Effects of environmental estrogens on reproductive biology of the fathead minnow

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Effects of environmental estrogens on reproductive biology of the fathead minnow

by

Robert Bruce Bringolf

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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ABSTRACT

Since the early 1990s, environmental estrogens have been recognized as an important environmental threat. Wastewater of 10 aerated lagoon treatment facilities in Iowa was evaluated for estrogenic activity using a short-term caged fathead minnow exposure and a plasma vitellogenin (Vtg) assay. Plasma Vtg results indicated that wastewater entering the three-lagoon systems was estrogenic to male fish, but with serial passage through the lagoons, the estrogenic activity decreased to a level that was not sufficient to induce vitellogenesis. Wastewater retention time in the lagoons may have been a key treatment factor.

Feral fathead minnows captured at aerated lagoon wastewater treatment facilities (WWTFs) exhibited plasma Vtg trends similar to those of the caged fish. Incidence of ovotestes in feral fish was low (1 of 65; 1.5%) and similar to that of fathead minnows captured at a reference site (national wildlife refuge). The results of both the caged and feral fish studies indicate that effluents from aerated lagoon WWTFs are low in estrogenic activity, but that raw wastewater was estrogenic to fish. Thus, the potential exists for release of estrogenic effluents from these systems if the treatment is not complete.

The plasma Vtg response of male fish exposed to estradiol for 10 days was dose-dependent and predictable ($R^2 = 0.988$) through the range of estradiol exposure concentrations tested. The lowest observed effects concentration (LOEC) for induction of plasma Vtg was 50 ng/L. The dose-response curve from this study may be used in conjunction with exposure of male fish to surface water or wastewater to estimate the magnitude of the estrogenic potency of the water in terms of "estradiol equivalents."
Atrazine did not cause overt reproductive toxicity to adult fathead minnows in a short-term reproduction assay. However, decreasing trends in relative testis weight, testis maturity, and percent embryo fertilization suggest that further investigation is warranted. Nearly all endpoints concerning fish exposed to estradiol (positive control) were significantly different from atrazine-exposed fish and control fish. The results suggest that atrazine did not have strong estrogenic effects and did not cause endocrine system disruption in fathead minnows at environmentally relevant concentrations.
GENERAL INTRODUCTION

Dissertation Organization

This dissertation is organized into four papers. I am the primary author and my major professor, Dr. Robert C. Summerfelt, is the secondary author for all four papers. A fellow graduate student, Jason Belden, is a co-author on one of the papers. I conceived the overall dissertation goals and objectives in close collaboration with Dr. Summerfelt. We worked together to design the projects and write grant proposals to fund the research. Expertise provided by Dr. Gary J. Atchison was essential for design of laboratory toxicity tests. Dr. Atchison also provided some laboratory space, equipment, and supplies. This research was supported by funding from the Center for Health Effects of Environmental Contamination, the Iowa Science Foundation, and the Iowa Agriculture and Home Economics Experiment Station. Project 3480. The Interdepartmental Program in Toxicology provided assistantship support for the first year. The Department of Animal Ecology (now Natural Resource Ecology & Management) provided a research/teaching assistantship and laboratory and office space for three years.

Each of the four papers follows the format required by the journal to which submission was made, or is proposed. Submission formats have been modified to allow inclusion of tables and figures at the most convenient location as soon after they are cited within the body of the paper. This format results in a document that would resemble the paper when published, rather than in submission form which requires all tables and figures to be placed on a separate sheet of paper at the end of the text.
The four papers are preceded by a literature review related to the goal of the research, which is to obtain a greater fundamental understanding of environmental estrogens and their effects on the reproductive biology of fish, with emphasis on effects of municipal wastewater exposure on physiological and histological biomarkers of exposure to environmental estrogens, the dose-response relationship between waterborne estradiol and biomarkers, and use of a screening-level fish reproduction assay to evaluate a potential environmental estrogen.

The first paper, "Reduction of estrogenic activity of municipal wastewater by aerated lagoon treatment facilities," describes use the fish Vtg bioassay to characterize the estrogenic activity of wastewater as it passes through aerated lagoon wastewater treatment facilities (WWTF) in Iowa and to evaluate WWTF variables (i.e., population served, lagoon volume, inflow, lagoon retention time, water quality parameters, rainfall) that may be related to the efficiency of the treatment system for reducing the estrogenic activity of the wastewater before discharge.

The second paper, "Occurrence of ovotestes and plasma vitellogenin in feral fathead minnows from lagoons of municipal wastewater treatment facilities in central Iowa," further examines the estrogenic activity of municipal wastewater by comparing estrogenic effects in caged and feral fish in the lagoons.

The third paper, "Plasma vitellogenin in fathead minnows exposed to waterborne 17β-estradiol," describes the dose-response relationship between exposure to a model estrogen and a physiological biomarker of exposure. This study was devised to determine the
"estradiol equivalents" required to induce a physiological response similar to that of the fish exposed to wastewater in the first paper.

The final paper, "Effects of atrazine on fathead minnow in a short-term reproduction assay," was designed to assess the potential risk for endocrine disruption in fish exposed to atrazine, a common herbicide that has recently been reported to cause sexual disruption in amphibians at very low exposure concentrations. This study was designed to include physiological biomarkers of exposure to environmental estrogens as well as population-level reproductive effects in the fish.
Since the early 1990s, endocrine disrupting compounds have been recognized as an important environmental threat (Colborn et al. 1996). Mounting evidence suggests a strong link between environmental pollution and the increasing incidence of reproductive disorders and behavioral and developmental abnormalities in humans, fish, and wildlife (for a review see Colborn et al. 1993; NRC 1999). "Endocrine disruptors" (EDs) are a diverse array of chemicals found in the environment that have the capacity to interfere with the endocrine system of vertebrates and invertebrates. The endocrine system produces various releasing factors and hormones that affect or control growth, maturation, reproduction, metabolism and other functions. The EDs enter the environment via municipal and industrial effluents, and agricultural pesticides and feedlot runoff, among other routes. Because of reported and potential effects of EDs, a U.S. Environmental Protection Agency (EPA) advisory committee, called the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), has recommended that EPA commence screening of the circa 66,000 chemicals currently in commerce for their endocrine-disrupting potential. EDSTAC also recommended that EPA fund studies of the effects of these chemicals on endocrine systems of fish and wildlife (EDSTAC 1998). In 1998, the EPA and other agencies allocated $8-10 million for much needed research on endocrine disrupters, thus illustrating the high priority of this topic. In a report released in 2002, the World Health Organization's International Programme on Chemical Safety concluded, "There is a need to identify life stages and species that are more vulnerable to the effects of endocrine-disrupting compounds and to understand how this
mechanism of toxicity may affect individual populations and communities”
(http://www.who.int/pcs/emerg_site/edc/global_edc_TOC.htm).

Environmental estrogens (EEs) are a sub-category of EDs that are known for their
estrogenic or anti-androgenic effects on organisms. This vast array of compounds includes
both natural and synthetic chemicals. Natural (endogenous) estrogens excreted by women
include $1\beta$-estradiol, estrone, and estriol. Women who take oral contraceptives or estrogen
replacement therapy also excrete the synthetic estrogen, $17\alpha$-ethinyl estradiol. Other
chemicals in the environment that have been found to have estrogenic activity include many
pesticides, polychlorinated biphenyls (PCBs), dioxins, alkylphenols (surfactants), plasticizers
(phthalates and bisphenol), and phytoestrogens, among others.

Screening of chemicals with estrogenic activity has been accomplished with a number of
in vitro and in vivo assays (for a review see NRC 1999). In vitro (cell culture) assays have
also been used extensively to test for estrogenic activity of chemicals. The most commonly
used assays include: hormone receptor binding assays, cell proliferation assays, and gene and
protein expression assays, among others. In vivo assays, such as the plasma vitellogenin
assay, gonad size, population sex ratios, sex hormone ratios, egg production, and
developmental abnormalities, have been used to identify chemicals or waters polluted with
chemicals that affect the endocrine system. A combination of the various in vivo and in vitro
tests is desirable for characterization of compounds with hormonal activity and their effects
on the exposed organism.

Field and laboratory studies have demonstrated that fish and wildlife exposed to EEs may
develop various reproductive abnormalities. For example, frogs exposed to atrazine (a
pesticide) developed gonads with both testicular and ovarian tissue (intersex) (Hayes et al. 2002), alligators exposed to DDT and its degradation products were reported to have smaller penises and altered steroid hormone levels (Guillette et al. 1996), and fish exposed to various EEs have developed intersex gonads, smaller testes, altered steroid hormone levels, and production of the yolk protein precursor, vitellogenin (Purdom et al. 1994; Harries et al. 1996, 1997, 1999; Jobling et al. 1996). Plasma vitellogenin (Vtg) is among the most commonly measured in vivo biomarkers that have been used routinely to diagnose exposure of oviparous vertebrates (i.e., fish, birds, and reptiles) to EEs. Normally, Vtg is produced in females when estrogens stimulate cells of the liver, but males will also produce Vtg if they are exposed to sufficient concentrations of EEs. Thus, measurement of plasma Vtg (in males especially) has become a standard biomarker for assessment of exposure to estrogenic chemicals, and induction of plasma Vtg in male egg-laying vertebrates is regarded as a definitive measure of sexual disruption (Ng and Idler 1983).

Other biomarkers of exposure to EEs include quantification of plasma zona radiata proteins (Arukwe et al. 1997) and alkaline-labile phosphate (Kramer et al. 1998). In addition, molecular biomarkers, such as Vtg mRNA (Folmar et al. 2000; Korte et al. 2000; Hemmer et al. 2001) and use of cDNA microarrays (Nancy Denslow, University of Florida, personal communication) to identify mRNA expression that changes with exposure to estrogenic compounds, are becoming more common. Plasma steroid hormone ratios (estradiol:testosterone) and relative gonad weight (GSI), have also been commonly measured in exposed fish and wildlife.
**Estrogenicity of municipal wastewater**

Widespread sexual disruption of riverine fish has been documented in the United Kingdom (UK) (Jobling et al. 1998). Investigators reported reproductive and developmental effects in fish from exposure to ambient levels of estrogenic chemicals present in British rivers. Researchers were first made aware of the problem when anglers reported catching fish with both male and female characteristics from effluent lagoons of wastewater treatment facilities (WWTF) (Purdom et al. 1994). Exposure of male rainbow trout (*Oncorhynchus mykiss*) to effluents of 15 different WWTFs throughout the UK resulted in pronounced increases (500 to 100,000-fold, depending on the site) in plasma Vtg concentrations to levels only observed in mature females during egg formation (Purdom et al. 1994). Subsequently, studies by Harries et al. (1996, 1997, 1999) have demonstrated that most, if not all, WWTF effluents in the UK are estrogenic to fish. Their findings also indicated that stretches of these rivers become estrogenic downstream from WWTF discharges (Harries et al. 1996, 1997).

A national survey of endocrine disruption in fish in U.S. rivers found hermaphroditism and unusual steroid hormone ratios in a few carp (*Cyprinus carpio*) (Goodbred et al. 1996). The estrogenic activity of WWTF effluents and effluent-receiving waters has not been thoroughly surveyed in Iowa. However, elevated levels of Vtg and abnormal steroid hormone ratios were found in male carp collected from the Mississippi River downstream of the Minneapolis-St. Paul (MN) WWTF (Folmar et al. 1996). Nichols et al. (1999) reported that effluents of small, but advanced WWTFs in Michigan did not induce elevated levels of plasma Vtg in fish, but they reported abnormal hormone ratios in fish from some test sites.

To date, the studies of estrogenicity of WWTF effluent in the U.S. have focused on metropolitan areas, which generally use advanced secondary (trickling filter and/or activated
sludge) and tertiary (reverse osmosis and/or activated carbon) processes for wastewater treatment; however, smaller communities (population < 10,000) rarely employ tertiary treatment and often rely only on the simplest forms of secondary treatment. The EPA estimates that by the year 2016, 71% of all WWTFs in the U.S. will serve communities of 10,000 or fewer people (U.S. EPA 1996), and that in six states, (Iowa included) more than 90% of WWTFs will serve communities of 10,000 or fewer people. Many of these small rural communities utilize the simplest form of treatment, which is multiple flow-through earthen lagoons, some of which may be aerated (Crites and Tchobanoglous 1998). Nearly 5,000 lagoon WWTFs exist in the U.S. (Grady et al. 1999) and there are currently 147 municipal aerated lagoon WWTFs in Iowa, each of which continuously discharges effluent into the aquatic environment (Iowa Department of Natural Resources 2002, http://www.state.ia.us/epd/wastewtr/wpermit/wpermit.htm). Lagoons are also used extensively for treatment of industrial and agricultural wastes. Identification of treatment processes that are not effective for elimination of estrogenic chemicals from effluent is essential.

*Ovotestis in feral fish*

An intersex (ovotestes) condition has been reported in fish exposed to estrogenic hormones and hormonally-active agents (EEs) in laboratory settings (Westor and Canton 1986; Gimeno *et al.* 1997; Gray and Metcalfe 1997; Hartley *et al.* 1998); however, ovotestes have also been reported in fish captured from polluted rivers and lakes. The "background" incidence of ovotestes in fish is not well characterized, but has been reported as high as 5% in control populations of carp (Komen *et al.* 1989), so it seems reasonable that a low level of
intersexuality could be considered "natural." Regardless, the finding of a single intersex fish has been reported in the literature for a variety of species including chinook salmon (*Oncorhynchus tshawytscha*) (Barnes et al. 2001), roach (*Rutilus rutilus*) (Arne 1965; Jaffri and Ensor 1979), and western mosquitofish (*Gambusia affinis*) (Teh et al. 2000).

Jobling et al. (1998) examined gonads of feral roach from a wide range of typical rivers throughout the British Isles, all of which receive municipal wastewater effluent. The authors cited "strong evidence of adverse reproductive health effects in wild populations of … roach." The investigators reported that whole fish were examined macroscopically, and all appeared either male or female; however, upon histological examination of the gonads, they determined a surprisingly large proportion of the males to be intersex, as defined by the presence of both male and female gonadal tissue. The occurrence of intersex fish and documented presence of estrogenic chemicals in wastewater (Desbrow et al. 1998), suggests that the intersexuality was due to feminization of genetically male fish rather than the masculinization of genetically female fish. The data suggests that a component of the WWTF effluent had disrupted steroid hormone levels in the fish and resulted in development of the intersex condition.

