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Determining a Developmental Window for the Effect of Polychlorinated Biphenyl (PCB) on Ultrasonic Vo

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Determining a Developmental Window for the Effect of Polychlorinated Biphenyl (PCB) on Ultrasonic Vocalization (USV) in Sprague-Dawley Rat Pups

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HONORS PROJECT

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Abstract

Commercial manufacturing and widespread use of polychlorinated biphenyl (PCB) in the United States has left lasting negative effects on the environment. These persistent contaminants continue to bioaccumulate in the food web because of their stable structure, long half-life, and high lipophilicity. Adding PCB into the diet of Sprague-Dawley rats during gestation and lactation alters the thyroid status of offspring, as well as the behavior of both dams and pups. To determine a critical period or "window" of development with the greatest impact of PCB exposure of females on offspring fitness, female Sprague-Dawley rats were mated and fed PCB diet (25 ppm PCB 47 and PCB 77 in standard rat chow) during one of five, two week "development windows", and one of three, one week "developmental windows." Ultrasonic vocalizations (USVs) were recorded on pup postnatal day (PND) 3, 7, 14, 21, and 22. Grooming behavior (PND 14), open field behavior (PND 21), and play behavior (PND 22) was also observed in pups. Blood serum was also collected on PND 3, 7, 14, 21, and 22 for thyroid hormone analysis. Preliminary data show that there is a significant difference in the number of USVs emitted from pups among the window groups ($F(5,23)=4.203$, $p<0.01$) and also between developmental days with an increase from PND 3 to PND 7 ($F(1,23)=14.461$, $p<0.001$). Those pups exposed to PCB the last week of pregnancy and the first week of lactation (4-PCB) emit a significantly greater rate of USVs on both PND 3 and PND 7 while those pups exposed to PCB the first two weeks of lactation (5-PCB) emit a significantly lower rate of USVs on both PND 3 and PND 7. It is anticipated that those pups from dams given PCB diet from the start of gestation to early lactation will emit a significantly greater rate of isolation USVs, as well as altered grooming, open field, and play behavior. Further behavioral testing as well as analysis of

thyroid hormone status will likely demonstrate that PCB exposure during a particular "window" of development has the greatest impact on pup behavior.

Introduction

Commercial manufacturing of polychlorinated biphenyl (PCB) in the United States until the late 1970s has left us with lasting negative effects today. These compounds, a group of persistent environmental contaminants known for their stability and heat resistant properties, were primarily used in electrical capacitors, transformers, plastics, flame retardant liquids, hydraulic fluids, and sealants[5]. These compounds continue to bioaccumulate in both the environment and in the food chain because of their stable chemical structure, long half-life, and high lipophilicity[20]. If humans consume tissue from other contaminated animals, such as fish, PCB can be ingested and begin to collect in the fatty tissues, causing potentially negative health effects over repeated, long-term exposure. Major developmental effects of PCB can be seen when it is eaten during the gestational period and early post gestational period in humans and other mammals.

PCB was primarily produced as a mixture of various congeners and widely used in many commercial and industrial applications. The congener conformation is based on the position and number of chlorine substituents around the biphenyl structure. Less chlorinated PCBs are colorless, odorless, and tasteless, whereas the more chlorinated PCBs are a deeper yellow and often viscous liquid. The coplanar congeners have received much attention because of their ability to mimic various hormones and bind to the aryl hydrocarbon receptor (AhR) which could enable them to disrupt various organ systems[7]. The non-coplanar congeners are also important as they contribute to much of the environmental contamination of PCBs. These congeners are

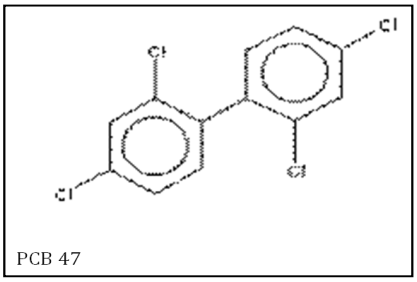
known to interfere with neuronal transmission, disrupt cell membranes, and enzyme systems in various model organisms[18]. Both the co-planar and non-coplanar congeners have shown to be disruptive to neurobehavioral development at least partially resulting from the ability of PCB to reduce thyroid hormone availability during gestational and early post-gestational development[10]. Proper thyroid hormone concentrations are crucial during the sensitive windows of development for normal brain development to occur[11].

