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## Highlighted Article

### II: Effects of a dopamine receptor antagonist on fathead minnow dominance behavior and ovarian gene expression in the fathead minnow and zebrafish

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## ABSTRACT

Neurotransmitters such as dopamine play an important role in reproductive behaviors and signaling. Neuroendocrine-active chemicals in the environment have potential to interfere with and/or alter these processes. A companion study with the dopamine 2 receptor antagonist, haloperidol, found no evidence of a direct effect of the chemical on fish reproduction. This study considered haloperidol's potential effects on behavior and ovarian gene expression. Male fathead minnows exposed to 50 µg haloperidol/L for 96 h were found to be significantly more dominant than control males. In terms of molecular signaling, investigated using oligonucleotide microarrays, there was little similarity in the identity and functions of genes differentially expressed in the ovaries of fathead minnows (*Pimephales promelas*) versus zebrafish (*Danio rerio*) exposed under the same conditions. Results suggest that non-lethal concentrations of haloperidol do not induce ovarian molecular responses that could serve as biomarkers of exposure to D2R antagonists, but may impact behavior.

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## 1. Introduction

Neurotransmitter systems play critical roles in regulating both social behaviors and physiological signaling which are essential for reproductive success. For example, the dopaminergic system exerts inhibitory control over gonadotropin release from the pituitary (Dufour et al., 2005). It also plays a role in modulating social dominance and aggressive behavior in fish and other vertebrates (Dennis et al., 2006; Höglund et al., 2005; Kudryavtseva et al., 1999; Schwartz et al., 2009; Winberg and Nilsson, 1992). There has long been evidence that exposure to a wide variety of environmental contaminants can alter the abundance of neurotransmitters, such as dopamine, and various components of their associated neurotransmission systems, which may in turn lead to human disease, behavioral disorders, etc. (Aoki, 2001; Jones and Miller, 2008; Richardson et al., 2006). However, there is also growing concern over the presence of direct acting neuroendocrine-active chemicals in aquatic environments and their

potential effects on aquatic life and ecosystems. A broad diversity of human pharmaceuticals have been detected in both wastewaters and surface water (Fent et al., 2006). Human pharmaceuticals of concern include a number of antipsychotics which directly target dopamine receptors (e.g., phenothiazines like cyamemazine, butyrophenones like haloperidol; Besse and Garric, 2008). Additionally, in a recent study pulp mill effluents were shown to contain chemicals that could directly interact with dopamine 2 receptors (D2R; Basu et al., 2009), suggesting there may be a diversity of sources of neuroendocrine-active contaminants in aquatic environments. Thus, direct interaction of environmental contaminants with neurotransmitter systems, like the dopaminergic system, are potentially relevant modes of action for neuroendocrine disruption in aquatic organisms.

As part of a companion study (Villeneuve et al., 2010) we examined the effect of the model D2R antagonist haloperidol on fish reproduction using the fathead minnow 21 d reproduction assay developed for the US EPA Endocrine Disruptor Screening Program (<http://www.epa.gov/endo/>; Ankley et al., 2001). The assay provided little evidence for a direct effect on fish reproduction at non-lethal exposure concentrations (Villeneuve et al., 2010). However, the companion study did not examine behavioral

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effects that could affect important social dynamics related to successful reproduction. Furthermore, only supervised methods were employed in probing the potential effects of haloperidol exposure on signaling within the reproductive axis.

The goal of the present study was to extend our analysis of the potential neuroendocrine impacts of this model D2R antagonist. Specifically, we tested the hypothesis that exposure to the antipsychotic drug may impair an important reproductive behavior in the male fathead minnow, the ability to successfully occupy and defend a breeding substrate from rival males. Additionally, we employed unsupervised analysis with oligonucleotide microarrays to probe the potential effects of haloperidol exposure on downstream signaling within the reproductive axis. The aim was to determine whether there may be robust transcriptional alterations in the ovary of exposed females that might serve as useful markers of exposure and/or effects for D2R antagonism as a mode of action. For the purposes of this study, robust transcriptional alterations were viewed as those detected in two separate fish species (fathead minnow and zebrafish), exposed to the same concentration of haloperidol for the same duration. Given the mode of action, the direct effects of the chemical were expected to occur primarily within specific brain regions. However, molecular alterations localized to specific brain regions were considered to have less practical applicability than those in a more readily sampled tissue like ovary. Additionally, ovary tissue was a major focus for transcriptional network and molecular marker discovery in our broader research program (Ankley et al., 2009). Thus, ovary tissue was given priority for this work with the understanding that mechanistic interpretation of the responses in non-neural tissue would be difficult and that molecular alterations in ovary would potentially represent downstream effects of altered signaling within the reproductive axis.