Jobling et al. (1998) reported that the incidence of intersexuality was 4% in the laboratory and at the field control sites and 100% at two sites downstream from WWTF sites. The percentage of intersex fish at downstream sites ranged from 16% to 100%. Populations of fish downstream from WWTFs had significantly higher percentages of intersex fish than did control sites ($\chi^2; p < 0.0001$). There was a highly significant relationship ($R^2 = 0.683, p = 0.0002$) between the proportion of intersex fish and the proportion of the river flow from
effluent. These results suggest that the incidence of intersexuality in roach is considerably higher than expected and is associated with discharge from WWTFs.

Jobling et al. (1998) also reported that intersexuality in some roach was "slight", while in others it was 50% ovarian and 50% testes. Consequently, they developed a numerical "intersex index" ranging from 0 to 7 to describe the degree of feminization of each fish. A score of 0 is a completely male gonad, with testicular germ cells and a sperm duct. A testicular gonad with both a sperm duct and an ovarian cavity are present was given a score of 1. Indices of 2 or 3 described gonads that were intersex in terms of both the germ cells and the reproductive ducts. The absence of a sperm duct and the presence of an obvious ovarian cavity characterized the more extreme cases of intersexuality (scores 4-7). The score increased with increasing percentage of ovarian vs. testicular tissue. A score of 7 indicates that microscopically the gonad is 100% ovarian, oocytes may be primary or secondary, and there is an obvious ovarian cavity. The mean intersex index was significantly higher in fish collected downstream from WWTF outfalls than in fish from field control sites and laboratory controls. The incidence and severity of intersex fish in WWTF lagoons in the U. S. has not been described.

**Dose-response relationship**

Some municipal and industrial effluents have been found to be estrogenic to fish (Purdom et al. 1994; Folmar et al. 1996; Harries et al. 1996, 1997, 1999; Jobling et al. 1998), but efforts to identify compounds in the estrogenic fraction of the effluent are limited. Among the compounds commonly found in municipal effluents is 17β-estradiol (E\(_2\)) (Lee and Peart
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1998; Desbrow et al. 1998; Routledge et al. 1998; Belfroid et al. 1999; Ternes et al. 1999b; Rogers-Gray et al. 2000; Huang and Sedlak 2001; Spengler et al. 2001), a natural human steroid estrogen. Human females excrete relatively large quantities of \( E_2 \), most potent of three natural human estrogens, ranging from 0.01 - 0.1 mg/day (Williams and Stancel 1996) and pregnant women discharge as much as 30 mg/day (Arcand-Hoy et al. 1998). Steroid hormones such as \( E_2 \) are excreted as inactive glucuronide or sulfide conjugates, but the hormones are rapidly deconjugated back to active forms by microbes in the wastewater treatment process (Ternes et al. 1999a). Reports of measurable concentrations of \( E_2 \) in WWTF effluents reinforce the need for basic toxicity research of \( E_2 \) as an aquatic contaminant.

The fathead minnow is a widely used species for aquatic toxicity testing; however, to date, efforts to describe effects of estrogenic substances have involved other species such as rainbow trout (Lech et al. 1996; Purdom et al. 1994; Schultz et al. 2001; Sheahan et al. 1994), carp (Harries et al. 1995; Gimeno et al. 1997; Schwaiger et al. 2001), channel catfish (\textit{Ictalurus punctatus}) (Nimrod and Benson 1996), sheepshead minnow (\textit{Cyprinodon variegates}) (Folmar et al. 2000; Hemmer et al. 2001), Japanese medaka (\textit{Oryzias latipes}) (Gray and Metcalfe 1997; Hartley et al. 1998; Metcalfe et al. 2001; Papoulias et al. 1999; Scholtz and Gutzeit 2000; Seki et al. 2002), flounder (\textit{Paralichthys dentatus}) (Folmar et al. 2001; Mills et al. 2001), goldfish (\textit{Carassius auratus}) (Bjerselius et al. 2001), and others.

Many of these and other studies have reported Vtg induction in male fish exposed to wastewater effluent; however, only Panter et al. (1998) and Folmar et al. (2000) describe the dose-response relationship for Vtg in fish exposed to waterborne \( E_2 \). Kramer et al. (1998) exposed fathead minnows to waterborne \( E_2 \) in a flow-through system at nominal
concentrations of 27, 272, and 2724 ng/L for 19 days. The authors quantified plasma alkaline labile phosphate (ALP) as an indicator of plasma Vtg expression and reported that the EC₅₀ (concentration causing a significant effect in 50% of the test animals) in males was 251 ng/L. Panter et al. (1998) used a continuous flow-through system to expose adult male fathead minnows to nominal concentrations of E₂ at 10, 32, 100, 320, and 1000 ng/L for 21 days to determine the dose-response relationship of E₂ and plasma Vtg. Actual E₂ exposure concentrations were not measured. The 21-day exposure was chosen to emulate the exposure period used for caged fish exposures in sewage effluent exposures. Panter et al. (1998) used a radioimmunoassay (RIA) developed for carp Vtg to quantify Vtg in the fathead minnow plasma. The authors reported that the lowest concentration of E₂ that resulted in significant elevation of Vtg in male fish was 32 ng/L. Panter et al. (1998) used seven fish per treatment and defined an individual fish as an experimental unit for statistical analysis, and thus reported seven replicates per treatment. Folmar et al. (2000) exposed sheepshead minnows (Cyprinodon variegatus) to nominal E₂ concentrations of 20, 200, 500, 1000 and 2000 ng/L. They found that 200 ng/L was the lowest concentration that induced significantly higher concentration of plasma Vtg than the controls, but concluded that the threshold E₂ exposure concentration is "undoubtedly lower" than 200 ng/L because of the large concentration gap between the lower exposure concentrations. Currently, there is no description of the Vtg response in fathead minnows exposed to E₂ for 10 d.

Beyond biomarkers—population level effects

Although the induction of biomarkers in fish exposed to EEs is well documented, only recently have studies examined the relationship of biomarkers with impairment of
reproduction and development (Kramer et al. 1998; Harries et al. 2000; Länge et al. 2001; Ankley et al. 2001; Seki et al. 2002). Kramer et al. (1998) described the correlation between egg production and concentrations of plasma ALP (indicative of Vtg) in both male and female fathead minnows. Harries et al. (2000) and Ankley et al. (2001) described the development of reproductive performance tests for identification of endocrine disrupting compounds. Länge et al. (2001) used a full-lifecycle test to evaluate the effects of the synthetic estrogen 17α-ethinyl estradiol on fathead minnow reproduction and development. Seki et al. (2002) examined the effects of 17α-ethinyl estradiol on reproductive success, gonad condition, and plasma Vtg of Japanese medaka. Each of these investigators concluded that physiological biomarkers (i.e., Vtg) were detectable at much lower exposure concentrations than the effects on egg production.

The EPA has developed and implemented a screening program for EDs that disrupt processes controlled by estrogen, androgen, or thyroid hormones (U.S. EPA 1998). Among the screening assays recommended by EDSTAC, is a short-term fathead minnow reproduction assay (EDSTAC 1998). The assay has been developed and tested by the EPA and involves exposure of adult fish to potential EDs followed by assessment of reproductive success and biological endpoints (U.S. EPA 2001; Ankley et al. 2001). To date, Ankley et al. (2001) provide the only report of the use of the assay for screening, and describe the effects of methoxychlor, a herbicide, and methyltestosterone, an androgenic steroid hormone. Effects of pesticides on the endocrine system of fish have been examined (for a review see Kime 1998), although a standard assay has been lacking until development of the short-term fathead minnow reproduction assay.
Atrazine, a triazine herbicide, has, for the past decade, been the most extensively applied pesticide in the U.S. (USDA 2000). Because of the widespread use of atrazine and its relative stability in the environment (Solomon et al. 1996), a high potential exists for contact with aquatic organisms. From 1992 to 1998, atrazine was detected in 85% of surface water samples collected year-round in both urban and agricultural areas, and was measured at > 0.1 μg/L in 34% of agricultural stream samples (USGS 2001). The maximum concentration of atrazine measured during the sampling period was 120 μg/L. Solomon et al. (1996), however, concluded that atrazine does not pose a significant ecological risk to the aquatic environment, even in high-use areas in the Midwestern U.S., because of the low acute toxicity relative to predicted environmental exposure concentrations.

Hayes et al. (2002), however, reported that larval frogs (*Xenopus laevis*) exposed to atrazine concentrations as low as 0.1 μg/L developed ovotestes, a biomarker of endocrine disruption. In the same study, male frogs exposed to atrazine concentrations of 1.0 μg/L had a significantly smaller larynx, an organ used to attract potential mates. The proposed mechanism of action for atrazine is upregulation of aromatase, the enzyme that converts testosterone into 17β-estradiol. To date, the potential endocrine-disrupting effects of atrazine in fish have not been described.

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REDUCTION OF ESTROGENIC ACTIVITY OF MUNICIPAL WASTEWATER BY AERATED LAGOON TREATMENT FACILITIES

A paper published in Environmental Toxicology and Chemistry

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Abstract—The estrogenic activity of municipal wastewater in aerated lagoon treatment facilities was evaluated using plasma concentrations of vitellogenin (Vtg) in male fathead minnows (Pimephales promelas). Caged fathead minnows were exposed for 10 to 12 d in three lagoons that are connected in series at each of 10 municipal wastewater treatment facilities in central Iowa during October and November of 2000. Fathead minnows held in the laboratory served as unexposed controls. Pooled \((n = 4 \text{ to } 10 \text{ fish})\) plasma Vtg, quantified by enzyme-linked immunosorbent assay (ELISA), was \(1.702 \pm 0.670\) \((\text{mean} \pm \text{standard error \(SE\)})\) \(\mu\text{g/ml}\) in the first lagoons \((n = 9)\), \(0.94 \pm 0.36 \mu\text{g/ml}\) in the second lagoons \((n = 10)\), and \(0.04 \pm 0.02 \mu\text{g/ml}\) in the third lagoons \((n = 8)\). Differences in mean fish plasma Vtg concentration among lagoons were highly significant \((p < 0.001)\). The mean concentration of plasma Vtg in fish in the third lagoons was not significantly different \((p = 0.990)\) from that of the control fish \((0.04 \pm 0.02 \mu\text{g/ml})\). Plasma Vtg concentrations of fish in the first lagoons were inversely correlated with wastewater retention time in the lagoons \((p = 0.002)\).

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Water temperatures of the final effluents during the study ranged from 9 to 12°C. General treatment efficiency of lagoons has been shown to be dependent upon temperature, so there is potential for decreased removal of estrogenic activity when water temperatures are lower (e.g., winter months) than the present study. In conclusion, wastewater entering aerated lagoon systems was estrogenic to fish but with serial passage through the lagoons the estrogenic activity decreased to a level that was not sufficient to induce vitellogenesis in male fathead minnows in a 10- to 12-d exposure.

**Keywords**— Fish, Vitellogenin, Environmental estrogens, Bioassessment, Municipal wastewater lagoons

**INTRODUCTION**

Since the mid 1990's, occurrence and effects of endocrine disrupting (or hormonally active) chemicals in the environment have gained considerable attention [for a review see 1]. Many estrogenic compounds are found in municipal sewage; effluents from metropolitan wastewater treatment facilities (WWTFs) in Europe [2-8] and the United States (U.S.) [9] have been demonstrated to be estrogenic to fish. A collaborative effort among European countries known as Community Programme of Research on Environmental Hormones and Endocrine Disruptors (COMPREHEND) has demonstrated that estrogenic effluents occur across mainland Europe and that these include both municipal and industrial waste waters.
The estrogenic activity of WWTF effluents and rivers receiving the effluents has been evaluated by fish Vtg assays [2-4,6-10], in vitro assays [11-14], and quantification of individual estrogenic compounds [12,15-20]. Few published studies have characterized the estrogenicity of raw wastewater and the treated effluent to determine efficiency of the WWTF for removal of estrogenic activity. Recently, however, investigators in Germany have quantified various estrogenic substances and estrogenic activity of wastewater with in vitro assays at various points in the treatment process at several metropolitan WWTFs [11-13,18]. Estrogenic effects on fish were not examined in those studies, but can be inferred from other studies [2,21].

The investigations of WWTFs in Germany have led to the conclusion that tertiary treatment (e.g., activated charcoal filtration) efficiently eliminates estrogenic activity from metropolitan WWTF effluents [13,20]. Communities in the U.S. with populations over 10,000 generally use advanced secondary (trickling filter and/or activated sludge) and tertiary treatment processes; however, communities with populations less than 10,000 generally do not use tertiary treatment. The U.S. Environmental Protection Agency (U.S. EPA) estimates that by the year 2016, 71% of all WWTFs in the U.S. will serve communities of less than 10,000 people [22]. In six states (Iowa included), more than 90% of WWTFs will serve communities of fewer than 10,000 people. These small, rural communities commonly utilize multiple flow-through earthen lagoons, some aerated, for treatment of wastewater [23]. Nearly 5,000 lagoon WWTFs exist in the U.S. [24]; there are 148 municipal aerated lagoon WWTFs operating in Iowa, each of which continuously discharges effluent into a surface water system (Iowa Department of Natural Resources,
Lagoons are also used extensively for treatment of industrial and livestock wastes.

Effluents from WWTFs of less-populated areas are not always estrogenic to fish [10], but the estrogenic activity was neither evaluated for the influent nor throughout the treatment process. Further investigation is warranted because of the sheer numbers of small, rural community treatment systems and variations in treatment facilities, practices, and raw wastewater components. Our objectives were to first characterize the estrogenicity of domestic wastewater of small (population < 3,500), rural communities without industrial effluents, then determine how well serial passage through a three-cell aerated lagoon system reduces the estrogenic activity of the wastewater before it is released into the environment, and finally identify community or lagoon system design variables correlated with estrogenic activity.