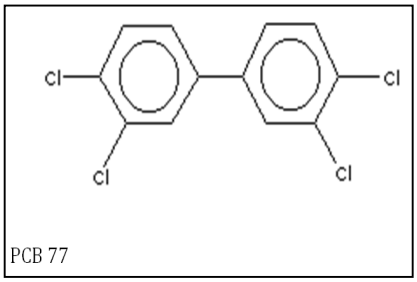
PCBs can traverse the placental barrier from mother to offspring passing into fetal circulation and have also been found in breast milk[9]. In several studies, PCB has been shown to cause altered maternal and pup behavior in Sprague-Dawley rats. Developing offspring exposed to PCB have shown alterations in locomotor activity, and impairments in learning and memory tasks[14]. In a recent study in our lab, PCB exposure during either all of gestation, all of lactation, or during both, was shown to alter rates of ultrasonic vocalizations by rat pups[15,17]. PCB has been shown to compromise the endocrine system by altering the structure of the thyroid gland, which interferes with the ability of this gland to respond to thyroid stimulating hormone[13]. PCB has also been shown to interfere with thyroid hormone metabolism, exhibiting the ability to displace T_4 from the binding proteins[13]. These effects have possible linkages to hypothyroidism and neurological, developmental, and learning deficits similar to those seen with attention deficit hyperactivity disorder (ADHD) and autism[12].

Ultrasonic vocalizations (USVs) are emitted by rats and other organisms to communicate emotional states to littermates and dams. USV communication is greatly increased during highly emotional states, which can be divided into two broad categories, the 50kHz (30-70kHz range) USVs and the 22kHz (18-32kHz range)[2]. These USVs are thought to reflect, respectively, a positive emotional state (seen in play behavior and social contact), and a distressed emotional

state (seen in a rat in an environment with a predatory cue or foot shock)[2]. A unique type of USV is emitted at 35-40kHz at a much greater rate when pups are separated from littermates and the dam. This type of USV is seen as a distress call, and is crucial to pup survival as the dam will perform searching and retrieving behavior when a pup becomes separated from the nest[3]. These isolation USVs are thought to express an increased state of emotional anxiety and an increased frequency of these USV emissions could give insight into the underlying level of anxiety a pup experiences upon separation from littermates and dam. It has been shown in previous studies that exposure to PCB can cause alterations in the frequency of these isolation USV emissions when compared to controls[15,17].

Our purpose in the proposed study was to determine whether there is a critical period, or "window" for the greatest effects of PCB exposure during pup development based on observation and testing of ultrasonic vocalizations (post natal day (PND) 3, 7, 14, 21, 22), grooming behavior (PND 14), open field behavior (PND 21), play behavior (PND 22), as well as thyroid hormone analysis. Pregnant dams were assigned to a "window" group in which they were fed a mix of tetrachlorinated PCB 44 and PCB 77 congeners (Figure 1) during two week developmental periods ranging from early gestation to pup PND 22 (Figure 2). It is anticipated that a critical developmental window for PCB exposure during early gestation will show the greatest alterations in pup behavior and altered thyroid hormone status.





PCB Diet

Combine:

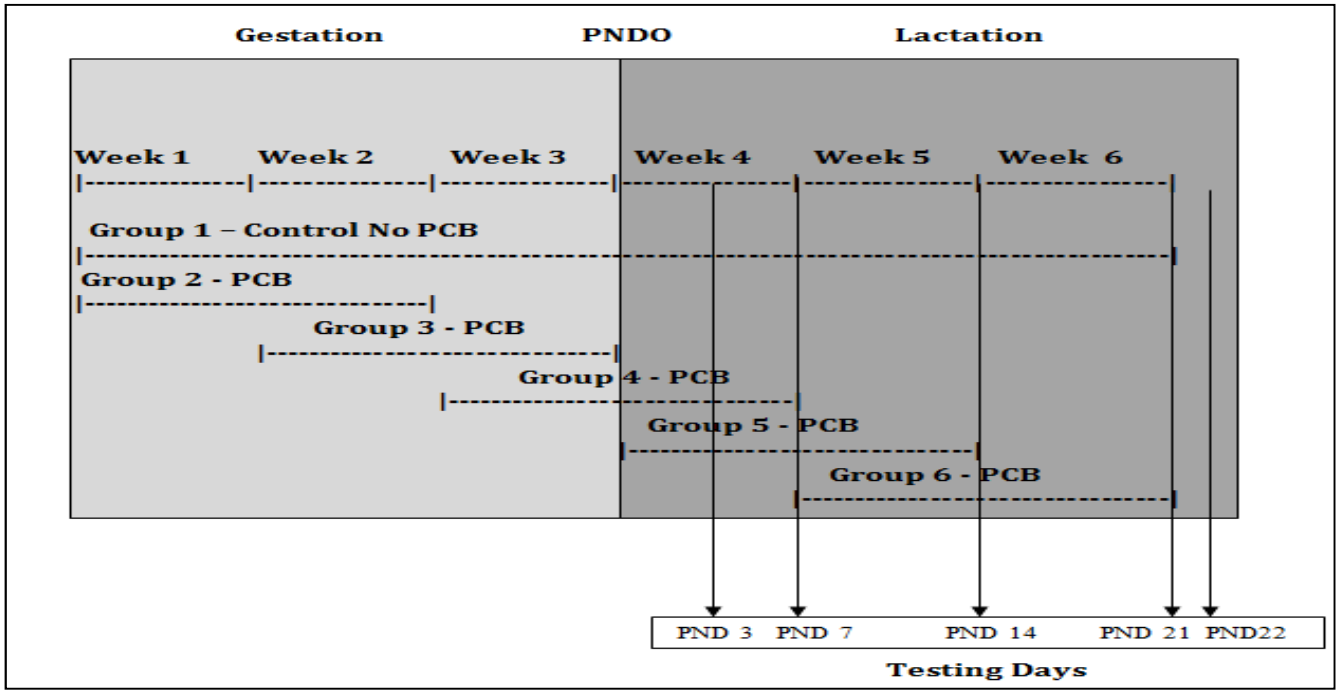
25g stock PCB 47

50g stock PCB 77

925g standard rat chow mash

Total: 1000g 25ppm PCB

Figure 1 PCB Congeners and Diet A mixture of PCB congeners 47 and 77 are used in PCB diet for experimental groups.



