Active Ingredient in Oral Contraceptives (17α-ethinylestradiol) Alters Male Competitive Courtship Behaviors and Secondary Sexual Characteristics in Fathead Minnows (*Pimephales promelas)*

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ABSTRACT

Wastewater effluents that are released into natural waterways contain quantifiable, biologically-relevant amounts of hormones and it is currently unknown how this effects fish populations, particularly the ability to reproduce. As a sample model, the effect of 17α-ethinylestradiol (EE_2) was studied on male fathead minnows, *Pimephales promelas*, and their ability to compete for breeding structures in a breeding scenario. EE_2 is the active ingredient in most birth control pills taken by millions of women. During breeding, male fathead minnows compete for, defend, and maintain structures for spawning. After exposing male fathead minnows to three different concentrations of EE_2 (10, 20, and 40 ng/L) for 21 days, fish were paired with an unexposed fish of equal length and age, one female (for stimulation), and a breeding structure. After a 24 h acclimation period, competitive behaviors between males were videotaped and analyzed. Spawning structure maintenance (cleaning the underside of the breeding structure) and aggressive behaviors were counted and the time each male spent under the shelter was recorded. Afterwards, fish were weighed, measured, and male secondary sex characteristics (dorsal epithelial pad and cephalic tubercles) were observed and ranked for size. In addition, the presence of an ovipositor, a female egg laying organ, was observed in exposed males and recorded. Non-exposed males were also examined as to rule out non EE_2 anomalies. Presence and rank of secondary characteristics, and ovipositor presence were directly related to the concentrations of EE_2 exposure. Behavioral results indicated that males exposed to EE_2 displayed reduced motivation to clean and defend the nest ($p < 0.05$). Exposed males were also less competitive and aggressive. Reduced aggressive and protective behaviors could directly affect the reproductive capabilities of an individual or population. These results may be applied to other aquatic organisms, illustrating that hormones in effluent and other pollutants can alter breeding behaviors, and the reproductive dynamics and gene flow in natural fish populations.

KEYWORDS: 17a-ethinylestradiol, fathead minnow, endocrine disrupting compounds, courtship behaviors

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1. INTRODUCTION

Endocrine disrupting compounds (EDCs) are substances that have been found to disrupt the hormone systems of humans and animals. EDCs can mimic the effects of a hormone, stop a hormone from being produced, or increase/decrease the rate of hormone production. EDCs are often found in a variety of common

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pollutants such as certain pesticides or sewage. Many industrial (i.e. Polychlorinated biphenyls), agricultural (i.e. Dichloro-Diphenyl-Trichloroethane), and natural compounds (i.e. human and animal waste) are considered estrogenic (Arcand-Hoy and Benson 1997, Gray et al. 1999, Rajapakse, Nissanka, Delia Ong, and Andreas Kortenkamp 2001). These pollutants are either directly added into waterways through discharges or indirectly runoff into water. Concentrations of pharmaceutical compounds are additionally added into receiving streams from municipal wastewater treatment plants which are not able to remove or only partially remove EDCs.

17α-ethinylestradiol (EE_2) is the active ingredient in oral contraceptives (birth control pills), and degrades very slowly in the environment. EE_2 is commonly prescribed to prevent unwanted pregnancies, and therefore is excreted in high concentrations by those who use oral contraceptives. EE_2 is considered to belong to a narrow class of estrogenic compounds called xenoestrogens because it imitates the effects of a natural estrogen. Oral contraceptives have become increasingly popular, with almost 82% of sexually experienced women using them at least once (Mosher 2004). EE_2 concentrations in wastewater effluent and water sources may also increase over time, causing concerns because of their non-degrading property. Wastewater treatment plants are the main source of EE_2 that enters rivers and streams (Verstraeten et al. 2003) and EE_2 has been discovered in concentrations up to 12 ng/L in the North America, and higher amounts in Europe and South America (Desbrow et al. 1998, Kolpin et al. 1999-2000, Larsson et al. 1999, Werner et al. 2006). Rising levels in the aquatic environment could cause a skew in population dynamics in fish, resulting in possible ecosystem alterations.

Male fish exposed in the laboratory and in the field develop varying degrees of female-like features organs after exposure to EDCs (Ankley 2004, Blazer 2002). Although these feminized males are prominent in local waters, research has yet to reveal what these physiological effects have on entire fish populations and the general aquatic ecosystem ecology. It is also unknown how EE_2 affects mating behavior: specifically, how it affects the ability of males to compete in courtship scenarios.

