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Full Length Research Paper

Developmental methylmercury exposure affects avoidance learning outcomes in adult zebrafish

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The present study investigated the neurobehavioral effects of embryonic exposure to methylmercury (MeHg) in zebrafish using avoidance conditioning as the behavioral paradigm. In this study, adult zebrafish developmentally exposed as embryos to 0.00, 0.01, 0.03, 0.1, or 0.3 μM of MeHg were trained and tested for avoidance responses. The results showed that control zebrafish hatched from embryos unexposed to MeHg learned avoidance responses during training and showed significantly increased avoidance responses during testing. Zebrafish developmentally exposed to MeHg as embryos were hyperactive as they frequently swam back and forth, and showed no significant changes in avoidance responses from training to testing. Results of the present study suggested that embryonic methylmercury exposure produced hyperactivity and impaired avoidance learning.

Key words: Active avoidance conditioning, methylmercury, zebrafish, fish shuttle-box.

INTRODUCTION

Human exposure to mercury (Hg^{2+}) is a worldwide problem due not only to the multiple pathways by which Hg^{2+} enters the environment, e.g., industrial, coal burning electrical power plants and mining, and its ability to biomagnify in higher trophic levels of the food chain; but also its propensity to disperse globally to become a transboundary contaminant (Ryaboshapko et al., 2007; Seigneur et al., 2003, 2004). Significant exposures have been recorded in a wide range of locales throughout the world (Barbieri et al., 2009; Bose-O'Reilly et al., 2010a, b; Fakour et al., 2010; Laks, 2009; Papu-Zamxaka et al., 2010). Moreover, it is possible that even low levels of Hg^{2+} exposure can lead to changes in behavioral responses (Echeverria et al., 1995).

Anaerobic bacteria, e.g., sulfate reducers, iron reducers and methanogens, have been linked to mercury methylation in aquatic systems (Avramescu et al., 2011). Methylmercury (MeHg) biomagnifies through the food

chain, and can reach human populations via the consumption of contaminated fish and seafood. MeHg exposure through maternal transfer can induce neurological damage to the developing fetus (Cagiano et al., 1990), and such deficits may not manifest themselves until much later (Rice, 1996). A variety of exposure regimens, therefore, has been used to identify adult learning deficiencies due to developmental exposures to MeHg. These experimental designs include gestational (Cuomo et al., 1984; Eccles and Annau, 1982; Fredriksson et al., 1996; Gilbert et al., 1993, 1996; Reed et al., 2008; Reed and Newland, 2007), perinatal (Falluel-Morel et al., 2007) and gestational through postnatal MeHg exposures (Goulet et al., 2003; Onischenko et al., 2007; Rice, 1992). Among the behavioral changes in adults that have been exposed to MeHg, are decreases in reference memory, short-term memory, avoidance and logical memory, perseverative behaviors and increases in anxiety levels (Chang et al., 2008; Hilt et al., 2009; Onishchenko et al., 2007; Rice, 1992), although some researchers report only small changes in these neuropsychological outcomes (Rohling and Demakis,

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2006). It is important, therefore, to further investigate whether these developmental exposures induce adult learning deficits.

Unlike mammalian systems in which there are linkages between maternal and fetal health during gestational MeHg exposures, fish, because of external fertilization and embryo development, allow researchers to use waterborne exposures with newly fertilized eggs to separate these potential interactive effects, e.g., maternal bioaccumulation or exposure to other environmental stressors, and isolate specific periods of development that are especially sensitive to toxic exposure. Smith et al. (2010) used zebrafish to identify critical windows of sensitivity to acute, environmentally relevant, developmental MeHg exposures; and evaluate long-term effects of the developmental exposure on spatial learning.

