Fundamental Toxicological Sciences (Fundam. Toxicol. Sci.)
Vol.2, No.2, 67-78, 2015

Original Article

Effect of prenatal methylmercury exposure on neurobehavioral development in male mice: comparison between methylmercury in fish and methylmercury chloride added to diets

Miyuki Iwai-Shimada[1,2,3], Michiaki Yamashita[4], Naoyuki Kurokawa[1], Kunihiko Nakai[1,5], Mitsuharu Ishida[6], Akira Naganuma[3] and Hiroshi Satoh[1,7]

[1]Environmental Health Sciences, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan
[2]Research Fellow of the Japan Society for the Promotion of Science, 8 Ichiban-cho, Chiyoda-ku, Tokyo 102-8472, Japan
[3]Laboratory of Molecular and Biochemical Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3 Aramakiazaaoba, Aoba-ku, Sendai, Miyagi 980-8578, Japan
[4]National Research Institute of Fisheries Science, 2-12-4 Fukuura, Yokohama, Kanagawa, 236-8648, Japan
[5]Present address: Department of Development and Environmental Medicine, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8575, Japan
[6]Department of Food Management, School of Food, Agricultural and Environmental Sciences, Miyagi University, 2-2-1 Hatatate, Taihaku, Sendai, Miyagi 982-0215, Japan
[7]Currently Professor Emeritus, Tohoku University, Sendai, Japan

(Received March 15, 2015; Accepted March 18, 2015)

ABSTRACT — While the primary source of human MeHg exposure is the consumption of fish contaminated with MeHg, it is unknown whether the toxicity of MeHg in fish is equivalent to that of MeHg chloride (MeHgCl) experimentally added to the diet. We investigated developmental and behavioral effects of MeHg derived from fish and MeHgCl added to various diets during the prenatal period in mice from GD 0 to GD 17. From 7 to 9 female C57BL/6NCr mice were assigned to each of the following exposure groups: Control (CL), CL+MeHgCl (CL+MeHg, 1.6 mgHg/kg), low MeHg tuna (LT, 0.2 mgHg/kg), LT+MeHgCl (LT+MeHg, 1.6 mgHg/kg), and high MeHg tuna (HT, 1.6 mgHg/kg). In pups, body weight was depressed and elevated by MeHg exposure in the CL+MeHg and the LT, respectively, compared with other three groups. In neurodevelopmental test, the righting reflex of 4 groups other than CL showed the facilitated developments compared to the CL. The cliff avoidance of the HT developed slower than in the CL+MeHg, LT and LT+MeHg. In water maze test, the swimming speed of the HT decreased in comparison with the CL in males but not females. The latency until falling from a rotating rod of the LT+MeHg was significantly shorter than that of the LT in males but not females. Our results are suggesting the possibility that the toxicological profiles of MeHg derived from fish and reagent MeHg are somewhat different. Our findings also provide evidence that males are more susceptible than females to prenatal MeHg exposure.

Key words: Methylmercury derived from fish, Prenatal exposure, Neurodevelopment, Mouse, Susceptibility of the male

INTRODUCTION

Methylmercury (MeHg) is a ubiquitous environmental neurotoxicant. The main route of exposure to MeHg is food, particularly fish and fish products. It is readily absorbed and distributed throughout the body, easily penetrating the blood-brain and placental barriers. The susceptibility of the developing central nervous system (CNS) to MeHg is well established through epidemiological and experimental evidence (NRC, 2000). The pri-

Correspondence: Miyuki Iwai-Shimada (E-mail: miyuki@m.tohoku.ac.jp)

mary dietary source of long-chain polyunsaturated fatty acids (PUFAs), which are critical for normal brain development (Innis, 2007), is also fish. Therefore it is important that fish consumption is such that benefits are maximised while risks are minimized (EFSA, 2015).

Despite the fact that the adverse effects of exposure to MeHg via fish and fish products on neurobehavioral development in humans are a serious concern, there have been few animal experimental studies to examine the effects of MeHg exposure using fish as the exposure source of MeHg. Typically, MeHg compounds are most often administrated subcutaneously, intraperitoneally, intravenously, or dissolved in drinking water, which are not the natural routes for MeHg entrance into the body. There was only one report by Olson and Boush (1975) to compare the effect between MeHg in fish and MeHg hydroxide added to diet. The results indicated that retarded maturation of offspring rats was observed in the group perinatally exposed to fish MeHg (2 mg/kg in diets) with respect to swimming behavior and righting reflexes compared to MeHg hydroxide added to diet and control diet groups. These findings suggested that the toxic effect of MeHg in fish might be different from the toxic effects due to the exposure to MeHg reagents experimentally administered. In the previous study using fish meat might have caused nutritional confounding against MeHg effects. Actually, the Seychelles Child Development Nutrition Study suggested that maternal nutritional status could modulate the relationship between prenatal MeHg exposure and developmental outcomes in children (Strain *et al*., 2012).

In the present study, we compared the developmental effects of MeHg derived from fish with those by methylmercury chloride (MeHgCl) added to diets. We chose two kinds of tuna with low and high MeHg contents for the MeHg source to minimize nutritional confounding of the diets. For assessment of MeHg-induced toxicity, we observed various developmental landmarks and reflexes before weaning. We also examined the animals using the open-field test, Moris' water maze test, and rotor rod test in immature stages after weaning.

MATERIALS AND METHODS

Preparation of the mouse diets

We prepared two kinds of granular myofibrillar protein from young Pacific bluefin tuna (*Thunnus olientalis,* about 1 year old and the average fish weight was 2.9 kg) and bigeye tuna (*Thunnus obesus*) by washing the minced meat. Moreover, these granules were lyophilized (FD-550, Tokyo Rikakikai Co., LTD., Tokyo, Japan), powderized using a crusher (IFM-800DG, Iwatani Corporation, Tokyo, Japan), and adjusted to a particle size of < 500 μm. The average total mercury (THg) concentrations in fresh fish meat of young Pacific bluefin tuna (PBT) and big eye tuna (BET) were 0.13 mg/kg-wet (n = 63) and 1.4 mg/kg-wet, respectively. The average THg (MeHg) concentrations in the powders of PBT and BET were mean 0.86 (0.83) μg/g-wet and 7.91 (7.61) μg/g-wet, respectively (n = 2). The percentage of MeHg in THg was more than 95%.

We used AIN93G (Oriental Yeast Co., LTD., Tokyo, Japan) as a purified diet for growth, pregnancy and lactation. AIN93G contains 20% casein as a protein source. Instead of the 20% casein in AIN93G, powdered PBT or BET, was added to the modified AIN93G (without 20% casein) along with required phosphorus (0.3 g/100 g). The measurements of nutrition and fatty acids were carried out by Japan Food Research Laboratories (Tokyo, Japan). Metals in the diets were analyzed by IDEA Consultants, Inc. (Tokyo, Japan). Methylmercury (II) chloride (purity 95%) was purchased from Kanto Chemical Co. Inc. (Tokyo, Japan) and added to these diets as necessary.

