

UC Irvine

UC Irvine Previously Published Works

Title

I. Effects of a dopamine receptor antagonist on fathead minnow, *Pimephales promelas*, reproduction

Permalink

<https://escholarship.org/uc/item/4s79p5dz>

Journal

Ecotoxicology and Environmental Safety, 73(4)

ISSN

0147-6513

Authors

Villeneuve, Daniel L
Garcia-Reyero, Natàlia
Martinović, Dalma
[et al.](#)

Publication Date

2010-05-01

DOI

10.1016/j.ecoenv.2009.09.007

Copyright Information

This work is made available under the terms of a Creative Commons Attribution License, available at <https://creativecommons.org/licenses/by/4.0/>

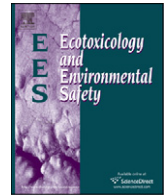
Peer reviewed



ELSEVIER

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Highlighted Article

I. Effects of a dopamine receptor antagonist on fathead minnow, *Pimephales promelas*, reproduction

Daniel L. Villeneuve^{a,*}, Natàlia Garcia-Reyero^{b,c}, Dalma Martinović^d, Nathaniel D. Mueller^a, Jenna E. Cavallin^a, Elizabeth J. Durhan^a, Elizabeth A. Makynen^a, Kathleen M. Jensen^a, Michael D. Kahl^a, Lindsey S. Blake^a, Edward J. Perkins^b, Gerald T. Ankley^a

^a US Environmental Protection Agency, Office of Research and Development, National Health and Environmental Effects Research Laboratory, Mid-Continent Ecology Division, 6201 Congdon Boulevard, Duluth, MN 55804, USA

^b US Army, Engineer Research and Development Center, Environmental Laboratory, 3909 Halls Ferry Road, Vicksburg, MS 39108, USA

^c Jackson State University, Department of Chemistry, Jackson, MS 39217, USA

^d University of St. Thomas, Department of Biology, St. Paul, MN 55105, USA

ARTICLE INFO

Article history:

Received 8 May 2009

Received in revised form

3 September 2009

Accepted 4 September 2009

Available online 26 September 2009

Keywords:

Endocrine disruption

Neurotransmitter

Antipsychotic

Neuroendocrine

Gonadotropin-releasing hormone

Fish

Fecundity

Secondary sex characteristics

ABSTRACT

Neurotransmitters such as dopamine play an important role in regulating fish reproduction. However, the potential for neuroendocrine active chemicals to disrupt fish reproduction has not been well studied, despite emerging evidence of their discharge into aquatic environments. This study is the first to apply the fathead minnow 21 d reproduction assay developed for the US Endocrine Disruptor Screening Program to evaluate the reproductive toxicity of a model neuroendocrine active chemical, the dopamine 2 receptor antagonist, haloperidol. Continuous exposure to up to 20 µg haloperidol/L had no significant effects on fathead minnow fecundity, secondary sex characteristics, gonad histology, or plasma steroid and vitellogenin concentrations. The only significant effect observed was an increase in gonadotropin-releasing hormone (cGnRH) transcripts in the male brain. Results suggest that non-lethal concentrations of haloperidol do not directly impair fish reproduction. Potential effects of haloperidol on reproductive behaviors and gene expression were examined in a companion study.

Published by Elsevier Inc.

1. Introduction

Reproduction in fish and other vertebrates is predominantly regulated by the hypothalamic–pituitary–gonadal (HPG) axis and a variety of environmental and social cues. Endocrine active chemicals (EACs) that disrupt the normal mechanisms of HPG axis function, or interfere with response to appropriate reproductive cues, have the potential to adversely impact reproductive success. If the impacts are sufficiently widespread, reproductive impairments in individuals can have significant population-level consequences (e.g., Kidd et al., 2007; Miller and Ankley, 2004). Thus, there is a need to characterize the diversity of mechanisms through which xenobiotics might disrupt vertebrate reproduction and develop appropriate techniques to screen chemicals for such activity (e.g., high throughput *in vitro* bioassays, quantitative structure–activity relationships) and/or detect their influence in the field (e.g., diagnostic biomarkers).

As part of an on-going research program aimed at systematic characterization of various modes of endocrine disruption using small fish (Ankley et al., 2009), we used a generalized model of the teleost HPG axis (Villeneuve et al., 2007b) to identify likely molecular targets through which xenobiotics could interact with the axis and potentially impair reproduction. This included some relatively well-studied targets such as the estrogen receptor, androgen receptor, and various enzymes involved in steroid biosynthesis. Fathead minnow (*Pimephales promelas*) reproduction assays showed that chemicals that interact with these targets reduce the fecundity of exposed individuals and produce a variety of molecular changes consistent with effects on HPG axis function (e.g., Ankley et al., 2002, 2003, 2005, 2007; Lange et al., 2001; Martinović et al., 2008). Other HPG axis components, such as the gonadotropin-releasing hormone (GnRH) receptors and gonadotropin (GtH) receptors, play key roles in regulating reproduction but were considered unlikely targets for direct modulation by xenobiotics since they bind peptide hormones, which do not have structural analogues that are environmental contaminants. In contrast, various neurotransmitter receptors (e.g., dopamine receptors, gamma amino butyric acid [GABA] receptors) bind

* Corresponding author.