Given the similarity of fundamental behavioral mechanisms across vertebrate phyla, zebrafish (*Danio rerio*) have become a widely used vertebrate model system for examining learning and memory (Gómez-Lapaza and Gerlai, 2009; Salas et al., 2006; Sison and Gerlai, 2010; Williams et al., 2002; Xu et al., 2007). Fish models of learning and memory have strong similarities to mammalian models in both phenotypic expression and underlying physiological mechanisms. The regions in the teleost brain responsible for directing those behaviors have many anatomical parallels to mammals, specifically the mammalian hippocampus and amygdala which are homologous to the dorsolateral and dorsomedial telencephalon, respectively, in fishes (Portavella et al., 2002). Similar to the role of the mammalian hippocampus in spatial learning, the dorsolateral region of the teleost telencephalon is critical for spatial learning to identify foraging areas, nesting locations, and landmarks (Dodson, 1988; Salas et al., 1996). Similar to the role of the mammalian amygdala in fear learning, the dorsomedial telencephalon is critical for avoidance learning as demonstrated in ablation experiments and microinjection studies using goldfish (Duran et al., 2008; Portavella and Vargas, 2005; Xu et al., 2003, 2009). Our previous work with zebrafish (Smith et al., 2010) showed that embryonic exposure to MeHg induced learning deficits in a spatial alternation task, a task that involves the dorsolateral telencephalon. To extend our previous work, the present study with zebrafish investigated the neurobehavioral effects of embryonic exposure to MeHg in active avoidance conditioning, a task that involves the dorsomedial telencephalon.

MATERIALS AND METHODS

Breeding and egg collection

Adult zebrafish (Ekkwill Waterlife Resources, Gibsonton, FL) were acclimated for several weeks prior to the initiation of experiments. Fish were maintained at 26 to 28°C on a 14-h light and 10-h dark cycle in a flow-through buffered, dechlorinated water system at the Aquatic Animal Facility of the University of Wisconsin-Milwaukee Children's Environmental Health Sciences Center. All experimental

procedures were approved by the University of Wisconsin-Milwaukee Animal Care and Use Committee. Zebrafish were bred in 2 L plastic aquaria with a 1/8" nylon mesh false bottom to protect fertilized eggs from being consumed by the adults. Eggs were collected \leq 2 h post fertilization (hpf) and placed into metal-free, glass culture dishes (100 mm diameter \times 50 mm depth) in E2 medium (each liter contains 0.875 g NaCl, 0.038 g KCl, 0.120 g MgSO₄, 0.021 g KH₂PO₄, and 0.006 g Na₂HPO₄; Nüsslein-Volhard 2002).

Exposure regimen

Methylmercury (MeHg; >98% purity) was obtained from ICN Biomedicals (Aurora, OH). Collected eggs (< 2 hpf) were rinsed twice in MeHg-free E2 medium (as determined by ICP-MS analysis) and transferred to metal-free glass dish (100 mm diameter \times 50 mm depth) containing 100 ml of E2 medium with MeHg at 0.0, 0.01, 0.03, 0.10, or 0.30 μ M MeHg. These levels of developmental MeHg exposures were found to alter adult zebrafish visual startle responses (Weber et al., 2008). Higher concentrations were not used as these were at or above the LC₅₀ (personal observation). At 24 h pf the embryos were rinsed in MeHg-free E2 medium and then raised in MeHg-free E2 medium. Fry were fed vinegar eels twice each day starting at day 5 post hatch regardless of treatment until large enough to consume *Artemia nauplii*. Juveniles and adults were fed Aquarian™ flake food (Aquarium Pharmaceuticals, Inc., Chalfont, PA) in the morning and *Artemia nauplii* in the afternoon. Based on this and previous studies, there are no significant differences in embryo, larval, juvenile, or adult mortality or number of developmental malformations at the stated concentrations of MeHg.

Housing during avoidance conditioning

During behavioral experiments, adult zebrafish hatched from the embryos described above were kept in individual compartments of partitioned tanks at 26 \pm 1°C with a 12 h light-dark cycle (0700 to 1900 light) at the fish laboratory of Grand Valley State University. The behavioral experiments were conducted during the light cycle and all experimental procedures were approved by the Grand Valley State University Institutional Animal Care and Use Committee.

Apparatus for avoidance conditioning

Zebrafish were trained and tested individually in two identical zebrafish shuttle-boxes connected to a programmer/shocker unit. The zebrafish shuttle-box consisted of a water-filled tank (18 cm in length \times 7.5 cm in width \times 10 cm in height) separated by an opaque divider (7.5 cm in width \times 10 cm in height) into two equal compartments. The divider was raised 0.6 cm above the floor of the tank during trials allowing zebrafish to swim freely from one side of the tank to the other. The crossing movement of zebrafish was monitored by infrared light beams and their corresponding detectors located on the long sides of the tank. There was a light at each end of the tank and there were two stainless steel electrode plates (6.5 cm in length \times 4 cm in height) at each of the long sides of each compartment.