To evaluate the estrogenicity of wastewater in aerated lagoon systems, we used induction of plasma Vtg in male fathead minnows (*Pimephales promelas*) as the test metric. Induction of Vtg in male fish is a standard biomarker of exposure to environmental estrogens [1,25,26]. We compared plasma Vtg in males exposed to the three lagoons at 10 WWTFs in Iowa, USA, to determine first if estrogenic activity was present, and second, if it was reduced before effluent was released to the environment. Further, we evaluated WWTF variables (i.e., population served, lagoon volume, inflow, lagoon retention time, water quality parameters, rainfall) that may be related to the efficiency of the treatment system for reducing the estrogenic activity of the wastewater before discharge.
MATERIALS AND METHODS

Study sites

The WWTFs sampled in this study fit the description by Crites and Tchobanoglous [23] of "multicellular partial-mix aerated lagoons," in contrast to the complete mix associated with the activated sludge process. The WWTFs of communities in a four-county region of central Iowa, USA, were chosen based on type of treatment, accessibility, cooperation by operators, and the ability of lagoons to permit fish survival for up to 12 d. Each facility consisted of three continuous discharge lagoons, the first two lagoons were aerated, and the third was a quiescent pond for sedimentation of suspended solids before final discharge. None of these facilities received industrial input but served small municipalities with populations of 362 to 3,497 (Table 1).

Table 1. Characteristics of municipal wastewater treatment facilities in central Iowa that were evaluated for estrogenic activity.

<table>
<thead>
<tr>
<th>Municipality</th>
<th>Population served</th>
<th>County</th>
<th>Receiving water</th>
<th>Caged fish exposure (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carlisle</td>
<td>3,497</td>
<td>Warren</td>
<td>Des Moines River</td>
<td>10</td>
</tr>
<tr>
<td>Elkhart</td>
<td>362</td>
<td>Polk</td>
<td>South Skunk River</td>
<td>10</td>
</tr>
<tr>
<td>Hartford</td>
<td>759</td>
<td>Warren</td>
<td>Middle River</td>
<td>10</td>
</tr>
<tr>
<td>Laurel</td>
<td>783</td>
<td>Marshall</td>
<td>Timber Creek</td>
<td>11</td>
</tr>
<tr>
<td>Maxwell</td>
<td>807</td>
<td>Story</td>
<td>Indian Creek</td>
<td>10</td>
</tr>
<tr>
<td>Mitchellville</td>
<td>1,715</td>
<td>Polk</td>
<td>Camp Creek</td>
<td>11</td>
</tr>
<tr>
<td>Pleasantville</td>
<td>1,539</td>
<td>Marion</td>
<td>Coal Creek</td>
<td>11</td>
</tr>
<tr>
<td>Polk City</td>
<td>2,344</td>
<td>Polk</td>
<td>Saylorville Lake</td>
<td>11</td>
</tr>
<tr>
<td>Roland</td>
<td>1,324</td>
<td>Story</td>
<td>Bear Creek</td>
<td>12</td>
</tr>
<tr>
<td>State Center</td>
<td>1,349</td>
<td>Marshall</td>
<td>North Timber Creek</td>
<td>11</td>
</tr>
</tbody>
</table>

*2000 U.S. Census data.*
The volume of lagoon 1 ranged from 4,160 m$^3$ at Elkhart and Laurel to over 117,700 m$^3$ at Carlisle (Table 2). There was similar variation in the volumes of lagoon 2 and lagoon 3 (Table 2). Wastewater retention times in lagoon 1 ranged from 31 d at Mitchellville to 111 d at Carlisle (Table 2). There was similar variation of retention times in lagoon 2; however, retention times in lagoon 3 ranged from only 3 d at Maxwell and Mitchellville to a maximum of 76 d at Carlisle (Table 2). Influent and effluent flow rates during the study period were less than 1,100 m$^3$/day at all facilities (Tables 3 and 4). Lagoon depths were determined from the facility design specifications and ranged from 2.1 to 4.0 m for lagoons 1 and 2, while lagoon 3 depths ranged from 0.3 to 3.6 m.

Table 2. Volume and wastewater retention time of aerated lagoons examined in the present study.

<table>
<thead>
<tr>
<th>Municipality</th>
<th>Volume$^a$ (m$^3$) of lagoon</th>
<th>Retention time$^b$ (days) in lagoon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Carlisle</td>
<td>117,700</td>
<td>125,680</td>
</tr>
<tr>
<td>Elkhart</td>
<td>4,160</td>
<td>4,160</td>
</tr>
<tr>
<td>Hartford</td>
<td>10,600</td>
<td>9,840</td>
</tr>
<tr>
<td>Laurel</td>
<td>4,160</td>
<td>4,160</td>
</tr>
<tr>
<td>Maxwell</td>
<td>9,840</td>
<td>9,840</td>
</tr>
<tr>
<td>Mitchellville</td>
<td>31,800</td>
<td>31,800</td>
</tr>
<tr>
<td>Pleasantville</td>
<td>2,710</td>
<td>26,120</td>
</tr>
<tr>
<td>Polk City</td>
<td>46,180</td>
<td>48,830</td>
</tr>
<tr>
<td>Roland</td>
<td>21,580</td>
<td>22,330</td>
</tr>
<tr>
<td>State Center</td>
<td>21,200</td>
<td>16,660</td>
</tr>
</tbody>
</table>

$^a$ Original design specifications.
$^b$ Lagoon volume divided by mean rate of inflow during the experiment.
$^c$ Wetland (volume calculated assuming a depth of 0.3 m).
Table 3. Mean of water quality parameters<sup>a</sup> (measured daily) of influent to lagoon 1 for 10- to 12-d interval when fish were held in aerated lagoons at wastewater treatment facilities.

<table>
<thead>
<tr>
<th>Facility</th>
<th>Inflow (m&lt;sup&gt;3&lt;/sup&gt;/d)</th>
<th>CBOD&lt;sup&gt;b&lt;/sup&gt; (mg/L)</th>
<th>TSS&lt;sup&gt;c&lt;/sup&gt; (mg/L)</th>
<th>pH</th>
<th>Temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carlisle</td>
<td>1,060</td>
<td>159</td>
<td>180</td>
<td>7.1</td>
<td>17</td>
</tr>
<tr>
<td>Elkhart</td>
<td>80</td>
<td>145</td>
<td>127</td>
<td>7.8</td>
<td>NA&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Hartford</td>
<td>269</td>
<td>375</td>
<td>138</td>
<td>8.0</td>
<td>18</td>
</tr>
<tr>
<td>Laurel</td>
<td>125</td>
<td>122</td>
<td>NA</td>
<td>7.3</td>
<td>15</td>
</tr>
<tr>
<td>Maxwell</td>
<td>284</td>
<td>201</td>
<td>111</td>
<td>8.0</td>
<td>17</td>
</tr>
<tr>
<td>Mitchellville</td>
<td>1,010</td>
<td>299</td>
<td>148</td>
<td>8.0</td>
<td>17</td>
</tr>
<tr>
<td>Pleasantville</td>
<td>560</td>
<td>260</td>
<td>341</td>
<td>7.2</td>
<td>16</td>
</tr>
<tr>
<td>Polk City</td>
<td>519</td>
<td>184</td>
<td>225</td>
<td>8.4</td>
<td>16</td>
</tr>
<tr>
<td>Roland</td>
<td>356</td>
<td>122</td>
<td>NA</td>
<td>7.5</td>
<td>17</td>
</tr>
<tr>
<td>State Center</td>
<td>439</td>
<td>148</td>
<td>184</td>
<td>7.3</td>
<td>16</td>
</tr>
</tbody>
</table>

<sup>a</sup> As reported by treatment operators at each facility to the Iowa Department of Natural Resources for National Pollution Discharge Elimination System permit requirements.

<sup>b</sup> Carbonaceous biological oxygen demand.

<sup>c</sup> Total suspended solids.

<sup>d</sup> Not available.

Table 4. Mean of water quality parameters<sup>a</sup> (measured daily) of effluent from lagoon 3 for 10- to 12-d interval when fish were held in aerated lagoons at wastewater treatment facilities.

<table>
<thead>
<tr>
<th>Facility</th>
<th>Flow (m&lt;sup&gt;3&lt;/sup&gt;/day)</th>
<th>CBOD&lt;sup&gt;b&lt;/sup&gt; (mg/L)</th>
<th>TSS&lt;sup&gt;c&lt;/sup&gt; (mg/L)</th>
<th>pH</th>
<th>Temp. (°C)</th>
<th>NH&lt;sub&gt;3&lt;/sub&gt;-N&lt;sup&gt;d&lt;/sup&gt; (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carlisle</td>
<td>1,044</td>
<td>4</td>
<td>11</td>
<td>7.3</td>
<td>12</td>
<td>NA&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Elkhart</td>
<td>NA</td>
<td>5</td>
<td>18</td>
<td>7.8</td>
<td>10</td>
<td>NA</td>
</tr>
<tr>
<td>Hartford</td>
<td>NA</td>
<td>10</td>
<td>13</td>
<td>7.8</td>
<td>11</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Laurel</td>
<td>8</td>
<td>&lt; 2</td>
<td>NA</td>
<td>7.2</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Maxwell</td>
<td>NA</td>
<td>4</td>
<td>16</td>
<td>8.0</td>
<td>10</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Mitchellville</td>
<td>NA</td>
<td>8</td>
<td>26</td>
<td>8.1</td>
<td>12</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Pleasantville</td>
<td>625</td>
<td>12</td>
<td>46</td>
<td>8.2</td>
<td>11</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Polk City</td>
<td>519</td>
<td>9</td>
<td>34</td>
<td>8.8</td>
<td>11</td>
<td>NA</td>
</tr>
<tr>
<td>Roland</td>
<td>242</td>
<td>13</td>
<td>NA</td>
<td>9.0</td>
<td>9</td>
<td>NA</td>
</tr>
<tr>
<td>State Center</td>
<td>341</td>
<td>3</td>
<td>4</td>
<td>8.1</td>
<td>10</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>

<sup>a</sup> As reported by treatment operators at each facility to the Iowa Department of Natural Resources for National Pollution Discharge Elimination System permit requirements.

<sup>b</sup> Carbonaceous biological oxygen demand.

<sup>c</sup> Total suspended solids.

<sup>d</sup> Unionized ammonia.

<sup>e</sup> Not available.
Caged fish exposures

Adult fathead minnows were hatched in March of 2000 and reared indoors on the campus of Iowa State University, Ames, IA, USA. These fish were the third generation from an original stock obtained from a local bait vendor. The fish were grown to adult size in 100-L tanks at 20-25°C and pH of 7 to 8. Prior to the study, plasma from 10 male fish was analyzed for Vtg to assure that male fish had negligible levels at the onset of the study. None of these fish had plasma Vtg concentration greater than 0.2 μg/ml.

In October and November 2000, fish were transported to the sewage lagoons in sealed, 2-L plastic bags with pure oxygen gas. In each lagoon, 25 fish of unknown gender were placed in a 129-L floating cage (61.0 cm x 45.7 cm x 45.7 cm) with 6.4-mm wire mesh for a 10- to 12-d exposure (Table 1). There was not a significant difference in length ($p = 0.843$) or weight ($p = 0.223$) among the 35 groups of fish stocked into lagoons and control aquaria. Mean ± SE length was 60.9 ± 0.5 mm and mean ± SE weight was 2.1 ± 0.1 g. Fish were not fed during the exposure period, but they may have eaten natural food (e.g., zooplankton and chironomids) present in the lagoon.

It was not possible to have a lagoon at each site without sewage; therefore our control group consisted of fish (25/replicate) stocked into 38-L aquaria. The control fish were from the same laboratory population as the fish placed in cages. They were transported to lagoon sites along with the fish that were stocked into lagoons, but without being stocked. They were returned to campus and maintained in aquaria at 16-18°C.
**Plasma collection**

After the 10- to 12-d exposure period, fish were removed from the cages and transported in lagoon water back to campus. Each fish was euthanized with 250 ppm tricaine methane sulfonate, weighed and measured. Control fish were treated similarly for all analyses. Blood samples (50-100 µl) were collected with heparinized (25 mg/L) 50-µl micropipettes by severing the caudal peduncle with a scalpel. Blood samples were kept separate on ice until gender was determined by gross examination of the gonad. Gonads were preserved in 10% neutral buffered formalin for eventual histological sectioning to verify the sex. Blood samples from same-sex fish in each lagoon were pooled in 1.5-ml microcentrifuge tubes. The pooled blood was then centrifuged (6400 RPM, 2000 x G) for 10 min and the plasma was decanted with a 50-µl micropipette. Plasma was aspirated into a 2.0-ml cryovial and frozen in liquid nitrogen until vitellogenin analysis.

**Vitellogenin assay**

Vitellogenin was quantified with a commercially available ELISA, following the protocol provided by the manufacturer (Biosense, Bergen, Norway). The analysis is based on a sandwich ELISA utilizing specific binding between antibodies and Vtg for quantification of Vtg in plasma from common carp (*Cyprinus carpio*) or fathead minnow. The capture antibody is a monoclonal anti-carp Vtg antibody, and the detecting antibody is a polyclonal anti-carp Vtg antibody. Vitellogenin provided with the kit was used to make standards that ranged from 0.24 to 62.5 ng/ml. All standards and samples were assayed in triplicate on each plate. Intraassay coefficient of variation was 8.0% (*n* = 12), and interassay
coefficient of variation was 9.1% \((n = 10)\). Recovery of Vtg from spiked (62 ng/ml) plasma samples was 93% \((\text{SE} = 5.0\%; n = 10)\).

**Statistical analyses**

The lagoons were connected in series by design; therefore, randomization of treatments to lagoons was not possible. Thus, statistical analysis was conducted with the SAS Mixed Procedure \([27]\) to account for correlation between lagoons at each facility. Comparison of the lagoon systems was done by treating the log Vtg values for the three cells in each facility as a multivariate normal random vector with a mean for each cell and an unstructured variance-covariance matrix. Tukey’s test was used for mean separation analysis.

There was no conceivable way to incorporate a control lagoon at the 10 sewage treatment facilities; therefore, we reared control fish in the lab. Independent-sample \(t\)-tests were used to compare mean Vtg from each of the three treatment levels to the mean Vtg of the control fish. The \(p\)-value for each \(t\)-test was multiplied by the total number of \(t\)-tests being performed (three) to conservatively account for any error introduced by performing multiple \(t\)-tests. All analyses were evaluated at the overall Type I error rate \((\alpha)\) of 0.05.

