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# Neuroendocrine biochemical effects in methylmercury-exposed yellow perch

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ABSTRACT

The present study used *in vivo* and *in vitro* methods to characterize the effects of MeHg on key neuroendocrine biochemicals. First, adult female yellow perch (*Perca flavescens*) were exposed to dietary MeHg (0, 0.5, 5, 50 ppm) for 16 weeks throughout the gonadal development period. In different brain regions and liver, GABA-benzodiazepine (BZ), dopamine (D2), estrogen (ERs) and androgen receptors (ARs), and monoamine oxidase (MAO) were measured. Saturation curves revealed a MeHg-dependent increase of the maximum binding (Bmax) of D2 receptor in the hypothalamus *versus* controls (284–606%), concomitant with a decrease in receptor affinity (up to 984% lower). The activity of MAO was enhanced in different brain regions of exposed fish (240–383% in the hypothalamus). MeHg concentrations were negatively correlated with the number of ERs in all brain regions except for the hypothalamus. For the 5 and 50 ppm exposure groups, MeHg concentrations were positively correlated with the number of ARs (130–329% and 225% for brain regions and liver, respectively compared to controls). *In vitro* exposures were conducted in parallel using different fish species (giant danio, goldfish, yellow perch, lake trout) to test the inhibitory potential of MeHgCl or $HgCl_2$ (0.01–320 μM). There was no evidence of impacts on receptors, but the activities of GS, GAD and MAO (except in yellow perch) were inhibited by both MeHgCl and $HgCl_2$ in a species-dependent manner. In general, these findings show that environmentally relevant dietary exposures to MeHg can affect key neuroendocrine receptors and enzymes important to fish reproduction.



## 1. Introduction

Methylmercury (MeHg) is a pervasive toxicant present in all fish. For example, total mercury (Hg) concentrations in tissues of predatory fish collected from hundreds of lakes and rivers across North America ranged from 0.02 to 6.61 μg/g wet weight (Depew et al., 2012; Stahl et al., 2009), and there is a growing database of similar values worldwide (Evers et al., 2012). Diet is the dominant route by which fish take up MeHg (Hall et al., 1997), and speciation measurements suggest that MeHg constitutes 82–100% of the total Hg in the muscle tissue and whole body (Bloom, 1992; Chumchal et al., 2011). Mercury burdens currently found in many fish populations are thought to be high enough to cause health effects that include impairment to growth, reproduction, and development (Crump and Trudeau, 2009; Depew et al., 2012; Wiener et al., 2003). While such effects may not cause direct mortality to individual fish, they can exert sublethal effects that may have repercussions at the population level (Murphy et al., 2008, 2009).

There is a growing body of evidence to link ecologically relevant MeHg exposure with reproductive impairment in many fish species. For example, spawning was delayed and reduced in fathead minnows (*Pimephales promelas*) that were fed MeHg-contaminated diets (0.9–3.9 μg MeHg/g) until sexual maturity. Moreover, Hg concentrations in the carcasses were inversely correlated with proper ovarian development, egg production and steroid hormone levels (Hammerschmidt et al., 2002; Sandheinrich and Miller, 2006). A subsequent experiment found reductions in testosterone and estradiol in male and female fathead minnows, respectively, upon exposure to a MeHg-supplemented diet (3.93 μg/g), concurrent with an inhibition of the gonadal development of females and spawning behaviour in males (Drevnick and Sandheinrich, 2003). In another study, Drevnick et al. (2006) chronically exposed fathead minnows to diets contaminated with MeHg and showed that increased ovarian follicular apoptosis was related to

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suppressed 17β-estradiol concentrations and smaller ovary size in female fathead minnows exposed to MeHg. This study suggests that increased apoptosis of steroidogenic gonadal cells as a possible mechanism for the suppression of sex steroid hormones. Despite such findings, little is known about the underlying mechanisms by which MeHg impairs fish reproductive systems and if such MeHg induced impairments are similar across diverse taxa.

In fish, reproduction is mediated by a multitude of classical hormones and neurotransmitters such as GABA, glutamate and dopamine (Popesku et al., 2008; Trudeau, 1997), and these represent potential targets for MeHg. For instance, MeHg exposures have been related to increased dopamine concentrations in brains of walking catfish after a 90-day exposure to 40 μg/L of MeHg (Kirubagaran and Joy, 1990), or in heads of 7- and 14-day post-hatch mummichog larvae exposed to MeHg (10 μg/L) (Zhou et al., 1999). MeHg exposures have been associated with reduced concentrations of monoamine oxidase (MAO), an enzyme that catalyzes the degradation of biogenic amines such as dopamine, in the brains of juvenile Atlantic salmon (*Salmo salar*) fed 10 μg/g MeHg for 4 months (Berntssen et al., 2003). While these studies provide some evidence that MeHg can disrupt fish neurochemistry, they do not adequately mirror ecological situations (*i.e.*, these studies tested high levels of MeHg and/or used waterborne *versus* dietary exposures).

The objective of the current paper was to characterize the effects of MeHg on neuroendocrine parameters in fish *via in vivo* and *in vitro* studies. First, *via* laboratory-based bioassays, female yellow perch undergoing gonadal maturation were exposed to MeHg. Following a four-month dietary exposure, fish were sacrificed and brains and livers collected for investigation. Neuroendocrine analyses were conducted on different brain regions, with a special attention to the hypothalamus, due to its major role in the regulation of reproductive hormone synthesis. Several measures of brain neurochemistry (*e.g.*, dopamine and GABA-benzodiazepine receptors, and MAO activity) and steroid hormone receptors (estrogen and androgen receptors) were evaluated, and ovarian somatic indices were calculated. Second, *in vitro* assays were conducted on cell-free extracts isolated from four different fish species: (giant danio (*Devario aequipinnatus*), goldfish (*Carassius auratus*), yellow perch (*Perca flavescens*), and lake trout (*Salvelinus namaycush*). It aimed to assess whether Hg compounds (MeHg chloride and mercuric chloride) were capable of directly impairing the binding of neuroendocrine receptors (dopamine, GABA-benzodiazepine, estrogen and androgen) or activity of neurotransmitter enzymes (glutamine synthetase, glutamate decarboxylase, GABA transaminase, MAO). This *in vitro* study was conducted to: 1) help identify potential neuroendocrine targets of Hg exposure; 2) characterize differences between organic and inorganic forms of Hg; and 3) explore possible inter-species differences.