To address these questions, several laboratories have engaged in on-site observations. In addition, models have been developed to experimentally determine the effects of different endocrine disrupters. Fathead minnows (*Pimephales promelas*) are an ideal piscine model to assess the effects of endocrine disrupters because their endocrinology and physiology are well documented (McMillan and Smith 1974). Additionally, it is easy to determine the gender of fathead minnows because each sex has distinctive physical features (i.e., they are sexually dimorphic). The females are of a lighter color than their male counterpart. Males develop a dorsal epithelial pad on the top of their head for breeding structure cleaning and pointed tubercles on the end of their snouts for nest defense. Additionally, when courting, males typically display a vertical dark striped pattern.

Figure 1 shows secondary sex characteristics of the fathead minnow.

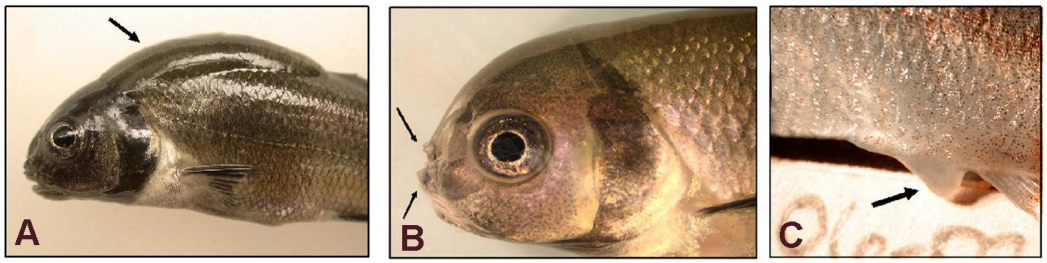

Figure 1. Secondary sex characteristics on male and female fathead minnows. Panel A shows a male with a well-developed dorsal epithelial pad that would be ranked as "3" on a scale of 0-3. Panel B indicates presence cephalic tubercles on a mature male. Panel C shows the ovipositor found in reproductive females but also sometimes in males exposed to estrogenic compounds.

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In the wild, the reproductive cycle of fathead minnows begins from mid-June to early July, and lasts about four months (McMillan and Smith 1972). Males select and defend a hollowed nest structure, such as the underside of floating objects, and begin cleaning the underside of it with their dorsal pad. They court females under the structure and position her against the nest to lay her eggs. The female is then chased out of the nest by the male who then fertilizes the eggs and defends his offspring from intruders. The ability of males to defend and maintain their nests is strongly correlated to the reproductive success of the entire population (Unger 1983). If males cannot produce the expected reproductive behaviors, they may be unable to successfully court a mate and produce viable offspring.

Although previous experiments have addressed effects of EE_2 on physiology and endocrinology, it is difficult to secure a large sample size when conducting behavioral assays. Each organism needs to be bred, maintained, and accounted for, and it is therefore unlikely that one will have a sample size larger than n=10, including a control group. This research project expanded on previous experiments (Majewski et al. 2002) with a larger sample size of n=12 for three concentrations of EE_2.

Previous research suggests that the level of the protein vitellogenin (an egg yolk precursor protein generally active in female fish and dormant in males) increases with exposure to estrogenic compounds and that the secondary sex characteristics (dorsal pad, tubercles, coloration) may be affected (Majewski et al. 2002, Robinson et al. 2003, Miles-Richardson et al. 1999). It is also unknown whether these exposures have a direct affect on courtship behavior or the extent of the effect on secondary sex characteristics.

My research sought to address the effect EE_2 has on the courtship behavior of fathead minnows, particularly in a competitive, male-dominated scenario. Two males were placed into a tank with one female (to provide a stimulus) to initiate male nest guard and courtship behaviors. A video recording technique was used to provide the most accurate display of specific behaviors for subsequent review and analysis. After exposure and placement of the males into the breeding tanks, the primary nest holder (based on time spent guarding the nest) was determined, in addition to their ability to care for and maintain the nest. Results indicated that males exposed to EE_2 displayed reduced nest maintenance and defense behaviors, and the implications are discussed.