1-CON	Control Group No PCB Diet
2-PCB	PCB Gestation Weeks 1-2
3-PCB	PCB Gestation Weeks 2-3
4-PCB	PCB Gestation Week 3 & Lactation Week 1
5-PCB	PCB Lactation Week 1-2
6-PCB	PCB Lactation Week 2-3

Fig 2 Window Group Designation Pregnant dams are assigned to one developmental window group and exposed to PCB accordingly.

Materials and Methods

Animals and Treatment

All animal experiments were conducted according to protocols approved by the Bowling Green State University Institutional Animal Care and Use Committee. Twenty adult female Sprague-Dawley rats (*Rattus norvegictus*) were purchased from Harlan Sprague-Dawley in Indianapolis, IN and housed in the Life Science Annex Animal Facilities at Bowling Green State University under a 12:12-h light-dark cycle with free access to food and water. After a week-long acclimation period, female Sprague-Dawley rats were randomly assigned to either a control or PCB exposure window group (Figure 2). Females were paired with adult male Sprague-Dawley rats for breeding purposes. Dams were deemed pregnant by acquisition of a sperm positive vaginal smear. Once pregnant, females were housed singly and given 100g of a standard chow

mash diet or PCB standard chow mash diet daily. PCB standard chow mash diet is a mixture of PCB 47 (2,2',4,4'-tetrachlorobiphenyl) and PCB 77 (3,3',4,4'-tetrachlorobiphenyl) dissolved in ethanol and added to standard chow mash diet to 25 ppm (25 mg/kg rat) PCB concentration(Figure 1). Dam weight and food consumption was monitored daily throughout gestation. The day of birth, post natal day (PND) 0, was noted and litters were left undisturbed. USV, behavioral testing, and blood serum collection testing schedules are indicated below (Figure 3).

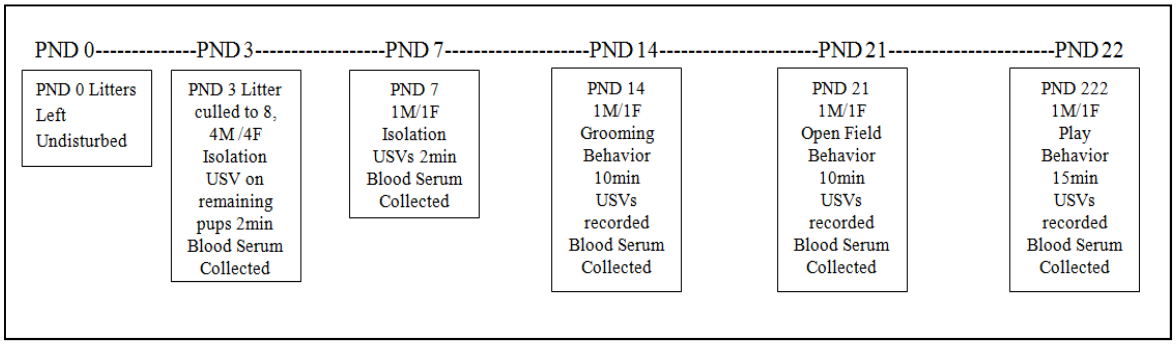


Figure 3 Testing Schedule Various behavioral tests were performed on pups on PND 3, 7, 14, 21, and 22.

Isolation Ultrasonic Vocalization (USV) Testing

On PND 3, litters were culled to 8 pups; 4 males and 4 females. On PND 3, remaining pups from culled litter were tested for USV emission. Pups were separated from dam and littermates and immediately taken to separate testing room. One by one, pups were placed in 500 ml beaker testing chamber within small animal cage. A microphone was suspended approximately 25 cm above the testing chamber and connected to a high frequency bat detector (Peterson D980 ultrasonic detector) which digitally records the USVs emitted at 196 kHz by pups during a 1 minute acclimation period and 2 minute analysis period. After USVs were recorded, pups were euthanized and blood serum was collected. Analysis of isolation USV calls

were viewed offline using a sonogram program (Avisoft Bioacoustics SAS Lab) and were counted for the 120s time period immediately following the habituation period (Figure 4). USV testing was performed on PND 7 in the same fashion, with 1 male and 1 female pup from culled litter. After USVs were recorded on PND7, pups were euthanized and blood serum was collected. USVs were also recorded on PND 14, 21, and 22 simultaneously with behavioral testing with the microphone placed near the various testing chambers and analyzed similarly for number of USVs emitted.