## 2. Materials and methods

### 2.1. Chemical and test organisms

Haloperidol was purchased from Sigma Aldrich (St. Louis, MO, USA; H1512; 99% pure). All fish were obtained from an on-site culture facility at the US EPA Mid-Continent Ecology Division (Duluth, MN, USA). Reproductively mature adult fathead minnows (6 months old) and ab wild-type zebrafish (5 months old) were used for these studies. For all experiments described below, fish were held at 25 °C under a 16:8 light:dark photoperiod and fed to satiation twice daily with either frozen (fathead minnow) or live (zebrafish) brine shrimp (*Artemia*). All animals were treated humanely with regard to 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. Fathead minnow and zebrafish behavior/microarray experiment

Fish for the behavior and microarray analyses were exposed to haloperidol in a 96 h, continuous flow-through experiment. Chemical delivery and exposure verification was accomplished using methods described in the companion paper (Villeneuve et al., 2010), noting that haloperidol spike recoveries in this experiment were  $99 \pm 3\%$ . Nominal treatment concentrations for the behavior/microarray experiment were 0 and 50  $\mu\text{g}$  haloperidol/L. The test concentration was selected based on results of a previous range-finding experiment (see Villeneuve et al., 2010) and consideration of the shorter exposure duration, compared with the companion 21 d reproduction assay. Chemical (or control water) delivery was initiated 24 h prior to adding fish to the tanks. Fathead minnows (four males, four females per tank) were added to each of three tanks per treatment group, while zebrafish (six males, six females per tank) were added to each of two tanks per group. The time of fish addition was staggered by replicate within each treatment such that all samples from a given exposure tank could be collected within 60 min of the intended 96 h exposure duration. After 96 h, male and female zebrafish and female fathead minnows were humanely terminated in buffered MS-222 and weighed. Whole gonads were removed, weighed, and preserved in RNAlater. Brains and pituitaries were also removed and transferred to RNAlater. Samples were stored at  $-20$  °C until extracted and analyzed. Male fathead minnows were kept

alive for use in a competitive behavior assay (below). All dissection tools were washed with RNaseZap between samples.

### 2.3. Behavior assay

A competitive behavior assay with male fathead minnows was adapted from the methods first described by Martinovic et al. (2007). After all females had been sampled, males were removed one at a time from their respective exposure tanks and anesthetized (non-lethally) in buffered MS-222. Whole body mass was measured and gross appearance of secondary sex characteristics (tubercles and dorsal pads) was scored. The top or bottom portion of the caudal fin was clipped, and the position of the clip was recorded. Fish were then transferred to a new aquarium (competition tank) receiving a continuous flow of control Lake Superior water and a single breeding substrate. One control male and one haloperidol-exposed male were assigned to each competition tank, taking care to match fish of approximately equal mass and masculinity (as judged based on secondary sex characteristics) and making sure to pair fish with oppositely positioned fin clips. Both the position of the fin clip and the order of addition of males to the competition tank were distributed equally across treatment groups to avoid confounding the independent variable of interest with either factor. The fish were then held without disturbance, other than to feed twice daily and observe nest-holding behaviors (Martinovic et al., 2007) for a 5 min period each day. All observations were made between 09:00 AM and 12:00 PM, prior to feeding the fish. Following behavioral observations on day 5 of competition, the males were anesthetized in a lethal dose of buffered MS-222, weighed, and tissues were collected as described above. Fisher's Exact test ( $p < 0.05$ ) was applied to test for significant differences in nest-holding ability between the control and haloperidol-exposed males.

### 2.4. RNA extraction and microarray analyses

#### 2.4.1. Fathead minnow

Total RNA was isolated from ovary samples using RNeasy kits (Qiagen, Valencia, CA, USA). RNA quality was assessed with an Agilent 2100 Bioanalyzer (Santa Clara, CA, USA) and quantity was determined using a Nanodrop<sup>®</sup> ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA from six fathead minnow ovaries per treatment group (two from each replicate per treatment) was labeled with cyanine 5 (Cy5) and reference samples, consisting of a pool of RNA from male and female fathead minnow gonad, liver, and brain tissue, with cyanine 3 (Cy3) following the manufacturer's kits and protocols (Agilent). Labeled samples were hybridized in a reference design format with experimental samples co-hybridized with reference RNA to fathead minnow  $4 \times 44$  K oligonucleotide microarrays containing approximately 22,000 elements, printed in duplicate, designed by EcoArray (Alachua, FL, USA) and purchased from Agilent. Hybridization, washing, scanning, and feature extraction were also conducted according to Agilent's recommended protocols. The fathead minnow microarray data and details regarding the methods employed for the microarray analysis are available in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO; Accession GSE15115; [www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/)).

