and then after depuration was that using each animal as its own control minimizes error variance from individual differences in behavior (Dytham, 2006). Independent t-tests for all behaviors confirmed that responses toward male and female intruders were not significantly different in any of the $EE_2$ exposure levels. The responses to both models were combined and were used in further data analysis.

To assess if $EE_2$ affects the ability for these fish to recover from the presence of a conspecific intruder, frequency and levels of behaviors in the aggression test w*e*re compared to those measured in the recovery period (mixed ANOVA). The proportionate data (*p*) were arcsine ($p^{1/2}$) transformed to stabilize variances. They were evaluated on both transformed and untransformed scales and, since the analyses were concordant, only the results from the untransformed scale are included.

## 5.2 Results: Aggression Experiment

The Effect of Testing Period on Aggression Levels

For approaches to the intruder, there was a significant interaction between testing period and $EE_2$ exposure level (mixed ANOVA, $F(3,58) = 4.40$, $p = 0.007$). Fish exposed to 10 ng/L of $EE_2$ approached intruders less often immediately post-exposure than after 100 d in clean water (post hoc Tukey, $p = .05$) (Figure 5.5). Figure 5.5 depicts the calculated effect of testing periods for each $EE_2$ exposure level (Mellor & Rockwell, 2006). Note that the confidence interval does not cross "0" for the 10 ng/L $EE_2$ treated fish $EE_2$ meaning that each group is significantly different from post-exposure to post-depuration.

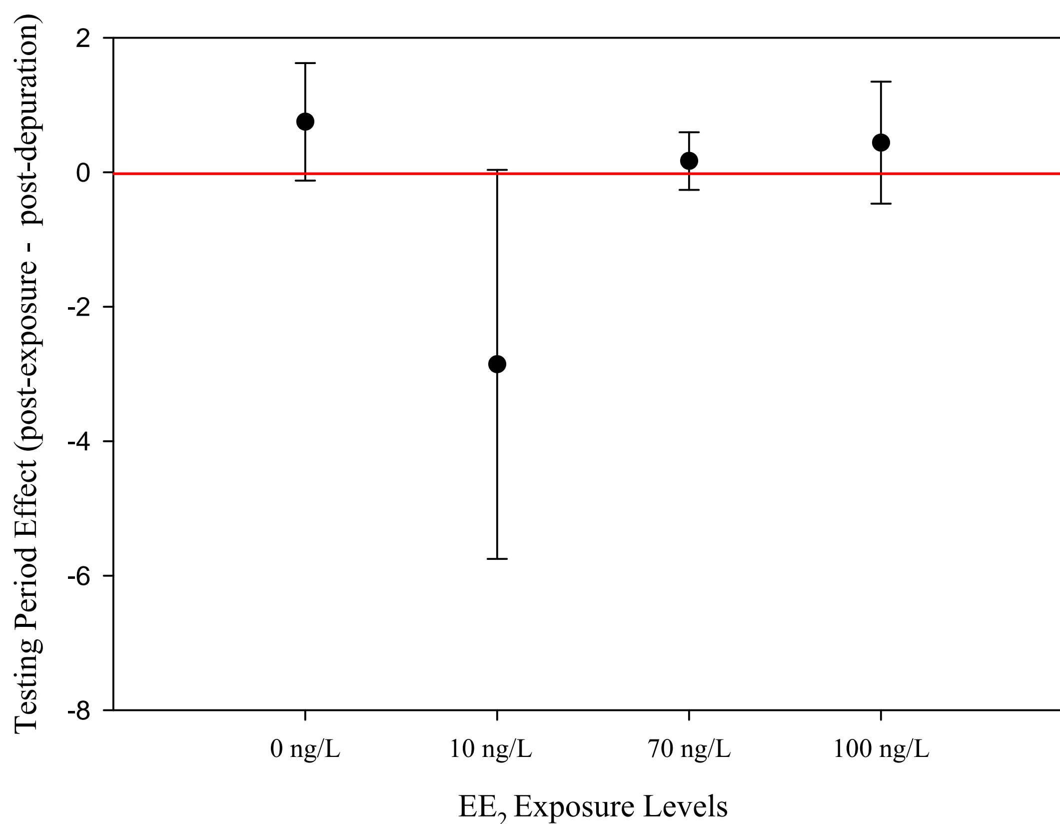


Figure 5.5: Average effects of testing period on approaches to intruder (males and females pooled), mean number of approaches to the intruder post-exposure – post-depuration for all $EE_2$ exposure levels. Error bars represent 95% confidence limits. Bars that do not cross 0 indicate a significant difference from post-exposure to post-depuration..

Time spent hiding was significantly different among $EE_2$ exposure levels (mixed ANOVA, $F(3, 58) = 3.94$, $p = 0.01$). Specifically, the 100 ng/L fish (M = 0.35) hid more than the 10 ng/L fish (M = 0.02) during both testing periods (post hoc Tukey, $p = 0.24$)

(Figure 5.6). There was no overall affect of the fish sex or testing period on time spent hiding.

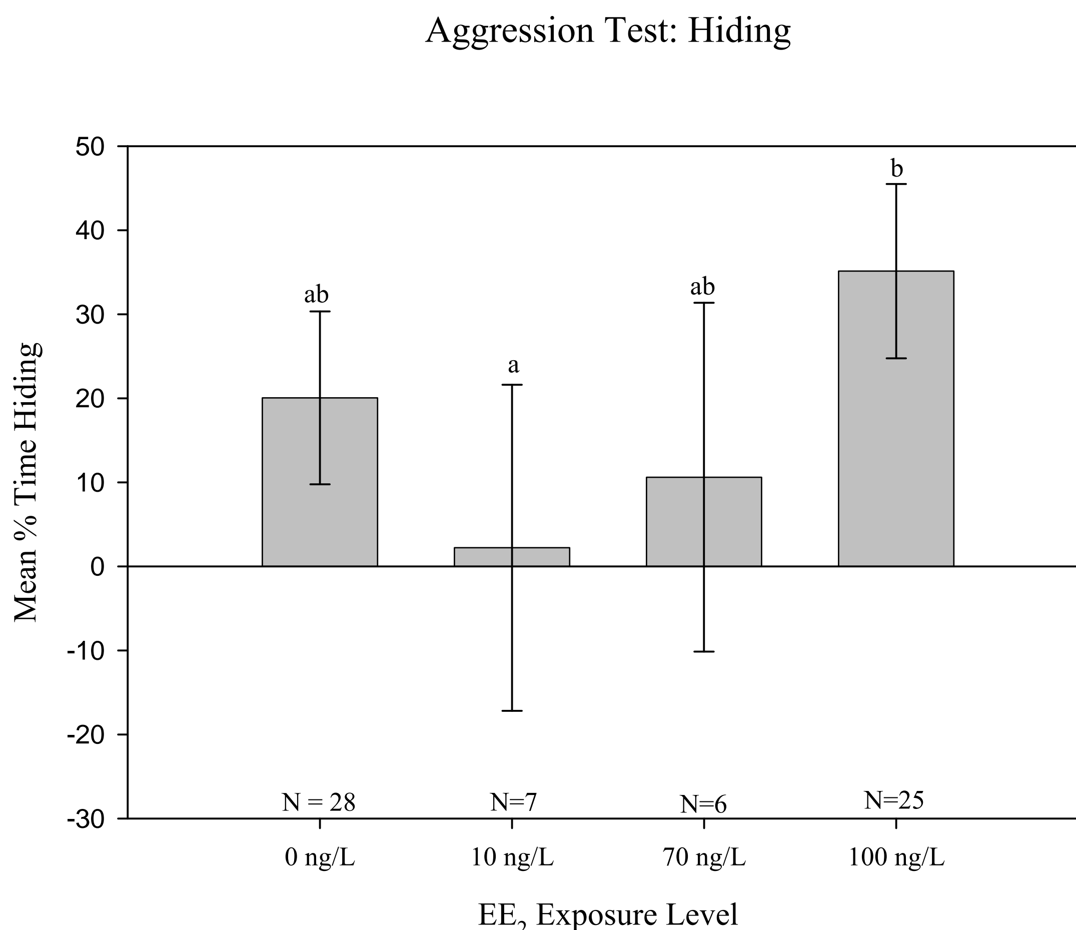


Figure 5.6 Mean % time hiding for fish (males and females pooled) over testing periods was significantly different among $EE_2$ exposure level, $F(3, 58) = 3.94$, $p = 0.01$. 100 ng/L fish (M = 0.35) hid more than the 10 ng/L fish (M = 0.02), $p = 0.24$. Error bars represent 95% confidence limits. Different letters, a and b, indicate $p < 0.05$. Paired letters indicate no significant differences.

Fish from all $EE_2$ exposure levels spent more time within one body length of the shelter post-exposure (overall $M = 0.63$) than at post-depuration (overall $M = 0.42$) (mixed ANOVA, $F(1,58) = 5.23$, $p = 0.026$).

Overall, fish differed in their time spent swimming among $EE_2$ exposure levels (mixed ANOVA, $F(3,58) = 3.55$, $p = 0.02$). The 10 ng/L fish spent significantly more time swimming ($M = 0.45$) than the 70 ng/L fish ($M = 0.06$) (post hoc Tukey, $p = 0.03$) (Figure 5.7).

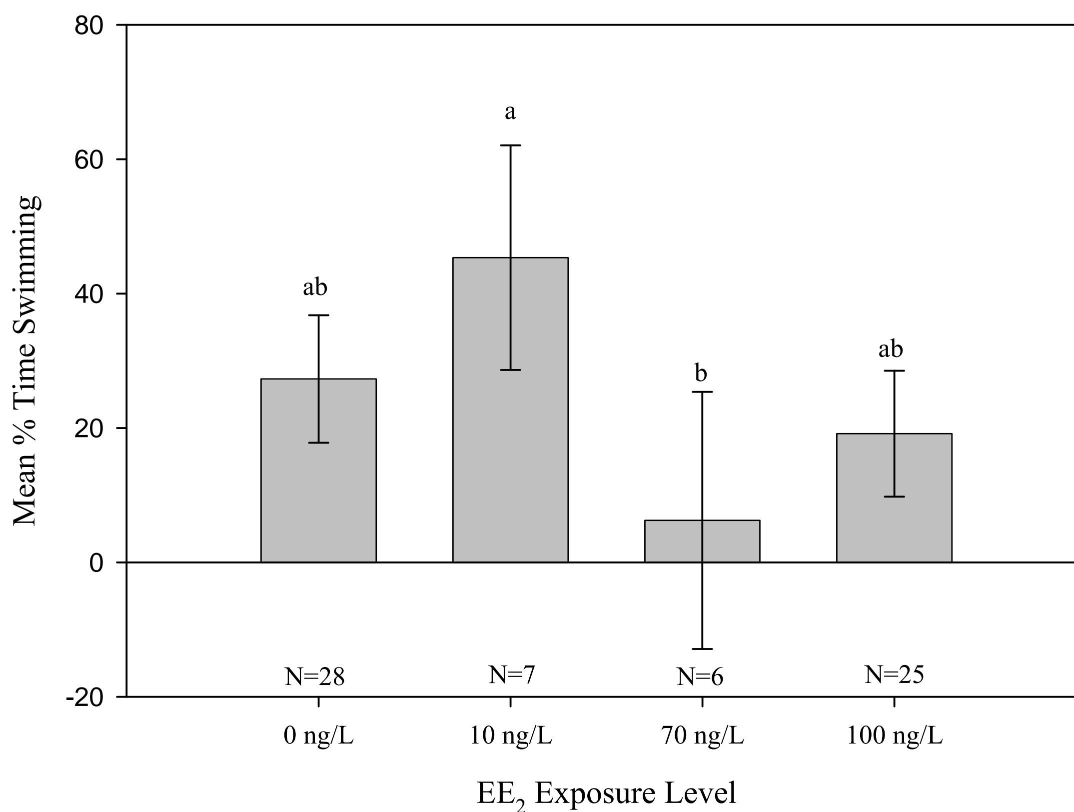


Figure 5.7: Mean % time swimming for fish (males and females pooled) over testing periods was significantly different among $EE_2$ exposure levels ($F(3,58) = 3.55$, $p = 0.02$). 10 ng/L fish swam more ($M = 0.45$) than the 70 ng/L fish ($M = 0.06$), (post-hoc Tukey, $p = 0.03$). Error bars represent 95% confidence limits. Different letters, a and b, indicate significant differences of $p < 0.05$.

There was no significant effect of $EE_2$ level, sex, or testing period on time spent within one body length of the intruder, the number of bites at the intruder, and the number of areas of the tank used.

The Effect of Recovery Period on Aggression Levels

Data from the 5-min encounter with the intruder fish were compared to data from the recovery period (mixed ANOVA). Post-exposure, time spent hiding increased in all $EE_2$ exposure levels from the test (overall $M = 0.20$) to the recovery period (overall $M = 0.36$) ($F(3,57) = 0.48$, $p = 0.04$). The number of areas used also increased in all $EE_2$ exposure levels from the test ($M = 1.39$) to recovery ($M = 2.68$) ($F(3,57) = 15.391$, $p < 0.0005$). $EE_2$ level, sex, and testing period did not affect time spent within one body length of the shelter, or time spent swimming post-exposure.