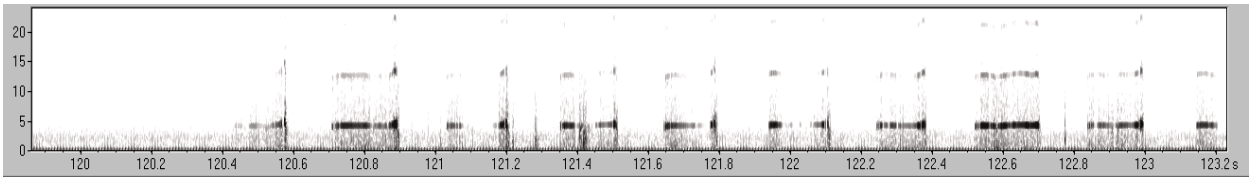


Figure 4 USV Spectrogram An example of a USV spectrogram generated by the Avisoft Bioacoustics Program used for counting USVs. Seen above is a 3 second section with a USV count of 10.

Behavioral and TH Hormone Testing

This project focused on analysis of Ultrasonic Vocalization testing and analysis. Behavioral testing and thyroid hormone analysis methods can be found in Appendix A.

Data Analysis

Statistical analysis was performed on isolation USV data using SPSS statistical analysis software. A series of variance (ANOVAs) and multi-factorial ANOVAs were performed with significance attributed to a p-value ≤ 0.05. If an overall significant effect of condition, day, or sex was found using the above ANOVAs, pair wise t-tests were performed in order to determine differences between the conditions and within-subject factors.

Results

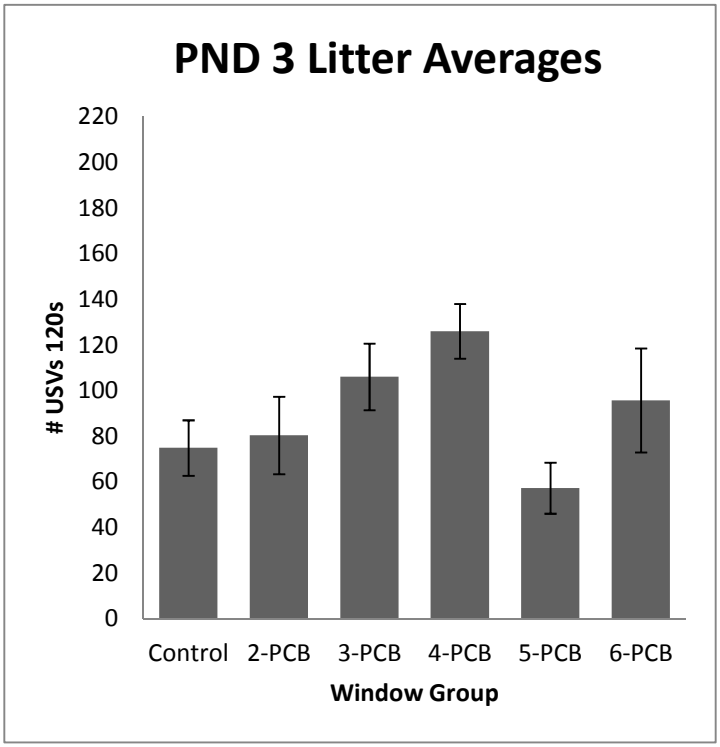
PCB Exposure Effects

A three-way ANOVA univariate analysis of variance revealed a significant difference among conditions, or window periods of PCB exposure ($F(5,23)=4.203$, $p<0.01$) on isolation USV production. Isolation USVs were affected by gestational and post-gestational exposure to PCB diet (Figures 5 & 7). Pair-wise t-tests were performed to determine if there were differences between experimental groups on both PND 3 and PND 7 (Figure 5). Those pups exposed to PCB the last week of pregnancy and first week of lactation (4-PCB) emitted a significantly greater rate of isolation USVs on PND 3 compared to the controls and those pups exposed to PCB the first and second week of lactation (5-PCB) emitted a significantly lower rate of isolation USVs on PND 3 compared to those pups exposed to PCB the last two weeks of pregnancy (3-PCB) and the last week of pregnancy and first week of lactation (4-PCB) (Figures 5-7). All other paired t-tests for PND 3 were not significantly different. Those pups exposed to PCB the first two weeks of lactation (5-PCB) emitted a significantly lower rate of isolation USVs on PND 7 when compared to pups exposed to PCB the last week of pregnancy and first week of lactation (4-PCB) (Figure 5). It appears that there is a trend between pups exposed to PCB the last two weeks of pregnancy (3-PCB) and the first two weeks of lactation (5-PCB) and the rate of isolation USV emission on PND 7 (Figure 5). All other paired t-tests for PND 7 were not significantly different.