E-mail address: villeneuve.dan@epa.gov (D.L. Villeneuve).

small organic ligands and also play important roles in regulating the reproductive axis (reviewed by Trudeau, 1997; Trudeau et al., 2000). However, potential adverse effects of neurotransmitter receptor agonists/antagonists in the environment on fish reproduction have not been widely studied from an ecotoxicological perspective.

In numerous teleost fish, as well as other vertebrates including certain amphibians, birds, mammals, and even some invertebrates, dopamine appears to exert direct inhibitory control of GtH release from the pituitary (reviewed by Dufour et al., 2005). In particular, dopaminergic regulation of GtH release in goldfish (*Carassius auratus*) and common carp (*Cyprinus carpio*) is well documented (Chang and Peter, 1983; Lin et al., 1988; Yu and Peter, 1990; Dufour et al., 2005), suggesting that the mechanism is likely conserved in closely related cyprinid species like the fathead minnow and zebrafish. *In vitro* experiments have suggested that the inhibitory effect of dopamine on luteinizing hormone (LH) release is primarily mediated through activation of a dopamine 2 receptor (D2R) in the pituitary (Vacher et al., 2000), although dopamine 1 receptor (D1R) activation in the pre-optic area of the brain may also play a role through the modulation of GnRH release (reviewed in Trudeau, 1997). A previous study in grey mullet (*Mugil cephalus*), an annual-spawning euryhaline marine teleost, demonstrated enhanced ovulation and spawning in fish treated with the dopamine antagonist domperidone (Aizen et al., 2005). However, fathead minnows are a repeat spawning species with asynchronous follicle development, so we postulated that any alteration in neuroendocrine regulation in this species could result in a hormonal imbalance that would disrupt some phase of the asynchronous ovarian development and/or spawning. Thus, the goal of the present study was to test the hypothesis that exposure to the D2R antagonist, haloperidol, would reduce cumulative fecundity and alter reproductive endpoints in fathead minnows exposed over a 21 d period.

Haloperidol (Drug bank id DB00502; www.drugbank.ca) is a butyrophenone antipsychotic drug used in humans for the treatment of schizophrenia. Like most traditional antipsychotics, haloperidol binds to D2R with greater affinity than dopamine and exerts its effects through competitive blockade of postsynaptic D2R (Seeman, 2002; www.drugbank.ca). Although we are not aware of any measured concentrations of haloperidol in surface waters, it has been included among candidate priority lists of potential human pharmaceuticals in surface waters (Besse and Garric, 2008). For our purposes, it was selected for these studies as a model chemical for examining whether similar pharmaceuticals or other environmental contaminants that antagonize the D2R may have relevance as reproductive toxicants in fish. Relatively recent evidence suggests that pulp mill effluents, for example, contain chemicals that can interact directly with the D2R (Basu et al., 2009).

2. Materials and methods

2.1. Chemical and test organisms

Haloperidol was purchased from Sigma Aldrich (St. Louis, MO, USA; H1512; 99% pure). The reproductively mature adult fathead minnows (6 months old) used in these experiments were obtained from an on-site culture facility at the US EPA Mid-Continent Ecology Division (Duluth, MN, USA). Fish were held at 25 °C under a 16:8 light:dark photoperiod and fed to satiation twice daily with frozen brine shrimp (*Artemia*). All animals were treated humanely with regard for alleviation of suffering, and all laboratory procedures involving animals were reviewed and approved by an Animal Care and Use Committee in accordance with Animal Welfare Act and Interagency Research Animal Committee guidelines.

2.2. Chemical delivery and exposure verification

Solvent-free, saturated aqueous stock solutions of haloperidol were prepared in UV-treated, filtered, Lake Superior water through continuous stirring in a 20 L glass carboy. Stock solution was diluted in Lake Superior water and delivered to 20 L tanks containing 10 L of water, at a continuous flow of 44 ± 1.5 ml/min (mean \pm SD) to achieve the appropriate nominal test concentrations. Water samples were collected from the stock solution and exposure tanks over the course of each experiment. Haloperidol concentrations in the water samples were directly analyzed using an Agilent (Wilmington, DE, USA) model 1100 LC-MSD equipped with an electrospray interface (API-ES). An aliquot of sample (30 μ l) was injected onto a Luna (Phenomenex, Torrance, CA, USA) CN column (2.0 \times 50 mm) and eluted isocratically at a flow rate of 0.3 ml/min. The mobile phase consisted 66% acetonitrile, 10 mM ammonium acetate buffer and 0.04% acetic acid. Haloperidol concentrations were determined using mass 376 (SIM positive ion mode) with an external standard method of quantitation. Routine quality assurance analyses (i.e., procedural blanks, spiked matrix, and duplicate samples) were conducted with each day's sample set. Spike recoveries averaged (\pm SD) $102 \pm 3\%$ and $96 \pm 6\%$ for the range finder and reproduction assay, respectively. Average (\pm SD) agreement among duplicate analyses of the same sample was $97.6 \pm 3.1\%$ ($n=24$). The method detection limit was 0.010 μ g haloperidol/L.

2.3. Range-finding experiment

A range-finding experiment was conducted to aid the identification of non-lethal exposure concentrations for use in the reproduction assay. The nominal (target) concentrations for the range-finding experiment were 0, 0.2, 2, 20, and 200 μ g haloperidol/L. There was one tank per test concentration and each tank contained three male and three female fathead minnows along with three breeding substrates (10 cm section of PVC pipe cut in half (Ankley et al., 2001)). The fish were exposed for 5 d, during which survival and spawning activity were monitored daily. Haloperidol concentrations in the exposure tanks were measured on days 1, 3, and 5 of the experiment. At the end of the experiment, surviving fish were humanely terminated in a buffered solution of tricaine methanesulfonate (MS-222; Finquel; Argent, Redmond, WA, USA).

