An investigation of the neurobehavioral and developmental effects of gestational exposure to lithium

By

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BACKGROUND

Since the 1950s, lithium has been prescribed to stabilize moods by decreasing severe swings between extreme depressive and manic states. Individuals diagnosed with bipolar disorder are more likely to be widowed, separated or divorced, and have a higher rate of drug and alcohol abuse or dependency. Bipolar disorder is a mental disorder characterized by cyclic periods of mania and depression, has a high chronic relapse rate, and a lifetime prevalence of ~1% (1,4,5). Suicide attempts are much higher for those suffering from bipolar disorder than in individuals who suffer from other mood disorders, and individuals who suffer from bipolar disorder and have suicidal thoughts are more likely to be treated with medication than those without suicidal thoughts (1). Lithium remains the most effective long-term therapy available, and greatly reduces the risk of suicide and short-term mortality in patients with bipolar disorder (2,3).

Bipolar disorder can be broken down into three different classifications: bipolar I vs. bipolar II, typical bipolar vs. rapid cycling bipolar, and mania with mixed features. Bipolar I is the most common type, with the recognized episodes of mania and depression. While bipolar II has episodes of depression, the manic episodes are brief, less intense, often unrecognized and therefore typically unreported by the individual. Individuals who suffer from typical bipolar disorder experience depressive and manic episodes for several months at a time, with normal moods occurring between. Rapid cycling bipolar is classified as two or more episodes of both depression and mania within a year, and can dramatically cycle from week to week or even day to day. Finally, mania with mixed features is described as individuals experiencing concurrent manic and depressive symptoms. Describing the varying types of bipolar disorder is important, as each type will be treated differently. Treatment aims to reduce current symptoms and to reduce the likelihood of relapse. Prevention of recurrence is a top priority, but due to the nature of the disorder, four out of five individuals will experience a relapse. Therefore, a treatment goal is to effectively reduce the number and frequency of relapses. Lithium is an important treatment option, as it provides relief for all types of bipolar disorder (6).

The average age of onset for bipolar disorder is approximately 25 years of age (7). While onset of bipolar disorder is most common in early adulthood, it can certainly occur during childbearing years, as well as later in life. The variability of timing and onset of bipolar disorder, combined with hormonal changes in women, can complicate medical management of symptoms, and childbearing women have a higher frequency of anxiety and mood disorders compared to nulliparous women (8,7). While men and women are equally affected, bipolar disorder presents differently in men vs. women in terms of age of onset, symptoms, the order in which symptoms present, and the overall subjective experience of bipolar disorder. Average age of onset for men

occurs in their late teens to early twenties, but women can commonly experience onset up to their fifth decade of life. Women also experience the first onset of symptoms an average of 3.2 years later than men. Onset of depression is 27.2 years of age for women vs. 22.4 years of age for men, while manic episodes are typically seen at 25.9 years of age for women vs. 21.8 years of age for men. The first symptom typically experienced with bipolar disorder is depression, but this also can vary between genders. Women tend to experience episodes of depression before mania, while men tend to experience manic episodes before depressive ones. Approximately 75% of women experience depression first. It should be noted that depressive features may be misinterpreted as a normal, hormonal mood swings in women, which can delay a bipolar diagnosis. Though there have been identified differences in depression, no studies have been able to find gender differences in the severity of manic episodes. The only identifiable difference is that women tend to experience depressive episodes more than manic episodes while men experience both equally (9).

Women suffering from bipolar disorder are more likely to experience co-morbidity with various medical and psychiatric disorders. Anxiety, substance abuse, eating disorders, and borderline personality disorder are most commonly associated with bipolar disorder in women (9). For both men and women, lithium is the first line pharmacotherapeutic treatment for bipolar disorder.