**RESULTS**

Fish survival

Survival of the 25 caged fish in each lagoon ranged from 16 to 100% during the 10- to 12-d exposures (Table 5), but survival was greater than 50% in all lagoons but one, the first
Table 5. Total fish survival (of 25 stocked per lagoon) and number of male fish surviving per lagoon at wastewater treatment facilities during 10- to 12-d exposure period.

<table>
<thead>
<tr>
<th>Municipality</th>
<th>Percentage fish survival and (males per lagoon)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lagoon 1</td>
<td>Lagoon 2</td>
<td>Lagoon 3</td>
</tr>
<tr>
<td>Carlisle</td>
<td>92 (10)</td>
<td>100 (10)</td>
<td>92 (10)</td>
</tr>
<tr>
<td>Elkhart</td>
<td>96 (10)</td>
<td>92 (10)</td>
<td>100 (4)</td>
</tr>
<tr>
<td>Hartford</td>
<td>88 (10)</td>
<td>88 (10)</td>
<td>100 (10)</td>
</tr>
<tr>
<td>Laurel</td>
<td>80 (10)</td>
<td>92 (10)</td>
<td>92 (10)</td>
</tr>
<tr>
<td>Maxwell</td>
<td>16 (4)</td>
<td>69 (9)</td>
<td>96 (10)</td>
</tr>
<tr>
<td>Mitchellville</td>
<td>52 (4)</td>
<td>88 (10)</td>
<td>96 (5)</td>
</tr>
<tr>
<td>Pleasantville</td>
<td>92 (10)</td>
<td>92 (10)</td>
<td>88 (5)</td>
</tr>
<tr>
<td>Polk City</td>
<td>76 (7)</td>
<td>60 (4)</td>
<td>80 (8)</td>
</tr>
<tr>
<td>Roland</td>
<td>68 (8)</td>
<td>88 (10)</td>
<td>76 (7)</td>
</tr>
<tr>
<td>State Center</td>
<td>88 (10)</td>
<td>88 (6)</td>
<td>88 (7)</td>
</tr>
<tr>
<td>Mean</td>
<td>75 (8)</td>
<td>86 (9)</td>
<td>91 (8)</td>
</tr>
</tbody>
</table>

lagoon at Maxwell, where only 4 of 25 (16%, all male) fish survived. Because of the uniquely low fish survival in this lagoon, the pooled plasma Vtg value for fish in this lagoon was not included in statistical analyses.

**Plasma vitellogenin**

Despite the substantial variability within treatment groups (Fig. 1), there were significant differences in mean fish plasma Vtg among the three lagoons of the aerated sewage treatment systems (Table 6). The mean plasma Vtg values decreased as wastewater passed through the serial lagoon systems: fish in lagoon 1 had the highest mean plasma Vtg, fish in lagoon 2 intermediate, and fish in lagoon 3 the lowest. There was a highly significant difference between plasma Vtg of fish in lagoon 1 and the control fish ($p < 0.001$) and also between
plasma Vtg of fish in lagoon 2 and the control fish \( (p = 0.018) \); however, the difference between mean plasma Vtg of fish in lagoon 3 and that of the controls was not significant \( (p = 0.990) \).

Fig. 1. Plasma vitellogenin (Vtg) concentration of male fathead minnows exposed to aerated lagoon wastewater treatment facilities in Iowa, USA. Each data point represents a pooled plasma sample from 4 to 10 male fish. Between mean plasma Vtg of fish in lagoon 3 and that of the controls was not significant \( (p = 0.990) \).
Table 6. Mean plasma vitellogenin (Vtg) of male fathead minnows exposed to aerated sewage lagoons

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number</th>
<th>Vtg ± SE(^a) ((\mu g/ml))</th>
<th>Tukey's(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lagoon 1</td>
<td>9</td>
<td>1,702 ± 670</td>
<td>A</td>
</tr>
<tr>
<td>Lagoon 2</td>
<td>10</td>
<td>0.94 ± 0.36</td>
<td>B</td>
</tr>
<tr>
<td>Lagoon 3</td>
<td>8</td>
<td>0.06 ± 0.02</td>
<td>C</td>
</tr>
<tr>
<td>Control</td>
<td>5</td>
<td>0.04 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Standard error.

\(^b\) Results of Tukey's test performed on log transformed Vtg means; different letters denote significant differences (\(\alpha \leq 0.05\)) among lagoon treatments.

\(^c\) Fish from the same population as those stocked into lagoons, but were stocked into laboratory aquaria for the 10- to 12-d exposure period.

The range in plasma Vtg values for lagoon 1 replicates was from < 5 \(\mu g/ml\) to > 6 mg/ml. Plasma Vtg of fish in lagoon 1 was not significantly correlated with percent fish survival, inflow rate, carbonaceous biological oxygen demand, total suspended solids, or total rainfall at the site during the study (\(p > 0.05\)). However, a significant inverse correlation was observed between plasma Vtg and municipality population (Fig. 2A), between plasma Vtg and lagoon volume (Fig. 2B), and between plasma Vtg and wastewater retention time (Fig. 2C). Communities with the largest populations had the largest lagoons (\(p < 0.001, r = 0.956\)) and the WWTFs with larger volumes had longer retention times (\(p = 0.002, r = 0.816\)). The coefficient of determination for retention time and fish plasma Vtg indicates that wastewater retention time accounts for nearly 77% of the variability in Vtg values observed in lagoon 1 at the WWTFs. The correlation between plasma Vtg of fish in lagoon 2 and plasma Vtg of fish in lagoon 1 (at the same WWTF) was not significant (\(p = 0.874, r = 0.062\)), nor was the correlation between plasma Vtg of fish in lagoon 2 and retention time in lagoon 2 (\(p = 0.228, r = 0.442\)).
Fig. 2. Relationship between concentration of plasma vitellogenin (Vtg) of male fathead minnows in lagoon 1 and community population (A), lagoon volume (B), and wastewater retention time (C) in the lagoon ($n = 9$). Each data point represents a pooled plasma sample of $n = 4$ to 10 male fish. The $p$-value is for the slope of the regression line.
DISCUSSION

The concentration of plasma Vtg in male fathead minnows observed in lagoon 1 implies that during October and November 2000, wastewater entering aerated lagoon WWTFs from small, rural communities in central Iowa was strongly estrogenic. The plasma Vtg levels in male fish exposed to lagoon 1 averaged 1.7 mg/ml, a value comparable to levels measured in gravid female fish [28] and similar to plasma Vtg concentrations reported by Folmar et al. [9] in male common carp exposed to metropolitan municipal sewage effluent. In a laboratory study with fathead minnows exposed to waterborne estradiol for 10 d (same as the lagoon exposures), approximately 200 ng/L of estradiol was necessary to induce similar levels of plasma Vtg in males [29]. Fathead minnows and other cyprinids are common in many of the effluent receiving waters and have been used to evaluate the estrogenic activity of water and wastewater in other studies [10, 30-32]. The fathead minnow has been used historically for a number of regulatory programs by the U.S. EPA [33-36], is one of the most extensively tested species of fish in the world, and is an appropriate species for screening of compounds that exert reproductive toxicity through alterations in endocrine function controlled by estrogenic or androgenic actions [30].

During our fall sampling period (October and November), the serial lagoon sequences successfully reduced the estrogenic activity from the wastewater before it was discharged to the environment from lagoon 3. The finding that aerated lagoon systems remove estrogenic activity from wastewater is supported by the findings of Bringolf [29], who reported plasma Vtg concentrations in feral male fathead minnows captured in aerated lagoons similar to concentrations measured in the caged fish in the present study. Mean plasma Vtg
concentration of caged fish in lagoon 1 and lagoon 2 was significantly different from controls. However, mean plasma Vtg concentration of male fish in lagoon 3 was not significantly different from that of the controls held in laboratory aquaria, indicating that a three-lagoon system was necessary to achieve a non-estrogenic discharge into surface waters and that partially treated wastewater may cause estrogenic effects in organisms in receiving waters. Other factors, however, such as dilution, adsorption, and degradation of estrogenic compounds in the receiving water, would reduce the likelihood of estrogenic effects and the area of the impact zone. While induction of Vtg in males is a biomarker of exposure to estrogenic compounds, a strong correlation between Vtg induction in males and population-level effects has not been demonstrated, although the body of literature examining the relationship between these variables is growing [30-32].

Since municipality population was inversely correlated with plasma Vtg concentration of fish in lagoon 1, we looked for differences in the lagoon systems based on population. We found that larger communities had lagoons with larger volumes and longer retention times. When lagoon volume was normalized for population, there was a strong correlation with retention time. There was a strong correlation of plasma Vtg concentration of male fish in lagoon 1 and wastewater retention time in lagoon 1. Wastewater retention time in a lagoon is dependent upon the lagoon volume and rate of flow through the lagoon. The reduced estrogenic activity of wastewater in lagoons with long retention times was likely due to the additional time for microbial degradation of estrogenic compounds in the wastewater.

Temperature is one of the most important physical factors influencing the overall efficiency of lagoons for converting wastes to stable end products; favorable temperatures for growth and activity of microbes are 10 to 40°C [37], although metabolic activities generally
increase with temperature within this range. All WWTF influent temperatures and most effluent temperatures in the present study were within this range. Recent studies have shown that natural estrogens, such as the hormones estradiol and estrone, are easily biodegradable in sewage treatment processes [38,39], but Rodgers-Gray et al. [8] found that while superior effluent treatment in the United Kingdom occurred between July and November, less efficient treatment occurred from December to March. They concluded that the seasonal variability of estrogenicity of WWTF effluent was caused by seasonal variation in temperature and thus microbial activity and overall WWTF efficiency. Reduced treatment efficiency of lagoon systems during cold weather is well documented and may result in a 50% decrease in removal of CBOD [23,37,40]. Similarly, the effluents of lagoon WWTFs examined in the present study generally have ammonia levels less than 1 mg/L in effluents from June to November; however, monthly reports at these facilities indicate that ammonia levels in January and February can reach 14 mg/L, indicating reduced microbial activity (nitrification) in the lagoons during winter months.

Future research with lagoon systems and their treatment of estrogenic substances should identify the efficiency of the systems during winter and early spring months. These times are especially critical because many species of fish (in receiving waters) are undergoing gonadal development and spawning and may be sensitive to subtle changes in endocrine system function. Caged fish exposures could be used to monitor estrogenicity of the final effluent throughout the year; however, the lagoons may not support fish survival during months when conditions (e.g., temperature, concentration of unionized ammonia) are unfavorable. Alternatively, in vitro bioassays can be used to monitor the estrogenicity of wastewater year-round [14], although there are disadvantages to in vitro bioassays as well [1].
To our knowledge, this is the first description of estrogenic activity of wastewater in lagoon WWTFs as well as reduction of estrogenic activity in serial passage through the systems. Although the latter findings were encouraging, the present study was carried out in months when water temperatures (9-18°C) were sufficient to support relatively high rates of metabolic activity by bacteria and other organisms in the lagoons. We caution that lagoon treatment efficiency for reduction of environmental estrogens may be dependent upon sufficient retention time and water temperature.

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REFERENCES


Since the early 1990s, endocrine disrupting compounds have been recognized as an important environmental threat. Male fish exposed to effluent from large, metropolitan municipal wastewater treatment facilities (WWTFs) have developed ovotestes and elevated levels of plasma vitellogenin (Vtg), a plasma protein typically produced by egg-laying females, among other reproductive abnormalities. In the summer of 2000, gonads and plasma Vtg concentrations were examined in feral male fathead minnows (*Pimephales promelas*) collected from lagoons of 11 small, rural municipal WWTFs and a reference site (a national wildlife refuge) in Iowa. Fathead minnows were captured in traps from five of the 33 lagoons (three per WWTF) sampled. No other fish species were captured. The five lagoons with fathead minnows were found at three WWTFs. Gonad histology indicated only one of 65 (1.5%) male fish living in the lagoons had ovotestes, which was similar to the incidence at a reference site (1 of 29, 3.4%). Plasma Vtg, however, was substantially higher
in fish from four of the five lagoons than in fish from the reference site, indicating that fish in lagoons were exposed to estrogenic substances.

INDEX DESCRIPTORS: Endocrine-disrupting compounds, environmental estrogens, fathead minnow, wastewater, ovotestes, vitellogenin.

Male feral fish captured downstream from effluent outfalls of municipal wastewater treatment facilities (WWTF) in Europe (Jobling et al. 1998, Allen et al. 1999) and the United States (Folmar et al. 1996, 2001) have been shown to develop symptoms of feminization, or demasculinization, as well as biomarkers of exposure to estrogenic substances. The estrogenic compounds most often identified in effluents and receiving waters include hormones excreted by human females (Desbrow et al. 1998, Lee and Peart 1998, Belfroid et al. 1999, Ternes et al. 1999, Johnson et al. 2000, Körner et al. 2000, Huang and Sedlak 2001), such as estrone and 17β-estradiol as well as 17α-ethinylestradiol, the synthetic estrogen used commonly in oral contraceptives and estrogen replacement therapy. Industrial compounds (i.e., alkylphenols) also have been identified in the estrogenic fraction of wastewater and receiving waters (Desbrow et al. 1998, Spengler et al. 2001).

The morphological endpoints and biomarkers of exposure to estrogenic substances reported in fish include gonad abnormalities, altered levels of steroid sex hormones (estrogens and androgens), and induction of female proteins (e.g., vitellogenin) in male fish. Entire populations of male roach (*Rutilus rutilus*), a cyprinid fish, in some rivers that receive a large percentage of their flow from WWTF effluent in the United Kingdom (U.K.) have
been shown to have ovotestes, a mixture of ovarian and testicular tissue (Jobling et al. 1998). The reproductive status of these fish currently is not known.