2.4. Fathead minnow 21 d reproduction assay

The basic experimental design for the fathead minnow 21 d reproduction assay was described by Ankley et al. (2001) except a paired, rather than group spawning approach was used (e.g., Ankley et al., 2005). Fathead minnows were paired (one male, one female) and placed in tanks at a density of two pairs per tank. Pairs were separated by a water permeable mesh divider and each had its own breeding substrate. The fish were held in the test system, receiving Lake Superior (control) water only, for a 14 d acclimation period during which the fecundity and fertility of each pair were assessed daily. After 14 d, exposures were initiated with pairs that had spawned successfully during acclimation. Five exposure concentrations (0, 0.02, 0.2, 2, and 20 μ g haloperidol/L, nominal) were tested. There were four replicate tanks per treatment and two pairs per tank for a total of eight pairs per treatment.

Over the course of the exposure, the total number of eggs spawned and the number of fertile eggs produced by each pair were recorded daily. Haloperidol concentrations in the test tanks were quantified approximately every 3 d over the course of the exposure (7/21 d total). Water quality characteristics (mean \pm SD) monitored regularly over the course of the study included temperature 25.3 ± 1.0 °C, dissolved oxygen 6.02 ± 0.57 mg/L, pH 7.39 ± 0.08 , hardness 45.6 ± 0.5 mg/L as CaCO₃, and alkalinity 47.4 ± 0.4 mg/L as CaCO₃. After 21 d of exposure, surviving fish were anesthetized in buffered MS-222. Immediately after anesthetization, blood was collected from the caudal vasculature using heparinized microhematocrit tubes. Plasma was separated by centrifugation and stored at -80 °C until extracted and analyzed. Following blood sampling, body mass (sans blood) was measured, secondary sex characteristics (i.e., nuptial tubercles) were evaluated, and gonads, dorsal fatpads (males only), brains, and pituitary glands were removed. Tubercles were scored based on their number and relative size (Jensen et al., 2001; US EPA, 2002). Gonad samples were weighed and divided into several pieces. A subsample (approximately 10 mg) was used for an *ex vivo* steroid production assay. The remaining gonad mass was divided into approximately equal thirds (females) or halves (males) with one portion preserved in Davidson's fixative for later histological characterization, one portion preserved in RNAlater (Sigma R0901), and one portion (ovaries only) snap frozen in liquid nitrogen. Dorsal fatpads were weighed and discarded. Brains and pituitaries, collected separately using fine forceps, were each transferred to pre-weighed tubes containing RNAlater. Samples in RNAlater were stored at -20 °C until extracted. Snap-frozen samples were stored at -80 °C. All dissection tools were washed with RNaseZap (Ambion, Austin, TX, USA) between samples to prevent cross-contamination or degradation by RNases.

Gonads preserved for histological analysis were embedded, stained, sectioned and analyzed as described previously (Villeneuve et al., 2008). *Ex vivo* steroid

production assays were conducted using methods adapted from (McMaster et al., 1995) as described previously (Martinović et al., 2008). Briefly, subsamples of gonad tissue from each fish were transferred to wells of a 48-well plate microplate (Falcon 35-3078, Beckton Dickinson, Franklin Lakes, NJ, USA) containing 500 μ l of Medium 199 (M2520; Sigma) supplemented with 0.1 mM isobutylmethylxanthine (IBMX; Sigma I7018) and 1 μ g 25-hydroxycholesterol (Sigma)/ml on ice. Samples were incubated at 25 °C overnight (16.5 h), after which media from each well was transferred to a microcentrifuge tube and stored at –20 °C until extracted and analyzed. The mass of each gonad subsample was weighed after collection of the media. Average (\pm SD) samples masses were 8.7 \pm 6.8 mg for testes and 28.8 \pm 13.6 mg for ovaries. Wells containing supplemented medium but no tissue were incubated, sampled, and analyzed along with experimental samples to serve as assay blanks. Steroids were extracted from medium samples (*ex vivo*) or plasma samples by liquid–liquid extraction with diethyl ether and quantified by radioimmunoassay (Jensen et al., 2001; US EPA, 2002). With the exception of cases where plasma volumes were limiting, both plasma estradiol and plasma testosterone concentrations were quantified. Plasma concentrations of the estrogen-inducible egg yolk precursor protein, vitellogenin (Vtg), were quantified by enzyme-linked immunosorbent assay using a polyclonal antibody to fathead minnow Vtg and purified fathead minnow Vtg as a standard (US EPA, 2002).