Post-depuration, the number of areas used increased for all $EE_2$ exposure levels from the test ($M = 1.42$) to recovery ($M = 2.60$) ($F(3,58) = 12.92$, $p = 0.001$). Time spent within one body length of the shelter, time spent swimming, and time spent hiding were not significantly different as a function of $EE_2$ level, sex or testing period nor were the interactions.

The Effect of Captivity on Aggression

None of the behaviors tested for the aggression experiment for “wild” male and female fish tested immediately after capture and again after 60 days were significantly different between testing periods (paired t-tests).

## 5.3 Discussion: Aggression Experiment

These results support the prediction that exposure to $EE_2$ can suppress aggressive behaviors in fish responding to a model conspecific intruder. The surprising finding was that depending on the level of exposure $EE_2$ also had a stimulating effect on behaviors related to aggression. Specifically, fish showed decreased levels of aggression when exposed to higher levels of $EE_2$ and increased levels of aggression at the lowest level of $EE_2$.

Testing period was another key factor that modulated behavior in this experiment. Importantly, only one behavior (number of approaches to the intruder) was affected by testing period. This lack of interaction between $EE_2$ exposure level and testing period for hiding and swimming means that depuration did not diminish the behavioral changes that $EE_2$ exposure produced in this behavior. Also, $EE_2$ exposure did not alter the ability for fish to recover from the presence of a conspecific intruder -- all fish swam in more spatial areas of the tank after the intruder was removed.

The finding that aggressive behaviors were not different between control or $EE_2$ exposed males and females was surprising given that male stickleback aggression is androgen dependent (Hoar, 1962) and that exposure to an anti-androgen reduces male aggression (Rouse, Coppenger, & Barnes, 1977). If exogenous estrogen causes down-regulation of androgen, $EE_2$ exposure could result in lower levels of aggression (Bell, 2001). As for the females, little is known about the hormonal basis of aggression except that female aggression levels are generally much lower compared to males (Adkins-Regan & Weber, 2002), and that aggression decreases from the juvenile stage to adulthood (Bakker, 1985). Conceivably the lack of difference between males and

females could be due to the naturally occurring low aggression levels in females in conjunction with the reduced aggressive behavior in males as a result of down-regulation of androgens after $EE_2$ exposure.

The reduced level of aggressive behavior at the higher levels of $EE_2$ used in this experiment is in agreement with data from studies on male threespine sticklebacks exposed to 15 n/L of $EE_2$ (Bell, 2001) and male *Betta splendens* exposed to 100 ng/L of 17b-estradiol (Clotfelter & Rodriguez, 2006). The higher level of aggressive behaviors in the fish exposed to the lowest level of $EE_2$ in this study compared to other research may be due to different species sensitivity or the variation in the duration of exposure to $EE_2$. Another possibility is that there is a hormetic response relationship (Calabrese & Baldwin, 2002) between aggression levels and $EE_2$ exposure at low doses, $EE_2$ stimulates aggressive behavior while at high-doses it inhibits aggressive behavior.

Sufficient levels of aggression are essential for acquiring territories (Whoriskey & FitzGerald, 1994) large enough to attract mates (Van den Assem, 1967), food acquisition, and the survival of offspring (Wooton, 1976). Consequently, decreased aggression in fish exposed to $EE_2$ or other estrogen mimics will have an effect on an individual's status, condition, and reproductive success. Changes in these factors could lead to population level effects over time. This is especially relevant since depuration did not mitigate the behavioral changes in aggression caused by $EE_2$ exposure.

## 6.0 Introduction: Predator Avoidance Experiment

The ability to survive encounters with predators is important to individuals, but it is also important at the population and community levels. For this reason, ecotoxicologists have used predator avoidance as a tool to measure potentially dangerous behavioral aberrations caused by exposure to environmental pollutants (Eroschenko, Amstislavsky, Schwabl, & Ingermann, 2002; Verrell, 2000). In this experiment, a number of predator avoidance behaviors were examined immediately after exposure to $EE_2$ and also after depuration to determine if predator avoidance can be modulated by $EE_2$ exposure in adult fourspine sticklebacks.

Studies on antipredator behavior in sticklebacks report that fish increase their chances of survival after simulated predator attacks by spending less time swimming and less time remaining out in the open (Huntingford, 1976b), freezing, or swimming away (Rodewald & Foster, 1998). Cautious fish take longer to approach exposed food (Wibe, Fjekl, Rosenqvist, & Jenssen, 2004), make fewer subsequent approaches to food (Huntingford, 1976b), take fewer bites at food (Bell, 2004), and have a heightened response to predators. The opposite scenario would be expected for bolder fish that often use fewer predator avoidance tactics (“risky” behavior).

Prior research on the effect of $EE_2$ exposure on fish reveals a potentially negative effect on survival by increasing risky behavior in the presence of a predator (Bell, 2004). In this case, risky behavior was defined as foraging and swimming around in the presence of predation risk. Other environmental contaminants have also been shown to significantly affect predator avoidance. Cadmium (fathead minnows, Sullivan, Atchison, Kolar, & McIntoch, 1978), pentachlorophenol (juvenile guppies, Brown, Johansen,

Colgan, & Mathers, 1985), lead (mummichog larvae, Weis & Weis, 1995), and organotin compound (TBTO) (threespine stickleback, Wibe, Nordtug, & Jenssen, 2001) all have documented effects on predator avoidance behavior.

### 6.1 Methods: Predation Avoidance Experiment

The objective of this experiment was to establish whether exposure to $EE_2$ alters predator avoidance in sticklebacks. Predation avoidance was measured by simulating a heron attack with a mechanical heron-skull replica (Bone Clones®) (Giles, 1984). Sixty-six fish were tested (Tables 6.1) although only half of the fish completed the trials (Table 6.2).

| | Post-Exposure | | | | Post-Depuration | | | |
|---|---|---|---|---|---|---|---|---|
| $EE_2$ ng/L | 0 | 10 | 70 | 100 | 0 | 10 | 70 | 100 |
| Males | 3/10 | 3/3 | 2/3 | 9/10 | 4/10 | 2/3 | 0/3 | 9/10 |
| Females | 11/18 | 3/4 | 1/3 | 13/15 | 8/18 | 2/4 | 1/3 | 14/15 |
| Total | 14/28 | 6/7 | 3/6 | 22/25 | 12/28 | 4/7 | 1/6 | 23/25 |

Table 6.1: Number of successful trials/number of attempted trials and number of subjects in each testing period, separated by $EE_2$ level and sex.

| $EE_2$ ng/L | 0 | 10 | 70 | 100 | Total |
|---|---|---|---|---|---|
| Males | 1 | 2 | 0 | 8 | 11 |
| Females | 6 | 2 | 0 | 13 | 21 |
| Total | **7** | **4** | **0** | **21** | **32** |

Table 6.2: Total number of subjects with data for both testing periods (post-exposure and post-depuration). The data for the control and 100 ng/L $EE_2$ treated fish were used for statistical analysis.

General procedure: Four fish were tested separately each day in an experimental predation tank. The day before testing, fish were removed from their home tank and

placed in their assigned experimental tank for acclimation. Fish were not fed the day before testing and were not given their daily ration of food until after the predation avoidance trial to keep the fish food motivated during the test. At the beginning of the trial, food was placed in a cup that was suspended in the middle of the tank. The food cup was precisely placed to encourage the fish to move to a position right under the target area of the heron skull (Figure 6.1).

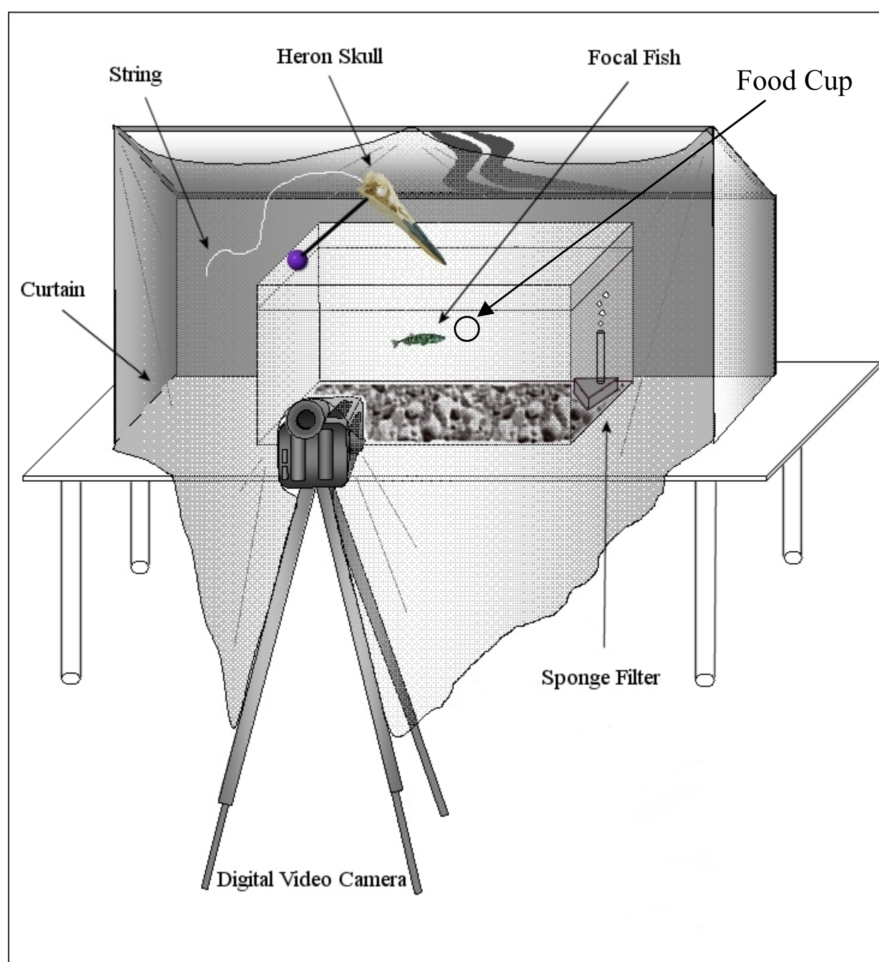


Figure 6.1: Predator Avoidance Test Experiment Set-Up (figure adapted from Crook, 2004). Tank dimensions = 40.6 x 20.3 x 25.4 cm (not to scale), camera positioned 50 cm from the front of the tank.

Testing: The predator avoidance test began with the addition of 0.1 g of frozen brine shrimp to a transparent food cup suspended in the middle of the top third of the experimental tank with the acclimated fish. The heron skull was attached to the tank by a 15 cm bar on a hinge and frame that fit around the lip of the tank (Figure 6.1). The frame positioned the heron bill so that when it was dropped from its upright position, it would hit the water surface in the middle of the tank directly above the food cup (Giles, 1984). The simulated heron attack was initiated once the fish came within one body length of the food cup. The heron skull was released twice, with a one-second interval between strikes, to simulate a natural attack. Video recording began once the food was added to the tank and continued for 5 minutes after the heron attack. If the fish did not approach the food cup within 10 minutes, the trial was aborted. Fish with aborted trials were re-tested the following day. Only one re-test was conducted for each fish per testing period. The following observations were recorded offline using frame-by-frame analysis and totaling the following behaviors in one-minute bins:

1) time spent swimming
2) number of bites at food
3) # of approaches to the food cup (an approach was defined as directional movement towards the food cup, subsequent approaches were counted if the fish turned away and approached again)
4) latency time (time lapsed (s) until first food-cup approach)
5) response to attack (1=no response, 2=small movement (slow movement less than a body length), 3=jumping in place, 4=escape from site)
6) number of feeding bouts
7) time spent within 1 body length of the food cup

(Huntingford, 1976b (#1, 3, 7); Wibe, Nordtug, & Jenssen, 2001 (#5); Bell, 2004 (#2); Wibe, Fjekl, Rosenqvist, & Jenssen, 2004 (#4).)

**Data Collection and Analysis for Predator Avoidance Experiment**

The predator avoidance test recordings were scored by viewing the tapes frame-by-frame offline, by K. K. and observers blind to the treatment. The proportional behavioral measures (time spent swimming and time spent within 1 body length of the food cup) were defined as the average number of seconds fish spent engaged in each behavior in the 5 min trial and was calculated as follows:

Let $t_1$ = time (s) engaged in behavior for the first minute, $t_2$ = time (s) engaged in behavior for the second minute, etc.

$$\text{Trial average} = \frac{t_1 + t_2 + t_3 + t_4 + t_5}{300s}$$

The count data [number of bites at the food cup, latency time (time lapsed until the fish approached the food cup), number of approaches to the food cup and number of feeding bouts] were summed for the entire 5 min trial. Lastly, the response of the fish to attack was evaluated and recorded as: 1=no response, 2=small movement (slow movement less than a body length), 3=jumping in place, and 4=escape from site (Wibe, Nordtug, & Jenssen, 2001).

In this experiment fish had to approach the food cup for the trial to start. In both testing periods many fish did not approach the food cup within the 10 min limit and these trials were considered aborted (see Tables 6.1 & 6.2). Data for fish exposed to 10 ng/ and 70 ng/L of $EE_2$ were not included in the statistical analysis due to the low numbers of fish that completed the trials; thus, only data for the control and 100 ng/L were analyzed and compared. Males and females were pooled for statistical analyses.

