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Long-term sex selective hormonal and behavior alterations in mice exposed to low doses of chlorpyrifos *in utero*

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ABSTRACT

Chlorpyrifos, O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate, is an organophosphate insecticide known to be present in human urine. *In utero* exposure to chlorpyrifos may cause long-term hormonal and behavior alterations. In this study mice were exposed to 0, 1 or 5 mg/kg chlorpyrifos on gestational days 17–20. *In utero* exposed mice were then tested in a novel foraging behavior maze and assayed for thyroid hormones. Free Thyroxine Index increased significantly in females, but not males. Learning latency and reduced learning ability was evident during training sessions 5–9 in female mice exposed to 1 or 5 mg/kg chlorpyrifos. No learning deficiencies were observed in male mice. No differences were seen in behavior when using a *standard* radial arm maze during the nine training sessions. These data suggest that mice are susceptible to neuro-endocrine reprogramming by chlorpyrifos, and demonstrate the efficacy of the novel foraging maze as an efficient behavior assay tool.

1. Introduction

Chlorpyrifos, O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate, is a broad-spectrum organophosphate insecticide used widely for the control of agricultural and domestic insect pests [1]. Chlorpyrifos (CPF) functions as a cholinesterase inhibitor in animals, the enzyme is necessary for the inactivation and subsequent breakdown of acetylcholine, an important neurotransmitter. If given at large enough doses, CPF and its metabolites allow for the buildup of acetylcholine in the synaptic cleft leading to paralysis and eventually death [2]. More importantly, at low doses the chlorpyrifos oxon, a bioactivated metabolite of chlorpyrifos, disrupts microtubule polymerization by forming adducts with specific tyrosine residues in tubulin [3]. Despite increased regulation by the Environmental Protection Agency (EPA), CPF continues to be used in residential, public, and agricultural settings for insect control (USEPA). Moreover, the EPA has focused its attention on regulating work related exposure due to the application of CPF rather than on where the insecticide is being applied (USEPA). Although there has been some effort to limit the amount of pesticide sprayed on crops commonly consumed by children such as apples and grapes, CPF continues to be used in the residential setting by pest control companies [4]. Coupled with the consumption of conventional diets, exposure to skin from surfaces containing CPF in the home poses a significant risk for children and pregnant women [5]. This is especially of concern considering that the safe re-entry period based on air concentrations [6] is reported to be 1 day after broadcast application of CPF. However, due to its semi-volatile nature CPF residues may persist on surfaces for more than 2 weeks [7].

Residential and/or dietary exposure to CPF may be significant. Children consuming conventionally grown foods have been shown to have CPF metabolites in their urine. Feeding organic diets for as little as 1 week to these same children caused the CPF metabolites present in urine to drop to the level of children consuming an all-organic diet. When the children were returned to the conventional diet the CPF metabolites in the urine were restored to preexisting levels [8].

Recent research has focused on *in utero* chlorpyrifos exposure at doses that are far below those needed to produce overt toxicity. Levin et al. [9] demonstrated sex selective impairment of neural activity in rats exposed to chlorpyrifos on gestational days 17–20. The offspring of dams dosed with only 1 mg/kg chlorpyrifos, a dose insufficient to cause fetal acetylcholinesterase inhibition [10], showed the greatest impairment in working and reference memory [9]. Exposure to chlorpyrifos during the late gestational window reprograms serotonin and dopamine neural pathways in a sex selective manner at similar doses [11]. Behavioral alterations induced by the exposure include reference and working memory errors, hyperactivity and reduced radial arm maze habituation in females, but not males [9].

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In this study we examine the long-term hormonal and behavioral changes in mice exposed to CPF *in utero*. Behavioral testing in mice was performed using a novel Foraging Maze, developed in our laboratory, designed to exploit the mouse's natural foraging behaviors. We demonstrate a long-term, dose dependent, sex selective impairment of foraging behavior and as well as learning latency in female mice exposed to CPF *in utero*. These behavioral alterations can be quantified in as little as nine sessions using our novel foraging maze compared to 18 sessions with the standard radial arm maze.