Relative abundance of selected mRNA transcripts in ovary, brain, or pituitary samples from the reproduction assay was quantified using quantitative real-time PCR (QPCR). Gonad or brain samples in RNAlater were transferred to TRI Reagent[®] (Sigma) and total RNA was extracted from the tissue according to the manufacturer's protocol. Total RNA was extracted from individual pituitary samples using RNeasy micro kits (Qiagen, Valencia, CA, USA). RNA concentration and quality was evaluated using a Nanodrop[®] ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). The 260/280 nm absorbance ratio for all samples was between 1.7 and 2.2. Total RNA samples were diluted to either 10 ng/ μ l (brains, gonads) or 1 ng/ μ l (pituitaries) for use in QPCR assays. Relative abundance of mRNA transcripts coding for fathead minnow follicle-stimulating hormone β subunit (FSH β), luteinizing hormone β subunit (LH β), and follicle-stimulating hormone receptor (FSHR) was determined using primers, probes, and protocols described previously (Villeneuve et al., 2007a, 2007b). Partial cDNA sequences for fathead minnow GnRH (c, chicken-type isoform; *gnrh2*) and D2R were determined using methods similar to those described elsewhere (Villeneuve et al., 2007a, 2007b). Gene-specific primers and probes corresponding to the partial sequences were developed (Table 1) and applied, using the previously described QPCR protocol (Villeneuve et al., 2007a), to measure the relative abundance of cGnRH and D2R transcripts.

Data from the reproduction assay were tested for normality and homogeneity of variance. When data conformed to parametric assumptions, one-way analysis of variance (ANOVA) was used to test for differences across treatments and a post-hoc Duncan's test was applied to determine which groups differed ($p < 0.05$). When data did not conform to parametric assumptions, they were either transformed (e.g., log 10) or analyzed using a non-parametric Kruskal–Wallace test ($p < 0.05$). *Ex vivo* steroid production data were analyzed by general linear models ANOVA using both haloperidol concentration and the mass of each gonad subsample as independent variables. Pair was treated as the unit of replication for fecundity and fertility. However, statistical conclusions were the same whether pair or tank was viewed as the unit of replication. Individuals were considered as the unit of replication for all other endpoints, as no tank effects were evident. All statistical analyses were conducted using SAS 9.0 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Toxicity

In the range-finding experiment, 200 μ g haloperidol/L was lethal to six of six adult fathead minnows within 3 d. Exposure to

Table 1
GenBank accession numbers for partial fathead minnow gonadotropin-releasing hormone (c, chicken-type isoform; cGnRH) and dopamine 2 receptor (D2R) cDNA sequences and corresponding primers and probes used for real-time PCR assays.

Gene/protein	cGnRH	D2R
Accession no.	EF693733	EF693735
Fw primer ^a	TTGCTGTGTTTAAAGTGCCAGTT	GACGGGTTCGCTCTGATGATC
Probe ^b	AGCTCTCAGCACTGGT	CCATCGTCTGTTTC
Rv primer ^a	TCCAGGGTACCAAGCATGA	GGCAGGAATGGCAAATGAC

^a Sequences 5' \rightarrow 3'; Fw=forward primer, Rv=reverse primer.

^b Dual-labeled DNA probe with 5'-FAM[™] (6-carboxyl-fluorescein), 3'-Black Hole Quencher-1[®] (Integrated DNA technologies, Coralville, IA, USA).

20 μ g haloperidol/L or less was not lethal over the 5 d range-finding experiment. Based on these results, 20 μ g/L was identified as the maximum target test concentration for the reproduction assay.

3.2. Fathead minnow 21 d reproduction assay

Average (\pm SD) haloperidol concentrations measured in water samples collected from the reproduction assay test tanks were 0.017 \pm 0.003, 0.204 \pm 0.019, 2.27 \pm 0.27, and 22.0 \pm 2.10 μ g/L for the 0.02, 0.2, 2.0, and 20 μ g/L nominal treatment groups, respectively. No haloperidol was detected in the control tanks, or in Lake Superior water blanks. There were no significant differences in measured concentrations between replicate tanks within a treatment.

One female from the control group died on the final day of exposure prior to sampling, but there were no mortalities in any of the haloperidol treatments. Cumulative fecundity was greatest for control fish and lowest for fish exposed to 2.0 μ g haloperidol/L, but there were no statistically significant differences among treatments (Fig. 1), nor were there significant differences in the fertility of spawned eggs (data not shown). Similarly, there were no significant treatment-related differences in fish weight, gonad weight, gonadal somatic index [gonad wt. \times body wt.⁻¹ \times 100%], or male tubercle scores, dorsal pad weight, or dorsal pad index [dorsal pad wt. \times body wt.⁻¹ \times 100%] (Table 2). Plasma estradiol, testosterone, and vitellogenin concentrations were not