Fisher's Exact Probability Tests for independence were used to determine if controls or fish treated with 100 ng/L of $EE_2$ were more likely to approach the food cup

within the 10 min trial limit for each testing period. Mann-Whitney U Tests were used to compare the control and 100 ng/L $EE_2$ treated fish in their responses to the attack at each testing period. To compare the response of fish to the heron attack at post-exposure and post-depuration, Wilcoxon Signed Rank Tests were used. Parametric tests were used for all other behaviors. Paired t-tests were conducted to track any differences in behaviors as a function of testing period among control and 100 ng/L $EE_2$ treated fish. Independent t-tests were used to determine if the control and 100 ng/L $EE_2$ treated fish behaved differently after exposure to $EE_2$ and after depuration.

Data Transformation

The proportionate data (*p*) were arcsine ($p^{1/2}$) transformed to stabilize variances. They were evaluated on both transformed and untransformed scales. As transformed and untransformed scales were concordant, only untransformed scale results are presented here.

## 6.2 Results: Predator Avoidance Experiment

After exposure to $EE_2$, the 100 ng/L treated fish were more likely to approach the food cup than the control fish (Fisher's Exact Test, two-sided, $p = 0.004$) (Table 6.3). This difference continued post-depuration (Fisher's Exact Test, two-sided, $p < 0.0005$) (Table 6.4) (Figure 6.2).

| $EE_2$ Exposure Level | No | Yes | Total |
|---|---|---|---|
| 0 ng/L | 14 (50%) | 14 (50%) | 28 |
| 100 ng/L | 3 (12%) | 22 (88%) | 25 |

Table 6.3: Post-Exposure: Number of fish that did and did not approach the food cup (% of total in each exposure level). Fisher's Exact Test, $p = 0.004$

| $EE_2$ Exposure Level | No | Yes | Total |
|---|---|---|---|
| 0 ng/L | 16 (57.1%) | 12 (42.9%) | 28 |
| 100 ng/L | 2 (8%) | 23 (92%) | 25 |

Table 6.4: Post-Depuration: Number of fish that did and did not approach the food cup (% of total in each exposure level). Fisher's Exact Test, $p < 0.0005$

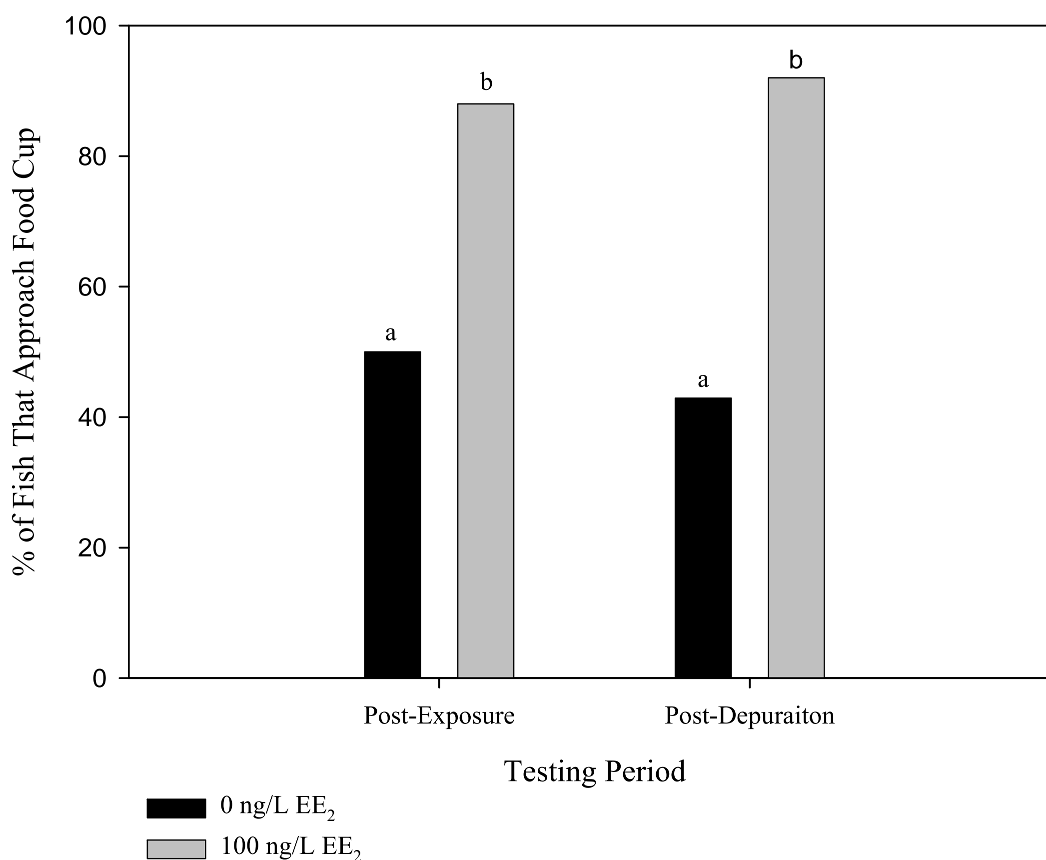


Figure 6.2: Fish that approached the food cup, post-exposure and post-depuration. Fisher's Exact Probability Test, different letters indicate $p < 0.005$.

The response to the simulated heron attack was not different between the control and the 100 ng/L treated fish post-exposure (control $N = 14$, 100 ng/L $N = 22$, Mann-Whitney U

Test, $Z$ = -0.23, $p$ =0.62) or post-depuration (control $N$ = 12, 100 ng/L $N$ = 23, $Z$ = -0.23, $p$ = 0.96. Post-exposure (control $N$ = 14, 100 ng/L $N$ = 22) the number of bites at the food were significantly different between control ($M$ = 0.21) and 100 ng/L $EE_2$ treated fish, ($M$ = 6.68; independent t-test, $p < 0.0005$) (Figure 6.3).

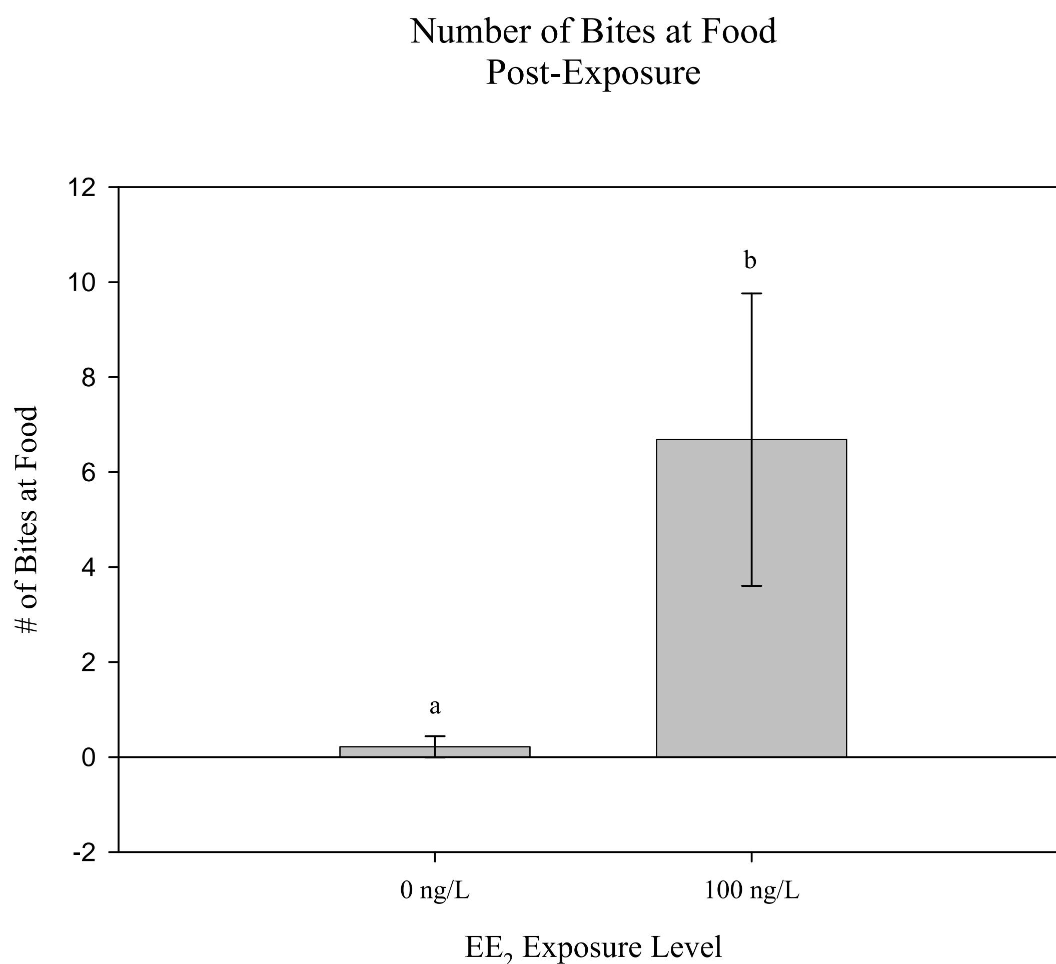


Figure 6.3: Post-Exposure: Number of Bites at food, different letters, a and b, indicate differences at $p < 0.05$, independent t-test. Error bars represent 95% confidence limits.

The time spent swimming was also significantly different between control ($M$ = 0.60) and 100 ng/L $EE_2$ treated fish ($M$ = 0.89), independent t-test, $p$ =0.02) (Figure 6.4).

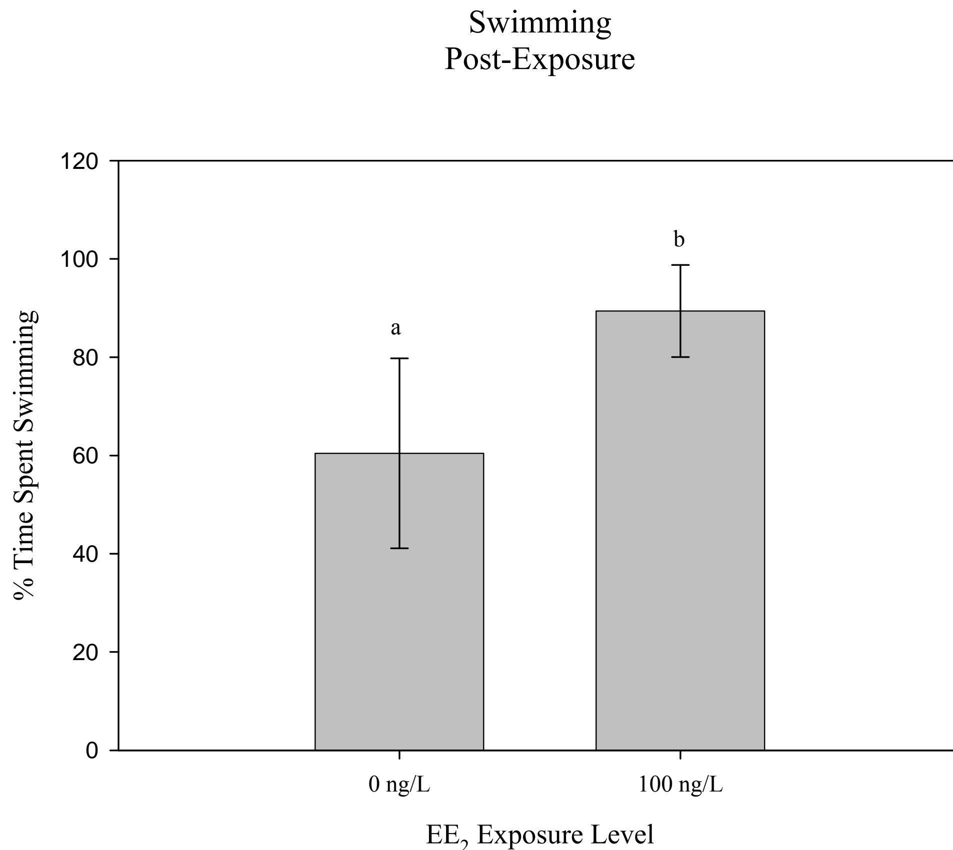


Figure 6.4: Post-Exposure: Time spent swimming; different letters, a and b, indicate significant differences of $p < 0.05$, independent t-test. Error bars represent 95% confidence limits.

Post-depuration (control $N = 12$, 100 ng/L $N = 23$) the latency to approach the food cup was much longer in the controls ($M = 378.58$) compared to the 100 ng/L $EE_2$ treated fish ($M = 93.04$; independent t-test, $p < 0.0005$) (Figure 6.5).