Animals and exposure

Male and female C57BL/6NCr mice were purchased from a commercial breeder (Nippon SLC Co. Ltd., Hamamatsu, Japan) at the age of 9 weeks. Upon arrival at our laboratory, all the mice were housed individually in plastic cages. They were kept in a temperature-controlled room (23 ± 2°C) with a 12-hr light-dark cycle (light phase, 08:00-20:00). The AIN93G diet and tap water were available *ad libitum*. Mating began 7 days after arrival when each female mouse was paired with an unexposed male mouse. The same male-female pairs were housed together until confirming the presence of a vaginal plug, designating gestational day (GD) 0.

From 7 to 9 female mice were assigned to each of the following exposure groups: (1) AIN93G (control, CL), (2) AIN93G+MeHgCl (to make 1.6 mgHg/kg-dry, CL+MeHg), (3) modified AIN93G (no casein and added phosphorus) +PBT (LT: low Hg tuna, 0.2 mgHg/kg-dry), (4) LT+MeHgCl (to make 1.6 mgHg/kg-dry, LT+MeHg), and (5) modified AIN93G+BET (HT: high Hg tuna, 1.6 mgHg/kg-dry). The pregnant mice were fed one of the diets from GD 0 to GD 17. Thereafter, they were given the control diet (AIN93G). The day of parturition was defined as PND 0. On PND 4, the litter size was adjusted to 4-6 mice and approximately half the mice of both sexes were assigned for preweaning developmental assessment and postnatal behavioral tests. The offspring mice were

weaned on PND 21. All the dams were killed at weaning and implantation sites were counted. At 5 weeks of age, mouse behaviors are steady and reproducible. Thus, two mice of each sex from each litter were used for the three behavioral tests.

All animals were handled by the same experienced person to accustom them to being handled. This study was carried out in accordance with the guidelines of the Guide for Animal Experimentation of Tohoku University Graduate School of Medicine.

Neurobehavioral test

Preweaning developmental assessment

Before weaning, physical and neurobehavioral development were assessed on PNDs 7, 10, 12, 14 and 16 for the mice of both sexes. For the assessment of physical development, the following were observed and recorded; pinna detachment (complete detachment of the pinna of both ears was observed), hair growth (hair growth in the trunk was evaluated), eye opening (bilateral eye opening was evaluated), and incisor eruption (the occurrence of incisor eruption was evaluated visually). For the neurobehavioral development, the pups were assessed according to the method described by Sugawara *et al*. (2008). The tests were conducted during the light phase between 10:00 and 18:00, with each mouse subjected to a test at approximately the same time of day. The following reflexes and responses were examined; the walking (a pup moves using its four feet with its abdomen not touching the ground), righting reflex (a pup quickly returns to its four feet when placed on its back), negative geotaxis (a pup turns around 180° when placed on a board inclined at 45° with its head pointing downward), cliff avoidance (a pup turns and crawls away from the edge of a cliff when placed with its forepaws beyond the edge) , and grasp reflex [a pup holds itself from a steel wire (diameter, 1.0 mm) using its forepaws]. Since there was no significant difference in the body weight between males and females during preweaning developmental assessment, we did not separate the sexes in the analyses.

Open-field test

The open-field test was carried out as described by Sugawara *et al*. (2008) on PND36. The apparatus used was a square white wooden device (50 cm × 50 cm × 33 cm). During the experiment, the open field was illuminated at 800 lx using a fluorescent lamp placed 1.5 m above the apparatus. Each mouse was transferred from its home cage directly to the center of the open field, and being covered with a small box made of opaque plexiglas. After the box was removed, the movement of each mouse was recorded for 2 min using a CCD camera. The recorded movement was then analyzed using an image analyzer (LimeLight, Neuroscience Inc., Tokyo, Japan). The latency before the start of walking was determined, a distance traveled by the mouse and mean walking speed were calculated over the period of 2 min. The frequencies of defecation and urine traces in the open field were determined by the observer. Before each trial, the floor was cleaned with 70% ethanol and then wiped with wet cotton to prevent possible bias due to odor clues left by previous mice. To minimize the possible effects of circadian changes on open-field behavior, all trials were carried out between 11:00 and 14:00.

Water maze test

Two days after the open-field test, the mice were subjected to the water maze test as described by Sugawara *et al*. (2008). The water maze consisted of a circular white plastic pool 100 cm diameter. The plastic pool was filled with tap water adjusted to approximately 20°C. A 10-cm-diameter white platform was placed 0.5 cm beneath the water surface at the center of one quadrant of the pool. Different visual cues were placed around the pool. Each mouse was released into the water at a fixed position inside the pool wall. A CCD camera mounted above the center of the pool was used to record the movements of each mouse. Then, the recorded movements were analyzed using an image analyzer (WaterMaze, Neuroscience Inc.). The time taken and distance traveled to reach the submerged platform, and the mean swimming speed were calculated. The mice practiced swimming in the pool on the first day. From the next day, the mice were subjected to three trials per day for 5 days. When a mouse could not find the platform within 2 min, the test was discontinued and the mouse was placed on the platform and left there for 10 sec. In this case, the time to reach the platform was considered to be 2 min.

Rotor rod test

For assessment of the rotor rod performance, we referred to the method described by Bellum (2007); however, we did not use a plastic cover on the rod. Balance and motor coordination were evaluated using rotor rod (47600, Ugo Basile Srl., Varese, Italy). The mice were placed on a rod that rotated at 3 rpm at 0 min, and accelerated to 26 rpm after 3 min of run time, which continued for 5 min. Starting at PND44, the time until falling was measured for 3 consecutive days, with each mouse experiencing four trials per day with intertrial intervals of 10 min.

Mercury and selenium analyses

With 0.5-1 mL of distilled water, tissues were homogenized before analyzing. The sample analyses for THg and selenium (Se) in the diets, mouse brain and blood were performed at our laboratory. THg was measured by cold vapor atomic absorption spectrometry (CVAAS, HG-201, Sanso Seisakusho Co. Ltd., Tokyo, Japan). The analytical method of CVAAS has been described elsewhere (For details, see Ministry of the Environment, Japan [2004]). Se was measured fluorometrically as a 2,3-diaminonaphtalene derivative (Watkinson, 1966). The MeHg concentrations in the fish powders and diets were determined at the International Mercury Lab, Ltd. (Minamata, Japan). MeHg in these samples was measured by gas chromatography with electron capture detection (Akagi *et al*., 2000). Sample analysis was carried out in duplicate, and reanalysis was done when the difference from the average was more than 5%. Accuracy was ensured using a certified reference material (DOLT-4: dogfish liver, NRC, Canada) as the quality control material. The THg concentration (CV, *n*) was determined to be 2.69 ± 0.06 ng/mg (2.2%, n = 4) as compared to the recommended value of 2.58 ± 0.22 ng/mg. When we used another certified reference material (TORT-2: Lobster hepatopancreas, NRC, Canada), the THg level was determined to be 0.29 ± 0.01 ng/mg (3.4%, n = 4) as compared to the recommended value of 0.27 ± 0.06 ng/mg, and the Se concentration was 5.39 ± 0.18 ng/mg (3.3%, n = 6) as compared to the recommended value of 5.63 ± 0.67 ng/mg. We additively used a certified reference material with low Se (IAEA-A-13: animal blood, IAEA), and the Se concentration was determined to be 0.21 ± 0.01 ng/mg (4.8%, n = 6) as compared to the recommended value (range) of 0.24 (0.15-0.31) ng/mg.