PND 3	Control	2-PCB	3-PCB	4-PCB	5-PCB	6-PCB
Control						
2-PCB	0.67					
3-PCB	0.076	0.26				
4-PCB	0.003	0.04	0.29			
5-PCB	0.44	0.31	0.02	<0.001		
6-PCB	0.61	0.89	0.41	0.083	0.27	

PND 7	Control	2-PCB	3-PCB	4-PCB	5-PCB	6-PCB
Control						
2-PCB	0.39					
3-PCB	0.096	0.27				
4-PCB	0.093	0.35	0.69			
5-PCB	0.4	0.1	0.051	0.014		
6-PCB	0.64	0.79	0.31	0.28	0.23	

Figure 5 Effect of PCB Exposure among Conditions Pair-wise t-test p values for PND 3 (left) and PND 7 (right) comparison of isolation USV emission rates compared between groups.



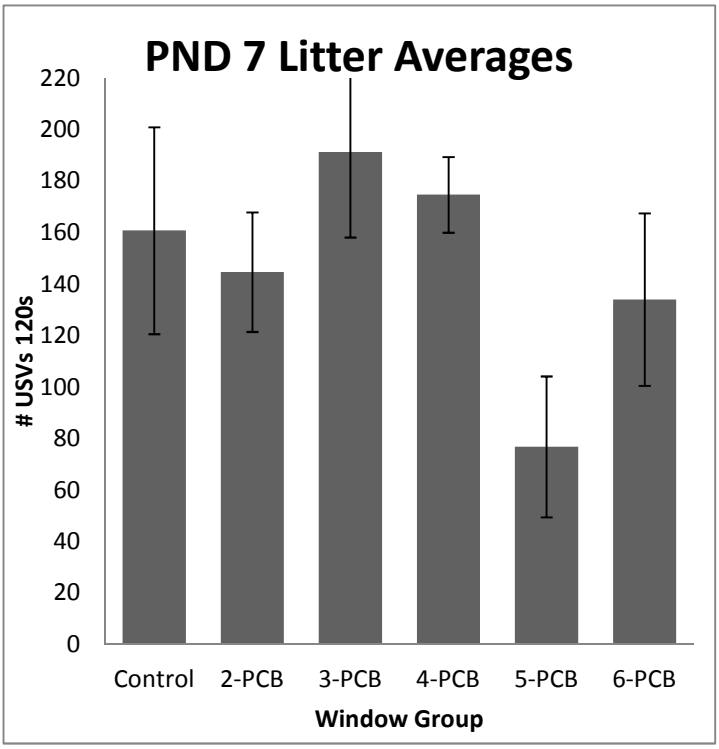
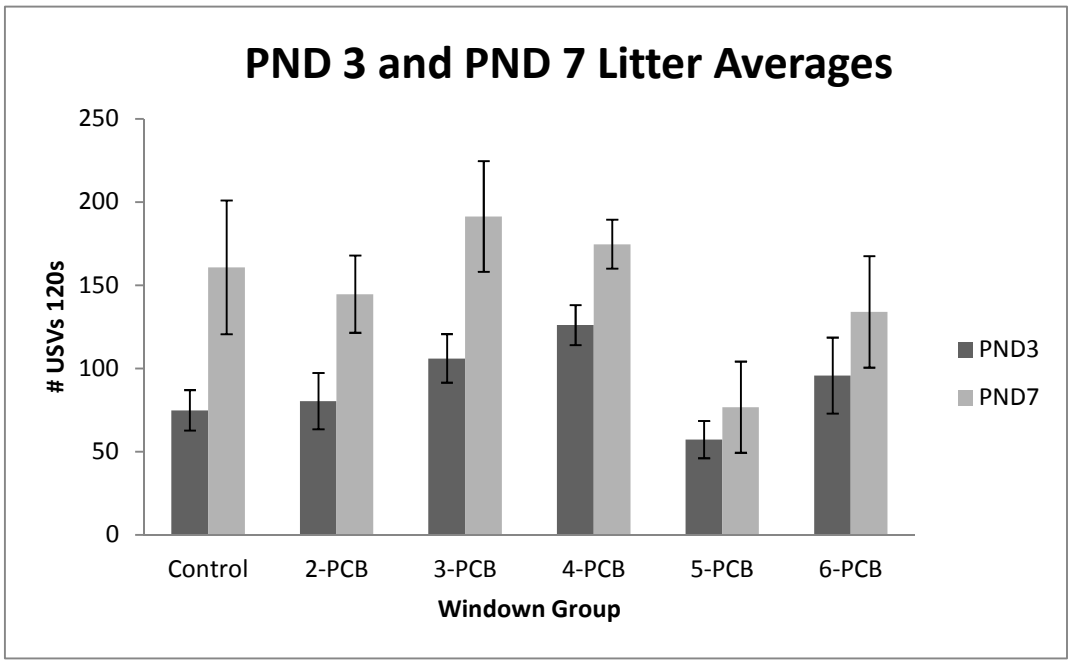


Figure 6 PND 3 and PND 7 Litter Averages Total litter averages for USV counts on developmental test days 3 and 7.