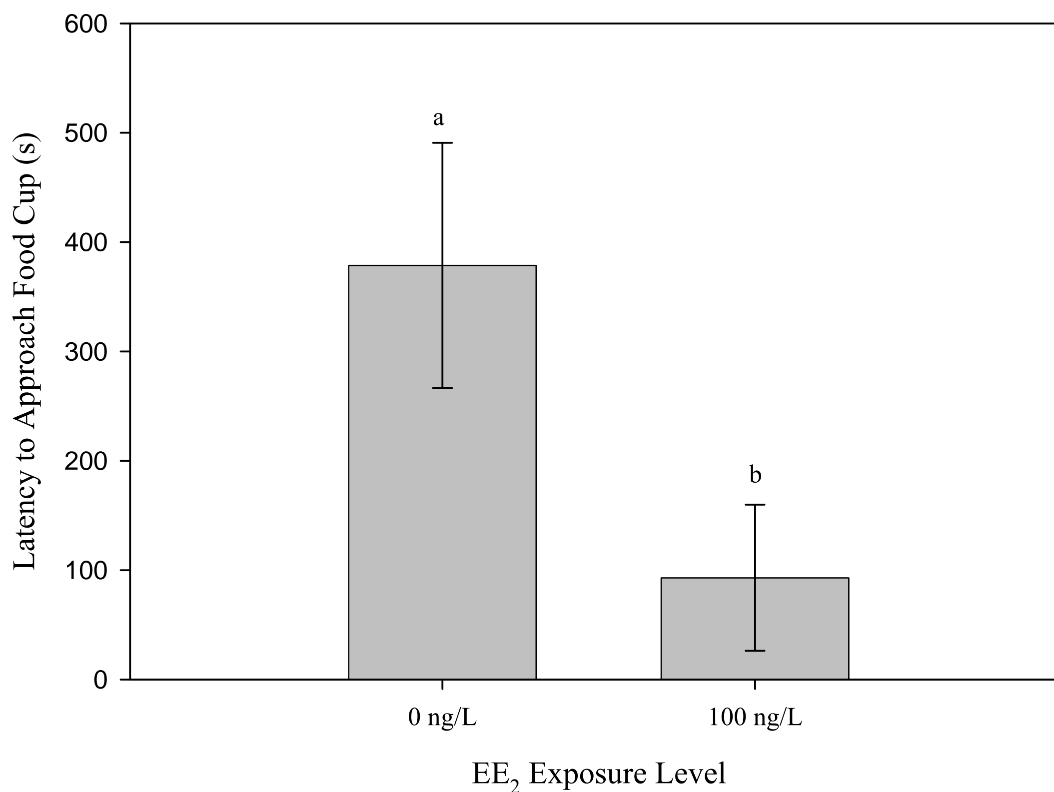


Figure 6.5: Post-Depuration: Latency to approach the food cup; different letters, a and b, indicate significant differences of $p < 0.05$, independent t-test. Error bars represent 95% confidence limits.

Controls also spent less time within one body length of the food cup ($M$ = 0.01) compared to the fish exposed to 100 ng/L $EE_2$ ($M$ = 0.08; independent t-test, $p$ =0.03 (Figure 6.6).

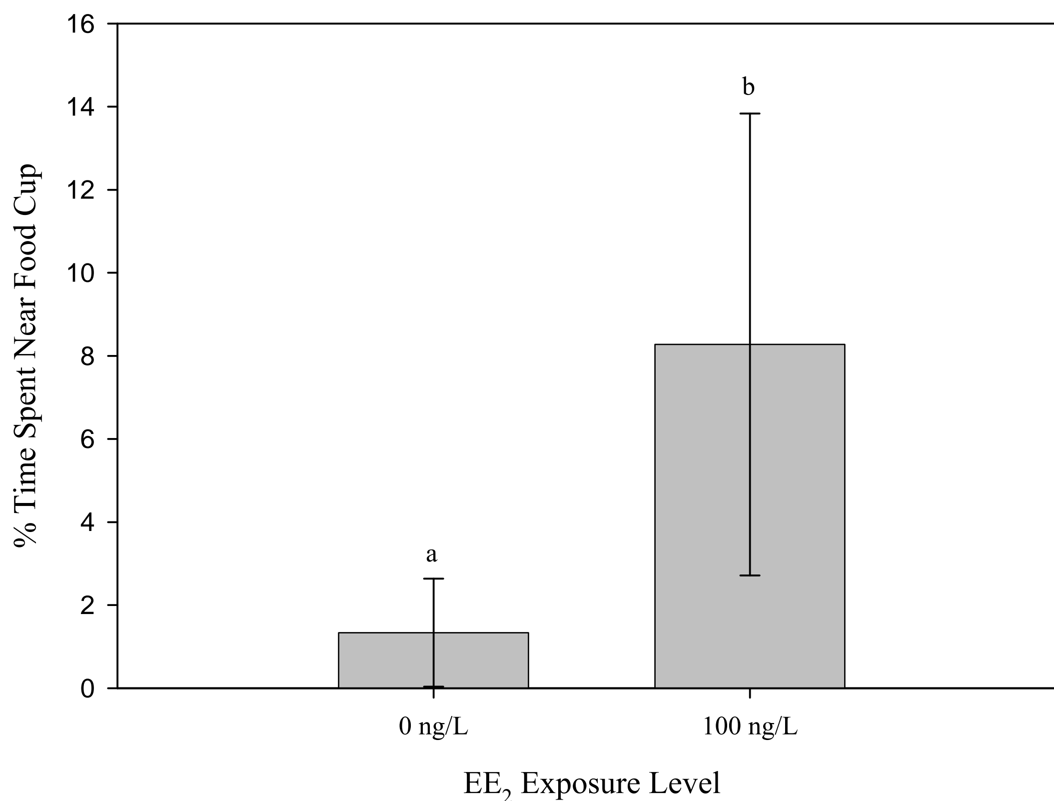


Figure 6.6: Post-Depuration: % time spent within one body length of the food cup; different letters, a and b, indicate significant differences of $p < 0.05$, independent t-test. Error bars represent 95% confidence limits.

Testing period did not have an effect on the response to the attack for controls ($N = 7$, Wilcoxon-Signed Rank Test, $Z = -0.38$, $p = 0.71$) or for fish treated with 100 ng/L of $EE_2$ ($N = 21$, $Z = -0.05$, $p = 0.96$). None of the control fish behaviors changed from post-exposure to post-depuration ($N = 7$). Testing period did have an effect on some of the behaviors of fish exposed to 100 ng/L $EE_2$ ($N = 21$). The number of bites at the food was

higher post-exposure than post-depuration (*M* = 7.00 to *M* = 1.76; paired t-test, $p = 0.009$) (Figure 6.7).

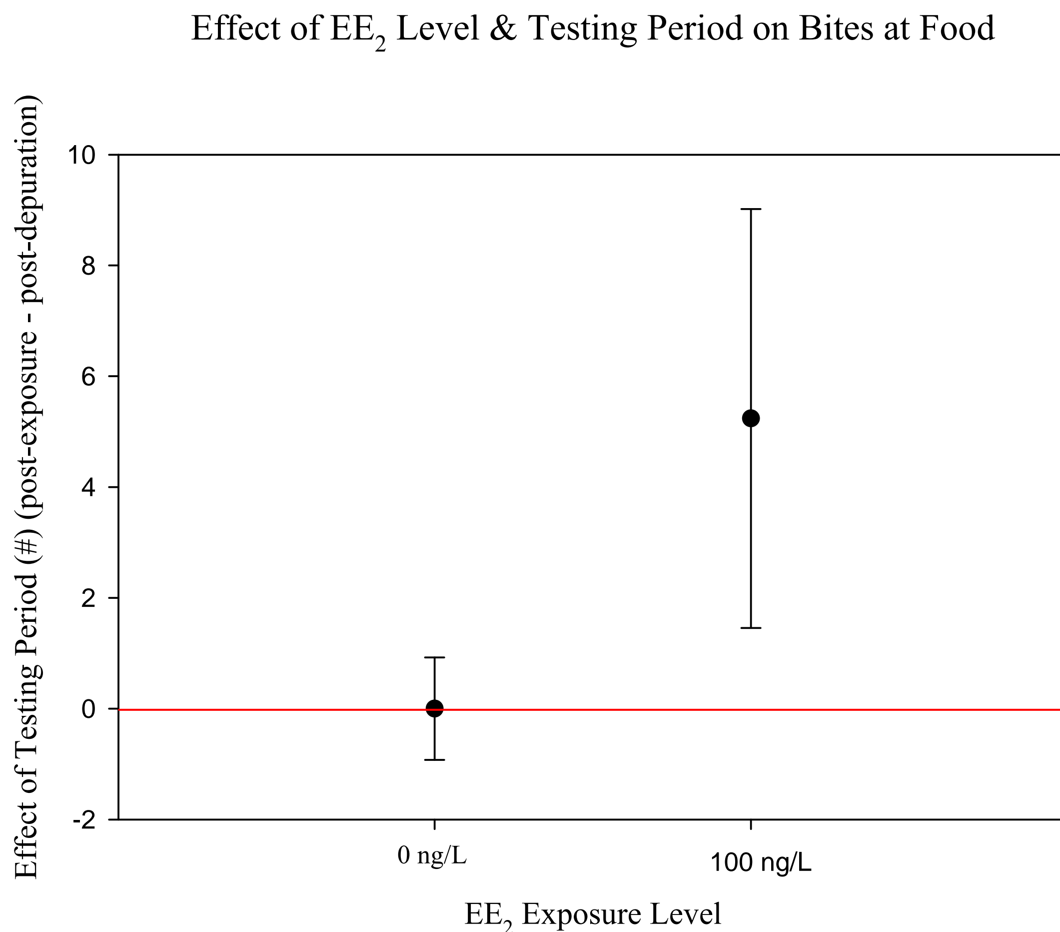


Figure 6.7: Average effects of testing period (males and females pooled). Mean number of bites at food, post-exposure – post-depuration (males and females pooled). Error bars represent 95% confidence limits.

The number of approaches to the food cup for the 100 ng/L treated fish was also higher post-exposure than post-depuration ($M = 6.52$ to $M = 3.48$; paired t-test, $p = 0.02$) (Figure 6.8).

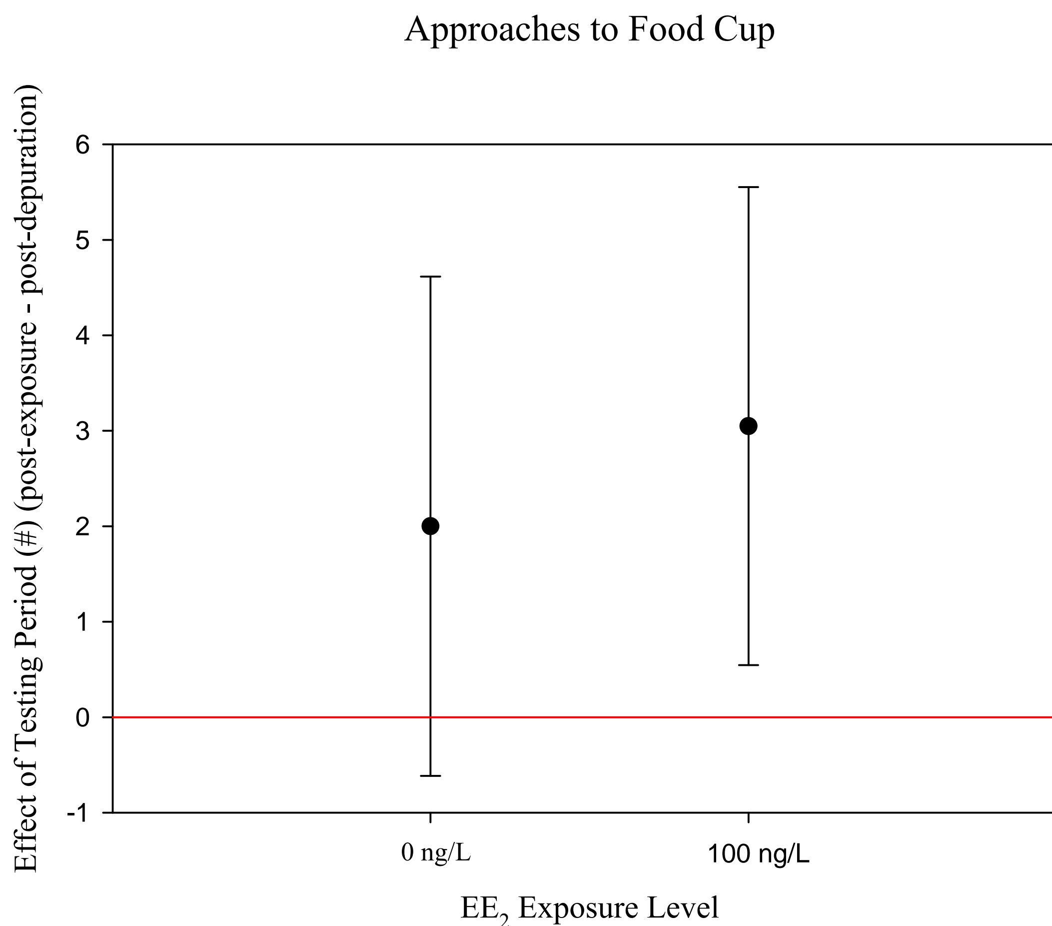


Figure 6.8: Average effects of testing period (males and females pooled). Mean number of approaches to food cup, post-exposure – post-depuration (males and females pooled). Error bars represent 95% confidence limits.

In addition, the proportion of time spent swimming decreased from post-exposure to post-depuration ($M = 0.93$ to $M = 0.65$; paired t-test, $p = 0.0003$) (Figure 6.9).