#### 2.4.2. Zebrafish

Ovary samples were transferred to TRI Reagent<sup>®</sup> (Sigma), homogenized, and extracted following the manufacturer's protocol. Total RNA was quantified using a Nanodrop<sup>®</sup> ND-1000 spectrophotometer, and the integrity and purity of the RNA samples were evaluated using an Agilent 2100 Bioanalyzer and a RNA Nano Chip Kit (Agilent). Absorbance ratios (260/280 nm) for all samples were between 1.97 and 2.08 and RNA integrity numbers (RIN; Agilent) ranged from 5.7–9.3. Aliquots of six ovary RNA samples per treatment group, generally those with greater RIN and three from each replicate tank, were submitted to an Agilent certified facility, MOgene, LC (St. Louis, MO, USA) for microarray analysis, along with a reference pool of RNA prepared from adult male and female zebrafish liver, brain, and gonad tissues (Martinovic et al., 2009). Agilent Oligo Microarray Zebrafish slides, with approximately 21,000 genes represented, were used for the microarray analyses ( $4 \times 44$  K format, product 015064). Samples were hybridized using a reference design where RNA from experimental samples was labeled with Cy5 and reference RNA with Cy3. Amplification, labeling, hybridization, scanning, and feature extraction were performed as described elsewhere (Martinovic et al., 2009). Additional methodological details and the zebrafish microarray data can be found in GEO (GEO Accession GSE14861).

### 2.5. Microarray data analysis and bioinformatics

As noted above, microarray data were generated for six independent samples (each representing a different fish, with equal distribution of fish from different replicate tanks) per treatment group (control, haloperidol). Zebrafish and fathead minnow microarray data were analyzed using Rosetta Resolver<sup>®</sup> 7.2 (Rosetta Biosoftware, Seattle, WA, USA). Data for each species were treated as separate experiments, but uniform analysis and bioinformatics procedures were applied to

each data set. Three separate statistical tests were applied in determining differentially expressed genes between the haloperidol and control treatments; (1) a two-sample *t*-test, (2) one-way error-weighted ANOVA, and (3) textbook ANOVA. Since all three statistical tests were valid for the design, but provided slightly different results in terms of numbers of differentially expressed genes, results of each analysis were overlapped using a Venn diagram, and only those genes identified as statistically significant using all three approaches were regarded as differentially expressed. Statistical analyses were conducted at both  $\alpha=0.05$  and  $\alpha=0.01$  and no multiple test correction was applied. Two-dimensional agglomerative clustering, using an average-link heuristic criteria and an error-weighted cosine correlation similarity metric, was applied to genes identified as differentially expressed at  $p < 0.05$  (includes all genes differentially expressed at  $p < 0.01$ ). Results of the cluster analysis were used to discriminate genes up-regulated versus down-regulated in the ovaries of haloperidol treated fish, relative to controls.

All homologous features on the Agilent zebrafish and Ecoarray fathead minnow microarrays were identified using a sequence-based reciprocal best hit approach (Moreno-Hagelsieb and Latimer, 2008; bioinformatic analyses conducted by Dr. Li Liu, Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville, FL, USA). In total, 10,902 homologous features (not all unique) were identified. Probe identifications associated with the lists of differentially expressed genes from the zebrafish and fathead minnow experiments were compared against the list of homologous features to identify the subset of differentially expressed genes common to both species.