Lithium has a half-life of 24 hours, with distribution to the brain occurring within the 24 hours. Therapeutic oral administration causes peaks in serum concentrations at approximately 1-2 hours, with complete absorption from the gastrointestinal tract at about 4-8 hours (10). Lithium pharmacokinetics include an equilibrium between intravascular and extracellular fluid compartments, but due to renal clearance, the process of eliminating lithium is slower, as lithium is typically exclusively excreted by the kidneys (10). Pharmacodynamic studies show it is capable of interacting with various neurotransmitters and cell signaling pathways, and has an effect on cytoskeletal phosphorylation, which is believed to induce a neuroplastic change leading to mood stabilization. When lithium is absorbed, it distributes throughout the central nervous system, interacting with various neurotransmitters and receptors to decrease norepinephrine release and increase serotonin synthesis. Serotonin is an important neurotransmitter for mood stabilization and it is hypothesized that a deficit of serotonin is a prime foundation for the onset of depression (11).

Lithium is thought to be effective for bipolar disorder treatment due to its ability to increase the serotonin concentration in the brain to stabilize the mood swings. Lithium also inhibits glutamatergic neurotransmission through interaction with N-methyl-D-aspartate (NMDA)

receptors. NMDA is an amino acid derivative that binds to and regulates the NMDA receptor, which is responsible for excitotoxicity. The binding is important because overactivity of NMDA receptors kills nerve cells by overexcitation. When lithium interacts with NMDA receptors, the cell death is prevented because the receptor cannot bind to NMDA (11). There is believed to be a negative interaction between the NMDA-receptor combination and lithium, but the overall mechanism of interaction is unknown. Lithium efficacy for bipolar disorder is dose dependent, so more severe cases of will likely need a higher dose to have a positive effect (12). However, lithium also has a narrow therapeutic window, meaning there is little room between therapeutic and toxic doses. Potential adverse effects are also believed to be dose dependent, but researchers argue that dose does not have an impact on severity of adverse effects; even a small exposure to lithium could result in any degree of complications (10,13). Lithium also has an effect on sodium channel activity. Therefore, it is important to avoid sodium depletion because it is likely to enhance the potential toxicity of lithium (3).

Lithium maintenance therapy, therefore, requires constant physician supervision due to the high risk of toxicity and to monitor and maintain proper dosing in order to safely avoid severe side effects while effectively stabilizing mood. Relapse rates are 2.3 times greater after discontinuation of a mood stabilizing treatment (85%) than those who receive continuous treatment (37%) (5). It is estimated that 50-60% of woman suffering from bipolar disorder will relapse during pregnancy if they are taken off lithium treatment. Abrupt termination of therapy causes relapse and usually results in greater swings from depression to mania (13). This relapse potential and the risk of severe mood swings leaves physicians hesitant to discontinue treatment at any point in time, including when the patient is pregnant.

In addition to baseline gender differences, for women bipolar disorder presents uniquely in its course due to the reproductive cycle. Hormonal changes across the phases of the reproductive cycle may worsen symptoms, particularly postpartum, but also during the phases of the menstrual cycle, peri-menopause, and menopause. A meta-analysis review identified that approximately 36% of women will experience onset of bipolar disorder during puerperium. The review also stated one-third of women with bipolar disorder experience manic episodes temporally related to childbirth, 67% of women experienced postpartum mood episodes, (predominately depression), and there was a 64% rate of recurring postpartum depression episodes for subsequent pregnancies (9).

Due to the potential for onset to occur around reproductive age, balancing the risks and the benefits of treatment with lithium versus untreated illness during pregnancy creates a troubling case. Healthcare professionals recommend that family planning and birth control use should be

discussed, but often pregnancies are unplanned (6). Once pregnancy is identified, maintenance of lithium treatment is brought into the forefront of prenatal care. Some women want to be taken off lithium, while others feel it is necessary to stay on, which then raises the issue of the risk of teratogenic effects to the fetus. If a woman is not treated with medication for bipolar disorder at the beginning of pregnancy, a woman will likely not be prescribed a new medication. For those on medication, ceasing treatment during the first trimester is recommended because this is the most critical period for growth and organ development of the fetus. Carbamazepine, lamotrigine, valproate, atypical antipsychotics and electroconvulsive therapy are all potential options for treatment, but are shown to not be as effective, and may carry risks of their own (6). Each treatment option has positives, but all have significant side effects that vary from person to person. If a woman chooses to stay on medication, selective serotonin uptake inhibitors (SSRIs) may be prescribed, as they have been found to have no significant associations with fetal birth defects (as compared to lithium). However, SSRIs are not as effective as lithium treatment for bipolar disorder (9).