2. Materials and methods

All animal experiments were approved by the College of Letters and Science animal care and use committee. Sexually mature male and female Swiss Webster (ND4 strain) were obtained from Harlan (Indianapolis, IN) and individually housed in polycarbonate shoebox cages. After 1 week of acclimation to the environment, male mice were moved into the female cage for mating. Female mice were checked for mating plugs each morning. When a mating plug was found the male was removed and the time was designated gestational day 0.5. On GD 17–20 female mice were injected subcutaneously with a dimethyl sulfoxide carrier containing 0, 1 or 5 mg CPF/kg body weight. Each litter was considered as a statistical unit and there were eight litters per treatment group. All litters were randomly culled to eight pups to insure adequate postnatal nutrition. Dams were allowed to bring up their young to weaning at 4 weeks of age. Young were weighed three times per week for the first 4 weeks. Approximately 60-day old, *in utero* exposed, male and female young were used for maze testing. At approximately postnatal day 150, F1 mice were euthanized and blood was collected for measurement of thyroid hormone levels.

2.1. Foraging maze

The foraging maze consisted of a 15.25-cm diameter central platform elevated 70-cm from the floor on a small movable table, containing eight 5 cm × 26 cm arms projecting radially. At the end of each radial arm two 5 cm × 8 cm choice arms projected in a 'Tee' format (Fig. 1). At the end of each 'Tee' in the choice arm a low barrier is placed on both sides that serves as a food cup, and conceals a large bait under a screen to mask olfactory clues. The maze was made from black plastic sides and clear overhead plastic and was housed in a lighted room with multiple extra-maze visual cues overhead.

Foraging behavior testing was carried out 3 days a week for 3 weeks, totaling nine sessions per mouse. Each of the same four arms was baited at the beginning of each session with a piece of organic Heritage O's cereal (each piece was 0.02 ± 0.002 g), leaving four arms "un-baited" (inaccessible bait below the cup screens). Baited arms were marked inside the maze with 0.5 cm radius pegs at the entrance to each radial arm. The fasted mouse was placed in a cylinder in the central starting area for 10 s to allow for orientation and to avoid biased arm entry. Timing began when the cylinder was lifted and the hole covered with a clear plastic disc. The mouse was allowed to roam freely about the maze for 5 min or until all four baits had been discovered. Radial arm choice was recorded if all four paws crossed the threshold of the arm. A choice arm 'look' was recorded if the nose of the mouse broke the plane defined by the bait cup barrier at the end of either side of each choice arm. Pre-reward and post-reward arm entries and looks were recorded in separate categories in baited arms. A note was made if the cereal was moved or eaten.

2.2. Radial arm maze

We tested the results of the foraging behavior maze against a conventional radial arm maze. The radial arm maze consisted of a 15.25-cm diameter central platform elevated 70-cm from the floor on a small movable table, containing eight 5 cm × 21 cm arms projecting radially, each with a food cup 1.5-cm from the distal end. The maze was made from black plastic with a clear plastic top and was housed in a lighted room with multiple extra-maze visual cues overhead. During the first session the mice were placed in a cylinder in the middle of the maze with six organic Heritage O's cereal pieces (each piece was 0.02 ± 0.002 g) to familiarize the mice with the food baits used in the task. The mice were given up to 5 min to eat all the pieces. Radial arm maze testing was performed in subsequent sessions.

Radial arm maze testing was carried out 3 days a week for 3 weeks, totaling nine sessions per mouse. The same six arms were baited with a piece of cereal at the beginning of each session leaving two arms "un-baited" as defined above. The mouse was placed in a cylinder in the starting area for 10 s to allow for orientation and to avoid biased arm entry. Timing began when the cylinder was removed and the hole covered with a clear plastic disc. The mouse was allowed to roam freely about the maze for 5 min or until all six baited arms had been entered. Arm choice was recorded if all four paws crossed the threshold of the arm and a note was made if the cereal was eaten. Repeated entries into an arm were not rewarded and were counted as working memory errors. Entries into un-baited arms were counted as reference memory errors. Response latency (seconds per entry) was calculated by dividing the total time of the session by the number of arms entered.