Statistical analyses

With regard to data on preweaning physical and neurobehavioral development, the appearance of complete somatic features and adultlike responses were evaluated by logistic regression analysis. To assess the differences between MeHg in fish and MeHgCl added to diets in logistic regression analysis, we make comparisons between each groups. We calculated regression coefficient (β), odds ratio (OR) and 95% confidence interval (CI).

ANOVA was performed to determine statistically significant effects of exposures on reproductive performance, and open field test results (one-way), and on body weight, water maze test results, and rotor rod test data (two-way repeated measures). Thereafter differences among groups were assessed by Tukey multiple comparison. The software package JMP9.0 (SAS Institute Inc., Cary, NC, USA) was used to analyze the data. $P < 0.05$ was considered statistically significant.

RESULTS

The characteristics of the fish powder and the diets used are summarized in Tables 1 and 2. These diets were almost nutritionally equivalent. In the fish powder diets, the Se concentrations were slightly higher (Table 1). Table 1 also shows the metal concentrations in the diets. In additon, the fish-added diets characteristically contained fatty acids such as EPA and DHA derived from fish (Table 2). The average THg concentrations (SD, *n*) in the diet of CL+MeHg, LT, LT+MeHg and HT were 1.36 (0.03, 6), 0.17 (0.02, 6), 1.40 (0.02, 6) and 1.43 (0.03, 5) ng/mg-wet, respectively. THg in CL diet was not detected.

Table 3 shows the reproductive performances. We observed neglect of the pups and/or cannibalism in CL+MeHg and HT. No differences among groups were found for the length of pregnancy and the numbers of males and females pups. However, the numbers of implantation sites and surviving pups on PND4 were affected by the diet. In particular, the number of implantation sites in HT and the total number of surviving mice on PND4 were larger than those of CL ($P < 0.05$).

Figure 1 shows the changes in the body weight of dams during pregnancy (a) and offspring mice in the lactational period (b). The body weight gain of dams did not significantly differ among the groups during gestation. For the pups, ANOVA followed by the Tukey multiple comparison test showed that body weight was depressed and elevated in the CL+MeHg and the LT, respectively, compared with other three groups.

Different diets had no effect on the physical features observed before weaning, i.e., eye opening, pinna detachment, hair growth, or incisor eruption (data not shown). Figure 2 shows the neurobehavioral development in offspring mice. Logistic regression analyses of the results were judged by ORs. The walking test (Fig. 2a) revealed that the LT walked earlier than the CL and CL+MeH*g*. The righting reflex in the CL+MeHg, LT, LT+MeHg and HT developed earlier than in the CL (Fig. 2b). The negative geotaxis of the CL+MeHg and LT developed earlier than in the CL and HT (Fig. 2c). The cliff avoidance of the HT developed slower than in the CL+MeHg, LT and LT+MeHg groups (Fig. 2d). The development of grasp reflex in the LT+MeHg delayed in comparison with the CL+MeHg (Fig. 2e).

Figure 3 shows result in open-field test. The frequencies of defecation and urination showed no significant

Table 1. The nutritional compositions and metal concentrations in fish powders and diets.

	Lyophilized flesh		Diet		
	PBT	BET	AIN93G	AIN93G[a]+PBT	AIN93G[a]+BET
Basic nutrition					
Moisture (%)	9.3	8.1	14.8	21.7	17.3
Protein (%)	86.3	88.1	16.4	14.8	16.7
Fat (%)	6.3	4.8	4.4	6.6	6.3
Fiber (%)	< 0.1	< 0.1	1.3	1.1	1.0
Ash (%)	0.8	0.6	2.3	2.3	2.4
Calories (kcal/100g)	551	557	405	372	391
Vitamin B_6 (mg/100g)	N.A.	N.A.	0.59	0.80	0.75
Choline (%)	N.A.	N.A.	0.09	0.11	0.11
Metal concentration					
As (ng/g)	811	306	< 100	150	< 100
Ca (μg/g)	492	395	4930	4420	4670
Cd (ng/g)	8.3	72.8	7.5	9.6	19.3
Cu (ng/g)	2719	900	4305	6527	5752
Fe (μg/g)	15.2	17.6	40.4	37.0	40.5
K (μg/g)	N.A.	N.A.	3310	3760	3960
Mg (μg/g)	401	444	462	482	527
Mn (ng/g)	213	135	8726	8561	8548
P (μg/g)	2230	1250	3010	3050	3050
Pb (ng/g)	17.0	16.8	14.9	11.7	10.6
Se (ng/g)	1336	2548	209	378	519
Sn (ng/g)	65.8	23.9	< 20	< 20	< 20
Zn (ng/g)	15995	15875	32823	33597	33516

N.A.: not analyzed, PBT: Young Pacific bluefin tuna was used for the LT and LT+MeHg groups, BET: Big eye tuna was used for the HT group. [a] modified AIN93G: Instead of casein protein either fish powder was added.

Table 2. The fatty acid compositions in fish powders and diets.

Fatty acids (mg/100g)			Lyophilized		Diet		
			PBT	BET	AIN93G	AIN93G[a]+PBT	AIN93G[a]+BET
Saturated							
lauric		C12 : 0	0	0	4.7	0.0	0.0
myristic		C14 : 0	184.4	68.2	16.0	42.9	28.1
palmitic		C16 : 0	1376.6	1048.2	547.4	889.8	838.0
stearic		C18 : 0	412.4	335.1	220.6	334.0	320.8
arachic		C20 : 0	11.5	17.5	16.5	22.8	23.6
behenic		C22 : 0	0	0	18.6	24.6	23.8
lignoceric		C24 : 0	0	0	5.2	7.2	7.8
Monounsaturated							
myristoleic		C14 : 1	10.1	11.2	0.0	0.0	0.0
palmitoleic		C16 : 1	189.9	160.9	6.7	42.0	40.7
oleic		C18 : 1	544.5	1185	1073.5	1441.2	1490.5
eicosenoic		C20 : 1	113.3	133.5	9.2	34.9	37.9
erucic		C22 : 1	18.3	15	4.7	7.4	7.4
nervonic		C24 : 1	31.2	32	0.0	6.4	7.6
Polyunsaturated							
linoleic	n-6	C18 : 2	46.3	21.6	2580.5	3281.7	3190.3
γ-linolenic	n-6	C18 : 3	11	14.1	0.0	0.0	7.7
eicosadienoic	n-6	C20 : 2	17.9	14.6	0.0	5.6	5.3
arachidonic	n-6	C20 : 4	86.7	115.6	0.0	20.3	23.9
docosatetraenoic	n-6	C22 : 4	11	25.4	0.0	0.0	5.2
linolenic	n-3	C18 : 3	26.6	0	369.2	478.2	461.2
eicosapentaenoic[b]	n-3	C20 : 5	493.1	154.7	0.0	110.2	58.9
docosapentaenoic	n-3	C22 : 5	87.2	54.1	0.0	18.6	14.0
docosahexaenoic[c]	n-3	C22 : 6	1814.7	750.5	0.0	505.9	277.0

PBT: Young Pacific bluefin tuna was used for the LT and LT+MeHg groups, BET: Big eye tuna was used for the HT group. [a] modified AIN93G: Instead of casein protein either fish powder was added, [b]EPA, [c]DHA.