2. MATERIALS AND METHODS

Exposure to 17α-ethinylestradiol

Sexually mature male fathead minnows (age of about 7 months) were obtained from Aquatic Research Organisms (Hampton, New Hampshire) and were acclimated for one week in the laboratory. Acclimation consisted of transport to a 230L holding tank and daily feeding. After acclimation, 88 minnows were randomly assigned to exposure tanks. This way, the minnows who were easily caught (and therefore possibly weaker) would not all be placed in the same exposure group. For identification purposes during the behavioral observations, a randomly selected group of 44 fish were fin clipped on the right pectoral fin and the other half were clipped on the left pectoral fin. Although only 72 males were needed for the three trials, 4 additional males for each treatment (16 males total) were added for potential replacements in case of unexpected death or sickness. I prepared five 75L tanks and designated three as EE_2 exposure tanks, one for each exposure concentration, and two as non-exposure (control) tanks. Each of the exposure tanks contained 16 males while the control tanks contained 20 males. All tanks had an equal number of right- and left-clipped fish. During exposure fish were fed slow-sinking trout chow “crumble” (from Zielger Bros in Gardners, PA) each morning and were maintained under a 16:8 light:dark photoperiod.

Throughout the exposure period all tanks were siphoned to remove feces and debris, and 70% of the water was exchanged daily. A stock solution of 1.9 mg of EE_2 in 190mL of ethanol was prepared to maintain concentrations in the different exposure tanks over time. For the exposure tanks, 25µL, 50µL, or 100µL of

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the ethanolic EE_2 stock solution, respectively, was added as part of the static renewal water change, respectively, to maintain the desired nominal concentrations of 10 ng/L, 20 ng/L, and 40 ng/L. Concentrations were chosen to mimic those found in the environment; recent measurements have found 1-47 ng/L EE_2 downstream of waste water treatment plants in Brazil, Canada, and Europe (Werner et al. 2006). In order to ensure adequate water quality, the following parameters were measured on a weekly basis: pH, temperature (ºC), nitrite-nitrogen, nitrate-nitrogen, and unionized ammonia. All measurements remained within healthy parameters.

For each of three replicate, successive behavioral trials (i.e., triplicate runs), four 21-day exposed males from each exposure concentration, and an equal number control males, were randomly selected. Each exposed male was placed in a breeding aquarium with a similarly sized non-exposed male (length difference of <±5%, weight difference of ±10%, Figures 1 and 2) with the opposite fin clipped. One sexually mature female and an artificial breeding structure (made out of half of a PVC pipe) were also placed in each of the breeding aquaria. A total of 36 control males and 36 treated males were used for the triplicate runs. Females were not exchanged between trials.

Behavioral Observations

Competitive behaviors were recorded onto VHS tapes at four, 5-min periods for one day. Males were allowed to acclimate to their new tank and conspecifics for 24 hours prior to recording any behaviors. Behaviors were then recorded four times the next day using CCD cameras mounted below the breeding tanks (Figure 2). Cameras were connected to dedicated VCR decks that recorded the four 5-min video clips at 8:00, 10:00, 12:00, and 14:00. These observation parameters were established because it is known that competitive behaviors exhibited in the first 24 hours of competition predict long-term domination (Dalma et al. 2007).

The amount of time spent under the nest by each male was studied from video recordings by two observers who were "blind" to which treatment each fish received. During observation, behavior was recorded based on which side was fin clipped. Behaviors were later matched with the proper exposure group. The observers also recorded the number of times each male nibbled the underside of the nest, cleaned the nest with their dorsal pad (both of which will hereafter be referred to as nest maintenance behaviors), and head butted or chased the competing male (referred to as aggressive behaviors).