Active avoidance paradigm

Zebrafish were placed in the shuttle-boxes for 5 min, and then a trial began with the onset of the light, the conditioned stimulus (CS),

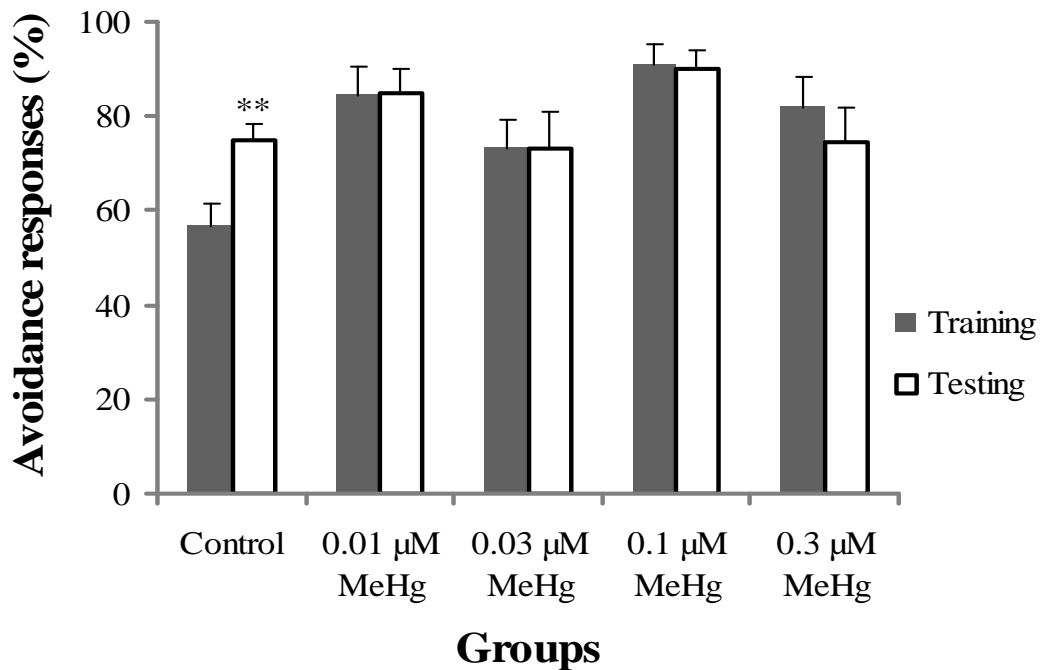


Figure 1. Avoidance responses of adult zebrafish hatched from embryos exposed to no MeHg or various levels of MeHg. Each bar represents the mean percentage of avoidance responses \pm SE for 8 to 29 fish. ** $p < 0.01$, compared testing against training of the same group.

on the side of the fish's location and the manually raised divider 0.6 cm above the floor of the tank. After the light was on for 12 s, a repetitive mild electrical shock (0.73 V/cm AC, pulsed 100 ms on and 1400 ms off), the unconditioned stimulus (US), was administered, along with the light, for 12 s through the water by means of electrodes. At the end of 24 s or at a crossing response by zebrafish during the 24 s, the trial ended with both the light and electrical shock switched off and the divider lowered. After an intertrial interval (ITI) ranging from 12 up to 36 s, another trial began.

Zebrafish initially swam through the raised divider only after receiving several shocks. The crossing response made after the onset of both light signal and electrical shock to escape the electrical body shock is defined as an escape response. During the training sessions, zebrafish gradually learned to swim from the lighted end to the dark end to avoid the electrical body shock. The crossing response made after the onset of the light signal, but before the onset of electrical shock to avoid the electrical body shock, is defined as an avoidance response. The time it takes for zebrafish to make the crossing response following the onset of the light signal is defined as crossing latency. The measurements were the number of avoidances and escapes; and crossing latency. Except the manually raised dividers, all experiments were automated through the programmer/shocker unit and a Gateway 2000 P5-100 computer that programmed stimuli, monitored and recorded behavior of zebrafish.

Zebrafish were trained on Behavioral Experimental Day 1, and tested on Behavioral Experimental Day 3. The training session consisted of 30 trials, and the testing session consisted of 10 trials. Percentage of avoidance responses and crossing latency were used as indicators of learning. Two-way ANOVAs with one between factor (different groups) and one repeated measures (training vs. testing) on the results were carried out first to determine possible significant differences, followed by one-way ANOVAs to determine

any significant differences among groups and correlated t-tests to determine any significant differences between training and testing.