Table 3. Reproductive performances.

Groups	No. of mated females	No. of dams who gave birth	No. of child rearing dams	Implantation site (n)	No. of surviving mice on PND4 (n)
CL	7	7	7	7.3 ± 0.5 (6)	6.0 ± 0.6 (6)
CL+MeHg	9	5	4	8.0 ± 0.6 (4)	7.0 ± 0.7 (4)
LT	8	7	7	9.3 ± 0.5 (6)	7.6 ± 0.6 (6)
LT+MeHg	8	5	5	9.4 ± 0.5 (5)	7.6 ± 0.6 (5)
HT	9	9	6	9.6 ± 0.5 (5)*	9.0 ± 0.6 (5)*

Data represent the means ± S.E.M. (n, number of mother), *Significantly different from control, $P < 0.05$. CL: control, LT: low MeHg tuna, HT: high MeHg tuna.

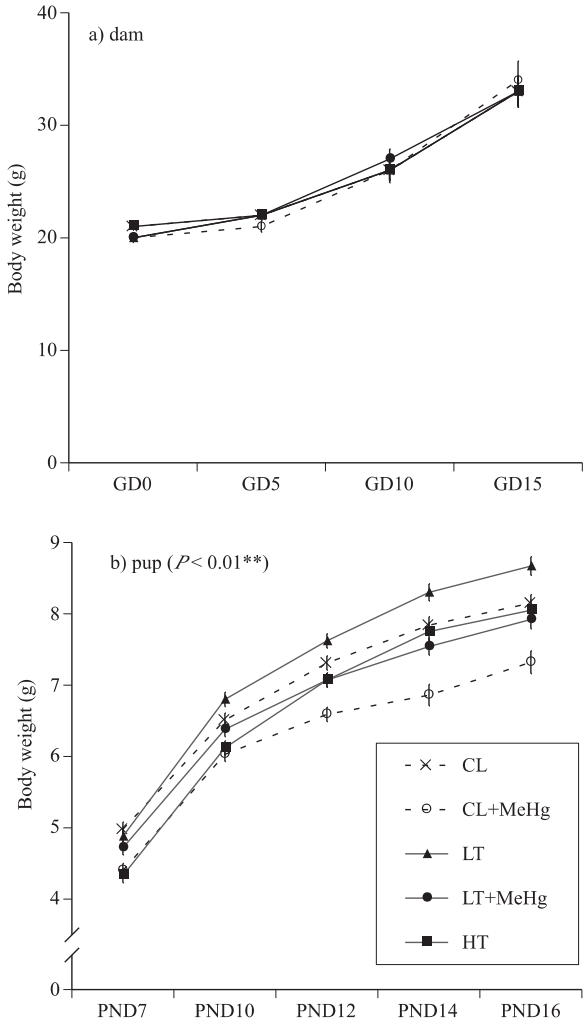


Fig. 1. Changes in the body weights of dams during pregnancy (a, n = 4-8) and offspring mice in lactation period (b, n = 19-31). Exposure groups: Control (CL), CL+MeHgCl (CL+MeHg), low MeHg tuna (LT), LT+MeHgCl (LT+MeHg), and high MeHg tuna (HT). Data represent the mean ± S.E.M.

differences (data not shown). The walking speed and distance (Figs. 3a-d) showed no significant differences in either males or females. The male CL+MeHg, LT, LT+MeHg and HT groups seemed to have shorter latency times than CL (Fig. 3e, $P = 0.08$).

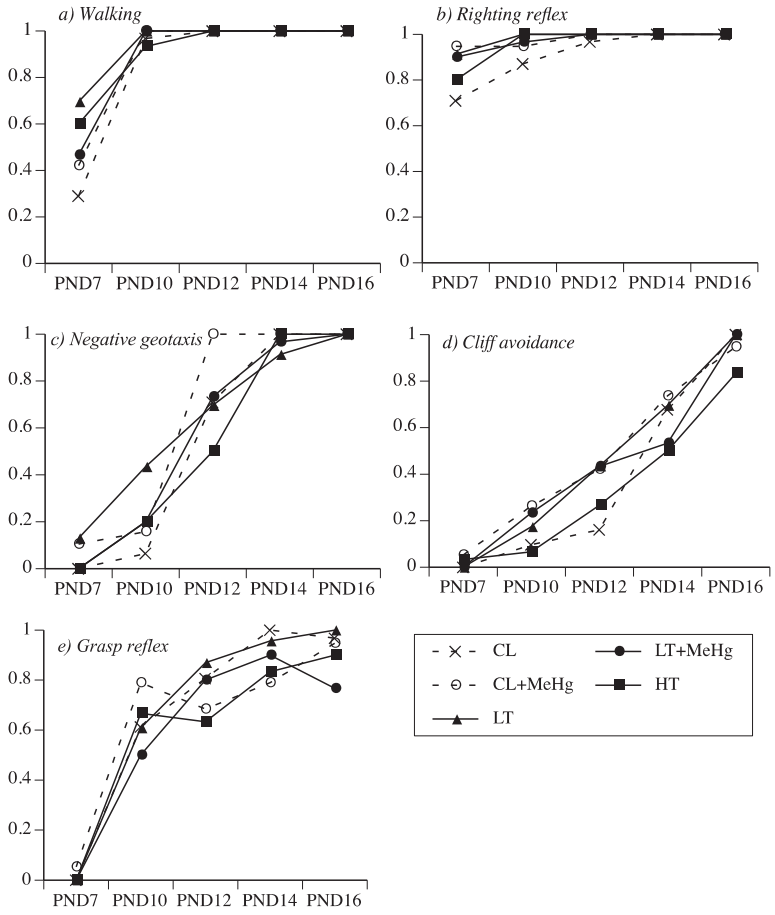
Figure 4 shows the time to reach the platform and swimming speed in the water maze test. The times needed to reach the platform showed no significant differences (Figs. 4a and b). The swimming speed of the males in HT was significantly delayed compared with CL (Fig. 4c).

Figure 5 shows the latency until falling from the bar for males in the accelerating rotor rod test. LT+MeHg males had a significantly shorter time than LT ($P < 0.05$). Among females there was no significant difference (data not shown).

Table 4 shows the THg and Se concentrations in the brain and blood of dams and offspring mice. On PND4, brain THg concentrations of LT+MeHg and HT offspring mice were about 1,200 ng/g. Although a sample was available, the brain THg concentration of CL+MeHg was within a comparable range. The brain THg concentrations became less on PND21 in all the three groups and on PND47, brain THg concentrations were below the detection limit.