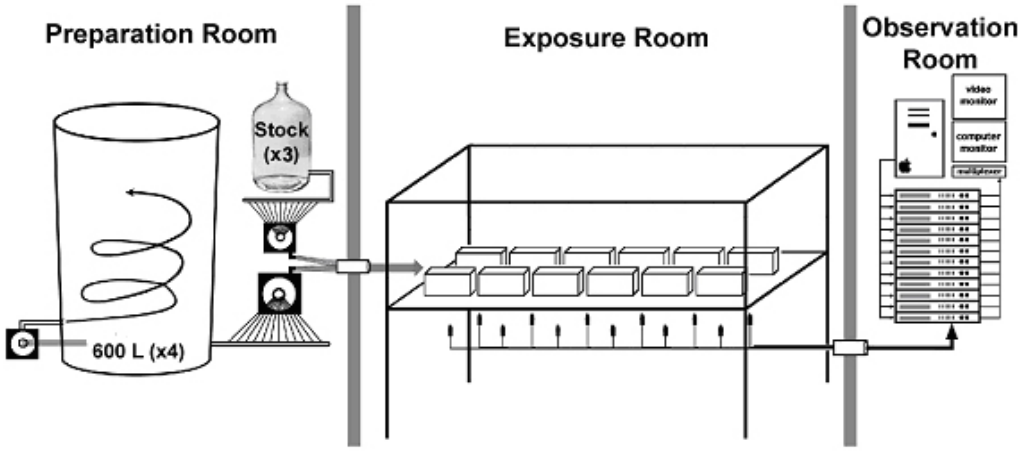

Figure 2. Schematic diagram showing behavioral observation system. 38-L glass aquaria were set up in the exposure room with below-mounted CCD cameras. Water for static renewal was made up and delivered from an adjacent preparation room. Cameras were connected to VCR decks that were computer-controlled to collect video data at discrete, multiple intervals.

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Observations of Secondary Sex Characteristics

At the beginning and conclusion of the behavioral observation period, the lengths (mm) and weights (g) and external physical characteristics of each fish were recorded. The dorsal epithelial pad was assessed for size on a rating scale from 0 to 3, 0 indicated no dorsal pad present, 1 indicated a minimal pad, 2 a moderate sized pad, and 3 a marked pad (Figure 1A). Tubercles were counted and categorized as small, medium, and large (Figure 1B). Ovipositors, the reproductive organ female used to lay eggs, have been found on male fish after exposure to estrogenic compounds (Figure 1C). The presence of previously non-existent ovipositors and reabsorbed tubercles (evidence that previous tubercles disappeared) were recorded for each male fathead minnow. All experiments were approved by the University of Maryland Animal Care and Use Committee (ACUC protocol #06-44).

3. DATA

Experimental data, including secondary sex characteristics, length, and weight of the male fathead minnows, were organized into a table similar to Table 1. Behavioral actions were recorded as long strings of characters, each character representing a corresponding action as seen in Table 2. These strings were entered into a self-written Java program that counted the number of each character and tallied the results (table 3).

Table 1. Secondary sex characteristics recorded after one-day male competition

Accession #	Tank #	Treatment	Fin Clip	Length	Weight	Fat Pad (0-3)	Tubercles			
							Total	Sm.	Med.	Lg.
APC 06-2-1	20 B	experiment	Right	56	2.4	0	16	9	7	0
APC 06-2-2	20 B	control	Left	62	3.2	3	20	4	10	6
APC 06-2-3	40 A	experiment	Left	73	4	1	4	4	0	0

Table 2. Behavioral data obtained from video footage

Camera	Tank	Group	VCR Time	Time of Day	Time (Left)	Time (Right)	Raw Data (Left)	Raw Data (Right)
1	20A	2	0:00-0:05	10:00	0:03	3:22	ss	bnnnsscncccnnnsss
			0:05-0:10	12:00	0:15	2:14	bccb	sscnnnnsssssn
			0:10-0:15	14:00	0:34	2:34	bssn	ssncccnnnsssssssn

Table 3. Data processed and tallied by program

Tank	Group	VCR Time	Time of Day	Right fin-clipped male				Left fin-clipped male			
				Scrub	Nibble	Chase	Butt	Scrub	Nibble	Chase	Butt
20A	2	0:00-0:05	10:00	2	0	0	0	5	7	4	1
		0:05-0:10	12:00	0	0	2	2	7	5	1	0
		0:10-0:15	14:00	2	1	0	1	9	5	3	0

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4. RESULTS

Length and Weight Data

Length and weight data from males randomly distributed between the different exposures and breeding tanks did not vary in their lengths or weights (Figures 3 & 4).