Some psychotropic agents used to treat mood disorders are known teratogens, which are drugs that can significantly disturb the development of the fetus, and may result in termination of the pregnancy (2,13). Lack of evidence does not imply safety - if a psychotropic agent is not classified as a teratogen, it may be due to the fact that there is no significant data to estimate the potential risk on the developing fetus. Lithium has long been identified as a teratogen. Exposure while pregnant can result in various complications for the child during development and growth such as skin and hair disorders, low birth weight, anxiety-like behavior, poor renal functioning, nephrogenic diabetes insipidous, or Ebstein's anomaly (2). A study from the International Register of Lithium Babies in 2005 showed 36% of infants were born prematurely, while 10% of infants are born prematurely in the unexposed pregnancies (7). Lithium pharmacokinetics can also change during pregnancy. The glomerular filtration rate increases by 50%, with a 75% increase in renal perfusion during early pregnancy, which then declines in the late third trimester. Shortly after parturition, glomerular filtration rate returns to pre-pregnancy levels (7). This is important to note as majority of the lithium is excreted by the kidneys. Not all complications, such as the skin and hair disorders, anxiety-like behaviors or the poor renal functioning, will occur at once, and it is a possibility that none of the complications will occur at all, but the chances of any of the listed complications, or others, are very likely due to the toxic nature of lithium. The risk for Ebstein's anomaly, a congenital heart defect affecting the right atrial tricuspid valve, is approximately 1 in 20,000 in the general population, but the risk increases to 1 in 1,000 for lithium exposed fetuses (13).

The objective of this study was to investigate the effects of gestational exposure to lithium on the short- and long-term neurodevelopment of the offspring using a mouse model.

INTRODUCTION

Fetal development occurs over nine months, divided by weeks into three trimesters. The first trimester is when overall organ development and migration occurs, so this is the time period with the highest risk for drug-induced teratogenic birth defects. Although much of the present drug research focuses on effects of exposure during the first trimester, the brain continues to develop throughout the entire pregnancy (14). Lithium is a potentially dangerous and toxic drug because it readily and completely passes through the placenta, resulting in the fetus receiving 100% of the drug the mother consumes. The estimated toxic level for lithium is >1.2 mEq/liter, and the mother to infant lithium ratio is 1.0 mEq/liter. According to a study done by Newport *et al.*, when examining human lithium serum levels in the umbilical cord and the mother at time of birth, the mean ratio was 1.05 mEq/liter, which was the average from nine mothers with varying dosages, therapy durations and demographics. In order to decrease lithium levels in the newborn, it is suggested to significantly reduce or completely stop lithium treatment for 24-48 hours before giving birth. Stopping for that amount of time can reduce the lithium serum levels by up to 0.30 mEq/liter (13).

It is believed that birth complications are correlated with the level of lithium pregnant women are exposed to during pregnancy, but any lithium exposure for an extended period of time can result in any number of complications (13). High serum levels of lithium at time of delivery are associated with more intense complications, which is why many believe it is important to cease lithium treatment 24-48 hours before birth if the mother cannot be taken off treatment for the length of the pregnancy (13). For example, “floppy infant syndrome” is a key identifier of fetal lithium toxicity marked by characteristics such as lethargy, tachycardia, respiratory distress and cyanosis (15). Infants may be chronically exposed to lithium during the third trimester of pregnancy, especially in the case of discontinuation of treatment during the first trimester (or a decreased dose) to avoid teratogenic effects. The reduction of dose or ceasing of treatment could result in a relapse within a few days or weeks into the pregnancy. Therefore, evaluating the exposure of lithium during the third trimester is important in order to determine whether this particular treatment regimen is associated with adverse effects on brain development that may contribute to long-term neurobehavioral deficits.