DISCUSSION

Despite the fact that the adverse effects of exposure to MeHg via fish and fish products on neurobehavioral development in humans are a serious concern, there have been few animal experimental studies examining the effects of MeHg exposure using fish as the exposure source of MeHg. Therefore, our present results contribute important information about the risk of eating fish. This study shows that male offspring of mice exposed *in utero* to a low dose of MeHg (1.6 mg/kg) from GD 0 to 17 displayed decrease in motor performance in immature stages. In contrast, no significant changes in motor performance were detected in the female offspring.



Developmental tests	Group	CL	CL+MeHg	LT	LT+MeHg	HT
a) Walking	CL	-	0.70 (0.20-2.44)	0.18** (0.06-0.55)	0.50 (0.18-1.35)	0.45 (0.15-1.33)
	CL+MeHg	1.42 (0.41-4.97)	-	0.26* (0.07-0.97)	0.71 (0.22-2.26)	0.64 (0.20-1.95)
	LT	5.43* (1.80-17.9)	3.82* (1.03-15.0)	-	2.69 (0.89-8.71)	2.43 (0.75-8.38)
	LT+MeHg	2.02 (0.74-5.67)	1.42 (0.44-4.62)	0.37 (0.11-1.12)	-	0.90 (0.32-2.53)
	HT	2.24 (0.75-6.87)	1.57 (0.51-4.89)	0.41 (0.12-1.34)	1.11 (0.40-3.11)	-
b) Righting reflex	CL	-	0.13* (0.02-0.60)	0.15* (0.02-0.58)	0.21* (0.05-0.65)	0.29* (0.09-0.88)
	CL+MeHg	7.51* (1.67-54.2)	-	1.09 (0.12-10.29)	1.55 (0.27-12.1)	2.16 (0.45-15.6)
	LT	6.89* (1.73-46.3)	0.92 (0.10-8.62)	-	1.42 (0.25-10.9)	1.98 (0.39-14.8)
	LT+MeHg	4.84* (1.54-18.7)	0.64 (0.08-3.67)	0.70 (0.09-3.92)	-	1.39 (0.36-5.90)
	HT	3.47* (1.14-11.7)	0.46 (0.06-2.21)	0.50 (0.07-2.54)	0.72 (0.17-2.76)	-
c) Negative geotaxis	CL	-	0.26** (0.10-0.66)	0.41* (0.17-0.97)	0.70 (0.32-1.52)	1.10 (0.51-2.41)
	CL+MeHg	3.86** (1.51-10.2)	-	1.60 (0.57-4.59)	2.71* (1.08-6.96)	4.26** (1.70-11.1)
	LT	2.41* (1.03-5.74)	0.62 (0.22-1.76)	-	1.69 (0.71-4.10)	2.66* (1.10-6.55)
	LT+MeHg	1.42 (0.66-3.10)	0.37* (0.14-0.92)	0.59 (0.24-1.41)	-	1.57 (0.72-3.44)
	HT	0.91 (0.42-1.97)	0.23** (0.10-0.59)	0.38* (0.15-0.91)	0.64 (0.29-1.38)	-
d) Cliff avoidance	CL	-	0.49 (0.23-1.03)	0.55 (0.27-1.09)	0.67 (0.35-1.26)	1.47 (0.78-2.79)
	CL+MeHg	2.06 (0.97-4.41)	-	1.13 (0.49-2.62)	1.38 (0.66-2.89)	3.02** (1.43-6.47)
	LT	1.82 (0.92-3.65)	0.89 (0.38-2.05)	-	1.22 (0.60-2.48)	2.68** (1.31-5.5)2
	LT+MeHg	1.49 (0.79-2.83)	0.73 (0.35-1.52)	0.82 (0.40-1.67)	-	2.19* (1.16-4.20)
	HT	0.68 (0.36-1.29)	0.33** (0.15-0.70)	0.37** (0.18-0.76)	0.46* (0.24-0.86)	-
e) Grasp reflex	CL	-	0.79 (0.37-1.68)	1.11 (0.54-2.27)	1.66 (0.88-3.15)	1.45 (0.76-2.77)
	CL+MeHg	1.26 (0.59-2.70)	-	1.4 (0.60-3.27)	2.09* (1.02-4.38)	1.83 (0.88-3.85)
	LT	0.90 (0.44-1.85)	0.72 (0.31-1.66)	-	1.50 (0.73-3.09)	1.31 (0.63-2.72)
	LT+MeHg	0.60 (0.32-1.13)	0.48* (0.23-0.98)	0.67 (0.32-1.37)	-	0.87 (0.47-1.63)
	HT	0.69 (0.36-1.31)	0.55 (0.26-1.12)	0.76 (0.37-1.58)	1.14 (0.61-2.14)	-

Fig. 2. Neurobehavioral development in offspring mice (n = 19-31). Exposure groups: Control (CL), CL+MeHgCl (CL+MeHg), low MeHg tuna (LT), LT+MeHgCl (LT+MeHg), and high MeHg tuna (HT). Whole model tests of all logistic analyses were significant (P < 0.01). *P* values of lack-of-fit (LOF) test were not significant. Logistic regression models adjusted for covariates available in the body weight and PND. Values are odds ratio (95% CI; confidence interval), $P < 0.05$*, $P < 0.01$**.