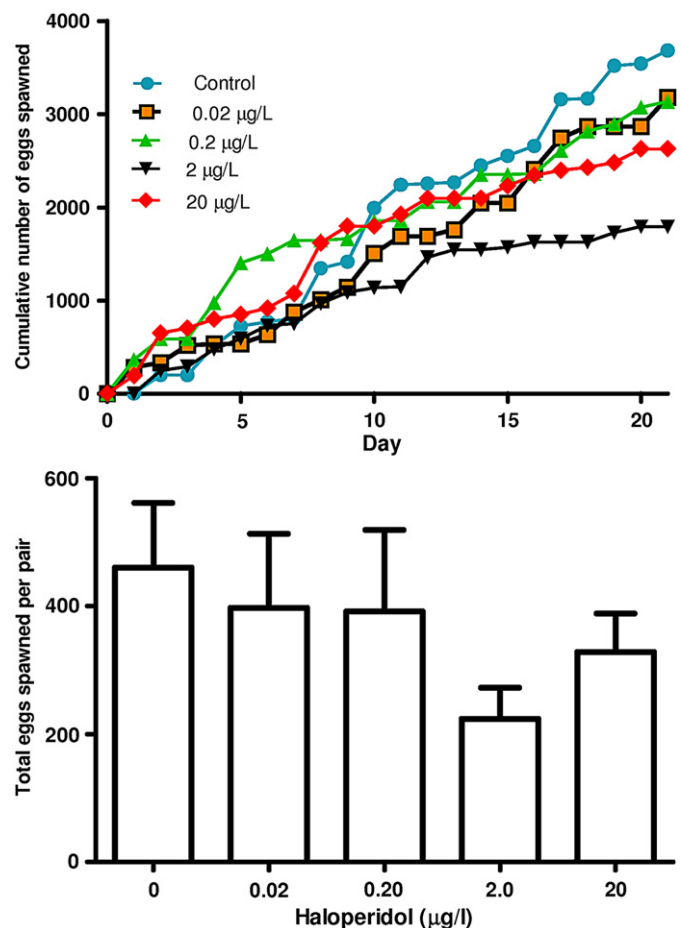


Fig. 1. Effects of haloperidol on fathead minnow fecundity in a 21 d test. Upper panel shows total cumulative fecundity per treatment group as a function of time. Lower panel shows mean (\pm SE) of the total number of eggs spawned per pair ($n=8$ pairs per treatment) over the 21 d exposure.

Table 2

Mean (\pm SD; $n=8$ except control females where $n=7$), fish mass, gonad mass, gonadal somatic index (GSI), dorsal pad mass^a, dorsal pad index (DPI)^a, and tubercle scores^a for male and female fathead minnows exposed to 0, 0.02, 0.2, 2.0, or 20 μg haloperidol/L for 21 d.

Endpoint	Sex	Haloperidol concentration (nominal; $\mu\text{g}/\text{L}$)				
		0	0.02	0.2	2.0	20
Mass (g) ^b	M	3.90 \pm 0.65	3.95 \pm 0.82	4.05 \pm 0.82	3.28 \pm 0.76	3.46 \pm 0.67
	F	1.52 \pm 0.24	1.48 \pm 0.24	1.57 \pm 0.24	1.43 \pm 0.27	1.51 \pm 0.25
Gonad mass (mg)	M	48 \pm 13	45 \pm 15	57 \pm 24	58 \pm 44	37 \pm 14
	F	183 \pm 52	173 \pm 101	192 \pm 58	161 \pm 75	189 \pm 63
GSI (%)	M	1.25 \pm 0.26	1.12 \pm 0.31	1.41 \pm 0.60	1.82 \pm 1.61	1.02 \pm 0.24
	F	12.1 \pm 2.5	11.2 \pm 4.3	12.5 \pm 3.7	10.9 \pm 3.2	12.3 \pm 3.1
Dorsal pad mass (mg)	M	140 \pm 71	163 \pm 69	114 \pm 32	78 \pm 36	122 \pm 73
DPI (%)	M	3.6 \pm 1.5	4.1 \pm 1.4	2.8 \pm 0.7	2.6 \pm 1.6	3.6 \pm 2.2
Tubercle score	M	33.1 \pm 6.4	29.6 \pm 5.6	31.9 \pm 6.2	34.0 \pm 5.1	32.7 \pm 8.5

^a Males only.

^b Whole body wet weight measured immediately following blood sampling.

Table 3

Mean (\pm SD; n) *ex vivo* steroid production and mean (\pm SD; n) concentrations of estradiol (E2), testosterone (T), and vitellogenin (Vtg) measured in the plasma of male and female fathead minnows exposed to 0, 0.02, 0.2, 2.0, or 20 μg haloperidol/L for 21 d.

Endpoint	Sex	Haloperidol concentration (nominal; $\mu\text{g}/\text{L}$)				
		0	0.02	0.2	2.0	20
<i>Ex vivo</i> T (ng/ml) ^a	M	2.61 \pm 1.24; 8	2.24 \pm 0.90; 8	2.28 \pm 1.24; 8	1.97 \pm 1.56; 8	2.75 \pm 1.03; 8
	F	0.47 \pm 0.39; 7	0.23 \pm 0.34; 8	0.23 \pm 0.21; 8	0.37 \pm 0.35; 8	0.39 \pm 0.35; 8
<i>Ex vivo</i> E2 (ng/ml) ^a	M	< 0.05 ^b ; 8	< 0.05; 8	< 0.05; 8	< 0.05; 8	< 0.05; 8
	F	2.55 \pm 0.69; 7	2.63 \pm 0.99; 8	2.03 \pm 0.35; 8	2.20 \pm 0.61; 8	1.82 \pm 0.74; 8
Plasma T (ng/ml) ^c	M	13.8 \pm 2.8; 8	11.1 \pm 5.1; 8	12.7 \pm 4.2; 8	14.1 \pm 6.6; 8	16.2 \pm 4.4; 8
	F	4.03 \pm 3.60; 5	4.63 \pm 3.62; 7	4.33 \pm 2.95; 6	7.20 \pm 4.75; 6	6.98 \pm 3.88; 6
Plasma E2 (ng/ml) ^c	M	0.43 \pm 0.28; 8	0.43 \pm 0.33; 8	0.36 \pm 0.30; 8	0.43 \pm 0.23; 8	0.37 \pm 0.37; 8
	F	6.99 \pm 3.55; 7	10.0 \pm 3.22; 7	9.63 \pm 3.61; 8	7.73 \pm 4.80; 7	9.58 \pm 3.83; 7
Plasma Vtg ($\mu\text{g}/\text{ml}$) ^d (mg/ml) ^d	M	1.58 \pm 1.30; 8	1.38 \pm 1.15; 8	1.66 \pm 1.54; 8	2.63 \pm 3.32; 7	3.15 \pm 3.76; 8
	F	18.0 \pm 9.54; 7	13.5 \pm 6.55; 8	14.0 \pm 5.60; 8	16.9 \pm 12.2; 8	17.5 \pm 9.00; 8

^a ng/ml of incubation medium, following 16.5 h of incubation.