Statistically enriched gene ontology (GO) categories associated with the differentially expressed genes ( $p < 0.05$ ) were identified using eGON v. 2.0 ([www.genetools.microarray.ntnu.no](http://www.genetools.microarray.ntnu.no); Beisvag et al., 2006). GenBank accession numbers corresponding to the sequences identified as differentially expressed (from EcoArray and Agilent microarray annotation files) were loaded as reporter lists in eGON. GenBank accession numbers corresponding to every feature on each array (Ecoarray or Agilent zebrafish) were loaded as master reporter lists. Master-target statistical analysis, employing Fisher's Exact test ( $p < 0.05$ ) was used to determine which, if any, GO terms associated with differentially expressed genes were statistically over- or under-represented compared with their overall representation on the entire microarray. The analyses examined over-/under-representation of molecular function, biological process, and cellular component GO terms. Interpretation focused on the biological process terms, as these were viewed as the most informative. In general, results are shown with auto grouping, limit set to 30, to reduce display of redundant categories; however, in all cases, the entire list of over-/under-represented GO terms was examined to confirm that the groupings were representative and did not omit any additional information that might impact interpretation or conclusions.

Over- or under-representation of differentially expressed genes in specific biological pathways (e.g., KEGG pathways) was evaluated using DAVID (<http://david.abcc.ncifcrf.gov/home.jsp>; Huang et al., 2009). Approximately 50% of the submitted accession numbers from the arrays were successfully mapped to homologous features in the DAVID database. Pathway enrichment analyses were conducted using DAVID's Functional Annotation tool with the default EASE score, a modified Fisher's Exact *p*-value, of 0.1 as a statistical cut-off for determination of pathway enrichment and a count threshold of 2 (the minimum number of genes belonging to any given annotation term considered).

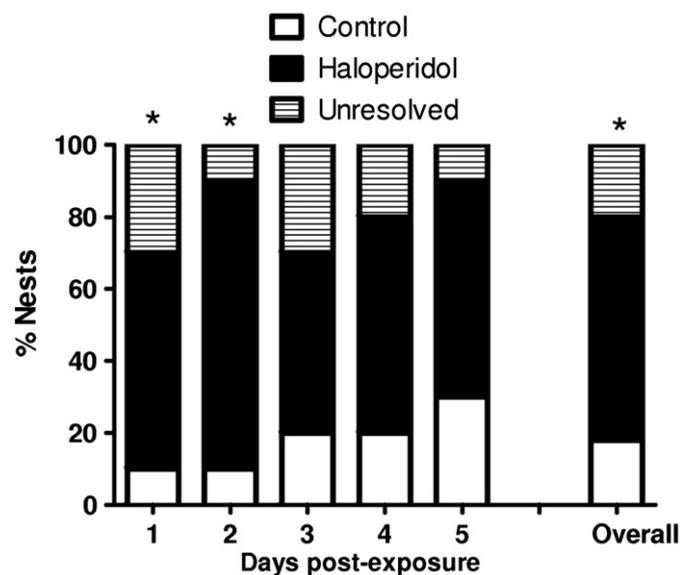
### 3. Results

#### 3.1. Behavior assay

The behavioral endpoint evaluated in this study was the ability of male fathead minnows to compete for and hold the breeding substrate/nest. On the first and second days of the post-exposure competition period, significantly more haloperidol-exposed (50  $\mu\text{g/L}$  nominal) than control males held the nest over the duration of the 5 min observation period (Fig. 1). On days 3–5 of competition, the haloperidol-exposed males continued to dominate nest-holding, but the percentage of nest-holding control males increased to the point where a statistically significant difference was not detected. Averaged over the 5-d competition period, haloperidol-exposed males were significantly more likely to hold a nest than their control counterparts (Fig. 1).

#### 3.2. Microarray experiment

Average ( $\pm$  SD) haloperidol concentrations measured in water samples collected from the microarray experiment test tanks were  $62.4 \pm 0.89$ ,  $54.0 \pm 3.8$ , and  $55.4 \pm 3.6$   $\mu\text{g/L}$  on days 0, 2, and 4 of



**Fig. 1.** Effects of 96 h of haloperidol exposure (50  $\mu\text{g/L}$ ) on the ability of male fathead minnows to compete for and defend a spawning substrate/nest. Percentage of nests ( $n=12$  competition tanks, 1 nest per tank) dominated by control males, haloperidol-exposed males, or not predominantly occupied by either male (unresolved) during a 5 min observation period, each day, for 5 d. Asterisks indicate significant differences in nest-holding ability as determined by Fisher's Exact test ( $p < 0.05$ ).

the experiment, respectively ( $n=5$  on each day). The measured haloperidol concentrations on day 0 were significantly greater than those on days 2 and 4, but there were no significant differences in the concentrations among replicate tanks (including both fathead minnow and zebrafish tanks) over the course of the exposure. No haloperidol ( $< 0.010$   $\mu\text{g/L}$ ) was detected in control tanks.