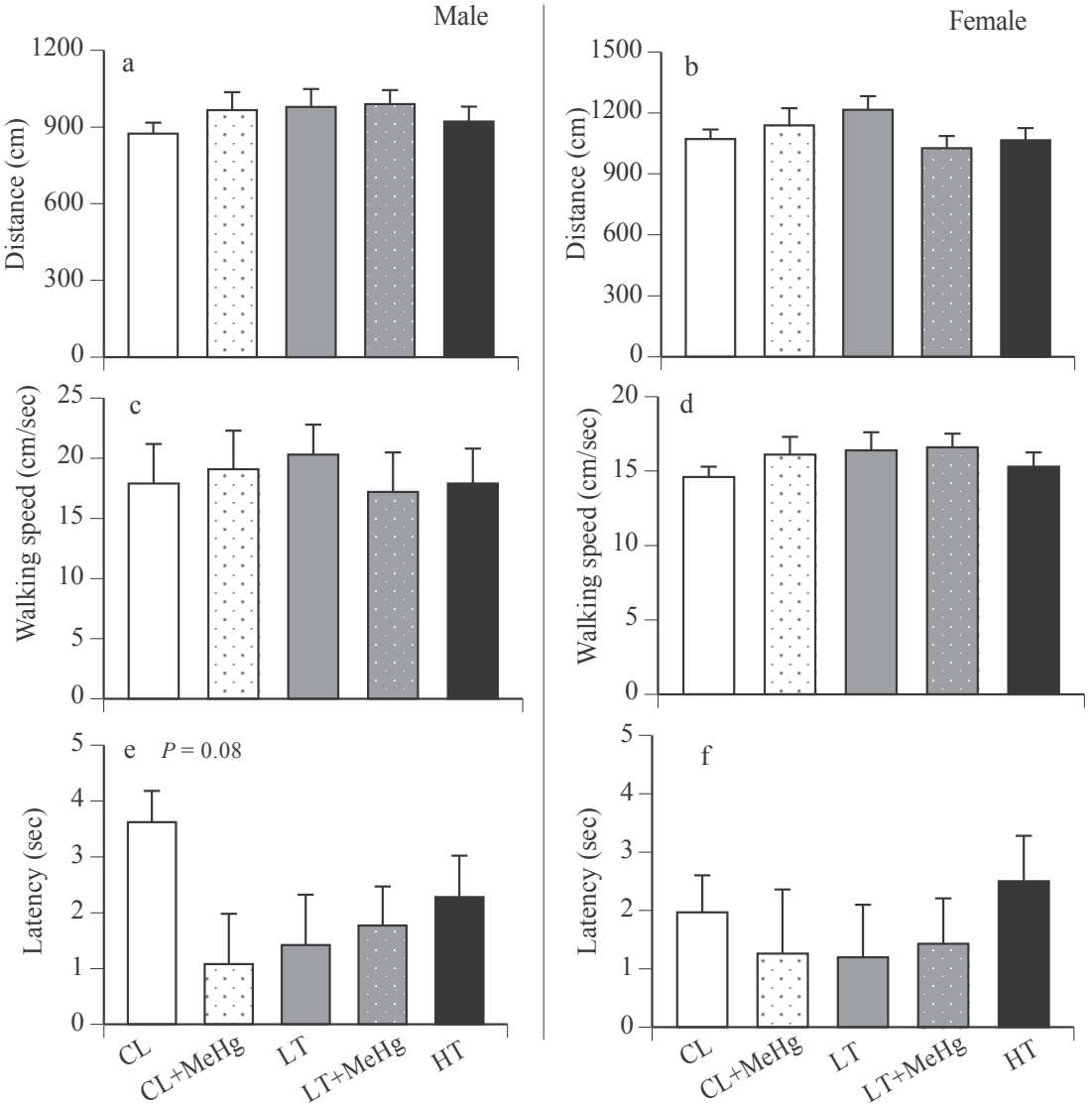


Fig. 3. Result in open-field test. Exposure groups: Control (CL), CL+MeHgCl (CL+MeHg), low MeHg tuna (LT), LT+MeHgCl (LT+MeHg), and high MeHg tuna (HT). Data represent the mean ± S.E.M (n = 5-16).

Several studies have reported the effect of prenatal MeHg exposure on male neurobehavioral development in human and animal studies. In the birth cohort study of the Seychelles Islands, increasing prenatal MeHg exposure was associated with decreasing activity levels in males as assessed in the Infant Behavior Record from the Bayley Scales of Infant Development at 29 months (Davidson *et al*., 1995). At 9 years of age, increased MeHg exposure was associated with decreased performance in the grooved pegboard for the nondominant hand in boys only (Myers *et al*., 2003). In the birth cohort study of the Faroe Islands, MeHg exposure was associated with poorer performance in the finger-tapping task (preferred hand) and slower reaction times for continuous performance tasks. The deficit on the finger-tapping test, particularly in boys, was confirmed in a later reanalysis of the data using a case-control design (Grandjean *et al*., 1998). In animal studies, prenatal MeHg exposure resulted in a trend towards hyperactivity in male rats with no changes in activity in the females (Buelke-Sam *et al*., 1985; Rossi *et al*., 1997). We also showed the effect of prenatal MeHg exposure on neurobehavioral development in male mice.

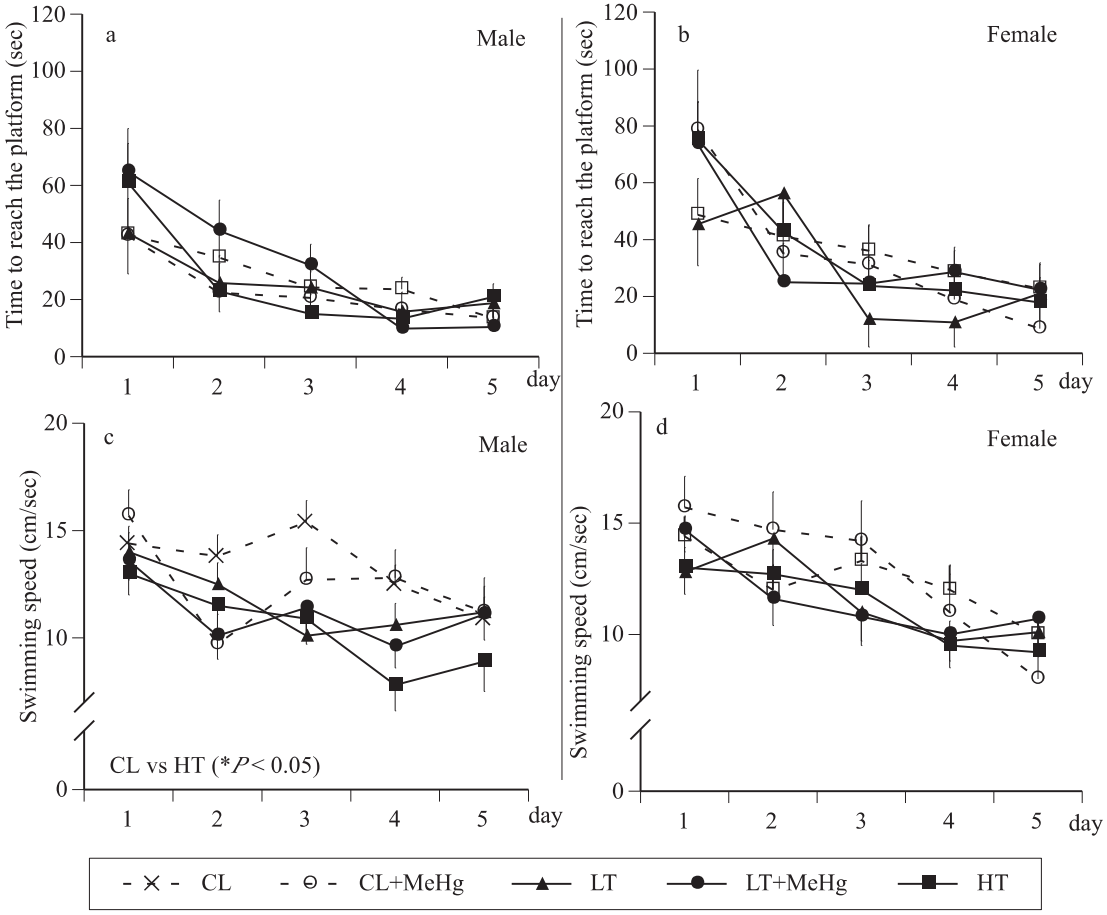


Fig. 4. Time to reach platform and swimming speed in water maze test. Exposure groups: Control (CL), CL+MeHgCl (CL+MeHg), low MeHg tuna (LT), LT+MeHgCl (LT+MeHg), and high MeHg tuna (HT). Data represents the mean ± S.E.M. *Significantly different from CL, $P < 0.05$ (n = 5-13).

Thus, males might be more sensitive to the prenatal exposure to MeHg than females.