^b Method detection limit for the E2 radioimmunoassay was 0.05 ng/ml. Testes samples generally did not produce detectable E2 concentrations over a 16.5 h incubation period.

^c ng/ml plasma.

^d Note, male and female Vtg concentrations are presented using different units as indicated.

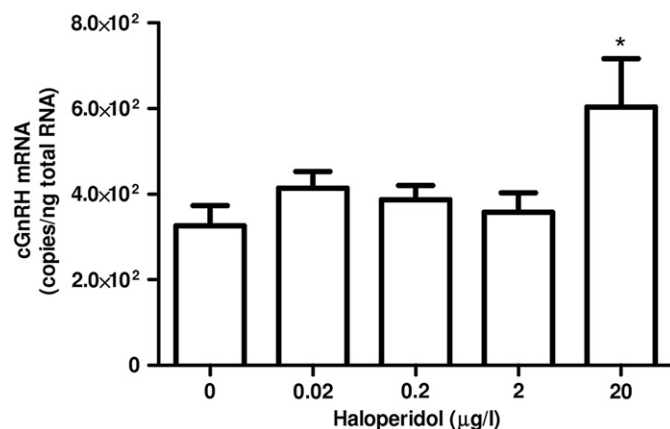


Fig. 2. Mean (\pm SE) abundance of mRNA transcripts coding for the c isoform of gonadotropin-releasing hormone (cGnRH) measured in the brain tissue of male fathead minnows exposed to haloperidol for 21 d ($n=6-8$ per treatment group). Asterisks indicate statistically significant difference from control ($p < 0.05$).

significantly affected by haloperidol treatment in either males or females, nor did *ex vivo* steroid production vary significantly with treatment (Table 3). Among the five gene expression endpoints examined, cGnRH transcript abundance in male brain was the only variable significantly affected by haloperidol exposure (Fig. 2; Supplementary Figs. S.1 and S.2). Expression of cGnRH mRNA in

the brain of male fish exposed to 20 μg haloperidol/L was increased compared to controls (Fig. 2).

Based on incidence and severity, there were no substantial differences in histological findings between exposed males or females and corresponding controls. Fungal elements surrounded by well-circumscribed clusters of macrophages, but no signs of other inflammatory cells or tissue damage, were detected in testes of three males from the 0.02 and 2.0 μg haloperidol/L groups. Additionally, unusual intermingling of yolk granules with cortical alveoli in mature vitellogenic oocytes was noted in the ovaries of three females from 0, 0.2, and 2.0 $\mu\text{g}/\text{L}$ groups. However, none of these unusual observations were considered exposure-related. Histologically determined gonad stage (Villeneuve et al., 2007c) was not significantly affected by haloperidol exposure.

4. Discussion

4.1. Reproductive endpoints

This study is the first to apply a fathead minnow assay developed by the US Environmental Protection Agency for its Endocrine Disruptor Screening Program (<http://www.epa.gov/endo/>; Ankley et al., 2001) to assess the potential reproductive toxicity of a chemical known to interact directly with a neurotransmitter receptor. Results of this study did not support the hypothesis that exposure to haloperidol would affect the

fecundity of fathead minnows exposed over a 21 d period. The average productivity of fish exposed to 2.0 µg/L was less than that of other groups, but the difference was not statistically significant, nor was there a clear concentration-dependent trend. Similarly, there was no evidence that exposure to a D2R antagonist enhanced spawning activity as had been reported for grey mullet (Aizen et al., 2005). The general lack of significant effects on reproductive parameters including secondary sex characteristics, gonad histology, and plasma steroid and Vtg concentrations was consistent with the lack of effect on fecundity or fertility.

4.2. Gene expression

Supervised analysis of the effects of haloperidol exposure on the abundance of mRNA transcripts coding for five different proteins was conducted by QPCR. Examination of D2R transcripts in brain did not provide any evidence to suggest that receptor expression had increased to offset the antagonistic effects of the chemical. Similarly, the lack of significant effects on the expression of GtH β subunits in the pituitary and FSHR in the testes did not provide any indication of dramatic shifts in GtH production or signaling, although we note that the regulatory effect of dopamine is thought to be at the level of GtH release, rather than transcription (Trudeau, 1997). Among the gene expression endpoints examined in the reproduction assay, the only suggestion that haloperidol may have been affecting the HPG axis of the exposed fish was a significant increase in cGnRH transcripts in the brain of males exposed to 20 µg haloperidol/L. This increase is broadly consistent with the idea that haloperidol exposure would release dopaminergic inhibition of GnRH synthesis and/or secretion. However, there was little corroborating evidence supporting that hypothesized effect. The same response could not be examined in the brains of females due to a technical mishap during RNA extraction. There were no significant changes in the abundance of LHβ or FSHβ transcripts in the pituitary tissue of exposed males or females. Finally, methods for measuring circulating peptide hormone concentrations (e.g., GnRH, GtH) are not currently available for the fathead minnow. Ultimately, whether haloperidol exposure modulated GnRH expression and/or secretion or not, results of the reproduction study did not indicate that haloperidol acted as a direct reproductive toxicant.