Elevated plasma vitellogenin (Vtg) concentration in males has become a standard biomarker of exposure to environmental estrogens (Heppel et al. 1995, Sumpter and Jobling 1995). Female fish (and other oviparous vertebrates) produce Vtg, the egg yolk protein precursor, in the liver in response to stimulation by estrogens from the ovaries (Le Guellec et al. 1988). Male fish possess the capability to produce Vtg but normally lack a sufficient estrogen stimulus; however, upon exposure to exogenous estrogens, males produce Vtg in a dose-dependent manner (Panter et al. 1998, Folmar et al. 2000, Bringolf 2002). A strong correlation of Vtg induction in males to population-level effects has not been demonstrated to date; however, the body of literature reporting a relationship between these variables is expanding (Kramer et al. 1998, Panter et al. 1998, Ankley et al. 2001).

Elevated levels of Vtg have been reported in fish exposed to effluent of large metropolitan municipal WWTFs, which often utilize advanced secondary and tertiary treatment processes. Small communities generally utilize simple treatment processes, often in the form of passage of wastewater through a series of lagoons. The estrogenic activity of wastewater in such systems has not been thoroughly examined. In the United States, there are over 7,000 lagoon systems in operation (Crites 1992). Aerated lagoon systems, in contrast to nonaerated systems, generally are operated as flow-through systems and release effluent continually into surface waters. There are currently 148 municipal aerated lagoon systems in Iowa that continually discharge effluent into the environment (Iowa Department of Natural Resources 2002).
The estrogenic activity of wastewater from small communities is not well characterized, although, Bringolf and Summerfelt (2003) reported that wastewater in the first and second lagoons of a three lagoon series at several aerated lagoon WWTFs in Iowa was highly to slightly estrogenic to male fish (Vtg assay), so the potential for effluent with estrogenic activity exists.

At this time there are no reports in the literature that have examined feral populations of fish living in WWTF lagoons in the U.S. for evidence of endocrine disruption. The objectives of the present study were to compare plasma Vtg concentrations and the incidence of ovotestis of male fish living in wastewater lagoons to fish in a reference population. This information was used to further characterize the risk of exposure to environmental estrogens in municipal wastewater effluent from aerated lagoon WWTFs.

METHODS

Sample Sites

The WWTFs sampled in the present study served the central Iowa communities of Carlisle, Elkhart, Granger, Hartford, Laurel, Maxwell, Mitchellville, Pleasantville, Polk City, Roland, and State Center. The WWTFs were selected based on location, type of treatment, accessibility, and previous cooperation between Iowa State University and the operators. Union Slough National Wildlife Refuge (NWR) in north central Iowa served as the reference site. From August to November 2000, two minnow traps were randomly placed within 1 m of shore (for 1 to 3 days at a time) in 33 lagoons at the 11 aerated lagoon WWTFs (three
lagoons per WWTF) and at the reference site. Each WWTF consisted of three continuous discharge lagoons: the first two at each site were aerated, and the third was a quiescent pond for sedimentation of suspended solids (clarification) before final discharge. The facilities fit the description by Crites and Tchobanoglous (1998) of “multicellular partial-mix aerated lagoons.” During the sampling period, retention time of wastewater in the three-lagoon systems ranged from 65 to 306 days, with a median of 103 days. None of these facilities received industrial wastewater; they served municipalities with populations of 362 to 3,497.

Collection of Gonads and Plasma

Captured fish were returned in lagoon water to Iowa State University, where they were anesthetized (with tricaine methane sulfonate), weighed, and measured. The caudal peduncle of each fish was severed with a scalpel, and whole blood was collected with heparinized 50-μl micropipettes. Blood samples were kept on ice throughout processing and were centrifuged (2000 x G) for 10 minutes to obtain plasma. Plasma samples were transferred to 250-μl microcentrifuge tubes and frozen in liquid nitrogen until Vtg analysis. The gonads were removed and fixed in 10% neutral buffered formalin until processing.

Gonad Histology

The fixed gonads were processed (dehydrated, embedded in paraffin, sectioned, and stained with hematoxylin and eosin) at the Veterinary Diagnostics Laboratory, Iowa State University, Ames, Iowa. Longitudinal sections (5 μm) of each gonad from each fish were examined for the presence of oocytes in otherwise testicular tissue and were rated according
to the 'intersex index' on a scale of 0 to 7, as described by Jobling et al. (1998), of which 0 was entirely male (testis) and 7 was entirely female (ovary).

**Vitellogenin Assay**

Plasma samples of male fish were thawed on ice for two hours and then pooled (n = 3 to 4 samples) with other fish plasma from the same lagoon. Plasma Vtg was quantified with a commercially available (Biosense, Bergen, Norway) enzyme-linked immunosorbent assay (ELISA) validated for use with common carp (*Cyprinus carpio*) and fathead minnow. Vitellogenin provided with the kit was used to make standards that ranged from 0.24 to 125 ng/ml. All standards and samples were assayed in triplicate on each plate, and each sample was assayed at three dilutions. Intraassay coefficient of variation was 8.5% (N = 6), and interassay coefficient of variation was 9.7% (N = 6). Mean ± standard deviation Vtg spike-recovery was 93.0% ± 13.5% (N = 4). Minimum detection limit (MDL) for plasma Vtg was 2.4 ng/ml.

**RESULTS**

**Fish Collection**

Fathead minnows were captured from the NWR and five of 33 lagoons at the 11 municipalities sampled. The five lagoons with fathead minnows were at three WWTFs (Carlisle: lagoons 2 and 3; Granger: lagoons 1 and 2; Pleasantville: lagoon 3). A total of 65 male fathead minnows (and no other species of fish) were captured from the lagoons and 29
from the NWR (Table 1). Female fathead minnows were also caught in the minnow traps, but were not evaluated for exposure to estrogenic compounds.

Table 1. Plasma vitellogenin (Vtg) concentrations of feral male fathead minnows captured in wastewater treatment lagoons and at a reference site (national wildlife refuge) in Iowa. Plasma Vtg values represent a mean of N pooled samples, each of 3–5 fish.

<table>
<thead>
<tr>
<th>Site</th>
<th>Lagoon captured</th>
<th>Total fish</th>
<th>Number (N) of pooled samples</th>
<th>Plasma Vtg (µg/ml) ± SE&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carlisle</td>
<td>2</td>
<td>19</td>
<td>4</td>
<td>3.4 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>19</td>
<td>4</td>
<td>&lt;MDL&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Granger</td>
<td>1</td>
<td>18</td>
<td>4</td>
<td>2,823 ± 1,393</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6</td>
<td>2</td>
<td>325.1 ± 65.0</td>
</tr>
<tr>
<td>Pleasantville</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>46.3</td>
</tr>
<tr>
<td>Union Slough National Wildlife Refuge&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29</td>
<td>29</td>
<td>4</td>
<td>&lt;MDL</td>
</tr>
</tbody>
</table>

<sup>a</sup>Reference site.  
<sup>b</sup>Standard error.  
<sup>c</sup>Below minimum detection limit (0.02 µg/ml).

**Gonad Histology**

Of the 65 fish sampled in lagoons, one (1.5%) had ovotestes and was rated as a 3 on the intersex index scale as described by Jobling et al. (1998). The testes of this fish had several structures that resembled primary oocytes scattered throughout the testicular tissue (Fig. 1). The ducts were not present in the histological sections and, hence, could not be evaluated. One fish of the 29 (3.4%) from NWR had an ovotestis; this fish was rated as a 4 on the
intersex index scale. Again, the ducts were not present in the tissue sections and, thus, could not be evaluated.

![Image of a testis section](image)

**Fig. 1.** Transverse section of a testis of a feral fathead minnow captured in an aerated wastewater treatment lagoon, showing several primary oocytes (arrows) embedded in seminiferous tubules that otherwise are filled with spermatocytes. Hematoxylin and eosin staining, x 40.

**Plasma Vitellogenin**

Mean plasma Vtg concentrations of feral male fathead minnows from the lagoons ranged from below MDL in the fish from Carlisle lagoon 3 to 2,823 μg/ml in fish from Granger lagoon 1 (Table 1). Concentrations of plasma Vtg of fish from the reference site were below MDL. The fish with an ovotestis captured at the reference site was in a pooled plasma sample that had a Vtg concentration below MDL. The fish from Granger lagoon 1 that had an ovotestis was in a pooled sample that had a Vtg concentration of 2112 μg/ml.
DISCUSSION

This is the first study from the U.S. or elsewhere to examine feral fathead minnows from WWTF lagoons for evidence of exposure to environmental estrogens. The study provides insight into the estrogenic activity of partially treated wastewater in simple WWTFs in Iowa. Nichols et al. (1999) used caged fathead minnows to investigate the estrogenicity of effluent from small WWTFs in the U.S., but the present study is the first to examine biomarkers of exposure to environmental estrogens in feral fish captured from treatment lagoons.

It is unknown how the feral fathead minnow populations became established in the lagoons, if they reproduce in the lagoons, or simply swim into the lagoons from the receiving water via the effluent flow. Once in the system, they conceivably could move between lagoons because the lagoons are connected in series by submerged pipes or culverts.

The small number of lagoons in which fish were captured prohibits broad generalizations about the effects of partially treated wastewater on fish; however, the plasma Vtg concentrations suggest that male fish in most of the lagoons were exposed to environmental estrogens and that Vtg concentrations were generally dose-dependent, e.g., Vtg concentrations were highest in lagoon 1 and less in each subsequent lagoon in the series, a trend similar to that reported by Bringolf and Summerfelt (2003) who exposed caged fathead minnows to aerated lagoon WWTFs for 10 to 12 days (Table 2). The Vtg concentrations of feral fish in the present study are greater than the concentrations measured in caged fish, but a plausible explanation for this difference may be in the duration of exposure. The range of concentrations of plasma Vtg in male feral fathead minnows in the present study is comparable to that reported by Folmar et al. (1996) for feral male common carp, also a
The highest plasma Vtg concentrations measured in the present study are in the range commonly measured in gravid female fish (Le Guellec et al. 1988).

Table 2. Comparison of plasma vitellogenin (Vtg) of caged male fathead minnows exposed to aerated lagoons of municipal wastewater treatment facilities (WWTF) for 10 to 12 days, to mean plasma Vtg of feral fathead minnows captured in WWTF lagoons.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Caged fathead minnows</th>
<th>Feral fathead minnows</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of Sites (N fish)</td>
<td>Mean plasma Vtg ± SE (μg/ml)</td>
</tr>
<tr>
<td>Lagoon 1</td>
<td>9 (83)</td>
<td>1.702 ± 670</td>
</tr>
<tr>
<td>Lagoon 2</td>
<td>10 (89)</td>
<td>0.94 ± 0.36</td>
</tr>
<tr>
<td>Lagoon 3</td>
<td>8 (76)</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>Control</td>
<td>5 (25)</td>
<td>0.04 ± 0.02</td>
</tr>
</tbody>
</table>

The incidence of ovotestes in lagoon fish was low and similar to that of fish captured from the NWR. There are no reports in the literature of the normal incidence of hermaphroditism in fathead minnow, however, incidence of intersexuality (ovotestes) has been reported as high as 5% in control populations of carp (Komen et al. 1989) and 4% in control populations of roach (Jobling et al. 1998), so it seems reasonable that a low incidence of ovotestes in fathead minnow could be considered "natural." Regardless, the finding of a single intersex fish has been reported in the literature for a variety of species including...
chinook salmon (*Oncorhynchus tshawytscha*) (Barnes et al. 2001), roach (*Rutilus rutilus*) (Arne 1965; Jaffri and Ensor 1979), and western mosquitofish (*Gambusia affinis*) (Teh et al. 2000).

Exposure of male fish to estrogen mimics during sexual differentiation has been shown to induce sex reversal and/or ovotestes (Gimeno et al. 1997, Gray and Metcalfe 1997, Jobling et al. 1996, 1998). In most teleost fish, sexual differentiation is a two-stage process that occurs early in life and involves gonadogenesis (development of the structural and supporting components of the gonad) and gametogenesis (proliferation and development of the germ cells). There is a labile period when sex differentiation is dependent upon the hormonal milieu in the fish (Kime 1998). The low incidence of ovotestes in fish captured from lagoons (1.5%) suggests that these fish were not living in estrogenic environments at the time of sexual differentiation; however, determination of onset and duration of exposure was not possible since it was not known how long the fish have lived in the lagoons or if they were able to reproduce in that environment.

Staging of gametocytes also has been used to indicate reproductive toxicity (Goodbred et al. 1996, Miles-Richardson et al. 1999, Van den Belt et al. 2002). Gonads of fish exposed to estrogenic compounds during sexual maturation tend to be in earlier stages of development than unexposed fish. This evaluation technique is particularly useful when fish are all of the same age and are sampled at the same time. It was not useful to compare spermatogenic stages in the feral fish of the present study because the fish were of unknown age (stage of sexual maturity) and were captured both during and after the spawning period. Relative size of gonads (gonadosomatic index; GSI) also is commonly used to indicate sexual disruption (Jobling et al. 1996); this endpoint was not evaluated for the same reasons as gamete staging.
The initial finding that feral fathead minnow populations exist in some wastewater treatment lagoons was surprising. The presence of these fish in partially treated wastewater suggests that the treatment process is effective, because the water quality (i.e., low dissolved oxygen and high ammonia levels) of raw wastewater is not favorable for most fish species. Wastewater retention time in lagoon treatment systems is a major factor for efficiency of removal of organic contaminants in lagoon systems (Middlebrooks et al. 1978, Tchobanoglous and Burton 1991), and estrogenic activity (fish Vtg assay) was inversely correlated with retention time in aerated lagoons (Bringolf and Summerfelt 2003). The relatively long retention times in the lagoon systems sampled (65+ days per system) certainly may contribute to the efficiency of degradation of estrogenic compounds in such systems. Therefore, findings of the present study indicate that the risk of exposure to estrogenic substances was small in effluent from aerated lagoon WWTFs during the time of the study.

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LITERATURE CITED


PLASMA VITELLOGENIN IN FATHEAD MINNOWS EXPOSED TO WATERBORNE 17β-ESTRADIOL

A paper submitted to Archives of Environmental Contamination and Toxicology

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Abstract. Vitellogenin (Vtg), a plasma protein, is used as a biomarker of exposure of male fish to estrogenic substances. One of the compounds most often identified in the estrogenic fraction of effluent from wastewater treatment facilities is 17β-estradiol (E2), a natural steroid hormone excreted primarily by females. To determine the potential magnitude of environmental exposure to E2, there is a need to understand the dose-response relationship of Vtg in fish exposed to waterborne E2. In the present study, male fathead minnows were exposed to E2 target concentrations of 10, 50, 100, 500, or 1000 ng/L for 10 days, at which time plasma samples were collected for quantification of concentrations of E2 and Vtg. The Vtg induction was dose-dependent and predictable through the range of waterborne E2 concentrations tested ($R^2 = 0.988$). The lowest observed effect concentration (LOEC) for induction of plasma Vtg was 50 ng/L. The plasma Vtg dose-response curve from this study may be used in conjunction with exposure of male fish to surface water or wastewater for a
short period to estimate the magnitude of the estrogenic potency of the water in terms of "estradiol equivalents."