RESULTS

Figure 1 showed avoidance responses of the five groups of zebrafish during both training and testing. A two-way ANOVA with one between factor (5 groups) and one repeated measures (2 sessions) on the avoidance responses indicated a significant group difference [$F(4, 75) = 3.381$, $p < 0.01$], and a significant group \times session interaction [$F(4, 75) = 3.211$, $p < 0.05$]. A one-way ANOVA with multiple comparisons on the avoidance responses of groups during the training session showed significant differences between the control group exposed to 0.0 μ M MeHg and MeHg exposed groups [$F(4, 75) = 5.596$, $p < 0.01$], while another one-way ANOVA with multiple comparisons on the avoidance responses of groups during the testing session showed no significant difference among groups. More importantly, correlated t-tests comparing testing and training of each group showed that the control fish learned avoidance responses during training and showed significant increases in avoidance responses during testing ($p < 0.01$) (Figure 1), while zebrafish developmentally exposed as embryos to MeHg were hyperactive as they frequently swam back and forth and showed no significant changes in avoidance responses from training to testing (Figure 1).

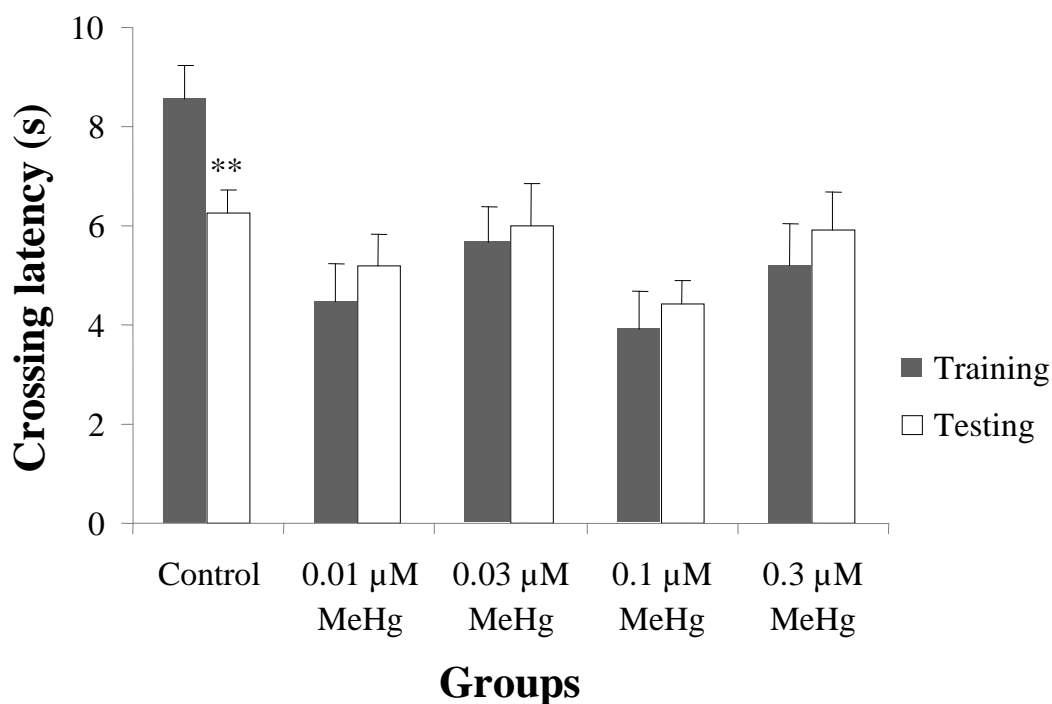


Figure 2. Crossing latency of adult zebrafish hatched from embryos exposed to no MeHg or various levels of MeHg. Each bar represents the mean crossing latency \pm SE for 8 to 29 fish. **, $p < 0.01$, compared testing against training of the same group.