5. Conclusions

Results of our study did not support the hypothesis that exposure to the D2R antagonist, haloperidol, would directly disrupt fish reproduction. The potential for D2R antagonism to interfere with behavior or other aspects of transcriptional signaling in reproductive tissues was examined in a companion study (Villeneuve et al., 2009).

Acknowledgments

The authors thank Katie J. Greene, Jeffrey D. Brodin, and Leah C. Wehmas for general laboratory assistance and Ann Linnum and Experimental Pathologies Laboratories for assistance with histological analyses. We thank J.J. Korte and L.W. Touart for comments on an earlier version of this manuscript. This work was supported in part by the US EPA National Center for Computational Toxicology and the Army Environmental Quality Program of the US Army Corps of Engineers. The manuscript has been reviewed in accordance with US EPA and US ACE guidelines and approved for publication. Approval does not indicate that the contents reflect the views of either Agency, nor does mention of trade names or

commercial products constitute endorsement or recommendation for use.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2009.09.007.

References

- Aizen, J., Meiri, I., Tzchori, I., Levavi-Sivan, B., Rosenfeld, H., 2005. Enhancing spawning in the grey mullet (*Mugil cephalus*) by removal of dopaminergic inhibition. *Gen. Comp. Endocrinol.* 142, 212–221.
- Ankley, G., Jensen, K., Kahl, M., Korte, J., Makynen, E., 2001. Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). *Environ. Toxicol. Chem.* 20, 1276–1290.
- Ankley, G., Jensen, K., Makynen, E., Kahl, M., Korte, J., Hornung, M., Henry, T., Denny, J., Leino, R., Wilson, V., Cardon, M., Hartig, P., Gray, L., 2003. Effects of the androgenic growth promoter 17-β-trenbolone on fecundity and reproductive endocrinology of the fathead minnow. *Environ. Toxicol. Chem.* 22, 1350–1360.
- Ankley, G., Kahl, M., Jensen, K., Hornung, M., Korte, J., Makynen, E., Leino, R., 2002. Evaluation of the aromatase inhibitor fadrozole in a short-term reproduction assay with the fathead minnow (*Pimephales promelas*). *Toxicol. Sci.* 67, 121–130.
- Ankley, G.T., Bencic, D., Breen, M., Collette, T.W., Connolly, R., Denslow, N.D., Edwards, S., Ekman, D.R., Jensen, K.M., Lazorchak, J.M., Martinović, D., Miller, D.H., Perkins, E.J., Orlando, E.F., Villeneuve, D.L., Wang, R.L., Watanabe, K.H., 2009. Endocrine disrupting chemicals in fish: developing exposure indicators and predictive models of effects based on mechanisms of action. *Aquat. Toxicol.* 92, 168–178.
- Ankley, G.T., Jensen, K.M., Durhan, E.J., Makynen, E.A., Butterworth, B.C., Kahl, M.D., Villeneuve, D.L., Linnum, A., Gray, L.E., Cardon, M., Wilson, V.S., 2005. Effects of two fungicides with multiple modes of action on reproductive endocrine function in the fathead minnow (*Pimephales promelas*). *Toxicol. Sci.* 86, 300–308.
- Ankley, G.T., Jensen, K.M., Kahl, M.D., Makynen, E.A., Blake, L.S., Greene, K.J., Johnson, R.D., Villeneuve, D.L., 2007. Ketoconazole in the fathead minnow (*Pimephales promelas*): reproductive toxicity and biological compensation. *Environ. Toxicol. Chem.* 26, 1214–1223.
- Basu, N., Ta, C.A., Wayne, A., Mao, J., Hewitt, M., Arnason, J.T., Trudeau, V.L., 2009. Pulp and paper mill effluents contain neuroactive substances that potentially disrupt neuroendocrine control of fish reproduction. *Environ. Sci. Technol.* 43, 1635–1641.
- Besse, J.P., Garric, J., 2008. Human pharmaceuticals in surface waters: implementation of a prioritization methodology and application to the French situation. *Toxicol. Lett.* 176, 104–123.
- Chang, J.P., Peter, R.E., 1983. Effects of pimoizide and des Gly10,[D-Ala6]luteinizing hormone-releasing hormone ethylamide on serum gonadotropin concentrations, germinal vesicle migration, and ovulation in female goldfish, *Carassius auratus*. *Gen. Comp. Endocrinol.* 52, 30–37.
- Dufour, S., Weltzien, F.A., Seibert, M.E., Le Belle, N., Vidal, B., Vernier, P., Pasqualini, C., 2005. Dopaminergic inhibition of reproduction in teleost fishes: ecophysiological and evolutionary implications. *Ann. N.Y. Acad. Sci.* 1040, 9–21.
- Jensen, K., Korte, J., Kahl, M., Pasha, M., Ankley, G., 2001. Aspects of basic reproductive biology and endocrinology in the fathead minnow (*Pimephales promelas*). *Comp. Biochem. Physiol. C* 128, 127–141.
- Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. *Proc. Natl. Acad. Sci. USA* 104, 8897–8901.
- Lange, R., Hutchinson, T.H., Croudace, C.P., Siegmund, F., Schweinfurth, H., Hampe, P., Panter, G.H., Sumpter, J.P., 2001. Effects of the synthetic estrogen 17 alpha-ethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). *Environ. Toxicol. Chem.* 20, 1216–1227.
- Lin, H.R., Van der Kraak, G., Zhou, X.J., Liang, J.Y., Peter, R.E., Rivier, J.E., Vale, W.W., 1988. Effects of [D-Arg6, Trp7, Leu8, Pro9NET]-luteinizing hormone-releasing hormone (sGnRH-A) and [D-Ala6, Pro9NET]-luteinizing hormone-releasing hormone (LHRH-A), in combination with pimoizide or domperidone, on gonadotropin release and ovulation in the Chinese loach and common carp. *Gen. Comp. Endocrinol.* 69, 31–40.
- Martinović, D., Blake, L.S., Durhan, E.J., Greene, K.J., Kahl, M.D., Jensen, K.M., Makynen, E.A., Villeneuve, D.L., Ankley, G.T., 2008. Reproductive toxicity of vinclozolin in the fathead minnow: confirming an anti-androgenic mode of action. *Environ. Toxicol. Chem.* 27, 478–488.
- McMaster, M.E., Munkittrick, K.R., Jardine, J.J., Robinson, R.D., Van Der Kraak, G.J., 1995. Protocol for measuring *in vitro* steroid production by fish gonadal tissue. Canadian Technical Report of Fisheries and Aquatic Sciences, vol. 1961, pp.1–78.
- Miller, D., Ankley, G., 2004. Modeling impacts on populations: fathead minnow (*Pimephales promelas*) exposure to the endocrine disruptor 17β-trenbolone as a case study. *Ecotoxicol. Environ. Saf.* 59, 1–9.
- Seeman, P., 2002. Atypical antipsychotics: mechanism of action. *Can. J. Psychiatry* 47, 27–38.

