Journal of the Iowa Academy of Science: JIAS

Volume 110 | Number 1-2 Article 6

2003

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Jour. Iowa Acad. Sci. 110(1,2):17–21, 2003

Occurrence of Ovotestes and Plasma Vitellogenin in Feral Male Fathead Minnows from Lagoons of Municipal Wastewater Treatment Facilities in Central Iowa

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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 reproductive abnormalities including ovotestes and elevated levels of plasma vitellogenin (Vtg), a plasma protein typically produced by egg-laying females. 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 routinely identified in the estrogenic fraction of wastewater and receiving waters (Desbrow et al. 1998, Spengler et al. 2001).

The morphological changes 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 plasma 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 potential for 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, Max-

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 samples, each a pooled sample of 3–5 fish.

Site	Lagoon	Total Fish Captured	Number (N) of Pooled Samples	Plasma Vtg (μg/ml) ± SE[b]
Carlisle	2	19	4	3.4 ± 2.9
	3	19	4	<MDL[c]
Granger	1	18	4	2,823 ± 1,393
	2	6	2	325.1 ± 65.0
Pleasantville	3	3	1	46.3
Union Slough Wildlife Refuge[a]		29	4	<MDL

[a]Reference site.
[b]Standard error.
[c]Below minimum detection limit (0.02 μg/ml)

well, 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 the 33 lagoons of the 11 aerated lagoon WWTFs (three lagoons per WWTF) and at the reference site. Each WWTF consisted of three serially connected 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 effluent 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 × 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 testes 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 gonads from each phenotypic male 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 5 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 Vtg. 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 ± SE Vtg spike-recovery was 93.0% ± 6.7% (N = 4). Minimum detection limit (MDL) for plasma Vtg was 0.02 μg/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.

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.

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.

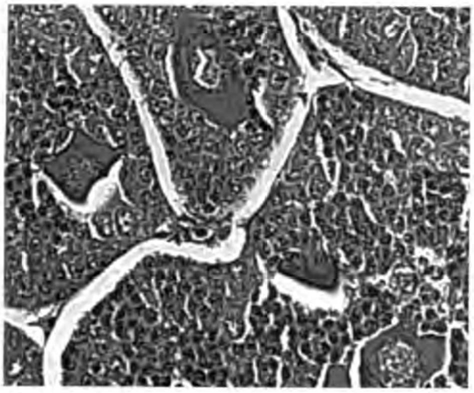

Fig. 1. Transverse section of a testis of a feral fathead minnow captured in an aerated wastewater treatment lagoon, showing several primary oocytes (arrows) embedded in seminiferous tubules that otherwise are filled with spermatocytes. Hematoxylin and eosin staining, ×40.

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 further insight into the estrogenic activity of wastewater in aerated lagoon WWTFs in Iowa. Nichols et al. (1999) used caged fathead minnows to investigate the estrogenicity of effluent from small WWTFs in the U.S., and Bringolf and Summerfelt (2003) exposed caged fathead minnows to aerated lagoon wastewater, but the present study is the first to examine feral fish captured from treatment lagoons for biomarkers of exposure to environmental estrogens.

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 estrogenic compounds and that Vtg concentrations were generally dependent upon the stage of wastewater treatment, e.g., Vtg concentrations were highest in lagoon 1 (receives raw wastewater) 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 to12 days (Table 2). Plasma 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 and/or timing of exposure.

The range of concentrations of plasma Vtg in male feral fathead minnows in the present study is comparable to that of other feral male cyprinids exposed to estrogenic compounds. Folmar et al. (1996) reported that plasma Vtg concentrations in feral male common carp captured near a metropolitan WWTF effluent in Minnesota were 0 to 10,000 μg/ml (Folmar et al. 1996). Concentrations of plasma Vtg in feral roach exposed to wastewater in the UK were generally lower than the present study but significantly higher than control fish, and ranged from approximately 0.3 to 2 μg/ml (Jobling et al. 1998). The highest plasma Vtg concentrations measured in male fish in the present study are in the range commonly measured in gravid female fish (Le Guellec et al. 1988). Although the induction of plasma Vtg is well documented in estrogen-exposed male fish, only recently have studies examined the histopathological effects of Vtg in males (Folmar et al. 2001) and the relationship of Vtg 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).

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." 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 (Arme 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

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 plasma Vtg of feral male fathead minnows captured in WWTF lagoons.

	Caged[a]		Feral	
Treatment	Number of Sites (N of fish)	Mean Plasma Vtg ± SE[b] (μg/ml)	Number of Pooled Samples (N of fish)	Mean Plasma Vtg ± SE (μg/ml)
Lagoon 1	9 (83)	1,702 ± 670	4 (18)	2,823 ± 1,393
Lagoon 2	10 (89)	0.94 ± 0.36	6 (25)	110.1 ± 70.0
Lagoon 3	8 (76)	0.06 ± 0.02	5 (22)	9.3 ± 9.3
Control	5 (25)	0.04 ± 0.02	4 (29)	<MDL[c]

[a]Bringolf and Summer felt (2003)
[b]Standard error
[c]Below minimum detection limit (0.02 μg/ml)

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 (Jobling et al. 1998). This evaluation technique is particularly useful when all fish are 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 reason(s) that fish are found in some lagoons and not others is unknown, but may be related to water quality parameters such as dissolved oxygen or ammonia concentrations. It is also 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 presence of these fish in partially treated wastewater suggests that the lagoon treatment process is effective, because the water quality 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.

ACKNOWLEDGMENTS

This is Journal Paper No. J-19859 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa, Project No. 3480, and supported by Hatch Act and State of Iowa funds. This project was funded in part by a grant from the University of Iowa Center for Health Effects of Environmental Contamination (CHEEC) and the Department of Animal Ecology, Iowa State University. We thank the municipal WWTF operators, and the staff at Union Slough National Wildlife Refuge for their cooperation with this project. Gary Atchison reviewed an earlier version of this manuscript.

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