Using the statistical criteria established for this study, a total of 326 genes expressed in the fathead minnow ovary were identified as differentially expressed between haloperidol-exposed and control females ( $p < 0.05$ ; Supplementary Table S.1). Among these, 164 (50.3%) were down-regulated (by haloperidol) and 162 (49.7%) were up-regulated. At the more stringent statistical cut-off of  $p < 0.01$ , there were 45 significant differences, with 24 (53.3%) up-regulated genes and 21 (46.7%) down-regulated genes. Of the 326 genes identified as differentially expressed in fathead minnow ovary, 143 had at least one homologous probe on the zebrafish microarray. Accounting for fathead minnow microarray features that mapped to more than one homologous zebrafish probe, a total of 227 homologous zebrafish features were identified.

In the zebrafish, fewer total genes were detected as differentially expressed in the ovaries of haloperidol-exposed females. At  $p < 0.05$ , 197 zebrafish genes were identified as differentially expressed; 106 (53.8%) up-regulated and 91 (46.2%) down-regulated (Supplementary Table S.2). Using the more stringent cut-off ( $p < 0.01$ ), only 25 genes were significantly differentially expressed; 17 (68%) up-regulated and 8 (32%) down-regulated. Of the 197 zebrafish genes identified as differentially expressed, 98 mapped to at least one homologous fathead minnow probe, with five zebrafish genes mapping to two probes, for a total of 103 homologous fathead minnow probes identified.

Out of a possible 330 homologous features that could be cross-referenced between the fathead minnow and zebrafish gene lists (Supplementary Tables S.1 and S.2), only two homologous genes (0.6%), aconitase 2 (*aco2*) and syntaxin 3A (*stx3a*), were

**Table 1**

Statistically-enriched biological process gene ontology (GO) categories<sup>a</sup> associated with genes identified as differentially expressed (up- or down-regulated;  $p < 0.05$ ) in the ovaries of fathead minnows exposed to 50  $\mu\text{g}$  haloperidol/L (nominal).

GO	Name <sup>b</sup>	FHMECOARRAY22K	FHMHALP05	p-Value
GO:0008150	biological_process	3191	76	
GO:0001558	regulation of cell growth	15	3	0.005
GO:0040008	regulation of growth	22	3	0.014
GO:0008361	regulation of cell size	19	3	0.010
GO:0016049	cell growth	19	3	0.010
GO:0051649	establishment of localization in cell	157	8	0.031
GO:0046907	intracellular transport	139	8	0.016
GO:0006886	intracellular protein transport	84	7	0.003
GO:0065002	intracellular protein transport across a membrane	15	2	0.048
GO:0006605	protein targeting	34	5	0.001
GO:0051169	nuclear transport	24	3	0.018
GO:0046164	alcohol catabolic process	28	3	0.028
GO:0006006	glucose metabolic process	31	3	0.036
GO:0019320	hexose catabolic process	27	3	0.025
GO:0046365	monosaccharide catabolic process	27	3	0.025
GO:0044262	cellular carbohydrate metabolic process	53	4	0.036
GO:0044275	cellular carbohydrate catabolic process	30	3	0.033
GO:0007399	<u>nervous system development</u>	168	0	<u>0.033</u>
GO:0045184	establishment of protein localization	143	11	0.000
GO:0015031	protein transport	143	11	0.000
GO:0017038	protein import	22	3	0.014
GO:0033036	macromolecule localization	158	11	0.001
GO:0008104	protein localization	154	11	0.001
GO:0016052	carbohydrate catabolic process	30	3	0.033
GO:0031644	regulation of neurological system process	10	2	0.022
GO:0051606	detection of stimulus	6	2	0.008

**FHMECOARRAY22K** indicates the number of features on the microarray annotated with the corresponding GO term.

**FHMHALP05** indicates the number of features identified as differentially expressed by haloperidol exposure that were annotated with the corresponding GO term.

Underlining indicates categories identified as statistically under-represented in the differentially expressed gene list.

<sup>a</sup>Categories with only one differentially expressed gene represented were excluded except in the case of categories identified as significantly under-represented.

<sup>b</sup>Indentation is intended to convey the hierarchical nesting of closely related GO terms.

differentially expressed in both species. Even for those two, the effect of haloperidol treatment was not consistent. Aconitase 2 was up-regulated in zebrafish but down-regulated in fathead minnows while *stx3a* was up-regulated in fathead minnows but down-regulated in zebrafish.