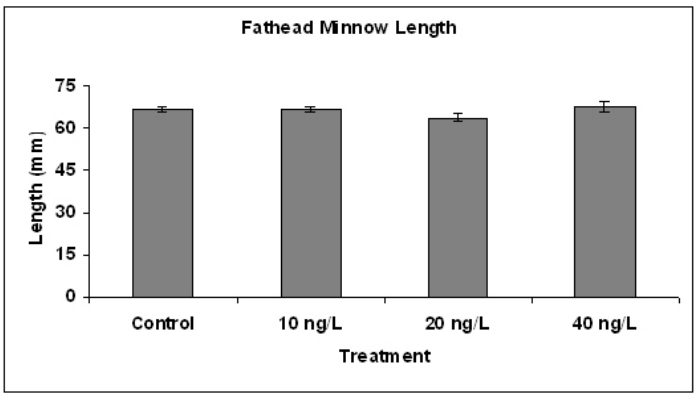

Figure 3. Fathead minnow length for all treatments. No lengths were statistically significant (p>0.05).

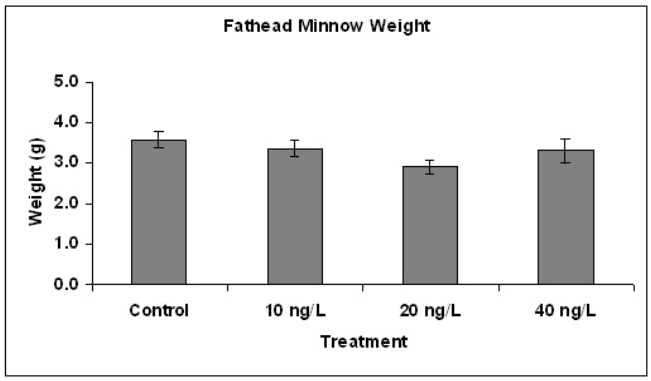

Figure 4. Fathead minnow weight for all treatments. No weights were statistically significant (p>0.05).

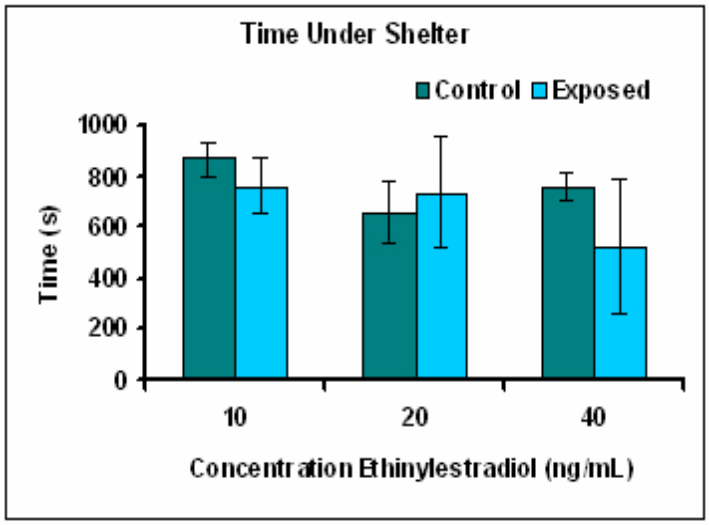

Figure 5. Time spent under sheleter by males exposed and unexposed to various concentrations of 17α-ethinylestradiol.

Behavior

Male fathead minnows displayed a higher frequency of nest maintenance behaviors than aggression behaviors across all treatments. This pattern agrees with previous competitive fathead minnow research and may be explained by the natural behaviors of the minnows or the decreased necessity of aggression due to the weakened exposed males (Dalma et al. 2007). Control males outcompeted EE_2-exposed males in nest maintenance, although there was not sufficient data to tell whether this was true for aggressive behaviors as well. The control and exposed fish did not spend a statistically different amount of time under the shelter (ANOVA, $p > 0.05$, Figure 5), but there were differences in nest upkeep behaviors, particularly cleaning. The total frequencies of scrubs (the rubbing of the dorsal pad against the nest structure) by exposed fish were significantly lower than those of the controls, throughout all concentrations (ANOVA, $p < 0.05$, Figure 6A). Nibble and chase frequencies were reduced in the males exposed to the 10 and 40 ng/L EE_2 exposures, with significant decreases in the 20 ng/L exposed males (ANOVA, $p < 0.05$, Figures 6B and C). The variance in the behavior of the control fish in nibble frequency can be explained by the large standard error in the first competitive group. Butting behaviors were reduced in the exposed fish when compared to controls, but due to high variability, no significant differences were found (ANOVA, $p > 0.05$, Figure 6D).