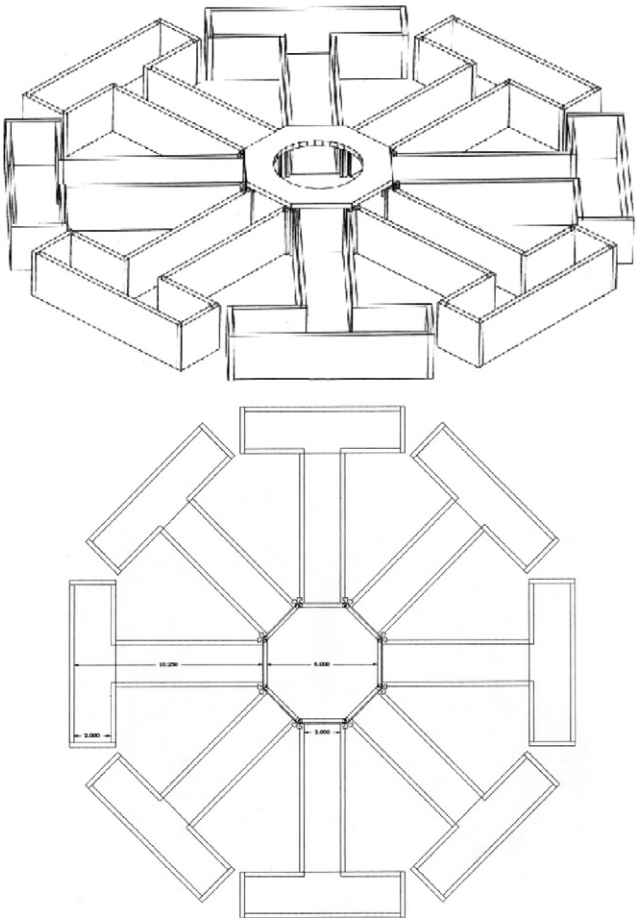

Fig. 1. Schematic representation of the Foraging Behavior Maze. The foraging maze consists of a 15.25-cm diameter central platform elevated 70-cm from the floor on a small movable table, containing eight 5 cm × 26 cm arms projecting radially. At the end of each radial arm two 5 cm × 8 cm choice arms projected in a 'Tee' format. At the end of each choice arm a low barrier is placed that serves as a food bait cup, and conceals a large bait either under a screen to mask olfactory clues "un-baited" or on top of the screen "baited".

2.3. Thyroid hormone assay

At the time of euthanasia, blood was collected from *in utero* exposed adult mice for quantification of serum thyroid hormones (total tetraiodothyronine and total triiodothyronine). Anesthetized animals were exsanguinated by axillary bleeding. Blood was left to coagulate at room temperature for 2 h and then centrifuged at 2000 rpm for 10 min. The sera were kept at −80 °C until analyses were performed. ELISA kits specific for mouse total T_4, total T_3, or T_3 Uptake were obtained from MonoBind Inc. (Lake Forest, CA). Serum was analyzed according to the manufacturer's instructions.

2.4. Statistics

Foraging maze and radial arm maze data were analyzed using the mixed procedure accounting for autocorrelation of repeated measures. ANOVA with least significant differences (LSD) post hoc analysis was performed on the thyroid hormone assay. Differences were considered significant at $p \leq 0.05$.

3. Results

Chlorpyrifos exposure during late gestation had no effect on the reproductive success of the dams. Young from all treatment groups had similar growth rates (data not shown).

In utero exposure to chlorpyrifos altered bioactive thyroid hormone only in females, and not in male mice exposed to chlorpyrifos (Table 1). Vehicle injected mice had similar total serum thyroid hormone levels to control mice reported in the literature [12,13]. Vehicle injected and 1 mg/kg chlorpyrifos exposed female mice had

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Table 1
Serum thyroid hormone in adult mice exposed to CHP in utero.