Endocrine disrupting compounds (EDCs) are among six high priority research issues identified by the U.S. Environmental Protection Agency (U.S. EPA 1996). "Endocrine disruptors" are identified by their capacity to interfere with endocrine influences on growth, maturation, reproduction, and metabolism. The most studied category of EDCs is the "environmental estrogens" (EEs), which mimic endogenous estrogens and can be potent at nanogram per liter concentrations. The effects of EEs have been most notable in male fish, where induction of high concentrations of the egg yolk protein precursor, vitellogenin (Vtg), has often been described, but other effects include depression of secondary sexual characteristics, induction of ovotestes, and even complete sex reversal (Purdom et al. 1994; Jobling et al. 1998; Panter et al. 1998; Routledge et al. 1998; Miles-Richardson et al. 1999). Elevated plasma Vtg has become a standard biomarker of exposure to estrogenic substances (Sumpter and Jobling 1995; Folmar et al. 1996, 2001a; Goodbred et al. 1996; Harries et al. 1996, 1997, 1999; Purdom et al. 1994; Nichols et al. 1999; Sherry et al. 1999; Irwin et al. 2001; Bringolf and Summerfelt 2003).

One identified source of EE contamination of aquatic systems is municipal wastewater effluent (Shore et al. 1993; Purdom et al. 1994; Folmar et al. 1996, 2001a; Harries et al. 1996, 1997, 1999; Jobling et al. 1998; Routledge et al. 1998; Körner et al. 1999). Of the three natural human steroid estrogens, the most potent is 17β-estradiol (E₂). It is among the
compounds most frequently identified in the estrogenic fraction of wastewater effluent (Lee and Peart 1998; Desbrow et al. 1998; Belfroid et al. 1999; Ternes et al. 1999a; Rogers-Gray et al. 2000; Huang and Sedlak 2001; Spengler et al. 2001). Human females excrete relatively large quantities of E₂, ranging from 0.01 - 0.1 mg/day (Williams and Stancel 1996) and pregnant women discharge as much as 30 mg/day (Arcand-Hoy et al. 1998). Steroid hormones such as E₂ are excreted as inactive glucuronide or sulfide conjugates, but the hormones are rapidly deconjugated back to active forms by microbes in the wastewater treatment process (Ternes et al. 1999b).

Many of these and other studies have reported Vtg induction in male fish exposed to wastewater effluent; however, only Panter et al. (1998) and Folmar et al. (2000) describe the dose-response relationship for Vtg in fish exposed to waterborne E₂. Folmar et al. (2000) exposed sheepshead minnows (Cyprinodon variegatus) to E₂ concentrations of 20, 200, 500, 1000, and 2000 ng/L for 16 d. Panter et al. (1998) exposed fathead minnows to E₂ concentrations of 10, 32, 100, 320, and 1000 ng/L for 21 d. In a previous study, we found that plasma Vtg was induced in caged fathead minnows (Pimephales promelas) that had been exposed (10 d) to aerated wastewater treatment lagoons in central Iowa (Bringolf and Summerfelt 2003). The present study was devised to describe the plasma Vtg response in fish exposed to E₂ for 10 days, which is the same duration of exposure of fathead minnows in WWTF lagoons in Iowa, and to determine the exposure concentration of E₂ necessary to induce plasma Vtg responses similar to those of male fathead minnows exposed to wastewater lagoons.
Methods

The range of E$_2$ exposure concentrations used in the present study represents levels previously reported in wastewater effluent (Lee and Peart 1998; Desbrow et al. 1998; Belfroid et al. 1999; Ternes et al. 1999a; Rogers-Gray et al. 2000; Huang and Sedlak 2001; Spengler et al. 2001) and concentrations that have been used to induce an in vivo vitellogenic response in minnow species (Panter et al. 1998; Folmar et al. 2000). The exposure duration was chosen to emulate the period used for caged fish exposures in wastewater treatment lagoons of a previous study (Bringolf and Summerfelt 2003).

Fish Husbandry

Male fathead minnows (18 months old) used in this study were hatched and reared in the laboratory at Iowa State University, Ames, IA, USA. The fish were third generation from an original stock obtained from a local bait vendor. The fish were reared from hatch to adult size in 100-L tanks at 20–25°C on a diet of live brine shrimp and a commercial microparticulate feed (BioKyowa). At the beginning of the experiment, the fish had a mean length ± standard error (SE) of 77.4 ± 0.6 mm and mean weight ± SE of 4.4 ± 0.1 g. At the end of the experiment, 17 d later, fish lengths and weights had changed little and there was no significant difference (p > 0.05) in the lengths or weights among treatment groups. During the experiment, fish were fed at a rate of 3% body weight per day. Water temperature was maintained at 24 ± 1°C with a photoperiod of 16L:8D. Dissolved oxygen was > 6.0 mg/L in all aquaria (monitored daily). Alkalinity (range 40–60 mg/L) and pH (range 6.7–7.2) were monitored every other day.
Experimental Design

Three male fish were placed in each of 24, 4-L aquaria and acclimated for 7 days. Each aquaria was assigned to one of eight treatments (in triplicate) representing nominal waterborne E$_2$ concentrations of 10, 50, 100, 500, 1000 ng/L (stock solution dissolved in acetone), control (culture water only), solvent control (0.5 ppm v/v acetone in culture water), and day 0 controls (sampled at the time the exposure period began). All E$_2$ exposure doses were achieved by adding E$_2$ stock solution to the aquaria. The solvent controls received 0.5 ppm (v/v) acetone because this was the highest acetone concentration used in the E$_2$ exposures. A static-renewal procedure was employed for the 10-d exposures. Every 24 hours, 90% of exposure water (nominal E$_2$ concentration) was replaced.

Plasma Collection

After the 10-d exposure period, fish were anesthetized with the standard fish anesthetic (tricaine methanesulfonate), weighed, measured, and bled. A mixed arteriovenous sample of blood (50-100 µl) was collected from the caudal sinus with heparinized (25 mg/l) 50-µl micropipettes after severing the caudal peduncle with a scalpel. Gross examination of the gonads was used to verify the sex of the fish. Blood from the three fish in each aquarium was pooled in a 1.5-ml microcentrifuge tube. The pooled blood samples were centrifuged to obtain plasma, which was frozen in liquid nitrogen until Vtg analysis.
Plasma Vitellogenin Quantification

Plasma Vtg was quantified with a commercially available ELISA kit (Biosense) developed for use with common carp (Cyprinus carpio) and fathead minnow, as previously described by Bringolf and Summerfelt (2003). The limit of detection for Vtg in plasma samples was 0.020 µg/ml. Mean (± SE) recovery of Vtg from spiked (3,125 µg/ml) plasma samples was 92 ± 5.8% (n = 6). Mean (± SE) CV for duplicate measurements of Vtg in each plasma sample was 5.6 ± 0.8 (n = 24). Duplicate samples with a coefficient of variation (CV) greater than 10% (n = 1) were excluded from statistical analyses.

Statistical Analysis

Plasma Vtg values were log transformed to achieve homogeneity of variance for statistical analysis; fish length and weight data did not require transformation. Statistical analysis of Vtg concentrations was performed with a least squares means general linear model one-way analysis of variance (ANOVA) (SAS Institute 1999) followed by Dunnett’s test to compare the treatment means to the control. Tukey’s test was used to determine significance of difference among fish lengths and weights. All statistical tests were evaluated at the α = 0.05 level of significance.
Results and Discussion

Plasma Vtg concentration increased with exposure to increasing concentrations of waterborne E₂ during the 10-d exposure period (Figure 1). Fish in the three control groups had low (0.22 – 1.02 μg/ml), but detectable levels of plasma Vtg. The 50 ng/L E₂ treatment was the lowest concentration that induced a significantly greater ($p = 0.009$) concentration of plasma Vtg from that of the controls; fish exposed to E₂ concentrations greater than 50 ng/L.

![Graph showing plasma vitellogenin concentration](image)

**Fig. 1.** Plasma vitellogenin (± standard error) concentration in male fathead minnows after a 10-day exposure to waterborne 17β-estradiol. An initial control group (day 0) was sampled at the start of the study and the solvent control group (SC) was exposed to 0.5 ppm (v/v) acetone. * Significant difference ($p <0.01$) from controls, and *** significant difference ($p <0.0001$) from controls.
had significantly ($p < 0.0001$) more plasma Vtg than the controls. Plasma Vtg concentrations exceeded 300,000 μg/ml in fish exposed to 1000 ng/L of E$_2$. The dose-response relationship between waterborne E$_2$ and plasma Vtg was logistic, indicating that responses to the exposure concentrations were well separated (Fig. 2). The coefficient of determination (i.e., $r^2 \times 100$) indicates that E$_2$ exposure accounted for nearly 99% of the variability in the plasma Vtg values.

![Graph showing dose-response regression analysis for waterborne 17β-estradiol and plasma vitellogenin concentration in male fathead minnows following a 10-d exposure period.](image)

**Fig. 2.** Dose-response regression analysis for waterborne 17β-estradiol and plasma vitellogenin concentration in male fathead minnows following a 10-d exposure period.

Panter et al. (1998) reported a significant Vtg induction in male fathead minnows at 100 ng/L E$_2$ (nominal) after a 21-d exposure, but the Vtg response was not significant at 32 ng/L.
Collectively, the findings of Panter et al. and those of the present study indicate that after a 10 to 21 day exposure, the lowest observable effects concentration (LOEC) for vitellogenin induction in male fathead minnows is between 32 and 50 ng/L E$_2$. Measured concentrations of E$_2$ in wastewater effluent are reported as highs of 88 ng/L (Rodgers-Gray et al. 2000), 64 ng/L (Ternes et al. 1999a) and 48 ng/L (Desbrow et al. 1998), but are commonly in the range of 1-15 ng/L (Lee and Peart 1998; Belfroid et al. 1999; Baronti et al. 2000; Johnson et al. 2000; Matsui et al. 2000; Huang and Sedlak 2001; Spengler et al. 2001). Results of the present study indicate that the concentrations of E$_2$ reported in some effluents are sufficient to induce plasma Vtg in male fish after a 10 to 21 day exposure.

In a previous study (Bringolf and Summerfelt 2003), plasma Vtg in caged male fathead minnows exposed to partially-treated municipal wastewater for 10 days averaged 1700 μg/ml. From the dose-response regression analysis of the present study, approximately 160 ng L of E$_2$ would be necessary to induce 1700 μg/ml of plasma Vtg in a 10-d exposure. Given that concentrations of E$_2$ greater than 100 ng/L have not been reported for raw wastewater or influent, it may be that there was an additive effect of combinations of other estrogenic compounds (e.g., alkylphenols or the synthetic estrogen ethynylestradiol) that was responsible for the induction of plasma Vtg in that study. Whatever the compound or combination of compounds in the wastewater, the plasma Vtg dose-response evaluation provides an indication of the relative estrogenic potency of the water in terms of "E$_2$ equivalents."

Measures of the plasma Vtg biomarker have often been obtained from fish exposed to estrogens for relatively short intervals (i.e., 7 to 21 days) (Purdom et al. 1994; Harries et al. 1996, 1997, 1999; Panter et al. 1998; Nichols et al. 1999; Bringolf and Summerfelt 2003).
The short exposure period is adequate for assessment of EE exposure because continuous exposure to a relatively constant concentration of estrogens results in a sigmoid-shaped response curve, and maximum plasma Vtg levels are generally reached within 7 to 10 days (Sumpter and Jobling 1995; Folmar et al. 2000). Furthermore, the plasma Vtg response in male fish to a single exposure of environmental estrogens is generally logarithmic for the first several days, but also reaches an asymptote within 7 to 10 days and can remain elevated for 40 days (Purdom et al. 1994; Sumpter and Jobling 1995; Sherry et al. 1999; Schultz et al. 2001). Considering those findings, the Vtg response of fish in the present study would likely have reached an asymptote by the end of the 10-d exposure period, even if the nominal concentrations of waterborne E2 were only achieved for a short period at the beginning of the study.

Plasma Vtg results of the present study are comparable to those of Folmar et al. (2000) who exposed sheepshead minnows (Cyprinodon variegatus) to nominal E2 concentrations of 20. 200, 500, 1000 and 2000 ng/L. They found that 200 ng/L was the lowest concentration that induced significantly higher concentration of plasma Vtg than the controls, but concluded that the threshold E2 exposure concentration is "undoubtedly lower" than 200 ng/L because of the large concentration gap between the lower exposure concentrations. Kramer et al. (1998) used plasma alkaline labile phosphate as an indicator of Vtg, and determined that the E2 EC50 (concentration that significantly affected 50% of the fish) for male fathead minnows was 251 ng/L, although 120 ng/L was reported as the EC50 for egg production. Rainbow trout (Oncorhynchus mykiss) have been reported to be more sensitive to E2 than cyprinids, showing a significant plasma Vtg response at 10 ng/L E2 (Purdom et al. 1994)
Levels of plasma Vtg commonly exceed 1000 µg/ml in gravid female fish but are generally less than 0.1 µg/ml in unexposed male fish (Le Guellec 1988). Exposure of female fish to EEs may not produce pathogenic effects because females transfer Vtg into developing oocytes; however, males lack such a reservoir and the absence of a clearing mechanism may result in various pathologies as Vtg accumulates in capillaries of tissues. Male summer flounder (Paralichthys dentatus) with plasma Vtg concentrations greater than 0.1 mg/ml had liver, kidney, and testicular pathologies (Folmar et al. 2001b). The population-level effects of exposure of fish to environmental estrogens are largely unknown; however, there has been some recent effort to describe the relationship of biomarkers of estrogen exposure (such as plasma Vtg) to impairment of reproduction and development (Kramer et al. 1998; Harries et al. 2000; Ankley et al. 2001; Länge et al. 2001; Seki et al. 2001).