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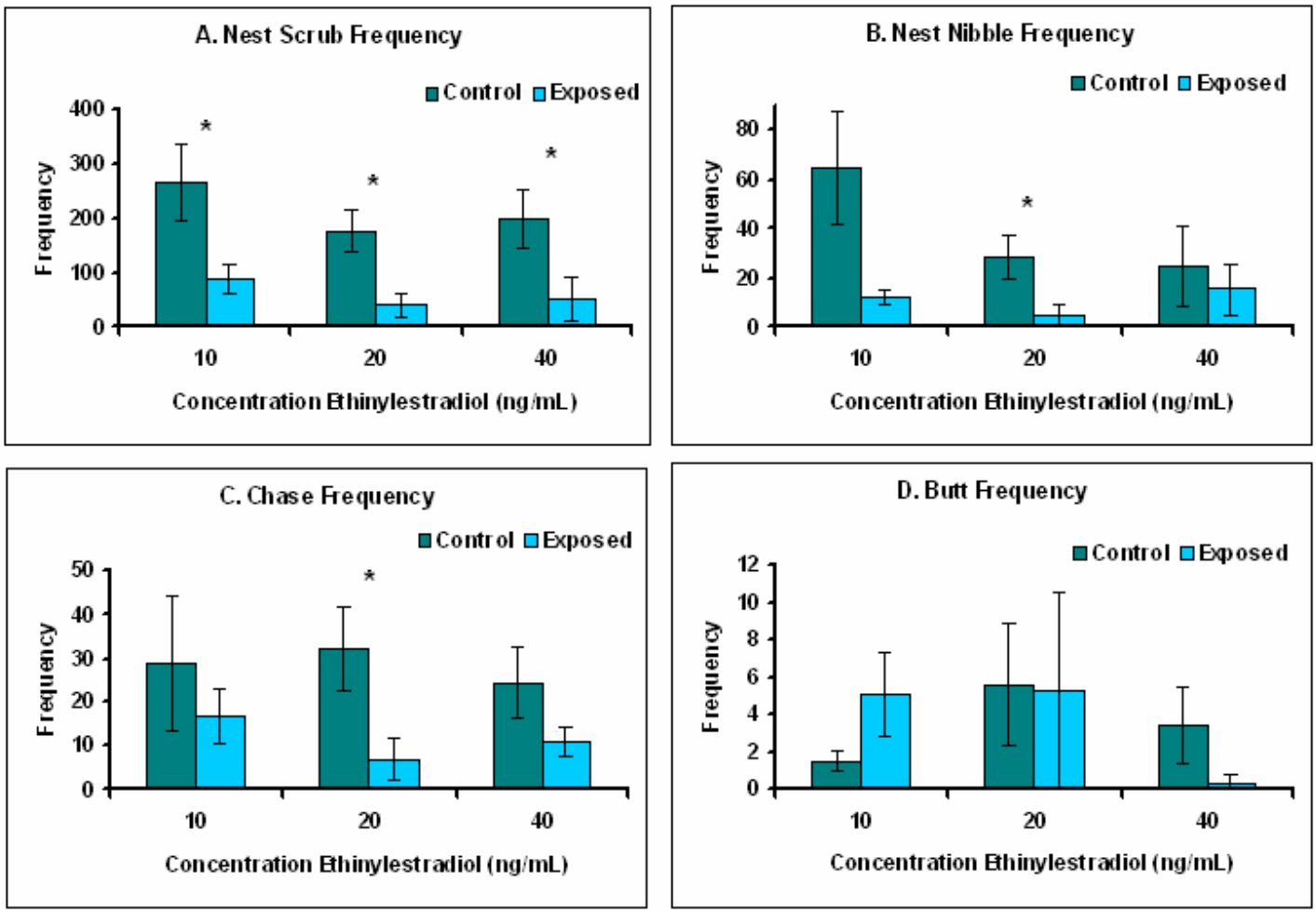

Figure 6. Frequencies of fathead minnow courtship behaviors including nest scrubbing (A), nest nibbling (B), aggressive chasing (C), and aggressive butting with tubercles (D). Frequencies were recorded for four five-minute video recorded sessions. An asterisk indicates a significant different from the control group ($p<0.05$).