Although gradual, complete cessation of lithium before pregnancy would be ideal for the development of the fetus, this is not always possible due to the high risk of relapse. Also, if the

mother chooses to breastfeed postpartum, it is recommended she remain off lithium. Just like in gestation, lithium can pass through the mothers' breastmilk 100% and be fully ingested by the newborn. However, another major concern of the mother remaining off the lithium treatment would be the increased likelihood of the development of postpartum psychosis (13). With the high chances of relapse during pregnancy and developing postpartum psychosis, it is likely that women will remain on lithium treatment during pregnancy and breastfeeding.

Although women are advised against staying on lithium while pregnant because of the severe increase in birth defects or miscarriages, sometimes it can be more detrimental to the mother's mental health to cease treatment, making it likely that women will take lithium throughout or at some point during pregnancy. As stated earlier, lithium pharmacokinetics can be altered by pregnancies due to hormonal and physical changes occurring in the body. The few studies that have been conducted to specifically address lithium exposure in human pregnancies suggest physiological changes that occur during pregnancy can cause changes in drug disposition therefore altering serum drug levels. It is estimated that a 50% decrease in lithium serum concentrations occurs in the mothers. Uncertain lithium concentrations in the mothers creates uncertainty about the lithium levels in the developing fetus. Analysis shows the lithium serum levels during the third trimester is reduced by 34% when compared to non-pregnant lithium serum levels. The reduction in of serum levels in humans is believed to be correlated to the increase renal clearance in pregnant women as lithium is not metabolized, and is excreted through the kidneys (16). Due to the uncertainty of lithium serum levels, effective treatment becomes complicated and therefore requires constant monitoring. If the serum levels are unstable in the mother, it is believed the lithium serum levels are constantly fluctuating in the child.

If lithium causes neurodevelopmental disturbances, this may be linked to its previously observed property of suppressing normal programmed cell death in mice (i.e. spontaneous apoptosis) during neurodevelopment (17). This effect on normal cell death, along with disruptions in neurotransmitter system development, could cause long-term social, emotional, and cognitive deficits, also known as behavioral teratogenicity. Such a finding would also warrant intensive investigation for the light it might shed on mechanisms underlying neurodevelopmental disorders. For example, neuroimaging studies have reported atypical volumetric differences in human adults and adolescents with autism, and abnormalities of some brain regions are implicated in the core autism deficits of social and repetitive/stereotyped behaviors (18,19,20). The volumetric changes in autistic children could be related to a disruption of developing networks during the critical brain growth spurt period. For example, if redundant or faulty neurons are not deleted from the developing brain, certain cortical regions would become over-sized and cluttered with

dysfunctional neurons. Continuous lithium exposure during neurodevelopment could cause long-term suppression of spontaneous neuroapoptosis, which might simulate this phenomenon, and cause the brain to become cluttered with dysfunctional neurons. The mechanism of action of lithium on serotonin is not yet fully understood, but lithium is believed to have an effect on the presynaptic serotonin receptor, the postsynaptic serotonin receptor, and serotonin production. Lithium is believed to down-regulate the presynaptic receptor in order to reduce re-uptake. It is hypothesized to up-regulate the postsynaptic receptor and serotonin production in order to produce more serotonin that would be taken up by the postsynaptic receptors since the presynaptic receptors are not as prevalent. It is also hypothesized that the presynaptic receptor is blocked by lithium so more serotonin is taken up postsynaptic (21).