The crossing latency results showed the similar pattern (Figure 2). A two-way ANOVA with one between factor (5 groups) and one repeated measures (2 sessions) on the crossing latency indicated a significant group difference [$F(4, 75) = 4.006$, $p < 0.01$], and a significant group \times session interaction [$F(4, 75) = 3.838$, $p < 0.01$]. A one-way ANOVA with multiple comparisons on the crossing latency of groups during the training session showed significant differences between the control group and MeHg exposed groups [$F(4, 75) = 6.174$, $p < 0.01$], while another one-way ANOVA with multiple comparisons on the crossing latency of groups during the testing session showed no significant difference among groups. Correlated t-tests comparing testing and training of each group showed that the control fish learned avoidance responses during training and showed significant decreases in crossing latency during testing ($p < 0.01$), while zebrafish developmentally exposed as embryos to MeHg showed slight, but not significant, increases in crossing latency from training to testing.

For the reason that MeHg exposed zebrafish displayed relatively high avoidance responses during the training session, the number of avoidance responses over six blocks of five trials during training were plotted in Figure 3. Figure 3 showed that the number of avoidance responses did not change from the block of the first five trials to the block of the last five trials except the 0.1 μM MeHg group that showed a decrease trend in avoidance

responses (Figure 3C).

DISCUSSION

The current study showed that adult control zebrafish that hatched from embryos unexposed to MeHg learned avoidance responses and displayed significant increases in avoidance responses from training to testing, confirming previous reports (Xu et al., 2007; Xu and Goetz, 2012). These zebrafish also showed significant decreases in crossing latency from training to testing, further indicating that these zebrafish learned to associate the CS of light with the US of shock and responded to the CS by readily crossing the divider to avoid the US. However, zebrafish hatched from embryos exposed to MeHg were hyperactive and showed no significant changes in either avoidance responses or crossing latency from training to testing.

The levels of developmental MeHg exposures from 0.01, 0.03, 0.1, to 0.3 μM used in the current study all produced impaired learning, even the lowest level. The same levels of MeHg exposures in adult zebrafish impair avoidance conditioning at the concentration of 0.03 μM or higher MeHg exposure in old adult and 0.1 μM or higher MeHg exposure in young adult zebrafish (unpublished results). Thus, zebrafish appear more sensitive to developmental MeHg exposure and show behavioral