Secondary Sex Characteristics

There was no statistical significance (Kruksal Wallace ANOVA, $p > 0.05$) between the dorsal pads of the males from each control treatment (Figure 7A). However, the total number and size distributions of tubercles were significantly reduced following EE_2 exposure (ANOVA, $p < 0.001$, Figure 7B). Blue, green, and red bars represent small, medium, and large sized tubercles respectively. Additionally, large tubercles were rarely present in any of the exposed minnows. In contrast, reabsorbed tubercles and ovipositors were only found in exposed males, percentages of which were highly significant (Kruskal Wallace ANOVA, $p < 0.001$), Figures 7C and 7D). These data suggest that the doses of EE_2 were bio-available and absorbed by the test fish.

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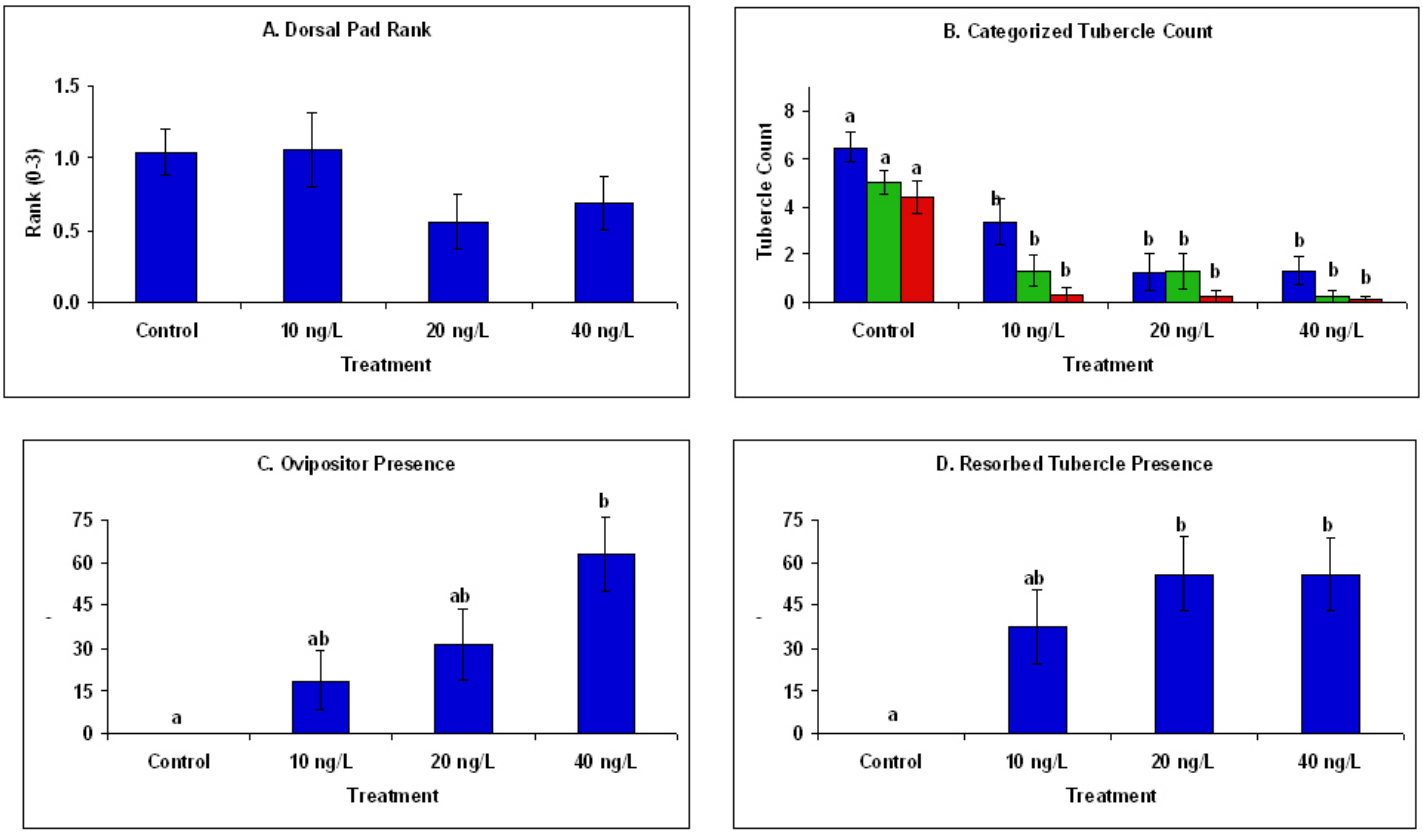