		Chlorpyrifos treatment		
		Vehicle	1 mg/kg	5 mg/kg
Female	tT3 (ng/ml)	0.76 (0.06)[a]	0.85 (0.06)[a,b]	1.048 (0.12)[b]
	tT4 (μg/dl)	6.36 (0.14)[a]	6.65 (0.34)[a,b]	7.61 (0.37)[b]
	T3U (%)	32.67 (0.21)[a]	32.79 (0.28)[a,b]	33.71 (0.37)[b]
	FTI	7.45 (0.15)[a]	8.01 (0.52)[a]	9.65 (0.60)[b]
Male	tT3 (ng/ml)	0.92 (0.10)	0.80 (0.11)	0.80 (0.13)
	tT4 (μg/dl)	5.74 (0.68)	6.47 (0.28)	6.29 (0.63)
	T3U (%)	31.07 (0.62)	32.00 (0.39)	31.93 (0.36)
	FTI	6.27 (0.88)	7.41 (0.48)	7.18 (0.87)

tT3: triiodothyronine; tT4: thyroxine; T3U: T3 uptake; FTI: free thyroid hormone index. Standard error of the mean in parentheses. Different superscript letters represent significant differences as with $p < 0.05$.

a Free Thyroxine Index (FTI) of 7.5 and 8, while the 5 mg/kg CPF exposed females had a significantly higher FTI of 9.7 (Table 1). All treatment groups had a similar FTI in male mice with means of 6.3, 7.4, and 7.1 for the vehicle, 1 mg/kg and 5 mg/kg treatments respectively (Table 1).

We assessed the natural foraging ability of mice exposed to CPF *in utero* using a novel behavior-testing maze designed to assess foraging behavior. The foraging maze has been validated in our laboratory using a benchmark study that indicated that the mouse's ability levels off at nine sessions in the maze (Fig. 2). In our CPF exposure study we further validated our maze by comparing our foraging maze to other behavior assays including the radial arm maze (Fig. 3), the t-maze (data not shown) and a standard wheel running activity test (data not shown). This comparison indicated full learning achievement could be detected by session 9 using our foraging maze. The conventional radial arm maze often takes 18 sessions to complete. As with the FTI, we observed sex selective differences in males and females. Females showed diminished foraging ability in a dose dependant manner due to *in utero* CPF exposure, while male foraging behavior was not diminished. Both the foraging maze and the food baits are a novel experience for mice. The number of food pieces found per pre-reward baited arm entries is a measure of food recognition in the maze. Fig. 4 shows

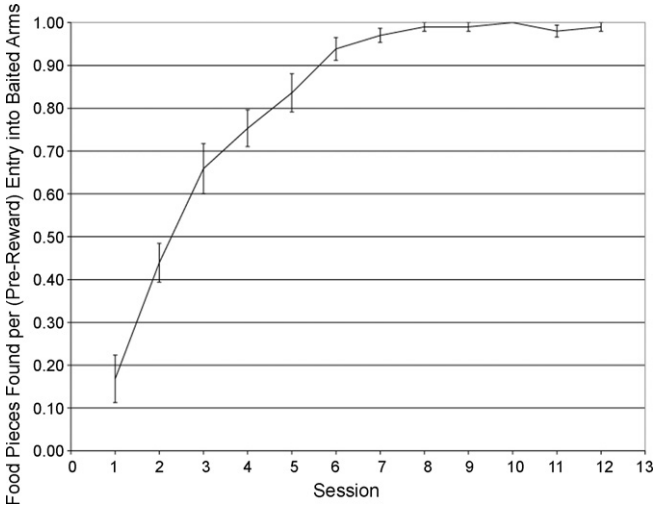

Fig. 2. Foraging maze food position learning in untreated mice over 18 sessions. Food position learning was defined as the number of looks into the left bait cup prior to finding the bait divided by the total number of pre-reward looks into bait cups in baited arms. A value of 0.5 represents no preference for looking left versus right. Values approaching 1.0 represent learning that baits are always in the left bait cup. This figure illustrates that food position learning reaches a plateau by session 9 in untreated mice, and is representative of all types of learning measurements taken in our benchmark study.