In terms of over-represented functional (biological process GO) categories, genes identified as differentially expressed in fathead minnows were associated with intracellular protein transport (Table 1; mix of up- and down-regulation; Supplementary Tables S.3 and S.4) cell growth (Table 1; primarily down-regulation; Supplementary Table S.4), carbohydrate metabolism (Table 1), and detection of stimulus (Table 1; primarily down-regulation; Supplementary Table S.4). Genes related to nervous system development were significantly under-represented (Table 1). In terms of pathways analyzed using DAVID, no KEGG pathways were detected as significantly over-represented when the entire fathead minnow differentially expressed gene list ( $p < 0.05$ , up- and down-regulated; 326 genes) or when only up-regulated genes were used for the analysis. However, when the analysis was restricted to genes identified as down-regulated in the ovaries of fathead minnows exposed to haloperidol, two over-represented KEGG pathways (<http://www.genome.jp/kegg/pathway.html>) were identified; ABC transporters (*dre02010*) and purine metabolism (*dre00230*). Five of the down-regulated genes were associated with overrepresentation of one particular reaction in the purine biosynthesis pathway (KEGG reaction R06605;  $p=0.0045$ ). These included genes homologous to adenosine deaminase, gmp synthetase, inosine-5'-monophosphate dehydrogenase, polymerase delta 2 regulatory subunit, and nucleoside diphosphate kinase-z6.

Biological process GO terms related to regulation of cell size/cell growth, neurogenesis and nervous system development, and general negative regulation of certain metabolic processes (Table 2) were identified as significantly over-represented in the list of genes differentially expressed in the ovaries of haloperidol-exposed zebrafish. Enrichment of neurogenesis and nervous system development terms was primarily associated with down-regulated genes (Supplementary Table S.5). Genes up-regulated in the ovary of haloperidol-exposed zebrafish were associated with negative regulation of cell growth and transcription (Supplementary Table S.6). No specific KEGG pathways or reactions were identified as being significantly over-represented in the lists of genes differentially expressed in zebrafish.

## 4. Discussion

### 4.1. Behavior

There is clear evidence in the literature that dopaminergic neurotransmission plays an important role in regulating the reproductive system of fish (Trudeau, 1997; Trudeau et al., 2000; Dufour et al., 2005). However, dopaminergic neurons also play a role in many other functions including motor control, vision, learning, and memory formation, to name a few (Gonzalez-Burgos and Fera-Velasco, 2008; Liss and Roeper, 2008; Mora-Ferrer and Neumeyer, 2008). Consequently, while sublethal concentrations of haloperidol may not directly disrupt or enhance spawning activity in a laboratory setting, it is plausible that haloperidol, or other

**Table 2**  
Statistically-enriched biological process gene ontology (GO) categories<sup>a</sup> associated with genes identified as differentially expressed (up- or down-regulated;  $p < 0.05$ ) in the ovaries of zebrafish exposed to 50 µg haloperidol/L (nominal).

GO	Name <sup>b</sup>	ZFARRAY_ALLGENES	ZFHALP05	p-Value
GO:0008150	biological_process	5061	55	
GO:0031324	negative regulation of cellular metabolic process	32	2	0.047
GO:0009892	negative regulation of metabolic process	33	2	0.049
GO:0045934	negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid...	25	2	0.030
GO:0016481	negative regulation of transcription	23	2	0.025
GO:0008361	regulation of cell size	24	2	0.027
GO:0022008	neurogenesis	90	4	0.016
GO:0030182	neuron differentiation	70	3	0.040
GO:0016049	cell growth	24	2	0.027
GO:0048646	anatomical structure formation	63	3	0.030
GO:0048514	blood vessel morphogenesis	28	2	0.037
GO:0007417	central nervous system development	139	5	0.017
GO:0007399	nervous system development	217	6	0.029

ZFARRAY\_ALLGENES indicates the number of features on the microarray annotated with the corresponding GO term.

ZFHALP05 indicates the number of features identified as differentially expressed by haloperidol exposure that were annotated with the corresponding GO term.

<sup>a</sup>Categories with only one differentially expressed genes represented were excluded.

<sup>b</sup>Indentation is intended to convey the hierarchical nesting of closely related GO terms.

D2R antagonists/agonists, could have behavioral effects with ecological relevance.