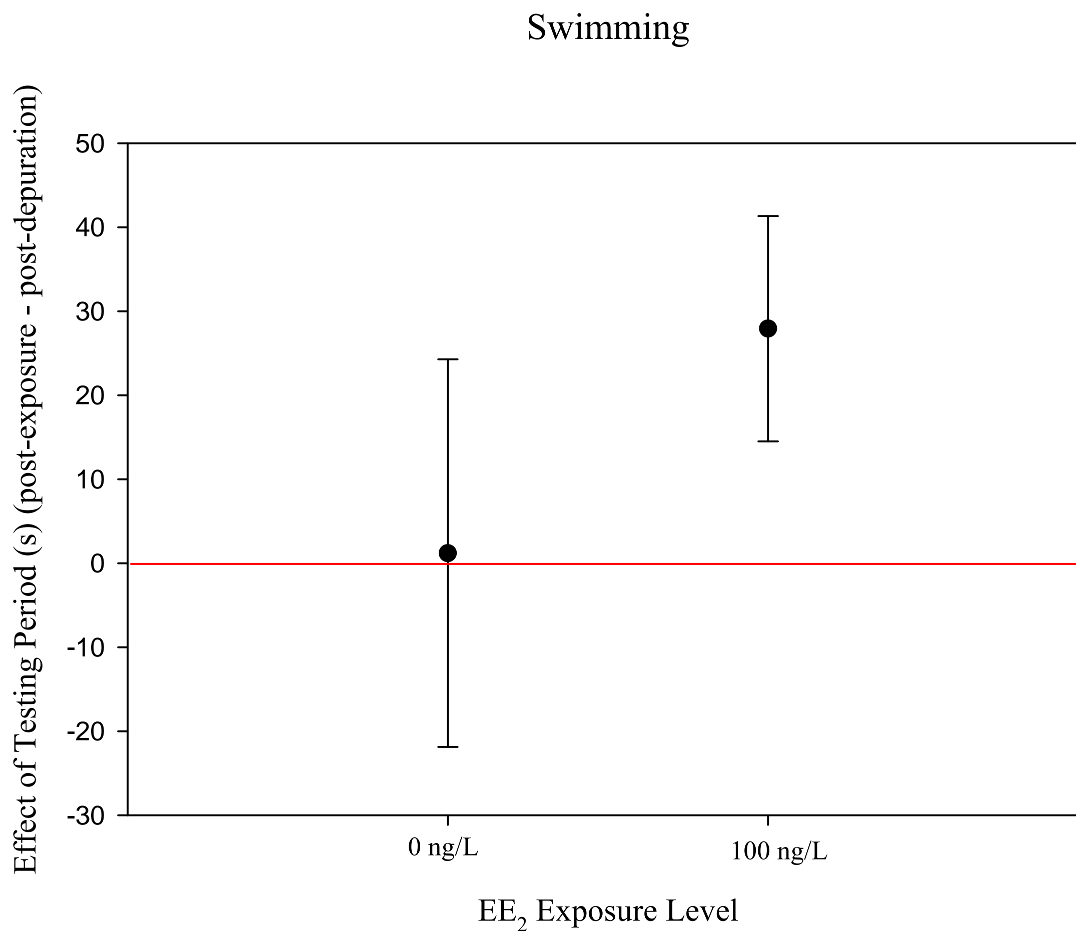


Figure 6.9: Average effects of testing period (males and females pooled). Mean proportion of time spent swimming, post-exposure – post-depuration (males and females pooled). Error bars represent 95% confidence limits.

Testing period did not have an effect on latency time to approach the food cup, the response to the attack, the number of feeding bouts or the time spent within one body length of the food cup.

The Effect of Captivity on Predator Avoidance

None of the behaviors tested for the predator avoidance experiment for "wild" male and female fish tested immediately after capture and again after 60 days were significantly different between testing periods (paired t-tests).

## 6.3 Discussion: Predator Avoidance Experiment

Exposure to $EE_2$ alters behaviors that contribute to successful predator avoidance. Fish exposed to $EE_2$ were more likely to approach the food cup, where the heron usually attacked, than controls. These changes in predation avoidance were evident even after exposure was terminated and fish were moved to clean water. Avoiding the food cup would allow control fish to incur survival benefits, while fish exposed to $EE_2$ increased their vulnerability to predators by engaging in risky behavior under predation risk. For instance, exposed fish took more bites at the food and swam more in the open after the simulated predator attack. This trend toward risky behavior in exposed fish continued even after depuration. Control fish did not change their behaviors after depuration, so this was not the result of learning or experience. Post-depuration, the fish exposed to 100 ng/L of $EE_2$ were quicker to approach the food cup, spent more time near it, took fewer bites at the food, and spent less time swimming.

Taken together these data imply that a nominal concentration of 100 ng/L of $EE_2$ has detrimental and long-lasting effects on predator avoidance. This is the first report on predator avoidance in adult fish exposed to $EE_2$ and it is in agreement with the observation that fish exposed to contaminants are more vulnerable to predator capture than fish that were not exposed (Smith & Logan, 1997). Similar results were reported by

Bell (2004) for threespine sticklebacks exposed to 100 ng/L of $EE_2$ from fertilization to the sub-adult stage (average of 211 days of exposure). In Bell's experiments, $EE_2$ exposure increased the swimming rate and the rate of foraging under the risk of predation. These behavioral changes render the fish more vulnerable to predation. Although Bell's work and the outcome from this study were comparable, the mechanism for the behavioral changes may not be identical given that the fish in Bell's study were exposed to $EE_2$ during the 'critical' period for sexual differentiation (Hahlbeck, Griffiths & Bengtsson, 2004) when organizational effects from $EE_2$ are likely. Changes in behavior from exposure to $EE_2$ in the adult stage are more likely the result of activational effects (Breedlove & Hampson, 2002; Cooke, Hegstrom, Villeneuve, & Breedlove, 1998). Although activational effects are often temporary, that does not seem to be the case here after 100 days of depuration. It is possible that the result would be different after a longer depuration period.

Other environmental pollutants have been reported to have detrimental effects on predator avoidance in fish and amphibians (Brown, Johansen, Colgan, & Mathers, 1985; Carlson, Bradbury, Drummond, & Hammermeister, 1998; Eroschenko, Amstislavsky, Schwabl, & Ingermann, 2002; Little, Archeski, Flerov, & Kozlovskaya, 1990; Sullivan, Atchison, Kolar, & McIntoch, 1978; Verrell, 2000; Weis & Weis, 1995; Weis & Weis, 1998; Wibe, Nordtug, & Jenssen, 2001). Few of these studies, however, also incorporated depuration in the protocol to asses the persistence of behavioral changes after exposure ends. Research that incorporated depuration as a part of the protocol reported diminishing effects on predator avoidance after depuration. Transitory decreases in predator avoidance from exposure to pollutants such as methyl-mercury

(mummichog embryos and larvae, Weis & Weis, 1995), lead (mummichog larvae, Weis & Weis, 1998), and bis(tributyltin)oxide (paint antifouling agent; adult male threespine stickleback, Wibe, Nordtug, & Jenssen) have been reported. Although there are certainly differences among the effects of different contaminants and exposure regimes, it is remarkable that $EE_2$ seems to have long-lasting effects on predator avoidance in fourspine sticklebacks.

Sticklebacks in the wild have variable anti-predator responses that are subject to a host of other factors including hunger (threespine stickleback, Milinski, 1993) and experience (threespine stickleback, Giles & Huntingford, 1984). The variability in these factors was minimized by collecting fish from the same habitat, keeping them under identical nutritional conditions, and testing the same fish at two testing periods (post-exposure and post-depuration). Comparisons of the same fish between the testing periods did not indicate that the behavioral differences were due to habituation -- the control fish did not demonstrate any significant differences in their behavior from post-exposure to post-depuration. Also, Wibe, Nordtug, & Jenssen (2001) reported that threespine stickleback did not habituate to an overhead predator attack even after tests were conducted once a day for 5 days. Given that the inter-test interval used in this experiment was 100 days, it is unlikely that habituation was a factor.

The evidence that fish exposed to $EE_2$ engaged in behaviors that made them vulnerable to predation may be a product of the stimulatory effects of $EE_2$ on growth (Bell, 2004; Johnsson & Bjornsson, 1994). A higher growth rate would increase nutritional needs and perhaps the risk that the fish were willing to take to obtain food

(Werner & Anholt, 1993). Since the fish used in this study were caught in the wild as adults their growth rate is unknown.

The ability to avoid situations that might result in being eaten is essential to survival. The results of this study suggest the possibility that altered predator avoidance behaviors in individual fish caused by $EE_2$ exposure can result in deleterious effects on populations as a whole and can be long lasting.

## 7.0 General Conclusions

Exposing stickleback fish to ethinyl estradiol adversely affects important behaviors and also markedly changes their gonads (Table 7.1). Exposed fish were significantly less active and did not actively avoid a predator as did unexposed animals. $EE_2$ affected aggression in sticklebacks as well, but this varied depending upon the level of exposure. Surprisingly, 100 days in clean water actually reversed the effects of $EE_2$ on activity. Depuration did not, however, reverse the detrimental changes in aggression or predator avoidance. These effects on individuals may ultimately affect their reproduction and survival. Changes in individual behaviors such as these can also have profound population-level consequences (Rose, Murphy, Diamond, Fuiman, & Thomas, 2003).

Activity, aggression, and predator avoidance were measured separately in this study, but under natural conditions these behaviors are not entirely independent of each other. Activity and risky behaviors likely affect many common behaviors, such as foraging, searching for territories and mates, and finding shelter. However, if all the changes measured in $EE_2$ exposed fish were entirely the result of an overall decrease in activity, animals would have remained relatively inactive during the aggression and predator avoidance tests as well – not displaying the risky behaviors seen in the aggression and predator avoidance tests. This was not the case; $EE_2$ exposure did not result in universal changes in activity levels or risky behavior across all behavioral contexts.

$EE_2$ exposure effected activity levels differently in each of the behavioral tests. Compared to the controls the fish that were exposed to 100 ng/L of $EE_2$ spent less time swimming in the activity test but more time swimming than the controls in the predator

avoidance test. This seemingly contradictory data indicates that the context in which the behavioral measure is taken is important. The predator avoidance test was administered when the fish were in a high state of hunger. This indicates that fish exposed to $EE_2$ are less active under satiated conditions but when starved can overcome the effect and exhibit poor predator avoidance to gain access to food. Fish exposed to 100 ng/L of $EE_2$ exhibited risky behavior in the predator avoidance test but not in the aggression test. Similar to the activity levels, this difference is likely due to the high state of hunger that the fish were in during the predator avoidance test.

In sticklebacks, exposure to $EE_2$ as adults resulted in abnormal testes and ovaries. In similar studies, gonadal abnormalities that accompanied deleterious behavioral changes also ultimately affected the reproductive fitness of guppies (Kristensen, Baatrup, & Bayley, 2004), threespine stickleback (Maunder, Matthiessen, Sumpter, & Pottinger, 2007), and medaka (Balch, Mackenzie, & Metcalfe, 2004). In this study, males exposed to $EE_2$ developed ovotestes and some expressed mature stages of spermatogenesis while females possessed more immature stages of oogenesis than controls. The presence of ovotestes and the differential timing of maturation in gonads could result in reproductive failure in this species as well.

Given that both oogenesis and spermatogenesis require a great deal of energy (Wooton, 1984), fish in active gametogenesis must spend extra time searching for food to meet their nutritional needs. Given this assumption, fish in active gametogenesis might be less active and display risky behavior in the presence of a predator than fish with quiescent gonads. That was not the case in this study, however, in either males or females. Exposed fish were less active and avoided a potential predator less than

unexposed fish. Perhaps the level of risk that fish were willing to take in the presence of a predator was the result of the presence of food during testing coupled with the nutritional demands of producing mature gametes. Since the activity experiment did not incorporate food as part of the protocol, animals might have been less willing to burn energy in the activity experiments.

To obtain an accurate picture of the full effects of EDCs on individuals and populations, both sexes and all life stages must be taken into account due to differences in the biotransformation (Winzer, Van Noorden, & Kohler 2002) and bioaccumulation (Johnston, Fisk, Whittle, & Muir, 2002) of pollutants among these groups (see review in Orlando & Guillette, 2007). The effects of EDCs on both sexes are rarely published because researchers either use only one sex or do not report sex as a factor in their statistical analyses (Burger, Fossi, McClellan-Green, & Orlando, 2007). Even though sex differences were not found in this study, it is still important to report the information to contribute to the body of work regarding sex differences in EDC research. It is equally important to look at how exposure at different life stages affects wildlife as most populations contain both young and adults and changes from exposure at early life stages may differ from those occurring later in life. Also, exposure to EDCs may be variable and may not encompass the entirety of a life stage since the total estrogenic input to a body of water is not constant, but varies with sewage outflow (Martinovic, Hogarth, Jones, & Sorensen, 2007) and precipitation.

Behavior is the culmination of interactions between the physiological state of an organism and its environment. If natural selection functions in a manner that produces an optimal phenotype for a particular environment, $EE_2$ and other EDCs can alter what is

"optimal" in contaminated environments (Adkins-Regan & Weber, 2002). Altering optimal phenotypes will not only affect individuals in a population, but can ultimately impact populations and entire aquatic communities (Kidd et al., 2007).