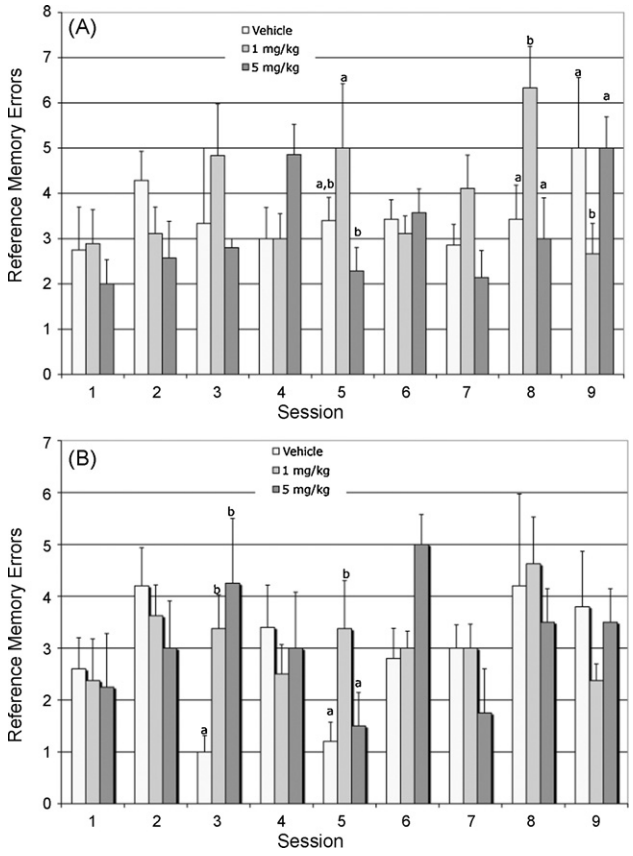

Fig. 3. Female and male radial arm maze reference memory errors versus chlorpyrifos exposure in mice. Dams were given a subcutaneous injection of either 1 or 5 mg/kg chlorpyrifos in DMSO or were injected with vehicle alone. After weaning mice were given ad libitum access to Purina 5002 certified chow diet and water. Sexually mature female (panel A) or male (panel B) ND4 mice were placed in an eight-arm radial arm maze and allowed to explore to find six food baits. Entries into un-baited arms were scored as reference memory errors. Data were analyzed using the SAS proc Mixed procedure accounting for autocorrelation of repeated measures. Letters represent significant differences with $p < 0.05$.

food recognition learning in mice exposed to CPF *in utero*. As mice improve their food recognition ability the value of this measure is expected to approach 1.0. In male mice CPF exposure may have enhanced their ability to recognize food in the early sessions of the foraging maze (Fig. 4B). Vehicle exposed female mice exhibited a steady improvement in the food recognition from session 1 (0.36) to session 7 (0.97) at which time they reached the plateau value of 1.0 (i.e. a food piece was found the first time a baited arm was entered every time). The 1 mg/kg CPF exposed females exhibited delayed food recognition from sessions 1 through 8, but were similar to the vehicle group by the 9th session. The 5 mg/kg CPF exposed group never attained the same level of food recognition learning as either the vehicle or the 1 mg/kg treatment groups (Fig. 4A).

Food position learning is an assessment of a mouse's ability to remember the food is always in the left side of the choice arm 'Tee' and is a more difficult task for mice than food recognition. Fig. 5 shows a measure of food position learning ("Pre-Reward Looks Left"/"Total Pre-Reward Looks in Baited Arms") in mice exposed to CPF *in utero*. During the first session both male and female mice chose right versus left randomly (as expected) and the food position measurement value is about 0.5 (Fig. 5A and B). As mice learn that food pieces are located only in the left 'Tee', the value of the measure will increase to a maximum value of 1.0. Male mice improved in the food position learning measurement from about 0.5 to about 0.8, and there was no effect due to the CPF exposure (Fig. 5B). Vehicle exposed female mice were similar to males in that there was