Developmental Test Day (PND) Effect

A three-way ANOVA analysis revealed a significant increase of isolation USVs emitted by pups tested on developmental test day, PND 3 to PND 7 ($F(1, 23)=14.461$, $p<0.001$). Window groups which showed a significant increase in rate of isolation USV emission from PND 3 to PND 7 include; pups exposed to PCB the first and second week of pregnancy (2-PCB) ($t(24)=2.156$, $p<0.05$), pups exposed to PCB the second and third week of pregnancy (3-PCB) ($t(24)=2.755$ $p<0.05$), and pups exposed to PCB the last week of pregnancy and first week of lactation (4-PCB) ($t(21)=2.116$ $p<0.05$) (Figure 7). Controls, and groups exposed to PCB the first and second week of lactation (5-PCB), and last two weeks of lactation (6-PCB) showed no significant difference in USV emission rates between developmental days PND 3 and PND 7 (Figure 7).

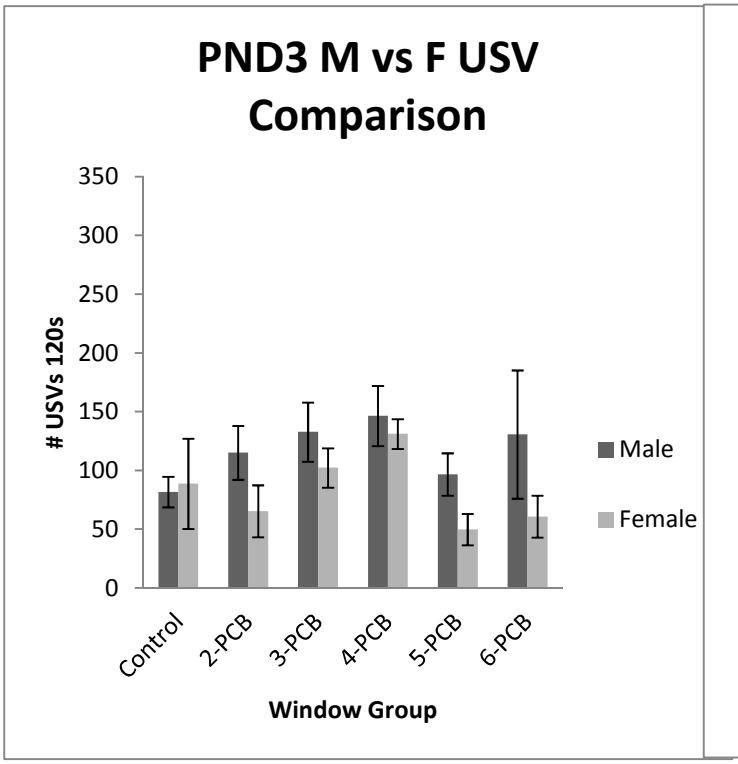


Group	p-value	df	t
Control	0.125	27	1.59
2-PCB	0.041	24	2.16
3-PCB	0.011	24	2.76
4-PCB	0.046	21	2.12
5-PCB	0.457	15	0.76
6-PCB	0.247	11	1.22

Figure 7 Developmental Main Effect between PND 3 and PND 7 Isolation USV emissions were significantly different between PND 3 and PND 7 pups tested in group 2-PCB, 3-PCB, and 4-PCB.

Effect of Sex on USV EmissionRates

Although it appeared as though there may be a difference or trend between the rate of isolation USV emission between male and female pups on PND 3 and PND 7 (Figure 8), ANOVA results showed no significant difference ($F(1,23)=0.094$ $p=0.759$).