Figure 7. Secondary sex characteristics: dorsal pad rank (A), tubercles categorized by size (B), ovipositor presence (C), and resorbed tubercle presence (D). Groups sharing common letters are not significantly different ($p>0.05$)

5. DISCUSSION

The objective of this experiment was to determine if exposure to environmentally relevant concentrations of EE_2 would hinder the ability of an ecologically relevant model, the fathead minnow, to compete and effectively maintain a breeding nest site. This study demonstrates that exposure to EE_2 significantly reduces the courtship behavior of fathead minnows in a competitive scenario. Male fathead minnows exposed to EE_2 appear to maintain breeding nests less frequently than unexposed conspecifics. Further, data from these experiments provide evidence of changes in essential reproductive behaviors resulting from EDC exposure, and may be useful in predicting reproductive alterations in an environmentally relevant setting.

Butting behavior in fathead minnows involves a male hitting competitors with the tubercles on its snout. Therefore, tubercle presence and/or size could directly influence the force with which the aggressor butts his opponent (McMillan and Smith 1974). Because male fathead minnows rub and nibble the underside of the nest structure with his dorsal pad and head, the effectiveness of these behaviors may also be influenced by the size and presence of the dorsal pad. In addition, reduced secondary sex characteristics resulting from EE_2 exposure may further be associated with reduced aggression.

If exposed male fathead minnows are unable to maintain a nest, as observed in this study, it is unlikely that the female's eggs will stick to the underside of the nest, therefore hindering reproductive success. Additionally, if the male cannot guard his nest from unwanted competitors, he would be an ineffective reproducer. This study reinforces previous conclusions by Majewski et al. (2002) where fathead minnows exposed to estrogenic compounds nest less frequently and are less aggressive.

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In future studies, it would beneficial to increase the sample size, and expose fish to a different range of EE_2 concentrations with shorter exposure times. One could also determine the amount in EE_2 in a certain wastewater effluent and expose the fathead model to that measured concentration. To expand this research further, water samples could be taken from various field locations and analyzed for EE_2 concentrations over a long period.

This study provides evidence of changes in essential reproductive behavior associated with environmentally relevant EDC exposure. Data from this study parallels other pilot studies conducted in the laboratory of Dr. Kane where similar exposures are also associated with reduced breeding success and altered plasma hormone levels in males (Kane et al. 2006). Specifically, testosterone and its metabolite, 11-ketotestosterone, were significantly reduced by exposure to EE_2. Therefore, it appears reasonable that exposure to xenoestrogens can indirectly alter 11-ketotestosterone, which in turn, is associated with the development of secondary sex characteristics and essential reproductive behaviors. This is environmentally relevant since alterations in male breeding success may be associated with increased energy to compete for "reproductive resources," and altered gene flow in natural populations.

6. ABREVIATIONS AND ACRONYMS

- **EE_2**: 17α -ethinylestradiol
- **EDC**: Endocrine Disrupting Chemical
- **ng/L**: nanogram per liter
- **VHS**: Video Home System
- **ACUC**: Animal Care and Use Committee
- **p**: the probability of obtaining a data value as extreme as the given one, assuming that value is a result of chance alone

7. ACKNOWLEDGEMENTS

Credits

I would like to acknowledge my mentors Dr. Andrew Kane and Dr. James Salierno at the University of Maryland Aquatic Pathobiology Center for their instruction and guidance throughout my research. I would also like to thank Dr. Glenda Torrence, my research teacher, for her scientific knowledge and continuous moral support.

Author

I am a graduating senior from the Math, Science, and Computer Science magnet at Montgomery Blair High School in Silver Spring, Maryland. I conducted my research during an internship at the Aquatic Pathobiology Center at the University of Maryland. I am an avid hiker and have always been interested in environmental research. Awards for my research thus far have included the United State Army Certificate of Achievement, the Central Intelligence Agency Award of Excellence, and the Sigma Delta Epsilon Graduate Women in Science-Certificate of Recognition. I also placed second at the National Junior Science and Humanities Symposium.

In my senior year of high school, I was an active member of the Chamber Choir, the chief of staff of the Student Government Association, and a delegate in Youth and Government. I co-founded and served as co-president of the Expedition Club—a club that leads outdoor-based adventures. I tutor and play recreational soccer outside of school. In my free time, I enjoy hanging out with friends, roaming the outdoors, and

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singing. In the fall, I plan on majoring in Environmental Engineering while preparing for med-school at Tufts University.

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