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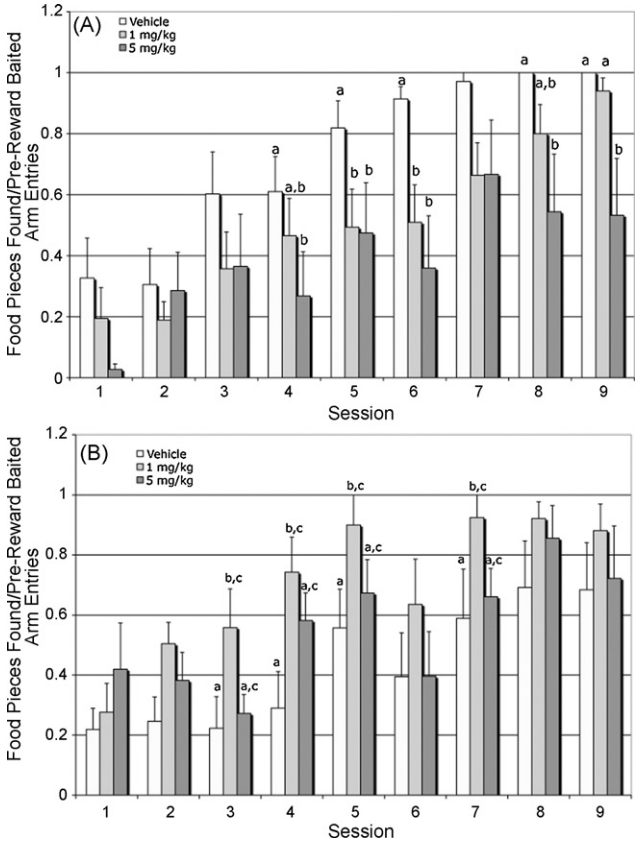

Fig. 4. Female and male foraging maze food recognition learning versus chlorpyrifos exposure in mice. Food recognition learning was defined as the number of food pieces found divided by the number of pre-reward arm entries. Values are expected to approach 1.0 as mice learn to find baits in baited arms. Female mice exposed to 1 mg/kg CPF exhibited a 3–4 session lag in learning of food recognition task compared to vehicle treated controls, however, by the 9th session the 1 mg/kg group were similar to control animals. The female 5 mg/kg CPF treated mice never reached the same level of task proficiency throughout the nine training sessions as the 1 mg/kg and vehicle control groups (panel A). Male mice exposed to 1 mg/kg CPF dose, demonstrated enhanced food recognition learning in sessions 3–7. By session 8 all males had similar food recognition abilities (panel B). Error bars represent the standard error of the mean. Data were analyzed using the SAS proc Mixed procedure accounting for autocorrelation of repeated measures. Letters represent significant differences between treatment groups within a single session with $p \leq 0.05$.