## 2. Methods

### 2.1. Fish exposure bioassays

Yellow perch were reared from eggs to adults under controlled environmental conditions at the University of Wisconsin-Milwaukee School of Freshwater Sciences aquaculture facility. For MeHg exposure, sexually mature female yellow perch were transferred from the aquaculture facility into 55-gal polyethylene tanks (polytanks, 12 fish/tank, 12 tanks) supplied with flow-through water at 15.5–20.5 ± 0.5 °C and photoperiods that parallel natural light cycles. Throughout the experiment (110 days total), fish were fed with food (Finfish Perch 45–12 5.0 mm Slow Sinking Food, Ziegler Bros., Inc. Gardners, PA) treated with MeHgCl (nominal concentrations of 0, 0.5, 5.0, or 50 ppm; using 100% ethanol as a vehicle) in separate tanks, with three experimental replicates (tanks) per condition. Total Hg concentrations in food pellets were monitored throughout the experiment by atomic absorption spectrophotometry in a Direct Mercury Analyzer (DMA-80, Milestone Inc., CT) according to methods previously described (Basu et al., 2009a; Rutkiewicz et al., 2011). The average measured values of THg in the feed (0.03, 0.40, 4.10 and 43.57 ppm) were similar to the intended values (Table 1). Total Hg concentrations in wet fish muscle were measured at the end of the exposure period using the method described above.

In North America, the gonads of yellow perch undergo maturation in the Fall (September–November) when water temperatures decrease (Dabrowski and Ciereszko, 1996). Thus, from July to October 2011, adult perch were environmentally manipulated to stimulate gonadal maturation to assess the possible effects of MeHg on reproductive maturation. This was achieved by decreasing the temperature of the flow-through water to mimic the seasonal water temperature decrease. Fish were held at ~20 °C for the first 12 weeks of the experiment and then water temperatures were decreased by 1 °C each week thereafter; thus, at the time of sampling at week 16 the tank water temperature was 16 °C. The photoperiod was maintained with *AstroDial Suntrackers* to remain in parallel with natural photoperiod changes throughout the treatment. After 16 weeks (in October 2011), four females were removed from each tank; this time period corresponds to their natural peak sexual maturation (3 replicates/MeHg dose = 12 fish total/dose/sampling time). Fish were anesthetized using tricaine methanesulfonate a concentration of 150 mg/L (MS-222, Argent Chemical Laboratories, Redmond, WA), as recommended by the Canadian Council on Animal Care and sacrificed by cervical spinal cord transection. Mean fish weights and lengths were 456.6 ± 8.3 g and 31.4 ± 0.2 cm, respectively. Brains were dissected from each fish to obtain the hypothalamus (HYP), optic tectum (OT), brain stem (BS), and telencephalon (Tel). Each tissue type was pooled together from the four fish removed from each tank (3 replicates/MeHg dose, with each replicate consisting of tissue pooled from four fish from a given exposure tank). Livers and ovaries were removed from each fish and ovary weights were recorded. The ovarian somatic indices (OSI) were calculated as gonad weight (g)/body weight (g).

### 2.2. Preparation of brain samples

Cellular homogenates and membranes of pooled brain regions were prepared according to Rutkiewicz et al. (2013) with slight modifications. Brains were homogenized in 1.5 mL (*i.e.*, 1:10 average weight to buffer volume) of 50 mM Tris buffer (50 mM Tris HCl, 50 mM Tris Base, pH 7.4). Next, 300 μL of each homogenate was centrifuged at 15,000 rpm for 15 min at 4 °C and then supernatant was immediately frozen at −80 °C until neuroendocrine enzyme analyses. Then, 1.2 mL of the homogenate was centrifuged at 48,000 g for 15 min at 4 °C to isolate the cellular membranes. The resulting pellets were resuspended in 10 mL of Tris buffer, and this operation was repeated twice to reach a total of three centrifugations per sample. After the third centrifugation, the final pellet was resuspended in 3 mL of Tris and aliquoted. Aliquots were immediately frozen at −80 °C until required for neuroendocrine receptor binding assays. The supernatant from the first centrifugation of the membrane preparation was sub-sampled (300 μL) and further centrifuged for 45 min at 30,000 rpm at 4 °C. The remaining supernatant was aliquoted and kept at −80 °C for estrogen (ER) and androgen (AR) receptor assays. A Bradford Protein Assay was performed on each sample to measure protein concentration. Ultimately, samples were diluted accordingly to yield a final concentration of 250 μg/mL for enzymes and receptors, and 400 μg/mL for ERs and ARs.

### 2.3. Receptor and enzyme assays

Binding assays specific for GABA-benzodiazepine (GABA BZ) and dopamine 2-like (D2) receptors were performed using cellular membranes according to previously published methods, using microplate wells containing a 1.0 μM GF/B glass filter (MultiScreenHTS, Boston, MA, USA) (Basu et al., 2009b). For GABA BZ, samples were incubated with [3H]-Flunitrazepam (2 nM), for 30 min at 4 °C with slow shaking. Non-specific binding was determined by incubating half the samples