This study demonstrates that even low levels of environmental contaminants can alter fish behavior and gametogenesis in a subtle but potentially negative manner. Importantly, these effects can be long-lasting. These data also support growing evidence demonstrating that $EE_2$ is a potent estrogen mimic and behavioral modifier in fish (Balch, Mackenzie, & Metcalfe, 2004; Bell, 2001, 2004; Kristensen, Baatrup, & Bayley, 2004). $EE_2$ exposure does not have to occur during development for these changes to occur. Activational effects of exposure change stickleback behavior in adults markedly as well. Using sensitive behavioral markers like those in this study will aid us in understanding the real-world impact of EDCs on wildlife.

| Experiment | Post-Exposure | Post-Depuration | Δ |
|---|---|---|---|
| Gonad Histology | • males: ovotestes in 3/7 $EE_2$ fish | • males: ovotestes in 1/6 $EE_2$ fish<br>• males GSI: 100 < C, 10, 70 | |
| | • females: 100 oocytes less mature<br>• GSI : 10 > 100 | • females: no difference | |
| Male Coloration | • no difference | • no difference | |
| Activity | • controls more active than 100 | • no difference | C ↓<br>100 ↑ |
| Aggression | • approaches: no difference | • 10 approach intruder more | 10 ↑ |
| | • hiding: 100 hides more than 10 | • hiding: 100 hides more than 10 | |
| | • swimming: 10 swims more than 70 | • swimming: 10 swims more than 70 | |
| Predator Avoidance | • likelihood to participate: 100 greater than C | • likelihood to participate: 100 greater than C | |
| | • bites at food: 100 more than C | • bites at food: no difference. | 100 ↓ |
| | • approaches to food cup: 100 more than C | • approaches to food cup: no difference | 100 ↓ |
| | • swimming: 100 more than C | • swimming: no difference | 100 ↓ |
| | • latency approach: no difference | • latency approach: 100 less than C | 100 ↓ |
| | • time near food: no difference | • time near food: 100 more than C | 100 ↑ |

Table 7.1: Summary of Results: Findings for each testing period and significant changes between them. C = control, 10 = 10 ng/L, 70 = 70 ng/L, and 100 = 100 ng/L of $EE_2$; Δ shows significant changes between testing periods.

**Literature Cited:**

Adkins-Regan, E., & Weber, D. N. (2002). Mechanisms of behavior. In G. Dell'Omo (Ed.), *Behavioural Ecotoxicology* (pp. 91-166). West Sussex: John Wiley & Sons Ltd.

Aherne, G. W., & Briggs, R. (1989). The relevance of the presence of certain synthetic steroids in the aquatic environment. *Journal of Pharmacy & Pharmacology, 41*, 735-736.

Andersson, C., Katsiadaki, I., Lundstedt-Enkel, K., & Orberg, J. (2007). Effects of 17[alpha]-ethynylestradiol on EROD activity, spiggin and vitellogenin in three-spined stickleback (*Gasterosteus aculeatus*). *Aquatic Toxicology, 83*(1), 33-42.

Andersson, E., Mayer, I., & Borg, B. (1988). Inhibitory effect of 11-ketoandrostenedione and androstenedione on spermatogenesis in the three-spined stickleback, *Gasterosteus aculeatus* L. *Journal of Fish Biology, 33*(6), 835-840.

Atkinson, S., Atkinson, M. J., & Tarrant, A. M. (2003). Estrogens from sewage in coastal marine environments. *Environmental Health Perspectives, 111*(4), 531-535.

Bakker, T. C. M. (1985). Two-way selection for aggression in juvenile, female and male sticklebacks, with some notes on hormonal factors. *Behaviour, 93*, 69-81.

Bakker, T. C. (1993). Positive genetic correlation between female preference and preferred male ornament in sticklebacks. *Nature, 352*, 155–156.

Bakker, T. C. M., & Sevenster, P. (1983). Determinants of dominance in male sticklebacks (*Gasterosteus aculeatus*). *Behaviour, 86*, 55-71.

Balch, G. C., Mackenzie, C. A., & Metcalfe, C. D. (2004). Alternations to gonadal development and reproductive success in Japanese medaka exposed to 17a ethinylestradiol. *Enviromental Toxicology and Chemistry, 23*(3), 782-791.

Baronti, C., Curini, R., D'Ascenzo, G., Corcia, A. D., Gentili, A., & Samperi, R. (2000). Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. *Environmental Science & Technology, 34*(24), 5059-5066.

Basil, J., & Sandeman, D. (2000). Crayfish (*Cherax destructor*) use tactile cues to detect and learn topographical changes in their environment. *Ethology, 106*(3), 247-259.

Bass, A. H. (1998). Behavioral and evolutionary neurobiology: A pluralistic approach. *American Zoologist, 38*, 97-107.

Bayley, M., Neilsen, J. R., & Baatrup, E. (1999). Guppy sexual behavior as an effect biomarker of estrogen mimics. *Ecotoxicology & Environmental Safety, 45*, 68-73.

Beauvais, S. L., Jones, S. B., Parris, J. T., Brewer, S. K., & Little, E. E. (2001). Cholinergic and behavioral neurotoxicity of carbaryl and cadmium to larval rainbow trout (*Oncorhynchus mykiss*). *Ecotoxicology and Environmental Safety, 49*(1), 84-90.

Bell, A. M. (2001). Effects of an endocrine disrupter on courtship and aggressive behaviour of male three-spined stickleback, G*asterosteus aculeatus*. *Animal Behaviour, 62*, 775-780.

Bell, A. M. (2004). An endocrine disrupter increases growth and risky behavior in threespined stickleback (*Gasterosteus aculeatus*). *Hormones and Behavior, 45*, 108-114.

Bell, M. A., & Foster, S. A. (1994). *The evolutionary biology of the threespine stickleback*. New York: Oxford University Press.

Beukema, J. J. (1968). Predation by the three-spined stickleback (*Gasterosteous aculeatus*): The influence of hunger and experience. *Behaviour, 31*, 1-126.

Bjerselius, R., Lundstedt-Enkel, K., Olsen, H., Mayer, I., & Dimberg, K. (2001). Male goldfish reproductive behaviour and physiology are severely affected by exogenous exposure to 17b-estradiol. *Aquatic Toxicology, 53*, 139-152.

Blair, R. M., Fang, H., Branham, W. S., Hass, B. S., Dial, S. L., Moland, C. L., et al. (2000). The estrogen receptor relative binding affinities of 188 natural and xenochemicals: Structural diversity of ligands. *Toxicological Sciences, 54*, 138-153.

Bond, G. W. (1968). *Breeding cycle and maturation of the sticklebacks, Apeltes quadracus* (Mitchell)*, Gasterosteus aculeatus* (Linnaeus) *and Pungitius pungitius* (Linnaeus)*, in two Rhode Island estuaries.* University of Rhode Island.

Borg, B. (1981). Effects methyltestosterone on spermatogenesis and secondary sexual characters in the three spined stickleback (G*asterosteus aculeatus*). *General and Comparative Endocrinology, 44*, 177-180.

Borg, B., & Mayer, I. (1995). Androgens and behaviour in the three-spined stickleback. *Behaviour, 132*(13-14), 1025-1035.

Bornestaf, C., Antonopoulou, E., Mayer, I., & Borg, B. (1997). Effects of aromatase inhibitors on reproduction in male three-spined sticklebacks, *Gasterosteus aculeatus*, exposed to long and short photoperiods. *Fish Physiology and Biochemistry, 16*(5), 419-423.

Breedlove, S. M., & Hampson, E. (2002). *Behavioral endocrinology* (Second ed.). Cambridge: MIT Press.

Brown, J. A., Johansen, P. H., Colgan, P. W., & Mathers, R. A. (1985). Changes in the predator-avoidance behavior of the juvenile guppies (*Poecilia reticulata*) exposed to pentachlorophenol. *Canadian Journal of Zoology, 63*, 2001-2005.

Burger, J., Fossi, C., McClellan-Green, P., & Orlando, E. F. (2007). Methodologies, bioindicators, and biomarkers for assessing gender-related differences in wildlife exposed to environmental chemicals. *Environmental Research, 104*(1), 135-152.

Calabrese, E. (2008) Hormesis: Why it is important to toxicology and toxicologists. *Environmental Toxicology and Chemistry, 27(7)*, 1451-1474.

Calabrese, E., & Baldwin, L. (2002). Defining hormesis. *Human and Experimental Toxicology 21*, 91–97.

Carlson, R. W., Bradbury, S. P., Drummond, R. A., & Hammermeister, D. E. (1998). Neurological effects on startle response and escape from predation by medaka exposed to organic chemicals. *Aquatic Toxicology, 43*, 51-68.

Chandra, A., Martinez, G. M., Mosher, W. D., Abma, J. C., & Jones, J. (2005). Fertility, family planning, and reproductive health of U.S. Women: Data from the 2002 national survey of family growth, national center for health statistics. *Vital Health Statistics, 23*(25).

Cleveland, L., Little, E. E., Ingersoll, C. G., Wiedmeyer, R. H., & Hunn, J. B. (1991). Sensitivity of brook trout to low ph, low and elevated aluminum concentrations during laboratory pulse exposures. *Aquatic Toxicology, 19*(4), 303-317.

Clotfelter, E. D., Bell, A. M., & Levering, K. R. (2004). The role of animal behaviour in the study of endocrine-disrupting chemicals. *Animal Behaviour, 68*(4), 665-676.

Clotfelter, E., & Rodriguez, A. (2006). Behavioral changes in fish exposed to phytoestrogens. *Environmental Pollution, 144*, 833-839.

Colborn, T., vom Saal, F. S., & Soto, A. M. (1993). Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Enviromental Health Perspectives, 101*(5), 378-384.

Cooke, B., Hegstrom, C. D., Villeneuve, L. S., & Breedlove, S. M. (1998). Sexual differentiation of the vertebrate brain: Principles and mechanisms. *Frontiers in Neuroendocrinology, 19*, 323–362

Craig-Bennett, A. (1931). The reproductive cycle of the three-spined stickleback, *Gasterosteus aculeatus* L. *Philosophical Transactions of the Royal* Society of *London*, B, 219, 197-279.

Crews, D. (1992). Diversity of hormone behavior relations in reproductive behavior. In J. B. Becker, S. M. Breedlove & D. Crews (Eds.), *Behavioral endocrinology* (pp. 143-186). Cambridge, MA: MIT Press.

Crews, D., Willingham, E., & Skipper, J. K. (2000). Endocrine disrupters: Present issues, future directions. *The Quarterly Review of Biology, 75*(3), 243-260.

Crisp, T. M., Clegg, E. D., Cooper, R. L., Wood, W. P., Anderson, D. G., Baetcke, K. P., et al. (1998). Environmental endocrine disruption: An effects assessment and analysis. *Environmental Health Perspectives, 106*(1), 11-46.

Crook, R. (Artist). (2004). *Experimental set up*

Czeczuga, B. (1980). Carotenoids in fish. *Pungitius pungitius* L. and *Gasterosteus aculeatus* L. (Gasterosteidae). *Hydrobiologia, 74*, 7-10.

Damstra, T., Barlow, S., Bergman, A., Kavlock, R., & Van der Kraak, G. (Eds.). (2002). *Global assessment of the state-of-the-science of endocrine disrupters*. Geneva, Switzerland: World Health Organization.

Denison, M. S., Chambers, J. E., & Yarbrough, J. D. (1981). Persistent vitellogenin-like protein and binding of DDT n the serum of insecticide-resistant mosquito fish (*Gambusian affinis*). *Comparative Biochemistry and Physiology, 69C*, 109-112.

Desbrow, C., Routledge, E. J., Brighty, G., Sumpter, J. P., & Waldcock, M. (1998). Identification of estrogenic chemicals in STW effluent. 1. Chemical fractionation and in vitro biological screening. *Environmental Science & Technology, 32*(11), 1549-1558.

Devlin, R. H., & Nagahama, Y. (2002). Sex determination and sex differentiation in fish: an overview of the genetic, physiological, and environmental influences. *Aquaculture, 208*, 191–364.

Donaldson, E. M., & Hunter, G. A. (1981). Sex control in fish with particular reference in salmonids. *Canadian Journal of Fisheries and Aquatic Sciences, 39*, 99– 110.

Dytham, C (2006) Choosing and using statistics. A biologist's guide. Blackwell Publishing, Oxford, UK.

Eroschenko, V. P., Amstislavsky, S. Y., Schwabl, H., & Ingermann, R. L. (2002). Altered behaviours in male mice, male quail, and salamander larvae following early

exposures to the estrogenic pesticide methoxychlor. *Neurotoxicology and Teratology, 24*, 29-36.

Food Quality and Protection Act of 1996, Pub. L. 104-170 (1996).

Fossi, M. C., & Leonzio, C. (1994). *Nondestructive biomarkers in vertebrates*. Lewis Publishers CRC Press.

Fowler, S. W. (1990). Critical review of selected heavy metal and chlorinated hydrocarbon concentrations in the marine environment. *Marine Environmental Research, 29*(1), 1-64.

Frischknecht, M. (1993). The breeding colouration of male sticklebacks (*Gasterosteus aculeatus*) as an indicator of energy investment in vigour. *Evolutionary Ecology, 7*, 439-450.

Giles, N. (1984). Development of the overhead fright response in wild and predator naive threespined sticklebacks *Gasterosteus aculeatus* L. *Animal Behaviour, 32*, 276-279.

Giles, N., & Huntingford, F. A. (1984). Predation risk and inter-population variation in anti-predator behavior in the three-spined stickleback (*Gasterosteus aculeatus* L.). *Animal Behaviour, 32*, 264-275.

Gomes, R. L., Scrimshaw, M. D., & Lester, J. N. (2003). Determination of endocrine disrupters in sewage treatment and receiving waters. *Trends in Analytical Chemistry, 22*(10), 697-707.

Gormley, K. L., & Teather, K. L. (2003). Developmental, behavioral, and reproductive effects experienced by Japanese medaka (*Oryzias latipes*) in response to short-term exposure to endosulfan. *Ecotoxicology and Environmental Safety, 54*, 330–338.