Roegge and Schantz (2006) reviewed the motor impairments reported in laboratory animals and humans following perinatal exposure to MeHg during development. In this review, MeHg exposure in animals has been shown to impair cerebellar-mediated motor functions, including walking ability (Inouye *et al*., 1985), hind-limb function (Spyker, 1975; Inouye *et al*., 1985; Magos *et al*., 1985; Sakamoto *et al*., 1993), and rotor rod performance (Sakamoto *et al*., 1993, 2004; Bellum *et al*., 2007; Huang *et al*., 2008; Montgomery *et al*., 2008), as well as swimming ability (Olson and Boush, 1975; Spyker, 1975). The majority of motor deficits observed in the MeHg epidemiological studies involve fine motor skills and hand tremors. Recently, in our birth cohort study in Japan, a negative relationship between the maternal hair mercury level and the motor cluster of the Neonatal Behavioral Assessment Scale (NBAS) was observed (Suzuki *et al*., 2010). We also found the declines of swimming ability and rotor rod performance in mice, indicating the effect of prenatal MeHg exposure on motor performance.

Animal experiments to compare differences in the adverse effects between exposure to MeHg derived from fish and reagent MeHg added to diet on neurobehavioral development have been reported in only one study. Olson and Boush (1975) reported that retarded maturation of offspring rats was observed in a Pacific blue marlin (*Makaira ampla*) diet group (2 mg Hg/kg in diets) with respect to swimming behavior and righting reflexes compared with "Geisha" brand canned albacore tuna (*Thunnus alalunga*) + methylmercury hydroxide diet (2 mg Hg/kg) and

Table 4. Mercury (THg) and selenium (Se) concentrations in brains and blood of dams and offspring mice (ng/g).

		CL		CL+MeHg		LT		LT+MeHg		HT	
	ng/g	blood	brain	blood	brain	blood	brain	blood	brain	blood	brain
Dam (at weaning)	THg	N.D.	N.D.	167 (2)	154 (2)	11.6 ± 4.3 (7)	9.5 ± 5.7 (9)	123 ± 19.3 (5)	58.5 ± 6.8 (5)	156 ± 61.4 (4)	60.3 ± 7.6 (4)
	Se	310 ± 20.2 (7)	178 ± 13.8 (7)	278 (2)	161 (2)	477 ± 16.7 (7)	180 ± 17.0 (7)	302 ± 9.2 (5)	188 ± 11.3 (5)	508 ± 6.7 (5)	208 ± 15.8 (5)
Pup-PND4	THg	N.A.	N.A.	398 ± 76.7 (3)[a]	1562 (1)[a]	N.A.	112 (2)	384 ± 120 (4)	1229 ± 104 (4)	434 ± 36.7 (3)	1256 ± 330 (3)
	Se	N.A.	N.A.	N.A.	N.A.	380 (2)	158 (2)	213 ± 16.5 (4)	152 ± 30.9 (4)	246 ± 19.1 (3)	179 ± 19.8 (4)
Pup-PND21	THg	N.D.	N.D.	33.4 ± 4.1 (4)	85.8 ± 8.1 (4)	N.D.	N.D.	21.9 ± 3.3 (8)	73.3 ± 16.8 (9)	28.2 ± 5.4 (11)	86.2 ± 14.7 (11)
	Se	273 ± 25.8 (9)	143 ± 12.1 (9)	251 ± 28.2 (4)	166(4) ± 0.3 (4)	256 ± 20.8 (15)	158 ± 15.5 (4)	266 ± 29.9 (9)	174 ± 13.2 (9)	286 ± 12.4 (11)	180 ± 33.6 (11)
Pup-PND47	THg	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
	Se	357 ± 47.0 (12)	121 ± 6.3 (12)	426 ± 90.7 (4)	99.8(4) ± 23.2 (4)	366 ± 47.7 (11)	114 ± 9.4 (11)	372 ± 31.0 (10)	127 ± 9.4 (10)	451 ± 109 (10)	118 ± 27.6 (10)

N.A.: not analyzed, N.D.: not detected.
Values are Mean ± S.D. (n), [a]PND3. CL: control, LT: low MeHg tuna, HT: high MeHg tuna.

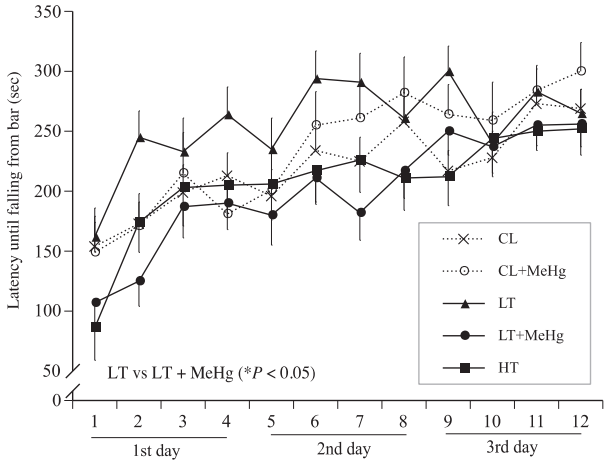


Fig. 5. Latency until falling from the bar of males in the accelerating rotor rod test. Exposure groups: Control (CL), CL+MeHgCl (CL+MeHg), low MeHg tuna (LT), LT+MeHgCl (LT+MeHg), and high MeHg tuna (HT). Data represent the mean ± S.E.M. *Significantly different from LT, $P < 0.05$ (n = 6-13).

control diet. The symmetrical maze testing produced evidence of a learning deficit in the MeHg exposure group derived from the Pacific blue marlin. Bourdineaud *et al*. (2011, 2012) reported the adult mouse experiment that fish was used as exposure source of MeHg. They reported that 2-month feeding of diets containing cod, tuna, and swordfish (35.75 ± 0.15 ng Hg/g) caused significant observable effects as compared to the control (2.3 ± 0.1 ng Hg/g) and the group with a diet containing salmon (1.15 ± 0.15 ng Hg/g). The observable effects consisted of decreased body growth, altered behavioral performance and an increased anxiety level in adult mice (Bourdineaud *et al*., 2011, 2012), though the dose of MeHg they employed was rather small and nutritional bases of diets might be different.

In our study, neurodevelopmental tests of walking, righting reflex and negative geotaxis of 4 groups other than CL showed tendencies of facilitated developments compared to the CL. These support facilitation of clinging ability by prenatal MeHg exposure observed by Sobotka *et al*. (1974). There might be a possibility that prenatal exposure to MeHg disrupts the normal developmental process.

It is of interesting that in the cliff avoidance test HT showed difference from other groups, though there was not statistically significant difference between HT and CL. These indicated MeHg derived from fish and reagent MeHg have neurodevelopmental effects on pups. However, it should be emphasized that the observed differences between CL and each of other groups were not identical, suggesting the possibility that the toxicological profiles of MeHg derived from fish and reagent MeHg are somewhat different. Actually, the Seychelles Child Development Nutrition Study suggested that maternal nutritional status such as PUFA could modulate the relationship between prenatal MeHg exposure and developmental outcomes in children (Strain *et al*., 2012). To clarify the association with effects of human MeHg exposure, further detailed studies are necessary to investigate the effects of long-term exposure to MeHg derived from fish on the motor development in mice.