- Trudeau, V., 1997. Neuroendocrine regulation of gonadotropin II release and gonadal growth in the goldfish, *Carassius auratus*. *Rev. Reprod.* 2, 55–68.
- Trudeau, V.L., Spanswick, D., Fraser, E.J., Larivière, K., Crump, D., Chiu, S., MacMillan, M., Schulz, R.W., 2000. The role of amino acid neurotransmitters in the regulation of pituitary gonadotropin release in fish. *Biochem. Cell Biol.* 78, 241–259.
- US EPA, 2002. A short-term test method for assessing the reproductive toxicity of endocrine-disrupting chemicals using the fathead minnow (*Pimephales promelas*). Duluth: US EPA Mid-Continent Ecology Division. Report no. EPA/600/R-01/067, 154pp.
- Vacher, C., Mañanos, E., Breton, B., Marmignon, M.H., Saligaut, C., 2000. Modulation of pituitary dopamine D1 or D2 receptors and secretion of follicle stimulating hormone and lutenizing hormone during the annual reproductive cycle of female rainbow trout. *J. Neuroendocrinol.* 12, 1219–1226.
- Villeneuve, D.L., Blake, L.S., Brodin, J.D., Cavallin, J.E., Durhan, E.J., Jensen, K.M., Kahl, M.D., Makynen, E.A., Martinovic, D., Mueller, N.D., Ankley, G.T., 2008. Effects of a 3beta-hydroxysteroid dehydrogenase inhibitor, trilostane, on the fathead minnow reproductive axis. *Toxicol. Sci.* 104, 113–123.
- Villeneuve, D.L., Blake, L.S., Brodin, J.D., Greene, K.J., Knoebel, I., Miracle, A.L., Martinovic, D., Ankley, G.T., 2007. Transcription of key genes regulating gonadal steroidogenesis in control and ketoconazole- or vinclozolin-exposed fathead minnows. *Toxicol. Sci.* 98, 395–407.
- Villeneuve, D.L., Garcia-Reyero, N., Martinović, D., Mueller, N.D., Cavallin, J.E., Durhan, E.J., Makynen, E.A., Jensen, K.M., Kahl, M.D., Blake, L.S., Perkins, E.J., Ankley, G.T., 2009. II. Effects of a dopamine receptor antagonist on fathead minnow dominance behavior and ovarian gene expression in the fathead minnow and zebrafish. *Ecotoxicol. Environ. Saf.*
- Villeneuve, D.L., Larkin, P., Knoebel, I., Miracle, A.L., Kahl, M.D., Jensen, K.M., Makynen, E.A., Durhan, E.J., Carter, B.J., Denslow, N.D., Ankley, G.T., 2007. A graphical systems model to facilitate hypothesis-driven ecotoxicogenomics research on the teleost brain-pituitary-gonadal axis. *Environ. Sci. Technol.* 41, 321–330.
- Villeneuve, D.L., Miracle, A.L., Jensen, K.M., Degitz, S.J., Kahl, M.D., Korte, J.J., Greene, K.J., Blake, L.S., Linnum, A.L., Ankley, G.T., 2007. Development of quantitative real-time PCR assays for fathead minnow (*Pimephales promelas*) gonadotropin beta subunit mRNAs to support endocrine disruptor research. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 145, 171–183.
- Yu, K.L., Peter, R.E., 1990. Dopaminergic regulation of brain gonadotropin-releasing hormone in male goldfish during spawning behavior. *Neuroendocrinology* 52, 276–283.