Gray, M. A., Niimi, A. J., & Metcalfe, C. D. (1999). Factors affecting the development of testis-ova in medaka, *Oryzias latipes*, exposed to octylphenol. *Environmental Toxicology and Chemistry, 18*, 1835-1842.

Hahlbeck, E., Griffiths, R., & Bengtsson, B. E. (2004). The juvenile three-spined stickleback (*Gasterosteus aculeatus* L.) as a model organism for endocrine disruption I. Sexual differentiation. *Aquatic Toxicology, 70*, 287-310.

Hahlbeck, E., Katsiadaki, I., Mayer, I., Adolfsson-Erici, M., James, J., & Bengtsson, B. E. (2004). The juvenile three-spined stickleback (*Gasterosteus aculeatus* L.) as a model organism for endocrine disruption II-- kidney hypertrophy, vitellogenin and spiggin induction. *Aquatic Toxicology, 70*, 311-326.

Hall, M. F. (1956). *A comparative study of the reproductive behaviour of the sticklebacks.* (Gasterosteidae). University of Oxford, Oxford.

Halldin, K., Berg, C., Brandt, I., & Brunstrom, B. (1999). Sexual behavior in Japanese quail as a test end point of endocrine disruption: Effects of *in ovo* exposure to ethinylestradiol and diethylstilbestrol. *Environmental Health Perspectives, 107*(11), 861-866.

Halling-Serensen, B., Nielsen, S. N., Lanzky, P. F., Ingerslev, F., Lutzheft, H. C. H., & Jergensen, S. E. (1997). Occurrence, fate and effects of pharmaceutical substances in the environment - A review. *Chemosphere, 36*(2), 357-393.

Hansen, P. D., Dizer, H., Hock, B., Marx, A., Sherry, J., McMaster, M., et al. (1998). Vitellogenin - a biomarker for endocrine disrupters. *Trends in Analytical Chemistry, 17*, 448-451.

Hoar, W. S. (1962). Hormones and the reproductive behaviour of the male three-spined stickleback (*Gasterosteus aculeatus*). *Animal Behaviour, 10*(3-4), 247-266.

Hohenblum, P., Gans, O., Moche, W., Scharf, S., & Lorbeer, G. (2004). Monitoring of selected estrogenic hormones and industrial chemicals in groundwaters and surface waters in Austria. *Science of The Total Environment, 333*(1-3), 185-193.

Holm, G., Lundstrom, J., Andersson, T., & Norrgren, L. (1994). Influences of halogenated organic substances on ovarian development and hepatic EROD activity in the three-spined stickleback, *Gasterosteus aculeatus*, and rainbow trout, *Oncorhynchus mykiss*. *Aquatic Toxicology, 29*, 241-256.

Huntingford, F. A. (1976a). The relationship between inter- and intra-specific aggression. *Animal Behaviour, 24*(3), 485-497.

Huntingford, F. A. (1976b). A comparison of the reaction of sticklebacks in different reproductive conditions towards conspecifics and predators. *Animal Behaviour, 24*(3), 694-697.

Ikonomou, M. G., Rayne, S., & Addison, R. F. (2002). Exponential increases of the brominated flame retardants, polybrominate diphenyl ethers, in Canadian Arctic from 1981-2000. *Environmental Science & Technology, 36*, 1886-1892.

Jobling, S., Coey, S., Whitmore, J. G., Kime, D. E., Look, K. J. W. V., McAllister, B. G., et al. (2002). Wild intersex roach (*Rutilus rutilus*) have reduced fertility. *Biology of Reproduction, 67*, 515-524.

Johnsson, J. I., & Bjornsson, B. T. (1994). Growth hormone increases growth rate, appetite and dominance in juvenile rainbow trout, *Oncorhynchus mykiss*. *Animal Behaviour, 48*, 177-186.

Johnston, T. A., Fisk, A. T., Whittle, D. M., & Muir, D. C. G. (2002). Variation in organochlorine bioaccumulation by a predatory fish; gender geography, and data analysis methods. *Environmental Science and Technology, 36*, 4238-4244.

Kasper, P., & Wintzel, H. (1985). Steroid binding to the cytosolic estrogen receptor from rat uterus. Influence of the orientation of substituents in the 17-position of the 8 beta-and 8 alpha-series. *Journal of Steroid Biochemistry, 23*(259-265).

Katsiadaki, I., & Scott, A. P. (2002). Detection of environmental androgens: A novel method based on enzyme-linked immunosorbent assay of spiggin, the stickleback (*Gasterosteus aculeatus*) glue protein. *Environmental Toxicology and Chemistry, 21*(9), 1946-1954.

Katsiadaki, I., Scott, A. P., & Mayer, I. (2002). The potential of the three-spined stickleback (*Gasterosteus aculeatus* L.) as a combined biomarker for oestrogens and androgens in European waters. *Marine Environmental Research, 54*, 725-728.

Kavlock, R. J., Daston, G. P., DeRosa, C., Fenner-Crisp, P., L. Earl Gray, J., Kaattari, S., et al. (1996). Research needs for the risk assessment of health and environmental effects of endocrine disruptors: A report of the U. S. EPA-sponsored workshop. *Environmental Health Perspectives, 104*(4), 1-26.

Khan, I. A., Hawkins, M. B., & Thomas, P. (1999). Gonadal stage-dependent effects of gonadal steroids on gonadotropin II section in the Atlantic croaker (*Micropagnias undulatus*). *Biology of Reproduction, 61*, 834-841.

Kidd, K. A., Blanchfield, P. J., Mills, K. H., Palace, V. P., Evans, R. E., Lazorchak, J. M., et al. (2007). Collapse of a fish population after exposure to a synthetic estrogen. *Proceedings of the National Academy of Sciences, 104*(21), 8897–8901.

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., et al. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in U. S. Streams, 1999-2000: A national reconnaissance. *Environmental Science & Technology, 36*, 1202-1211.

Kristensen, T., Baatrup, E., & Bayley, M. (2004). 17a-ethinylestradiol reduces the competitive reproductive fitness of the male guppy (*Poecilia reticulata*). *Biology of Reproduction, 72*(1), 150-156.

Kubo, K., Arai, O., Omura, M., Watanabe, R., Ogata, R., & Aou, S. (2003). Low dose effects of bisphenol A on sexual differentiation of the brain and behavior in rats. *Neuroscience Research, 45*, 345-356.

Kuch, H. M., & Ballschmiter, K. (2000). Determination of endogenous and exogenous estrogens in effluents from sewage treatment plants at the ng/l-level. *Fresenius' Journal of Analytical Chemistry, 366*, 392-395.

Kvarnemo, C., Forsgren, E., & Magnhagen, C. (1995). Effects of sex ratio on intra- and inter-sexual behaviour in sand gobies. *Animal Behaviour, 50*(6), 1455-1461.

Lange, R., Hutchinson, T. H., Croudace, C. P., Siegmund, F., Schweinfurth, H., Hampe, P., et al. (2001). Effects of the synthetic estrogen 17a-ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry, 20*(6), 1216-1227.

Larsson, D. G. J., Adolfsson-Erici, M., Parkkonen, J., Pettersson, M., Berg, A. H., Olsson, P. E., et al. (1999). Ethinyloestradiol - an undesired fish contraceptive. *Aquatic Toxicology, 45*, 91-97.

Laurence, G. C. (1972). Comparative swimming abilities of fed and starved larval largemouth bass (*Micropterus salmoides*). *Journal of Fish Biology, 4*(1), 73-78.

Lindsey, C. C. (1978). *Form, function and locomotory habits in fish* (Vol. VII). London: Academic Press.

Little, E. E. (2002). Behavioural measures of injuries to fish and aquatic organisms: Regulatory considerations. In G. Dell'Omo (Ed.), *Behavioural toxicology* (pp. 411-431). West Sussex: John Wiley & Sons Ltd.

Little, E. E., Archeski, R. D., Flerov, B. A., & Kozlovskaya, V. I. (1990). Behavioral indicators of sublethal toxicity in rainbow trout. *Archives of Environmental Contamination and Toxicology, 19*, 389-385.

Martinovic, D., Hogarth, W. T., Jones, R. E., & Sorensen, P. W. (2007). Environmental estrogens suppress hormones, behavior, and reproductive fitness in male fathead minnows. *Environmental Toxicology and Chemistry, 26*(2), 271-278.

Maunder, R. J., Matthiessen, P., Sumpter, J. P., & Pottinger, T. G. (2007). Impaired reproduction in three-spined sticklebacks exposed to ethinyl estradiol as juveniles. *Biology of Reproduction, 77*(6), 999-1006.

Mellor, A., & Rockwell, R. F. (2006). Habitat shifts and parasite loads of lesser snow geese (*Chen caerulescens caerulescens*). *Ecoscience, 13*(4), 497-502.

Michel, G., & Baulieu, E. E. (1980). Androgen receptor in rat skeletal muscle: Characterization and physiological variations. *Endocrinology, 107*(6), 2088-2098.

Milinski, M. (1993). Predation risk and feeding behavior. In T. J. Pitcher (Ed.), *Behavior of teleost fishes* (pp. 285-305). London: Chapman & Hall.

Milinski, M., & Heller, R. (1978). Influence of a predator on the optimal foraging behavior of sticklebacks (*Gasterosteus aculeatus* L.). *Nature, 275*, 642-644.

Muckensturm, B. (1965). L'epinoche et les leurres, complexite de la reaction. Comptes rendus de l'Académie des Sciences Paris*, 264*, 745-748.

Nash, J. P., Kime, D. E., Van der Ven, L. T. M., Wester, P. W., Brion, F., Maack, G., et al. (2004). Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. *Environmental Health Perspectives, 112*(17), 1725-1733.

National Research Council (1992). Environmental neurotoxicology. Washington, D. C.: National Academy Press.

Nelis, H. J. C. F., Lavens, P., Van Steenberge, M. M. Z., Sorgeloos, P., Criel, G. R., & Leenheer, A. P. D. (1988). Qualitative and quantitative changes in the carotenoids during development of the brine shrimp *Artemia*. *Journal of Lipid Research, 29*, 492-499.

Nielsen, L., & Baatrup, E. (2006). Quantitative studies on the effects of environmental estrogens on the testis of the guppy, *Poecilia reticulata*. *Aquatic Toxicology, 80*(2), 140-148.

Orlando, E. F., & Guillette, J. L. J. (2007). Sexual dimorphic responses in wildlife exposed to endocrine disrupting chemicals. *Environmental Research, 104*(1), 163-173.

Oshima, Y., Kang, I. J., Kobayashi, M., Nakayama, K., Imada, N., & Honjo, T. (2003). Suppression of sexual behavior in male Japanese medaka (*Oryzias latipes*) exposed to 17b-estradiol. *Chemosphere, 50*, 429-436.

Pandey, S. (1969). The role of pituitary and gonadal hormones in the differentiation of testis and secondary sex characters of the juvenile guppy *Poecilia reticulata* Peters. *Biology of Reproduction, 1*, 272–281.

Panter, G. H., Thompson, R. S., & Sumpter, J. P. (1998). Adverse reproductive effects in male fathead minnows (*Pimephales promelas*) exposed to environmentally relevant concentrations of the natural oestrogens, oestradiol and oesterone. *Aquatic Toxicology, 42*, 243-253.

Pawlowski, S., Aerle, R. v., Tyler, C. R., & Braunbeck, T. (2004). Effects of 17a-ethinylestradiol in a fathead minnow (*Pimephales promelas*) gondadal recrudesence assay. *Ecotoxicology & Environmental Safety, 57*, 330-345.

Pelkwijk, J. J. T., & Tinbergen, N. (1937). Eine reizbiologische analyse einiger verhaltensweisen von *Gasterosteus aculeatus* L. *Zeitschrift fur Tierpsychologie, I*, 193-204.

Peters, R. E. M., Courtenay, S. C., Cagampan, S., Hewitt, M. L., & MacLatchy, D. L. (2007). Effects on reproductive potential and endocrine status in the mummichog (*Fundulus heteroclitus*) after exposure to 17[alpha]-ethynylestradiol in a short-term reproductive bioassay. *Aquatic Toxicology, 85*(2), 154-166.

Phoenix, C., Goy, R., Gerall, A., & Young, W. (1959). Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. *Endocrinology, 65*, 369–382.

Picard-Aitken, M., Fournier, H., Pariseau, R., Marcogliese, D. J., & Cyr, D. G. (2007). Thyroid disruption in walleye (*Sander vitreus*) exposed to environmental contaminants: Cloning and use of iodothyronine deiodinases as molecular biomarkers. *Aquatic Toxicology, 83*(3), 200-211.

Porter, W. P., Jaeger, J. W., & Carlson, I. H. (1999). Endocrine, immune, and behavioural effects of aldicarb (carbamate), atrazine (triazine) and nitrate (fertilizer) mixtures at groundwater concentrations. *Toxicology and Industrial Health, 15*, 133-150.

Pressnell, J. K., & Schreibman, M. P. (1997). *Humanson's animal tissue techniques* (5 ed.). Baltimore: John Hopkins University Press.

Propper, C. R. (2005). The study of endocrine-disrupting compounds: Past approaches and new directions. *Integrative and Comparative Biology, 45*(1), 194-200.

Purdom, C. E., Hardiman, P. A., Bye, V. J., Eno, N. C., Tyler, C. R., & Sumpter, J. P. (1994). Estrogenic effects of effluents from sewage treatment works. *Chemistry and Ecology, 8*, 275-285.