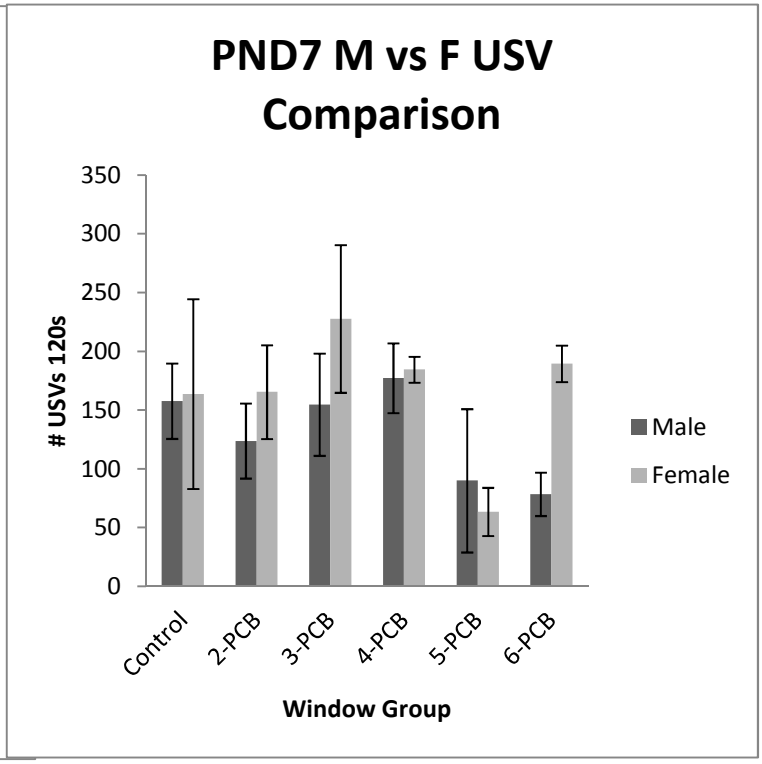


Figure 8 PND3 and PND7 Male vs. Female Litter Averages No significant difference was seen between averages in isolation USV emission between Males and Females within experimental groups.

Discussion

Communication between rodents and several other mammals occurs by way of ultrasonic vocalizations (USVs). This communication is crucial for offspring survival as it elicits maternal searching and retrieval behavior, often times when emitted as isolation USV when a pup is separated from dam or littermates. In the present study, isolation USVs were recorded on PND 3, 7, 14, 21, and 22 alongside several other behavioral and biochemical measures to determine whether there is a critical window for PCB exposure. Isolation USVs for PND 3 and PND 7 were measured analyzed over a 2 minute testing period on control pups and pups exposed to PCB during various two week developmental periods. We found significant differences among conditions as well as significant differences between developmental test days PND3 and PND 7 in regards to isolation USV emission rates. Pups tested in those groups exposed to PCB in groups 2-PCB (first two weeks of pregnancy), 3-PCB (second two weeks of pregnancy), and 4-PCB

(last week of pregnancy and first week of lactation) emitted a significantly higher rate of USVs on PND 7 compared to USVs emitted on PND 3. USVs emitted by PCB litters increased from PND 3 to PND 7 to a level significantly higher than the controls, which could be evidence of a strong anxiogenic effect during a specified period of development. A study by Mankin (2012) also found a significant PCB treatment effect shown by increased USV emissions on PND 10 among rats exposed to PCB during gestation and lactation, as well as those animals exposed solely during gestation. A study by Carden et. Al (1994), also found an increase in USVs in rat pups exposed to U50, 488 tested on PND 3, 10, and 18.

On PND 3, group 4-PCB emitted a significantly higher rate of USVs when compared to the control, 2-PCB, and 5-PCB group, suggesting there is a possible crucial developmental window within the group 4-PCB litters (exposed to PCB last week of pregnancy and first week of lactation). These pups exposed to PCB the last week of pregnancy and first week of lactation (4-PCB) appear to be more mature on both PND 3 and PND 7 with significantly more isolation USVs emitted during the testing period. Pups exposed to PCB during the first two weeks of lactation (5-PCB), emitted a significantly lower rate of isolation USVs on both PND 3 and PND 7, suggesting exposure to PCB during this developmental window causes disrupted slowed development, as they appear to be immature, and unable to call at a rate comparable to controls. Previous studies have shown that exposure to PCB during gestation can limit the levels of thyroxine to the developing pup which can greatly alter neurobehavioral development in both rats and humans. Overall, control animals and those fed PCB only post-natally showed no difference in isolation USV emission rates. Those animals exposed postally showed significant differences in isolation USV emission rates.

Further analysis of all ultrasonic vocalization recordings, grooming behavior, open field behavior, play behavior, and thyroid hormone status will allow further conclusions to be made regarding a critical window period for which PCB exposure has the greatest effects on rat pup behavior. It is thought that those pups exposed to PCB will exhibit altered behavioral measures when compared to a control, and will also have altered thyroid hormone status. When all data is combined, and analyzed, a developmental window could be shown in which PCB exposure has the greatest effects on pup development. Exposure to PCB during gestation or lactation could cause depressions in thyroid hormone levels, which are known to have direct linkages to neurological and behavioral development. The current experiment has clinical implications as detrimental consequences of PCB exposure are known both in model organisms and humans. A clearer understanding of a critical developmental window could shed light on methods to reduce PCB exposure, and the impact of its exposure on developing offspring.

Future Direction

In the present study, it appears that the group 4-PCB exposed pups are emitting a significantly greater rate of isolation USVs when compared to other groups, while the group 5-PCB pups are emitting a significantly lower rate. A current on-going study within our lab is exploring the same effects on pups exposed to PCB during one week developmental windows. Current litters have been tested for PCB exposure during the second week of gestation, third week of gestation, and first week of lactation. Further tests on pups exposed during the first week of gestation, and the second and third weeks of lactation could reveal a smaller developmental window when compared to the two week exposed pups in which PCB exposure has the greatest effect of pup behavior and thyroid hormone status changes.

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Appendix A

This project focused on isolation ultrasonic vocalization testing and analysis. The following behavioral and biochemical tests were performed according to the testing schedule (Figure 3). Future analysis of behavioral and biochemical data could help to determine a critical developmental window for rat pups on the effect of PCB exposure.