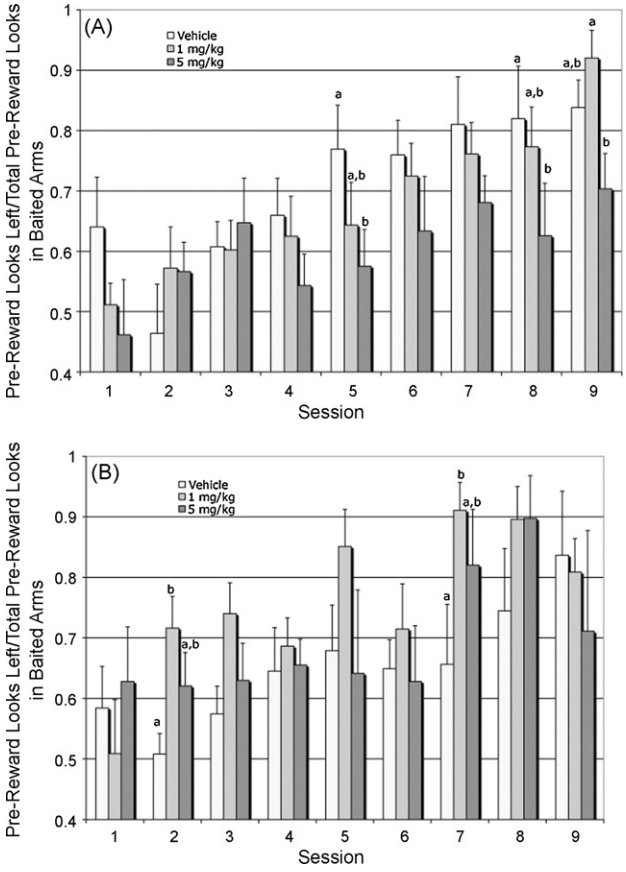

Fig. 5. Female and male foraging maze food position learning versus chlorpyrifos exposure in mice. Food position learning was defined as the number of looks into the left bait cup prior to finding the bait divided by the total number of pre-reward looks into bait cups in baited arms. A value of 0.5 represents no preference for looking left versus right. Values approaching 1.0 represent learning that baits are always in the left bait cup. Female mice exposed to 1 mg/kg CPF initially lagged vehicle treated controls in food position learning, however, by the ninth session the 1 mg/kg group were similar to control animals. The female 5 mg/kg CPF treated mice never reached the same level of task proficiency throughout the nine training sessions as the 1 mg/kg and vehicle control groups (panel A). There were spurious differences in food position learning in male mice throughout the nine training sessions; however, no definitive patterns were seen (panel B). Error bars represent the standard error of the mean. Data were analyzed using the SAS proc Mixed procedure accounting for autocorrelation of repeated measures. Letters represent significant differences between treatment groups within a single session with $p \leq 0.05$.

a steady improvement in the food position learning measure from 0.63 to 0.84 from sessions 1 to 9. The 1 mg/kg CPF exposed females exhibited delayed food position learning from sessions 1 through 5, but were similar to the vehicle group by the 9th session. The 5 mg/kg CPF exposed group never attained the same level of food position learning as either the vehicle or the 1 mg/kg treatment groups (Fig. 5A).

Although food recognition and food position learning were impaired in female CPF exposed mice, foraging rate was unaffected by treatment in both male and female mice. Fig. 6 shows time spent per arm entry for males (Fig. 6B) and females (Fig. 6A).

4. Discussion

Chlorpyrifos, O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl) phosphorothioate, is a broad-spectrum organophosphate insecticide used widely for the control of agricultural and domestic insect pests [1]. Chlorpyrifos may represent a significant source of in home pesticide exposure via direct contact and non-organic food consumption [14]. Authors in a broad literature review of chlorpyrifos and other organophosphorus pesticides show a long history of impacts on DNA synthesis in the brain, altered neurological functions, and impacts beyond the nervous system [15]. Garcia et al. [16] showed that chlorpyrifos could continue to affect glial cell development long after the conclusion of neurogenesis. Prenatal chlorpyrifos exposure may have long-term behavioral effects. Numerous studies including our current study have demonstrated that animals treated *in utero* are affected well into adolescence [9,11,16]. Thus the developmental neurotoxicity of chlorpyrifos is likely to extend beyond the prenatal period. Sex specific alterations in environmental and social cues in *adolescent* mice exposed to chlorpyrifos *in utero* may be related to altered glial cell development [17–19]. Even though organophosphates are thought to be cleared quickly, the consistent presence of chlorpyrifos metabolites in the urine of children on conventionally produced diets suggests frequent repeated exposures via diet [8].

Our lab has developed a superior method of detecting and assessing behavioral changes in mice relative to a standard radial arm maze (RAM). Our foraging maze is novel and superior to the RAM because it takes only nine sessions for mice to master memory tasks compared to at least 18 sessions needed in the RAM. Furthermore the foraging maze assesses multiple levels of behavior

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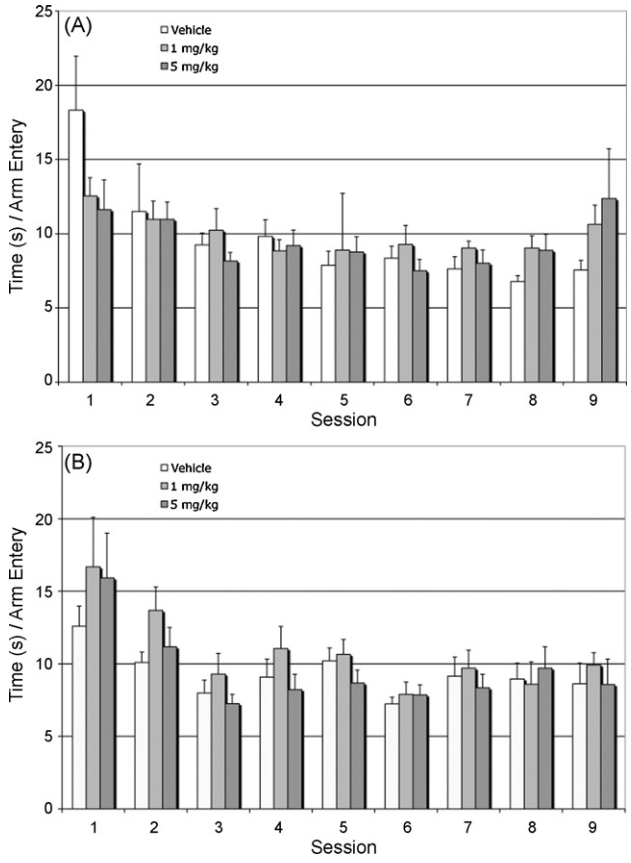