Rodewald, A. D., & Foster, S. A. (1998). Effects of gravidity on habitat use and antipredator behaviour in three-spined sticklebacks. *Journal of Fish Biology, 98*, 973-984.

Rodriguez-Mozaz, S., Lopez de Alda, M. J., & Barcelo, D. (2004). Monitoring of estrogens, pesticides and bisphenol A in natural waters and drinking water treatment plants by solid-phase extraction-liquid chromatography-mass spectrometry. *Journal of Chromatography A, 1045*(1-2), 85-92.

Rose, K. A., Murphy, C. A., Diamond, S. L., Fuiman, L. A., & Thomas, P. (2003). Using nested models and laboratory data for predicting population effects of contaminants on fish: A step toward a bottom-up approach for establishing causality in field studies. *Human and Ecological Risk Assessment, 9*(1), 231-257.

Rouse, E. F., Coppenger, C. J., & Barnes, P. R. (1977). The effect of an androgen inhibitor on behavior and testicular morphology in the stickleback *Gasterosteus aculeatus*. *Hormones and Behavior, 9*(1), 8-18.

Routledge, E. J., Sheahan, D., Desbrow, C., Brighty, G., Waldcock, M., & Sumpter, J. P. (1998). Identification of estrogenic chemicals in STW effluent. 2. In vivo responses in trout and roach. *Environmental Toxicology and Chemistry, 32*(11), 1559-1565.

Rowe, M. P., Baube, C. L., Loew, E. R., & Phillips, J. B. (2004). Optimal mechanisms for finding and selecting mates: How threespine stickleback (*Gasterosteus aculeatus*) should encode male throat colors. *Journal of Comparative Physiology A, 190*, 241-256.

Safe Drinking Water Act of 1996, Pub. L. 104-182 (1996).

Saglio, P., & Trijasse, S. (1998). Behavioral responses to atrazine and diuron in goldfish. *Archives of Environmental Contamination and Toxicology, 35*(3), 484 - 491.

Saligaut, C., Linard, B., Mananos, E. L., Kah, O., Breton, B., & Govoroun, M. (1998). Release of pituitary gonadotropins GtH I and GtH II in the rainbow trout (*Onchorhynchus mykiss*): Modulation by estradiol and catecolamines. *General and Comparative Endocrinology, 109*, 302-309.

Scholz, S., & Gutzeit, H. O. (2000). 17-[alpha]-ethinylestradiol affects reproduction, sexual differentiation and aromatase gene expression of the medaka (*Oryzias latipes*). *Aquatic Toxicology, 50*(4), 363-373.

Schultz, I. R., Orner, G., Merdink, J. L., & Skillman, A. (2001). Dose–response relationships and pharmacokinetics of vitellogenin in rainbow trout after intravascular administration of 17a-ethynylestradiol. *Aquatic Toxicology, 51*, 305–318.

Schulz, R. W., & Miura, T. (2002). Spermatogenesis and its endocrine regulation. *Fish Physiology and Biochemistry 26*, 43-56.

Scott, D. B. C. (1979). Environmental timing and the control of reproduction in teleost fish. *Symposia of the Zoological Society of London, 44*, 105-132.

Sheahan, D. A., Bucke, D., Matthiessen, P., Sumpter, J. P., Kirby, M. F., Neall, P., et al. (1994). The effects of low levels of 17a-ethinylestradiol upon plasma vitellogenin levels in male and female rainbow trout, *Oncorhynchus mykiss* held at two acclimation temperatures. In R. Muller & R. Lloyd (Eds.), *Subleathal and chronic effects of pollutants on freshwater fish* (pp. 99-112). Oxford, UK: Blackwell.

Shore, L. S., Gurevitz, M., & Shemesh, M. (1993). Estrogen as an environmental pollutant. *Bulletin of Environmental Contamination & Toxicology, 51*, 361-366.

Smith, E. H., & Logan, D. T. (1997). Linking environmental toxicology, ethology, and conservation. In J. R. Clemmonds & R. Buchholdz (Eds.), *Behavioral approaches to conservation in the wild* (pp. 277-302). Cambridge: Cambridge University Press.

Snyder, S. A., Keith, T. L., Verbrugge, D. A., Snyder, E. M., Gross, T. S., Kannan, K., et al. (1999). Analytical methods for detection of selected estrogenic compounds in aqueous mixtures. *Environmental Science & Technology, 33*, 2814-2820.

Southam, C., & Ehrlich, J. (1943). Effects of extracts of western red-cedar heartwood on certain wood-decaying fungi in culture. *Phytopathology, 33*, 517–524.

Steele, C. W. (1983). Effects of exposure to sublethal copper on the locomotor behavior of the sea catfish, *Arius felis*. *Aquatic Toxicology, 4*(1), 83-93.

Stumpf, M., Ternes, T. A., Haberer, K., & Baumann, W. (1996). Determination of natural and synthetic estrogens in sewage plans and river water. *Vom Wasser, 87*, 251-261.

Sullivan, J. F., Atchison, G. J., Kolar, D. J., & McIntoch, A. W. (1978). Changes in the predator-prey behavior in fathead minnows (*Pimephales promelas*) and largemouth bass (*Micropterus salmoides*) caused by cadmium. *Journal of Fisheries Research Board of Canada, 35*, 446-451.

Sumpter, J., & Johnson, A. C. (2005). Lessons from endocrine disruption and their application to other issues concerning trace organics in the aquatic environment. *Environmental Science & Technology, 39*(12), 4321-4332.

Svare, B. (1983). *Hormones and aggressive behavior*. New York: Plenum Press.

Tabak, H. H., Bloomhuff, R. N., & Bunch, R. L. (1981). Steroid hormones as water pollutants II: Studies on the persistence and stability of natural urinary and synthetic ovulation-inhibiting hormones in untreated and treated wastewaters. In *Developments in industrial microbiology: A publication of the Society for Industrial Microbiology* (pp. 497-519). Washington, D. C.

Ternes, T. A., Stumpf, M., Mueller, J., Haberer, K., Wilken, R., Servos, M., et al. (1999). Behavior and occurrence of estrogens in municipal sewage treatment plants. I. Investigations in Germany, Canada and Brazil. *Science of The Total Environment, 225*, 81–90.

Teyke, T. (1989). Learning and remembering the environment in the blind cave fish. *Journal of Comparative Physiology A, 164*, 655-662.

Tinbergen, N. (1951). *The study of instinct*. Oxford: Clarendon Press.

Toft, G., & Baatrup, E. (2001). Sexual characteristics are altered by 4-tert-octylphenol and 17[beta]-estradiol in the adult male guppy (*Poecilia reticulata*). *Ecotoxicology and Environmental Safety, 48*(1), 76-84.

Toft, G., & Baatrup, E. (2003). Altered sexual characteristics in guppies (*Poecilia reticulata*) exposed to 17b-estradiol and 4-tert-octyphenol during sexual development. *Ecotoxicology & Environmental Safety, 56*, 228-237.

Toft, G., & Guillette, J., Louis J. (2005). Decreased sperm count and sexual behavior in mosquitofish exposed to water from a pesticide-contaminated lake. *Ecotoxicology and Environmental Safety, 60*(1), 15-20.

United Nations. (2007). *Worldwide contraceptive use*. New York: Department of Economic and Social Affairs: Population Division.

USEPA, Office of Water. (2000). *National Water Quality Inventory: 1998 Report to Congress* (No. EPA841-R-00-001). Washington, DC: EPA.

Van den Assem, J. V. D. (1967). Territory in the threespined stickleback. *Behaviour, Supplement 16*, 1–164.

Van den Belt, K., Verheyen, R., & Witters, H. (2003). Effects of 17[alpha]-ethynylestradiol in a partial life-cycle test with zebrafish (*Danio rerio*): Effects on growth, gonads and female reproductive success. *The Science of The Total Environment, 309*(1-3), 127-137.

Van den Belt, K., Wester, P. W., Van der Ven, L. T. M., Verheyen, R., & Witters, H. (2002). Effects of ethynylestradiol on the reproductive physiology in zebrafish (*Danio rerio*): Time dependency and reversibility. *Environmental Toxicology and Chemistry, 21*, 767–775.

Van der Kraak, G., Hewitt, M., Lister, A., McMaster, M. E., & Munkittrick, K. R. (2001). Endocrine toxicants and reproductive success in fish. *Human and Ecological Risk Assessment, 7*(5), 1017-1025.

Van Iersel, J. J. A. (1958). Some aspects of territorial behavior of the male three-spined stickleback. *Archives Neerlandaises de Zoologie, 13*(1), 383-400.

Verrell, P. (2000). Methoxychlor increases susceptibility to predation in the salamander *Ambystoma macrodactylum*. *Bulletin of Environmental Contamination and Toxicology, 64*, 85-92.

vom Saal, F. S. (1995). Environmental estrogenic chemicals: Their impact on embryonic development. *Human Ecological Risk Assessment, 1*, 3-15.

vom Saal, F. S., Nagel, S. C., Palanza, P., Boechler, M., Parmigiani, S., & Welshons, W. V. (1995). Estrogenic pesticides: Binding relative to estradiol in MCF-7 cells and effects of exposure during fetal life on subsequent territorial behavior in male mice. *Toxicology Letters, 77*, 343–350.

Wai, E. H., Hoar, W. S. (1963). The secondary sex characters and reproductive behaviour of gonadectomized sticklebacks treated with methyl testosterone. *Canadian Journal of Zoology, 41*, 611-628.

Wallace, R. A., & Selman, K. (1979). Physiological aspects of oogenesis in two species of sticklebacks, *Gasterosteus aculeatus L.* and *Apeltes quadracus* (Mitchill). *Journal of Fish Biology, 14*, 551-564.

Weis, J. S., Smith, G., Zhu, T., Santiago-Bass, C., & Weis, P. (2001). Effects of contaminants on behavior: Biochemical mechanisms and ecological consequences. *BioScience, 51*(3), 209-217.

Weis, P., & Weis, J. S. (1974). DDT causes changes in activity and schooling behavior in goldfish. *Environmental Research, 7*(1), 68-74.

Weis, J. S., & Weis, P. (1995). Swimming performance and predator avoidance by mummichog (*Fundulus heteroclitus*) larvae after embryonic or larval exposure to methylmercury. *Canadian Journal of Fisheries and Aquatic Sciences, 52*, 2168-2173.

Weis, J. S., & Weis, P. (1998). Effects of exposure to lead on behavior of mummichog (*Fundulus heteroclitus* L.) larvae. *Journal of Experimental Marine Biology and Ecology, 222*(1-2), 1-10.

Werner, E. E., & Anholt, B. R. (1993). Ecological consequences of the trade-off between growth and mortality rates mediated by foraging activity. *American Naturalist, 142*, 242-272.

Whoriskey, F. G., & FitzGerald, G. J. (1994). Ecology of the threespined stickleback on the breeding grounds. In M. A. Bell & S. A. Foster (Eds.), *The evolutionary biology of the threespine stickleback* (pp. 188–206). Oxford: Oxford University Press.

Wibe, A. E., Fjekl, E., Rosenqvist, G., & Jenssen, B. M. (2004). Postexposure effects of DDE and butylbenzylphthalate on feeding behavior in threespine stickleback. *Ecotoxicology & Environmental Safety, 57*, 213-219.

Wibe, A. E., Nordtug, T., & Jenssen, B. M. (2001). Effects of bis(tributylin) oxide on antipredator behavior in threespine stickleback *Gasterosteus aculeatus* L. *Chemosphere, 44*, 475-481.

Wibe, A. E., Rosenqvist, G., & Jenssen, B. M. (2002). Disruption of male reproductive behavior in threespine stickleback G*asterosteus aculeatus* exposed to 17b-estradiol. *Environmental Research, 90*(2), 136-141.

Winzer, K., Van Noorden, C. J. F., & Kohler, A. (2002). Sex-specific biotransformation and detoxification after xenobiotic exposure of primary cultured hepatocytes of European flounder (*Platichthys flesus* L.). *Aquatic Toxicology, 59*(1-2), 17-33.

Wooton, R. J. (1976). *The biology of the sticklebacks*. London: Academic Press.

Wooton, R. J. (1984). *A functional biology of sticklebacks*. Berkeley: University of California Press.

Zala, S. M., & Penn, D. J. (2004). Abnormal behaviours induced by chemical pollution: A review of the evidence and new challenges. *Animal Behaviour, 68*(4), 649-664.

Zhou, T., & Weis, J. S. (1998). Swimming behavior and predator avoidance in three populations of *Fundulus heteroclitus* larvae after embryonic and/or larval exposure to methylmercury. *Aquatic Toxicology, 43*(2-3), 131-148.

Zhou, T., John-Alder, H. B., Weis, J. S., & Weis, P. (2000). Endocrine disruption: Thyroid dysfunction in mummichogs (*Fundulus heteroclitus*) from a polluted habitat. *Marine Environmental Research, 50*(1-5), 393-397.

Zillioux, E. J., Johnson, I. C., Kiparissis, Y., Metcalfe, C. D., Wheat, J. V., Ward, S. G., et al. (2001). The sheephead minnow as an in vivo model for endocrine disruption in marine teleosts: A partial life-cycle test with 17a-ethynylestradiol. *Environmental Toxicology and Chemistry, 30*(9), 1968-1978.

Zoeller, J. (1998). The top 200 drugs. *American Druggist, February*, 46-53.