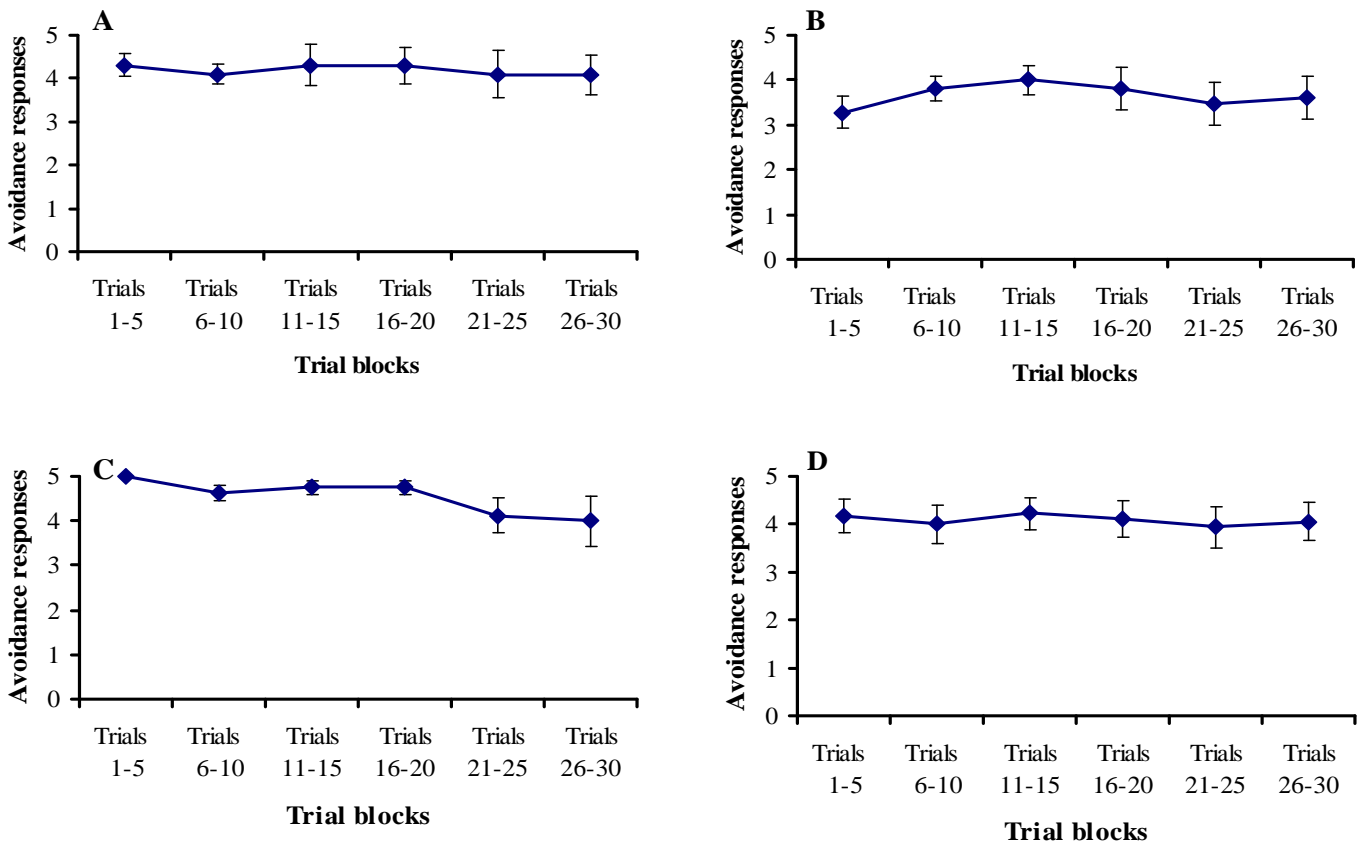


Figure 3. Avoidance responses of MeHg exposed zebrafish during the training session. Panels A, B, C, and D show avoidance responses of 0.01, 0.03, 0.1, and 0.3 μM MeHg groups, respectively. Each data point represents the mean number of avoidance responses in five trials ± SE for 8 to 18 fish.

impairments at lower levels of MeHg exposures. The developmental MeHg exposures also produced hyperactivity. Those zebrafish were observed to constantly swim back and forth during experiments, which confirmed the finding that developmental exposure to low doses of MeHg produces long-lasting hyperactivity in rats (Vitalone et al., 2010). In fact, the hyperactivity led to relatively high levels of avoidance responses displayed by MeHg exposed zebrafish throughout behavioral experiments. When the results from the 30 trials during the training session were divided into six blocks of five trials, the number of avoidance responses did not change over the six blocks in all MeHg exposed zebrafish except the 0.1 μM MeHg group that showed a slightly decrease trend in avoidance responses from the first block of five trials towards the last block of five trials. Thus, relatively high levels of avoidance responses displayed by MeHg exposed zebrafish during training was not the result of learning, rather due to general hyperactivity. Such general hyperactivity was not observed in adult zebrafish exposed to MeHg as adults (unpublished results).

The teleost telencephalon is a simple structure homologous to the limbic structures of higher vertebrates

(Northcutt and Bradford, 1980; Schroeder, 1980). Comparative studies suggest that the dorsolateral and dorsomedial areas of the teleost telencephalon are homologous to the mammalian hippocampus and amygdale, respectively. Dorsolateral ablation of the goldfish telencephalon impaired spatial learning but not avoidance learning, whereas dorsomedial ablation of the goldfish telencephalon impaired avoidance learning but not spatial learning (Portavella et al., 2002). Our previous work with zebrafish showed that developmental MeHg exposures impaired spatial learning (Smith et al., 2010). The current study showed that the same developmental MeHg exposures also impaired avoidance learning. Additionally, using a variety of learning paradigms, e.g., conditioned aversion, conditioned reinforcement tasks, and prey-capturing abilities, behavioral impairments in fish have been identified after exposure to environmental contaminants, including MeHg (Atchison et al., 1987; Hartman, 1978; Salzinger et al., 1973; Webber and Haines, 2003; Weir and Hine, 1970; Zhou et al., 2001). Thus, our data in conjunction with other studies suggest a widespread effect in the brain due to developmental MeHg exposures.

Zebrafish has become a useful organism for studying the effects of environmental contaminants on the neurobehavioral development of an organism due to its short generation times, high numbers of eggs per female, ease of breeding, and short developmental periods before hatching. It will be interesting to see how the neurobehavioral effects of embryonic MeHg exposure are manifested throughout the life span of zebrafish. Thus, future studies will examine learning and memory of zebrafish at the different stages of the life-span, e.g., from early to young to old adulthood, following embryonic MeHg exposure.

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