Fig. 6. Female (panel A) and male (panel B) foraging maze foraging activity versus chlorpyrifos exposure in mice. Foraging activity was defined as the time needed to complete the maze task (find all four baits or 5 min whichever is shorter) divided by the total number of arms entered during the task. No differences were seen in foraging activity between treatment groups in either males or females. Error bars represent the standard error of the mean. Data were analyzed using the SAS proc Mixed procedure accounting for autocorrelation of repeated measures.

such as exploratory behavior, memory acquisition and spontaneous activity. Thus the foraging maze allows for relationships to be made between previous data and the current data from this study. The foraging maze is an ideal tool to study the subtle changes in behavior that are expected in adult mice exposed to environmental contaminants *in utero*.

The importance of environmentally relevant exposures to chlorpyrifos to prenatal neuronal programming is recently being recognized. We have demonstrated environmentally relevant levels of *in utero* chlorpyrifos exposure cause a marked learning latency in females but not in males. Similar results including an impairment in radial arm maze working and reference memory, as well as hyperactivity were observed only in female rats given a low dose chlorpyrifos exposure during late gestation (GD 17–20) [9]. These rats also exhibited reduced habituation to the maze environment. Although we did not observe hyperactivity (Fig. 6), reference and working memory impairment is consistent with our data on food recognition, and food position learning in the foraging maze. Chlorpyrifos is a known acetylcholinesterase inhibitor at high concentrations; however, the *in utero* doses needed to alter behavior and alter memory are below the levels needed for acetylcholinesterase inhibition. *In utero* chlorpyrifos exposure (GD 17–20 in rats) has been shown to alter neuropeptide biomarkers, indicating a potential reduction in neuronal development [16]. Effects on neuropeptide level in mice following low dose *in utero* exposure are persistent into adulthood and show a dose and sex related response [20]. Furthermore, others have shown that the chlorpyrifos oxon, disrupts microtubule polymerization by forming adducts with specific tyrosine residues in tubulin [3] this further implicates a reduction in neuronal development as a possible cause for the learning latency observed. Our data suggest an impaired thyroid hormone signaling axis in affected females. Therefore it is possible that altered behavior and the thyroid hormone signaling are linked. In a recent study, it was found that late term gestational exposure to CPF alters thyroid tissues and thyroid hormone levels in adult mice. Similar to our results, this study reports differential regulation of thyroid hormone levels in males and females exposed to CPF [12]. Levin et al. [9], Garcia et al. [16] and our study suggest that while normal brains use cholinergic pathways, the CPF treated brain may form alternate noncholinergic neural pathways. This change in the way that the brain of CPF exposed mice forms may be an important factor in multiple phenotypic manifestations that are being found along the hypothalamic–pituitary–thyroid axis.

In conclusion, this study suggests that low dose prenatal chlorpyrifos (CPF) exposure causes sexually selective neural programming that causes behavioral and hormonal changes persisting into adulthood in female mice. Our study contributes to the growing body of evidence that demonstrates the need for CPF and other organophosphates to be analyzed for endocrine disruption during risk assessment analyses. Findings of sex selective behavioral changes coupled with findings of an elevated FTI in CPF exposed females, leads us to hypothesize that these altered neural pathways may be located in a brain region that controls both behavior and thyroid hormone levels. Our study adds to the body of literature that indicates late term gestational exposure to toxicants such as CPF is important for determining developmental effects of a toxicant. Regulatory approval processes for potentially toxic materials do not include tests for toxicity during late gestational exposure. This study reinforces the idea that low dose *in utero* exposures must be considered during toxicity assessment of materials.

Conflict of interest statement

The authors declare that there are no conflicts of interest regarding this work.

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