Outside of the laboratory, for most fish species reproduction involves intense competition for mates and/or spawning habitat (Balon, 1975; Kroon et al., 2000; Danylchuk and Tonn, 2001). Dominance and aggressive behaviors are particularly important to reproductive success in the male fathead minnow (Danylchuk and Tonn, 2001). Administration of the dopamine precursor L-dopa to Arctic charr (*Salvelinus alpinus*) increase aggressive behaviors and social dominance (Winberg and Nilsson, 1992). This result was in general agreement with evidence in lizards, birds, and mammals that increased dopamine is associated with aggressive behaviors, while dopamine receptor antagonists have been studied for their potential to reduce aggression (Dennis et al., 2006; Höglund et al., 2005; Kudryavtseva et al., 1999; Miczek et al., 2002; Schwartz et al., 2009; van Erp and Miczek, 2000). Consequently, we had hypothesized that haloperidol would impair the ability of male fathead minnows to successfully occupy and defend a breeding substrate compared with a control male.

In contrast, results indicated that male fathead minnows exposed to haloperidol for 96 h were significantly more likely to successfully occupy and defend a breeding substrate than were the unexposed males they competed against, particularly within the first 2 d following haloperidol exposure. This result was opposite of what was expected. At present, the reason for the discrepant behavioral response in the fathead minnow compared with other reports in the literature is unclear. However, it has been noted that pharmacological sensitivity of dopamine receptors can be altered by the behavioral history of individuals and many of the antipsychotic drugs may interact with other targets in addition to dopamine receptors, making it difficult to specifically attribute their anti-aggressive properties to effects on dopamine (Kudryavtseva et al., 1999; Miczek et al., 2002). Consequently, there are other complexities that may modulate the effects of haloperidol on behavior.

Regardless of the mechanistic basis for the effect, our results suggest that exposure to D2R-active compounds can affect fish behavior. A previous study by Martinovic et al. (2007) demonstrated that subtle behavioral impacts can be sufficient to affect reproductive fitness in scenarios where fish compete for reproductive opportunities. In the context of this study, our results suggest that males exposed to haloperidol may compete more effectively than naïve males and that the effect of haloperidol on male behavior is not a permanent one. Whether similar effects would be observed following exposure to other D2R antagonists, or whether the differences in behavior of similar magnitude would be sufficient to alter gene flow in a wild population (e.g., those exposed to D2R-active pulp mill effluents) remains to be determined. Nonetheless, the ability of the drug to significantly modulate fathead minnow behavior is of potential concern.

#### 4.2. Gene expression

Our on-going research program is aimed at systematic characterization of plausible modes of endocrine disruption in small fish and examining links between alterations at the molecular level (e.g., gene expression, proteins, metabolites) and reproductive outcomes (Ankley et al., 2001, 2009). Although it is mechanistically plausible that chemicals that interact with the D2R could disrupt or enhance reproduction, reproductive impacts at an apical level were not demonstrated in the 21 d assay (Villeneuve et al., 2010). Consequently gene expression changes observed in the present study would have limited or no utility as potential molecular markers that would be readily predictive of adverse effect. However, robust transcriptional responses to the

test chemical could still have value as potential biomarkers of exposure to D2R-active substances. Additionally, functional analysis of the altered transcripts could also provide insights into other potential roles of D2R within the reproductive axis.

Since the tissue examined (i.e., ovary) was peripheral to the presumed direct effects of haloperidol (i.e., on the brain), the transcriptomic analyses reported here were focused on identifying transcriptional responses common in two small fish models, fathead minnow and zebrafish. While there were some methodological differences in the sample processing (e.g., RNA extraction protocols, details of labeling and scanning protocols), the overall design, microarray platform, and analyses were consistent for the two species, as were the exposure concentrations and duration. To our knowledge, this is one of the first toxicogenomic studies to directly compare the transcriptional responses of two fish species exposed as part of the same experiment.

Relatively similar numbers of genes were identified as differentially expressed in ovary tissue of both species, with roughly 50% of those being up-regulated and 50% down-regulated. However, the number of homologous features identified as differentially expressed in both species was surprisingly low, just two out of 330 possible matches. This is a frequency that could reasonably be expected due to chance alone. Aconitase (EC 4.2.1.3) is an iron-sulfur protein that catalyzes conversion of citrate to isocitrate as part of the tricarboxylic acid cycle (Beinert et al., 1996). It has been linked to dopamine in the literature, primarily in the context of Parkinson's disease-like syndromes which are typically characterized by dopamine depletion and mitochondrial oxidative stress (Visser et al., 2002; Liang and Patel, 2004; Maguire-Zeiss et al., 2005). Certain syntaxins are thought to interact with dopamine transporter complexes and help regulate transmembrane proteins including neurotransmitter receptors (Torres, 2006; Carvelli et al., 2008). Consequently, plausible connections could be drawn between haloperidol exposure and the two genes that were differentially expressed in the ovary of both species. However, given potential for false discovery in microarray analyses and the differences in regulation (up versus down) between species, neither presents itself as a very robust ovarian marker of D2R antagonist exposure.