Materials and Methods (continued)

Grooming Behavior

On PND 14 two pups, one male and one female, from each litter were separated from the dam and littermates and were immediately taken to the testing room. A single pup was placed into the grooming apparatus and allowed a 5 minute habituation period. At the conclusion of this habituation period, approximately 1ml of water was placed on the dorsal side of the pup to elicit grooming behavior. Each pup remained in the grooming apparatus for 10 minutes to record grooming behavior. USVs were also recorded simultaneously with the microphone next to the grooming apparatus. After grooming behavior was recorded, animals were euthanized and blood samples were collected. Grooming video recordings were analyzed and scored for incomplete and completed grooming chains, as well as flexible grooming bouts (Figure 9).

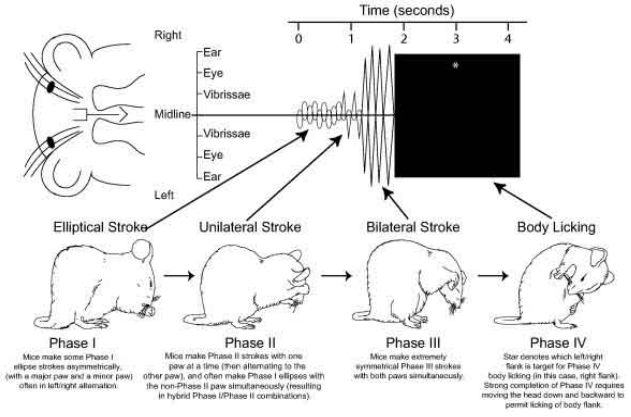

Figure 9 Grooming Chain Grooming behavior is monitored for complete and incomplete grooming chains through phases 1-IV as well as flexible grooming bouts.

Open Field Behavior

On PND 21 two pups, one male and one female, from each litter were separated from the dam and littermates and were immediately taken to testing room. A single pup was placed into the open field apparatus, a 40x50x20 cm box, clearly marked on the floor with 9 squares each with a dimension of 13.3x16.6 cm to monitor overall locomotion by the pups. Movement by each pup was video recorded for 10 minutes with simultaneous USV recording with microphone placed next to the open field apparatus. Horizontal movement (the number of line crossings), vertical movement (rearing), and the number of times the pup enters the center square were recorded. These movements were monitored and counted (each separate movement), and totaled at the end of the session. After open field behavior was recorded, pups were euthanized and blood samples were collected.

Play Behavior

On PND 21, the two remaining pups (1 male and 1 female) were separated from one another and the dam for 12 hours. On PND 22 after the 12 hour separation, pups were taken to the testing room and were reunited in the play arena. Play behavior was video recorded for 15 minutes with simultaneous USV recording with the microphone next to the testing apparatus. After play behavior was recorded, pups were euthanized and blood samples were collected. Video was scored for number of contacts, number of dorsal contacts, and number of pins.

Thyroid Hormone (TH) Determination

After each testing day, rat pups were euthanized by intraperitoneal injection of a pentobarbital based solution (100 mg/kg). Once a stage of deep anesthesia is reached, determined by absence of response to a toe pinch, pups were decapitated and blood collected. Blood was centrifuged and serum frozen for future thyroid hormone determination using a total thyroxine Enzyme Immuno Assay (EIA) coated plate kit. On PND 21 when the last 4 pups were separated from the dam, the dam was similarly euthanized and blood samples were collected for thyroid hormone determination.

Appendix B

Presentations and Publications

This project was started during the summer of 2012 with support from the SetGo Summer Research program. At the conclusion of the summer program, preliminary results were presented at the end of summer SetGo Summer Research Symposium.

I continued working on the project with Dr. Meserve and graduate student, Jeff Baldwin, throughout the 2012-2013 academic year while enrolled in Biology 4010 Independent Research.

I submitted an abstract for publication in The Ohio Journal of Science for the Abstracts 2013 volume produced for the 122nd Annual Meeting of the Ohio Academy of Science. My abstract was reviewed and accepted for publication in the April 2013 Ohio Journal of Science Abstracts edition for the Annual Meeting. I presented my research during a poster session at the 122nd Annual Meeting of The Ohio Academy of Science in April 2013 (University of Findlay Findlay, OH) with financial support from a BGSU Center for Undergraduate Research and Scholarship (CURS) travel grant.

In April 2013, I presented a poster at Posters at the Capitol at the State House in Columbus, Ohio.

I also presented a poster at BGSU's Undergraduate Research Symposium in April 2013.

A proposal was submitted and approved for a CURS Summer Research Grant for Summer 2013 to continue this project. I also received funding from the Suzanne K. Miller Biological Sciences Undergraduate Research Assistantship Award during the 2013-2014 academic year.

Work with this project is on-going. An abstract has been submitted and is under review for publication and presentation at the 2014 Ohio Academy of Science Annual Meeting. An abstract will be submitted for review and presentation for the 2014 International Congress of Endocrinology and The Endocrine Society Meeting in Chicago, IL June 21-24, 2014.