ACKNOWLEDGMENTS

The authors thank Yui Seino, Mai Satou, Narumi Takeya and Chieko Satoh for excellent technical assistance, Akinobu Koiwai and Jun Takahashi of medical students for experimental supports. This work was supported by grants from the Ministry of Agriculture, Forestry, and Fisheries of Japan (Regulatory science project, No. 2027).

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Akagi, H., Castillo, E.S., Corles-Maramba, N., Francisco-Rivera, A.T. and Timbang, T.D. (2000): Health assessment for mercury exposure among schoolchildren residing near a gold processing and refining plant in Apokon, Tagum, Davao del Norte, Philippines. Sci. Total. Environ., **259**, 31-43.

Bellum, S., Thuett, K.A., Grajeda, R. and Abbott, L.C. (2007): Coordination deficits induced in young adult mice treated with methylmercury. Int. J. Toxicol., **26**, 115-121.

Bourdineaud, J.P., Fujimura, M., Laclau, M., Sawada, M. and Yasutake, A. (2011): Deleterious effects in mice of fish-associated methylmercury contained in a diet mimicking the Western populations' average fish consumption. Environ. Int., **37**, 303-313.

Bourdineaud, J.P., Marumoto, M., Yasutake, A. and Fujimura, M. (2012): Dietary mercury exposure resulted in behavioral differences in mice contaminated with fish-associated methylmercury compared to methylmercury chloride added to diet. J. Biomed. Biotechnol., **2012**, 681016.

Buelke-Sam, J., Kimmel, C.A., Adams, J., *et al*. (1985): Collaborative behavioral teratology study: results. Neurobehav. Toxicol. Teratol., **7**, 591-624.

Davidson, P.W., Myers, G.J., Cox, C., *et al*. (1995): Longitudinal neurodevelopmental study of Seychellois children following in utero exposure to methylmercury from maternal fish ingestion: outcomes at 19 and 29 months. Neurotoxicology, **16**, 677-688.

EFSA (2015): Statement on the benefits of fish/seafood consump-

tion compared to the risks of methylmercury in fish/seafood. EFSA Journal, **13**, 3982.

Fredriksson, A., Dencker, L., Archer, T. and Danielsson, B.R. (1996): Prenatal coexposure to metallic mercury vapour and methylmercury produce interactive behavioural changes in adult rats. Neurotoxicol. Teratol., **18**, 129-134.

Geyer, M.A., Butcher, R.E. and Fite, K. (1985): A study of startle and locomotor activity in rats exposed prenatally to methylmercury. Neurobehav. Toxicol. Teratol., **7**, 759-765.

Grandjean, P., Weihe, P., White, R.F. and Debes, F. (1998): Cognitive performance of children prenatally exposed to "safe" levels of methylmercury, Environ. Res., **77**, 165-172.

Huang, C.F., Hsu, C.J., Liu, S.H. and Lin-Shiau, S.Y. (2008): Neurotoxicological mechanism of methylmercury induced by low-dose and long-term exposure in mice: oxidative stress and down-regulated Na+/K(+)-ATPase involved. Toxicol. Lett., **176**, 188-197.

Innis, S.M. (2007): Dietary (n-3) fatty acids and brain development. J. Nutr., **137**, 855-859.

Inouye, M., Murao, K. and Kajiwara, Y. (1985): Behavioral and neuropathological effects of prenatal methylmercury exposure in mice. Neurobehav. Toxicol. Teratol., **7**, 227-232.

Magos, L., Brown, A.W., Sparrow, S., Bailey, E., Snowden, R.T. and Skipp, W.R. (1985): The comparative toxicology of ethyl- and methylmercury. Arch. Toxicol., **57**, 260-267.

Ministry of the Environment, Japan. (2004): Mercury analysis manual. Ministry of the Environment, Japan, Tokyo.

Montgomery, K.S., Mackey, J., Thuett, K., Ginestra, S., Bizon, J.L. and Abbott, L.C. (2008): Chronic, low-dose prenatal exposure to methylmercury impairs motor and mnemonic function in adult C57/B6 mice. Behav. Brain Res., **191**, 55-61.

Myers, G.J., Davidson, P.W., Cox, C., *et al*. (2003): Prenatal methylmercury exposure from ocean fish consumption in the Seychelles child development study. Lancet, **361**, 1686-1692.

NRC (National Research Council) (2000): Toxicological effects of methylmercury. National Academy Press, Washington, DC.

Olson, K. and Boush, G.M. (1975): Decreased learning capacity in rats exposed prenatally and postnatally to low doses of mercury. Bull. Environ. Contam. Toxicol., **13**, 73-79.

Roegge, C.S. and Schantz, S.L. (2006): Motor function following developmental exposure to PCBS and/or MEHG. Neurotoxicol. Teratol., **28**, 260-277.

Rossi, A.D., Ahlbom, E., Ogren, S.O., Nicotera, P. and Ceccatelli, S. (1997): Prenatal exposure to methylmercury alters locomotor activity of male but not female rats. Exp. Brain Res., **117**, 428-436.

Sakamoto, M., Kakita, A., de Oliveira, R.B., Sheng Pan, H. and Takahashi, H. (2004): Dose-dependent effects of methylmercury administered during neonatal brain spurt in rats. Brain Res. Dev. Brain Res., **152**, 171-176.

Sakamoto, M., Nakano, A., Kajiwara, Y., Naruse, I. and Fujisaki, T. (1993): Effects of methyl mercury in postnatal developing rats. Environ. Res., **61**, 43-50.

Sobotka, T.J., Cook, M.P. and Brodie, R.E. (1974): Effects of perinatal exposure to methyl mercury on functional brain development and neurochemistry. Biol. Psychiatry, **8**, 307-320.

Spyker, J.M. (1975): Assessing the impact of low level chemicals on development: behavioral and latent effects. Fed. Proc., **34**, 1835-1844.

Strain, J.J., Davidson, P.W., Thurston, S.W., Harrington, D., Mulhern, M.S., McAfee, A.J., van Wijngaarden, E., Shamlaye, C.F., Henderson, J., Watson, G.E., Zareba, G., Cory-Slechta, D.A., Lynch, M., Wallace, J.M., McSorley, E.M., Bonham, M.P., Stokes-Riner, A., Sloane-Reeves, J., Janciuras, J., Wong, R., Clarkson, T.W. and Myers, G.J. (2012): Maternal PUFA status but not prenatal methylmercury exposure is associated with children's language functions at age five years in the Seychelles. J. Nutr., **142**, 1943-1949.

Sugawara, N., Ohba, T., Nakai, K., *et al*. (2008): Effects of perinatal coexposure to methylmercury and polychlorinated biphenyls on neurobehavioral development in mice. Arch. Toxicol., **82**, 387-397.

Suzuki, K., Nakai, K., Sugawara, T., *et al*. (2010): Neurobehavioral effects of prenatal exposure to methylmercury and PCBs, and seafood intake: neonatal behavioral assessment scale results of Tohoku study of child development. Environ. Res., **110**, 699-704.

Watkinson, J. (1966): Fluorometric determination of selenium in biological material with 2,3-diaminoaphthalene. Anal. Chem., **38**, 92-97.