In conclusion, plasma Vtg was induced in male fathead minnows exposed to environmentally relevant concentrations of waterborne E₂ after a relatively short-term exposure. The Vtg induction was dose-dependent and predictable through the range of E₂ concentrations tested. The plasma Vtg dose-response curve from this study may be used in conjunction with exposure of male fish to surface water or wastewater for a short period to estimate the magnitude of the estrogenic potency of the water in terms of "estradiol equivalents." Future research should evaluate the short- and long-term effects of elevated plasma Vtg on the health and reproductive fitness of male fish.

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EFFECTS OF ATRAZINE ON FATHEAD MINNOW IN A SHORT-TERM REPRODUCTION ASSAY

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Abstract—Atrazine is the most extensively used herbicide in the United States. Part per million concentrations of atrazine have been reported in agricultural runoff, it is detectable in surface waters and precipitation throughout the year, and it has been found in ground water sources of drinking water. Recent studies indicate that atrazine may be a potent endocrine disrupting compound to frogs exposed to part per billion (μg/L) concentrations. For these reasons, the effects of atrazine (5 and 50 μg/L) on several endpoints related to reproductive fitness were examined in fathead minnows (Pimephales promelas) in a 21-d static exposure. Estradiol (0.5 μg/L) was included as a positive control treatment. Endpoints examined in adult fish during and after the exposures included survival, egg production, number of spawns, eggs/spawn, relative gonad weight, gonad histology, number of nuptial tubercles, and plasma vitellogenin concentration. Eggs produced during the exposures were hatched
and reared in control water. The percentages of embryos fertilized and hatched, and larval survival were evaluated. There were decreasing trends in relative testis weight, testis maturity, and percent embryo fertilization, and suggest further investigation is warranted, but the differences in these and other endpoints were not statistically significant in the atrazine-exposed fish. Nearly all endpoints concerning fish exposed to estradiol were significantly different from atrazine exposed fish and control fish. These results suggest that atrazine did not have strong estrogenic effects in adult fathead minnows and did not cause overt reproductive toxicity at environmentally relevant concentrations.

Keywords— Fathead minnow reproduction endocrine disruption atrazine

INTRODUCTION

Endocrine disrupting chemicals (EDCs) have attracted substantial attention in the latter half of the past decade [1] because there is mounting evidence that they cause adverse reproductive and developmental effects in a variety of fish and wildlife species [2]. In the mid-1990s, researchers in Europe and the U. S. reported reproductive abnormalities such as altered sex hormone ratios, reduced gonad size, and induction of vitellogenin (Vtg) in fish exposed to environmental estrogens (EEs) [3-6]. Since then, studies worldwide have used induction of Vtg, the egg yolk protein precursor, in caged and wild fish to identify surface waters containing EEs [7-13]. Although the induction of biomarkers in EE-exposed fish is
well documented, only recently have studies examined the relationship of biomarkers with impairment of reproduction and development [14-18].

In response to a U. S. congressional mandate, the U.S. Environmental Protection Agency (U.S. EPA) developed and implemented a screening program for EDCs that disrupt processes controlled by estrogen, androgen, or thyroid hormones [19]. The U.S. EPA-convened Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) has currently recommended five in vivo assays for tier 1 screening, one of which is a short-term fathead minnow (Pimephales promelas) reproduction assay [20]. The assay has been developed and tested by the U.S. EPA and involves exposure of adult fish to potential EDCs followed by assessment of reproductive success and biological endpoints [17, 21].

Atrazine is the most extensively applied herbicide in the U. S. [22]. The triazine herbicide is used to control broadleaf and grass weeds. In 2000, it was applied to 84% (54 million acres) of corn acres at a rate of 1.14 kg/ha (1.01 lb/acre). Because it has widespread use and relatively long persistence in the environment [23], a high potential exists for aquatic organism exposure. Between 1992 and 1998, atrazine was detected in 85% of surface water samples collected year-round in urban and agricultural areas, and was measured at > 0.1 μg/L year-round in 34% of agricultural stream samples [24]. The maximum concentration of atrazine measured during the sampling period was 120 μg/L. The current drinking water standard for atrazine is 3 μg/L [25].

Based on acute toxicity data and predicted environmental exposure concentrations, Solomon et al. [23] concluded that atrazine does not pose a significant ecological risk to the aquatic environment even in high-use areas in the Midwestern U. S. However, Hayes et al. [26] reported that larval frogs (Xenopus laevis) exposed to atrazine at concentrations as low
as 0.1 μg/L developed into hermaphrodites (ovarian and testicular gonads). In the same study, frogs exposed to atrazine concentrations of 1 μg/L experienced a significant reduction in the size of the male larynx, an organ used to attract potential mates.

The objective of the present study was to evaluate atrazine as a potential EE in fish. The short-term fathead minnow reproduction assay developed by the U.S. EPA [21] as an endocrine disruptor screening tool was followed to integrate biomarkers of exposure with reproductive and developmental endpoints.

**METHODS**

*Experimental fish*

Fish used in the present study were adult (22 month old) fathead minnows (*Pimephales promelas*) hatched and reared in activated carbon filtered tap water in the laboratory at Iowa State University. The fish were third generation from an original stock obtained from a local bait vendor. At the time fish were allocated to treatment tanks, the mean weight ± standard error (SE) of male fish was 6.6 ± 0.2 g and mean weight ± SE of female fish was 3.1 ± 0.1 g. Preceding the experiment, the fish had no known chemical exposure or prior spawning activity.

*Experimental design and conduct*

In as much as possible, the experimental design followed that of a 21-d reproduction assay developed by the U.S. EPA [21] for screening of chemicals that exert reproductive
toxicity via the endocrine system. The major difference between the U.S. EPA protocol and that of the present study is that the U.S. EPA recommends flow-through exposures, while the present study was performed as a static-renewal assay. Briefly, the assay consisted of a 7-d acclimation period, a 21-d pre-exposure phase to establish the health and reproductive capacity of the test animals, and a 21-d exposure phase. The criterion for sufficient reproductive activity was that each experimental unit must produce a minimum of 12 eggs/female/day over the final 7 d of the pre-exposure period. The pre-exposure phase was conducted under the same conditions (feeding, temperature, photoperiod) as the exposure phase.

During the experiment, fish were fed live *Artemia* nauplii ad libitum twice daily. Photoperiod was 16:8 light:dark throughout the experiment. Water temperature was maintained at 25 ± 1 °C and dissolved oxygen was > 6.0 mg/L at all times (measured daily with a YSI Model 95 dissolved oxygen meter). Water samples were collected weekly from two replicates of each treatment for measurement of hardness (range: 156–180 mg/L as CaCO3), alkalinity (range: 29–44 mg/L), pH (range: 6.8–7.3), and total ammonia nitrogen (range: 0.2–1.48 μg/L).

Each experimental unit consisted of an aerated 39-L glass aquarium containing 18 L of water, four female fish, two male fish, and three halved 7.6 cm diameter PVC pipes (9 cm long) for spawning substrates. Experimental units were randomly assigned to one of five treatments (four replicates of each): control, solvent control (10 μL/L; 50:50 methanol:acetone), 5 μg/L atrazine, 50 μg/L atrazine, and 0.5 μg/L 17β-estradiol (E2). During the exposure period, fish were subjected to the chemicals via static exposure with
25% daily renewal. Atrazine (>98% pure; Chemservice) and E₂ (Sigma Chemical) stock solutions were prepared in a 50:50 v/v mixture of acetone and methanol so that each treatment received the same total volume (0.1 ml) of solvent daily.

**Chemical exposure validation**

Water samples were collected from aquaria (prior to 25% renewal) on days 1, 7, 14, and 21 of the exposure period. For atrazine analysis, water samples were extracted by solid phase extraction (SPE) and analyzed using gas chromatography with thermionic specific detection (GC-TSD) as previously reported [27]. Briefly, SPE columns (Envi-18, Supelco) were conditioned with ethyl acetate (3 ml), methanol (3 ml), and water (5 ml). Water samples (100 ml) from each aquaria (1000 ml for control aquaria) were extracted through SPE tubes (10 ml/min) and the tubes were allowed to air-dry for 10 minutes. Two 4-ml aliquots of ethyl acetate were then used to elute herbicides off the column. The extract was concentrated to 5 ml and analyzed by GC-TSD using a 30 m capillary column (DB5, Supelco). Extraction efficiency ± SE for this method was 94 ± 4% for atrazine, 89 ± 5% for deethylatrazine (DEA), 79 ± 6% for deisopropylatrazine (DIA). Reporting limits were 1.0 μg/L for treatment aquaria samples and 0.1 μg/L for control aquaria.

Waterborne E₂ was extracted from a 10-ml sample of aquarium water. SPE columns (Envi-18, Supelco) were preconditioned with 2.0 ml methanol followed by 4.0 ml distilled water. Flow rate of sample through the column did not exceed 5 ml/min. Following sample extraction, the column was rinsed with 6.0 ml distilled water then dried under vacuum for 10 min, wrapped in aluminum foil, and stored at −20 °C until elution and analysis.
For elution of E₂, SPE columns were thawed for 30 min at room temperature then placed under vacuum for 10 min to ensure dryness. Estradiol was eluted with 2 x 1.0 ml HPLC grade methanol and collected in 10 x 75 mm borosilicate glass culture tubes, which were then placed in a 45 °C waterbath. Methanol was evaporated under a gentle stream of nitrogen gas. The glass tubes were capped with parafilm and frozen at −20°C until analysis. At the time of analysis, samples were thawed for 1 h, resuspended in enzyme immunoassay (EIA) buffer, and assayed with an enzyme-linked immunosorbent assay (ELISA) kit (Oxford Biomedical Research) for quantitative analysis of E₂. Absorbance was measured at 450 nm with an automated microplate reader (THERMOMax, Molecular Devices). A standard curve (0.02 – 2.0 ng/ml) was prepared by dilution of the E2 standard (provided in the kit) in EIA buffer, and plotting absorbance vs. E₂ concentration on a semi-log scale. All samples were assayed in duplicate and absorbance was used to determine E₂ via the standard curve using microplate reader software (SOFTmax PRO, Molecular Devices). Limit of detection for E₂ was 1.0 ng/L in the 10.0 ml samples. Samples with a coefficient of variation greater than 10% were excluded from statistical analyses (n = 2). Mean ± SE recovery of E₂ from spiked samples was 70.2 ± 2.9% (n = 6) for distilled water samples spiked with 100 ng/L. Measured values were corrected for spike recovery.

Reproductive endpoints

Fish mortality, number of spawns, and number of eggs spawned (hereafter referred to as fecundity) were monitored daily during the pre- and post-exposure periods. Fecundity was monitored by removing the spawning substrates and counting all eggs adhered to the inside surface of the substrate. Each substrate with >25 eggs was placed on end in a 2000-ml
beaker in control water (25 ± 1 °C) with an air stone for gentle aeration of eggs and to help prevent fungus from becoming established. Incubation of eggs was used for assessment of fertilization success (number of eyed eggs by 48 hours postspawn), hatching success (percent of 50 eyed eggs that hatched by 120 hours postspawn), and larval fish survival (percent of hatched fish that survive to 48 hours posthatch).

On day 21 of the exposure period, coloration of male fish was evaluated in situ based on the presence or absence of light and dark vertical bands on the body surface and presence or absence of an ovipositor was noted for female fish. Following observation of secondary sexual characteristics, all fish were anaesthetized with tricaine methanesulfonate, weighed, measured, and bled. The caudal peduncle was severed with a scalpel and blood was collected with heparinized 50-μl micropipettes and aspirated into heparinized 250-μl microcentrifuge tubes. Fish sex was confirmed by gross examination of the gonads. Blood from same-sex fish in each aquarium was pooled into a single sample. Blood samples were kept on ice throughout processing and were centrifuged for 10 min at 6400 rpm (2000 x G). Plasma was decanted with a clean 100-μl micropipette and transferred to a 1.0 ml cyrovial for storage in liquid nitrogen until Vtg analysis. The gonads were removed, weighed (for GSI analysis), and fixed in 10% neutral buffered formalin until histological processing. Nuptial tubercles were counted under a dissecting microscope.

Plasma Vtg quantification

Plasma samples were thawed on ice for 2 h and plasma Vtg was quantified with a commercially available (Biosense) enzyme-linked immunosorbent assay (ELISA), following the protocol provided by the manufacturer, as previously described by Bringolf and
Summerfelt [13]. Intraassay coefficient of variation was 8.5% (N = 6), and interassay coefficient of variation was 15.1% (N = 4). Mean ± SE Vtg spike-recovery was 93.0% ± 6.8% (N = 4).

**Gonad histology**

Fixed gonads were dehydrated, embedded in paraffin, sectioned (5 μm) longitudinally (testis) or transversely (ovaries), and stained with hematoxylin and eosin (H &E). Sections of both gonads from each fish were mounted on glass slides, examined with no knowledge of the exposure group, and staged (0-3) as classified by Goodbred et al. [7]. One difference from the staging scale used by Goodbred et al. is that in the present study a “Stage 0” was included for males as well as females to indicate that the testes were undeveloped; i.e., they contained a thick germinal epithelium with no proliferation or maturation of spermatozoa. Fully mature gonads were designated as “Stage 3.”

**Statistical analyses**

As necessary, endpoint data were log-transformed to achieve homogeneity of variance. Differences among treatments were tested using analysis of variance followed by Tukey’s test for mean separation analysis, or Dunnett’s test for comparison of treatment means to control means. All statistical analyses were performed with SAS® 8.00 for Windows® (SAS Institute) and evaluated at the α = 0.05 level of significance.
RESULTS

Fish

At the end of the pre-exposure period, fecundity was below the predetermined threshold (12 eggs/female/day) in four of the 22 experimental units (aquaria). Fish survival was 100% during the chemical exposure period. Upon examination of gonads, it was noted that three fish were misidentified as females during the experiment. Mean fecundity values for each aquarium (eggs/female/day) were adjusted accordingly. There were no differences in length of males ($p = 0.548$) or females ($p = 0.140$), or weights of males ($p = 0.562$) or females ($p = 0.330$) at the end of the study.