Even though the individual genes identified as differentially expressed did not match to a great extent between the two species, it was possible that at a functional level there might be similarities, but that other genes performing related functions might have been affected in one species versus the other. This possibility was examined using functional analyses based on the available annotations for features that could be mapped to public databases. Functional analysis did not support the hypothesis that similar functional groups of genes were impacted in a similar manner in both species. Genes annotated with biological process GO terms related to cell growth were statistically over-represented in both the fathead minnow and zebrafish gene lists, but the associated genes were primarily up-regulated in fathead minnow, while in zebrafish the up-regulated genes were negative regulators of cell growth (Supplementary Table S.6). Differentially expressed genes associated with neuron differentiation, nervous system development, and neurogenesis were over-represented in zebrafish but under-represented in fathead minnow. The other enriched GO terms tended to be unique to each species. Consequently, based on the available biological process GO annotation, at a functional level there was relatively little similarity in the ovarian transcriptional response among species.

Some intriguing links between defects in purine recycling and degeneration of dopaminergic neurons have been observed (Egami et al., 2007; Lewers et al., 2008). In mammals, defects in hypoxanthine-guanine phosphoribosyl transferase (*hprt*), a purine recycling enzyme, are linked to dopamine loss associated with

several diseases (Egami et al., 2007; Lopez, 2008). Not only were genes related to the purine biosynthesis KEGG pathway over-represented in the list of genes down-regulated in fathead minnow ovary, but *hprt* was among the list of genes up-regulated in the ovaries of haloperidol-exposed fathead minnows (Supplementary Table S.1, up-regulated gene # 100). It is also notable that haloperidol has been previously reported to reduce expression of transcripts coding for ABC transporters in humans (Zhou and Wu, 2005; see curated entry in Comparative Toxicogenomics Database <http://ctd.mdibl.org/detail.go?type=chem&acc=D006220>), and ABC transporters have been identified as having particular relevance to the pharmacokinetics and dynamics of a number of antipsychotic drugs, including haloperidol (El Ela et al., 2004). These results suggest that although haloperidol did not cause reproductive toxicity, it was eliciting responses with known links to dopaminergic regulation, even in non-neural tissues.

However, as with the GO-based functional analysis, pathway-based functional analysis failed to identify any common pathways that were significantly affected in the ovaries of both species. From this study, it could not be determined whether the lack of any consistent transcription profile reflects species-specific differences in the toxicokinetics and/or toxicodynamics of haloperidol in these two model fish or other factors. It is possible that the apparent divergence between the profiles of altered transcripts may stem from the fact that no obvious apical effects were induced by haloperidol exposure. At these less severe levels of effect, there may be sufficient stochasticity in the response among organisms that no robust profile would be detected. Additionally, the sensitivity of the two species may vary such that the physiological response to the same concentration and duration of exposure is quite different. We hypothesize that chemicals and/or concentrations that elicit significant apical effects such as impaired spawning, altered ovarian histology, or significant inhibition of steroid biosynthesis, in both species would produce a more consistent profile of transcriptional changes between species. Comparison of these results to other studies conducted as part of our overall research program (Ankley et al., 2009) should provide an opportunity to test this hypothesis.

## 5. Conclusions

Results of our study provide evidence that D2R antagonism could cause behavioral alterations, which might have population-level implications under conditions where animals would need to compete for spawning opportunities. However, the current experiments do not directly establish a link to population-level consequences. Comparison of the effects of haloperidol on the ovarian transcriptome of fathead minnows and zebrafish did not identify any robust transcriptional responses with clear utility as potential molecular markers of exposure to a D2R antagonist or associated effects. Nevertheless, plausible connections between dopamine signaling and a number of the differentially expressed genes could readily be drawn from the open literature. This suggests that further mining of the microarray data may provide insights and/or testable hypotheses regarding the potential role(s) of dopamine signaling in ovarian tissue.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2009.09.018](https://doi.org/10.1016/j.ecoenv.2009.09.018).

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