Chemical exposure validation

Waterborne concentrations of atrazine on all four sample dates were 70–99% (mean = 86.8, SE = 1.6, n = 28) of nominal values and remained relatively constant during the exposure period (Table 1). Mean ± SE atrazine concentration was 4.32 ± 0.13 μg/L ($n = 12$) for the low treatment and 43.6 ± 1.0 μg/L ($n = 16$) for the high treatment. Concentrations of the atrazine metabolites, DIA and DEA, were below detection limit (1.0 μg/L) in the atrazine treatment samples. Atrazine was not detectable (< 0.1 μg/L) in water samples of control ($n = 4$) and solvent control ($n = 4$) treatments.

Measured water concentrations of E₂ ranged from 22–118% (mean = 59.7, SE = 6.8, n = 12) of nominal in E₂ treatments; mean ± SE concentration of E₂ was 298.6 ± 33.8 ng/L ($n = 12$) (Table 1). Levels of E₂ were below detection (1.0 ng/L) in water samples from control ($n = 4$) and solvent control ($n = 4$) treatments.
Table 1. Exposure water concentration (μg/L) of test chemicals during a 21-d fathead minnow reproduction assay.

<table>
<thead>
<tr>
<th>Treatment (nominal concentration)</th>
<th>Measured water concentration&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Day of exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Control (0)</td>
<td>&lt;MDL&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Solvent Control (0)</td>
<td>&lt;MDL</td>
<td></td>
</tr>
<tr>
<td>Estradiol (0.5)</td>
<td>0.219 (0.018)</td>
<td>0.386</td>
</tr>
<tr>
<td>Atrazine (5.0)</td>
<td>4.81 (0.03)</td>
<td>4.12</td>
</tr>
<tr>
<td>Atrazine (50.0)</td>
<td>44.61 (1.13)</td>
<td>41.11</td>
</tr>
</tbody>
</table>

<sup>a</sup> Water samples were collected at the end of the 24-h static exposure, prior to 25% renewal. Values are means (SE) of four replicates of each treatment except Estradiol and Atrazine (5.0), for each of which n = 3.

<sup>b</sup> MDL = minimum detection limit of atrazine assay (0.1 μg/L) and estradiol assay (1 ng/L).

**Reproductive endpoints**

Cumulative fecundity (total eggs spawned/treatment) was similar for all treatments prior to chemical exposure; however, upon initiation of chemical exposures, a rapid divergence in fecundity was evident in the E<sub>2</sub> treatment group (Fig. 1). No eggs were produced in the E<sub>2</sub> treatment group after the seventh day of the exposure period. Cumulative fecundity of fish
exposed to atrazine was slightly lower than controls, but the difference was not statistically significant. There was no significant difference between mean fecundity rate (eggs/female/day) of treatments and controls during the pre-exposure period, however, during the exposure period the E$_2$ treatment had significantly ($p < 0.05$) lower fecundity rate than the controls (Fig. 2A).

The decline in cumulative fecundity and fecundity rate of the fish exposed to E$_2$ was likely related to the reduced number of total spawns, although the number of eggs/spawn was not significantly different from the other treatments (Fig. 2B). The total number of spawns in the high atrazine was also lower, although it was not significantly different from controls (Fig. 2B). Fertilization success was reduced in the atrazine treatments, but the differences were not statistically different (Fig. 2C). There were no significant or obvious effects of atrazine exposure of adult fish on hatching success or larval fish survival to 48-h posthatch (Fig. 2C). Larvae were not evaluated beyond 48-h posthatch for possible effects of parental chemical exposure on growth, behavior, or development. Male and female GSI and gonad maturation were reduced by exposure to E$_2$ (Fig. 3A and 3B). To a lesser extent, atrazine exposure caused a decrease (not statistically significant) in male GSI. Gonads of male fish exposed to atrazine were rated less mature than controls, but not as immature as the gonads of fish exposed to E$_2$ (Fig. 4). Effects of atrazine on female GSI were not detectable in the low atrazine treatment but there was a reduction (non significant) in GSI in the high atrazine treatment. However, histopathological data did not reveal an effect of atrazine on gonad development, whereas the gonads of female fish exposed to E$_2$ were notably less mature than those of control fish. No mixed gonads (testis-ova) were identified in fish in any of the treatment groups.
Atrazine did not have an apparent effect on the secondary sexual characteristics of male fish, although the effects of E₂ exposure were evident. Exposure to atrazine did not reduce the number of breeding tubercles compared with the control fish (Fig. 5); however fish exposed to E₂ did not have breeding tubercles. It was not known if the fish exposed to E₂ had breeding tubercles that regressed in response to the E₂ exposure, or if the tubercles failed to develop. There were no obvious effects of atrazine on coloration of male fish, although all males exposed to E₂ lacked vertical banding patterns on their lateral body surfaces (data not shown).

Fig. 1. Cumulative fecundity (total eggs treatment) of fathead minnows during a pre-exposure period and exposure to estradiol (0.5 μg·L⁻¹) and atrazine (5.0 and 50.0 μg/L) in a short-term reproductive assay. Chemical exposures were initiated on day 0.
Fig. 2. Reproductive parameters of fathead minnows exposed to estradiol (0.5 μg/L) and atrazine (5.0 and 50.0 μg/L) in a short-term assay. Fecundity rate (A) and number of spawns and eggs/spawn (B) during the pre-exposure and exposure periods, and fertilization rate, hatch success, and survival (C) of embryos. Bars indicate standard error of the mean (n = 3 or 4). * Significant difference (p < 0.05) from controls.
Fig. 3. Effects of estradiol (0.5 μg/L) and atrazine (5.0 and 50.0 μg/L) on gonadosomatic index of female (A) and male (B) fathead minnows exposed to atrazine and estradiol in a 21-d reproductive assay. Bars indicate standard error of the mean (n = 3 or 4). * Denotes a significant difference (p < 0.05) from controls.

Plasma Vtg was significantly higher in male and female fish exposed to E2 than in all other treatments (Fig. 6A and 6B). There was also low-level induction of plasma Vtg in male fish exposed to both atrazine treatment concentrations, but similar concentrations of plasma Vtg were measured in fish of the solvent control treatment as well. The concentration (μg/ml) of plasma Vtg in atrazine-exposed and solvent-exposed male fish was 3 to 4 orders of magnitude lower than that of fish exposed to E2. In female fish, neither atrazine nor solvent affected plasma Vtg concentration.
Fig. 4. Development (Stage) of gonads of male (A) and female (B) fathead minnows after a 21-d exposure to estradiol (0.5 μg/L) and atrazine (5.0 and 50.0 μg/L). For males, n = 8, for females n = 12 to 14.

Fig. 5. Effects of estradiol (0.5 μg/L) and atrazine (5.0 and 50.0 μg/L) on number of nuptial tubercles on male fathead minnows after a 21-d exposure. Bars indicate standard error of the mean (n = 3 or 4). * Significant difference (p < 0.05) from controls.
Fig. 6. Plasma vitellogenin concentration of female (A) and male (B) fathead minnows after a short-term reproduction assay. Error bars indicate standard error of the mean (n = 3 or 4). Values that differ significantly from controls are noted as * (p < 0.05) and ***(p < 0.0001).
DISCUSSION

The exposure levels of atrazine used in the present study were chosen based on concentrations measured in the environment [24] during spring and early summer months, the time when fathead minnows begin to spawn in many parts of the country [28]. Concentrations of atrazine as high as 120 µg/L have been measured in the environment [24], and the half-life of atrazine in water has been reported to range from 1.7 d to 742 d, depending upon pH and concentration of humic acid (for a review see 23). For these reasons, we regard the 21 d exposures to the 5 and 50 µg/L atrazine concentrations used in the present study as environmentally relevant.

The positive control (E2) was chosen because of the implications that atrazine causes estrogenic effects in amphibians [26]. Effects of E2 were evident early in the exposure period, and in most endpoints examined. The rapid divergence of cumulative fecundity (Fig. 1), reduction of total number of spawns, as well as effects on male and female GSI, gonad maturity (histology), and male plasma Vtg, provided evidence that the fish reproduction assay is an effective screening method for EEs that affect processes influenced or controlled by steroid hormones. Ankley et al. [17] reported evidence that the assay is sensitive to androgenic compounds; however, the present study is the first to report that the assay is sensitive to compounds with estrogenic effects.

The present study provided no evidence of overt reproductive effects to fish from short-term (21 d) exposure to atrazine. There were no trends associated with atrazine for endpoints such as fecundity, percent hatch or larvae survival, or plasma concentrations of Vtg in male
and female fish. Atrazine did not visibly affect secondary sexual characteristics, including coloration of males and number of nuptial tubercles. Anecdotally, behavior was not notably different for male or female fish exposed to atrazine; males aggressively courted females and defended spawning substrates and eggs.

Though not statistically significant, there were dose-dependent trends associated with atrazine exposure and some endpoints, i.e., male GSI, testicular maturity, and percent of eggs fertilized. Also, female GSI was more variable and somewhat reduced in the high atrazine treatment, although female gonad histology results did not indicate an effect. In concurrence with the GSI results, gonads of male fish exposed to atrazine were less mature than those of control fish, although not as immature as gonads of fish exposed to estradiol. Effects of estrogenic compounds on gonad growth have been reported in other studies [29-32] and may likely be due to negative feedback of estrogens to the pituitary, resulting in suppression of gonadotrophin release and thus, reduced growth and maturation of the gonads [32-34]. The higher variability of some endpoints in the high atrazine exposure treatment may indicate that some individuals were susceptible to the atrazine exposure, while others were not.

Because plasma Vtg concentrations in atrazine-exposed fish were similar to those of fish in the solvent control group, the plasma Vtg induction cannot be attributed solely to atrazine exposure. Histopathological effects of high plasma Vtg concentrations in male fish have been reported [35], but the biological significance of low plasma Vtg concentrations, such as those measured in the atrazine-exposed male fish, is currently not known. In retrospect, the concentration of solvent used (10 μL/L) was relatively high and may have caused the unexpected induction of low concentrations of plasma Vtg; however this was the only endpoint that was significantly different between the solvent control and control groups. The
enigmatic plasma Vtg results, possible solvent-chemical interactions, and possible effects of solvent on the endocrine system, indicate that future studies should minimize or eliminate the use of solvents whenever possible.

Hayes et al. [26] reported a significant induction of testis-ova in frogs exposed to atrazine concentrations as low as 0.1 μg/L. The frogs were exposed as larvae, which may in part explain the difference to the results of the present study. Effects of atrazine exposure on larval fish development were not examined in this assay beyond measuring survival of prolarvae to onset of exogenous feeding, but larval fish were hatched and reared in control water, not exposed to waterborne atrazine. Fish are especially sensitive to exposure to endocrine-disrupting chemicals during a critical window of development prior to sexual differentiation [36,37], but also during other times of rapid sex-cell division, such as gonad recrudescence [34]. The goal of the present study was to use the short-term assay as a screen to determine if atrazine was detectable as an EE in adult fish; however the effects of atrazine on larval fish development, sexual differentiation, and eventual reproductive potential should be of high priority for future research.

In summary, it can be concluded that atrazine did not act as an endocrine-disrupting compound in a short-term fathead minnow reproduction assay and did not cause estrogenic effects in the adult fish. Although not statistically significant, there were dose-dependent trends in fish exposed to atrazine for endpoints such as % embryos fertilized, male GSI, and testicular maturity. The positive control, E2, induced significant differences in nearly all endpoints examined, providing evidence that the assay is sensitive for screening of compounds with estrogenic activity.
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REFERENCES


CONCLUSIONS

The study of the extent of environmental estrogens in the aquatic environment and their effects on fish has made substantial advances in the past decade, but there are still broad gaps in the basic science of this complex and controversial environmental issue. Investigation of the effectiveness of various processes used by wastewater treatment facilities to remove or inactivate endocrine active compounds has only just begun. The present study was the first to examine the change in estrogenic activity of wastewater passing through aerated lagoon systems. Investigation is warranted because of the sheer numbers of these small, rural community treatment systems and the variations in treatment facilities, practices, and raw wastewater components. I was able to demonstrate that wastewater entering aerated lagoon systems in Iowa was estrogenic to fish but with serial passage through the lagoons the estrogenic activity decreased to a level that was not detectable with a fish bioassay. The reduction in estrogenic activity of the water was strongly correlated with the retention time of the water in the lagoons.

The investigation of effects of wastewater on feral fish from aerated lagoons was the first of its kind in the U.S. and provided further evidence that there was not a substantial risk for release of estrogenic effluent from lagoon systems during the time of the study (October and November). The results of both the caged and feral fish studies indicate that effluents from aerated lagoon wastewater treatment facilities are low in estrogenic activity, but that raw wastewater was strongly estrogenic to fish. Thus, the potential exists for release of estrogenic effluents from these systems if the treatment is not complete. Aerated lagoon
systems are dependent upon biological treatment of wastes and the overall treatment efficiency of such systems declines with declining water temperatures. Based on these findings I recommend that future studies monitor the estrogenic activity of water in these systems throughout the year, especially during months when water temperatures are coldest.

The estradiol-vitellogenin dose-response study provided a reference point for the concentrations of estradiol necessary to induce the plasma vitellogenin in male fish exposed to lagoon water. The plasma vitellogenin dose-response curve from this study may be used in conjunction with other studies that use exposure of male fish to surface water or wastewater for a short period to estimate the magnitude of the estrogenic potency of the water in terms of “estradiol equivalents.” Future research should describe the time-course relationship of vitellogenin induction in fathead minnow exposed to environmental estrogens.

Although one previous study suggested that exposure to low concentrations of atrazine caused estrogenic effects in frogs, my study indicates that atrazine did not cause overt reproductive effects on fathead minnow at environmentally relevant exposure concentrations. However, there were decreasing trends observed for embryo fertilization, testes weight, and testes maturity, all of which may be related and warrant further investigation. Fish exposed to the positive control, estradiol, had significant changes in nearly all endpoints examined, demonstrating that the short-term fish reproduction assay was sensitive to estrogenic compounds. Future research should examine the effects of atrazine exposure over several generations of fish, with emphasis on effects of atrazine on larval fish from hatch through sexual differentiation.
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