Serotonergic neurons can first be identified as early as 5 weeks of gestation and by week 15, there is a typical arrangement of serotonin neurons. During development, serotonin is developing pathways that contribute to learning, thinking and stress reactivity. Serotonin has two important roles in fetal development; first it acts as a growth factor, regulating development of neural systems and secondly, it becomes a neurotransmitter in the mature brain to regulate cognition, attention, emotion, pain, sleep and arousal. Many questions have been raised about use of SSRI antidepressants treating maternal depression during pregnancy and how it will impact the subsequent behaviors and mental health of the unborn child across its lifespan. Serotonergic signaling has not been investigated electrophysically or neurochemically in intact, developing fetal brains thus functional consequences in the fetus remains unanswered (33). Serotonin is an important system for normal CNS function, thus disruption of development could result in impaired overall body and neuro-development. Psychiatric drugs studies have shown an alteration in serotonergic activity and create changes in the brain structure when exposed to long-term treatment. The immature brain is believed to overdevelop and produce excessive connections and cells. The connections and cells are later pruned according to their activity levels by apoptotic mechanisms which are guided by existing chemical systems. Human fetal research evidence suggests that serotonin promotes differentiation of cortical and hippocampal neurons (34). Although there is not enough current research on antidepressant impact on the serotonin system, it can be inferred that system would be greatly affected as a growth factor during fetal development as well as during long-term postnatal development.

Exactly how various regimens of lithium treatment during pregnancy affect the long-term development of newborn and child is largely unknown. We can only systematically address the question of whether or not lithium exposure during pregnancy causes short term developmental and long-term behavioral teratogenicity by conducting carefully controlled animal studies

designed to test for differences between lithium and control treatment groups. The hypothesis was that exposure to lithium during critical periods of development would have adverse effects on development and cause long-term behavioral teratogenicity. The experimental hypothesis are as follows:

- The neurodevelopment in offspring exposed to lithium is expected to be inhibited compared to the control pups and the exposed adult neurodevelopment is not expected to change, as majority of development has occurred before lithium exposure.
- The locomotor/exploratory behaviors of the exposed lithium pups are expected to be slower and experience delays compared to the control pups and the exposed adults should not experience any difference compared to adult controls.
- Exposed lithium pups will experience delays and difficulties in strength/balance/coordination compared to the control pups, as overall development has been inhibited.
- The exposed adult females will experience slight delays and difficulties compared to control adults as they have already developed but could still experiencing inhibition from the lithium.
- Reference memory and cognition will be affected for the exposed pups due to overall neurodevelopment being impaired and the lack of ability to create memories will be inhibited.
- Exposed adults will experience more difficulty compared to control adults but will not experience as much difficulty as the pups.

METHODS

In order to identify the effects of full gestational exposure to lithium, we designed a study using to examine the effects of fetal lithium exposure over the full mouse pregnancy. Unsuccessful breeding can be due to miscarriages or potential infertility due to ingestion of lithium. We were not able to identify the cause of unsuccessful breeding. Because the adult females fed lithium chow appeared unhealthy and exhibited abnormal behaviors, we designed a study to test their sensorimotor, cognitive, and social behavior. A new gestational exposure study was also designed to evaluate the effects of third trimester exposure to lithium on development and behavior of mouse pups treated with lithium during the neonatal period. The experimental design of each study is described as follows.

Study I: Full Gestational Exposure

Twenty Swiss Webster mice (Taconic Biosciences, Hudson, NY, USA) 10 females and 10 males, were separated by sex and housed in standard wire-top cages with 3-4 mice per cage. Males were used only as mating partners, they were not actually used in the experimentation. The females were randomly assigned to be fed lithium (7) (NIH-31 mouse lab chow supplemented with 0.3% Lithium, Envigo-Teklad) that or control chow (3) (NIH-31 mouse lab chow, Envigo-Teklad) produces blood concentrations at the upper level of the therapeutic (non-toxic) range for adult humans undergoing treatment for bipolar disorder (22). The lithium chow was introduced in small increments over the course of 1 week by adding lithium chow to the non-lithium chow until the mice were on a 100% lithium chow diet. To help combat potential toxicity, the mice on lithium chow were provided with drinking water containing 1.5% sodium chloride. Lithium displaces sodium absorption therefore adding salt to the water helps return the sodium levels back to a normal level (23). The litters born to the treatment and control dams were monitored closely from birth until adulthood by evaluating body weight, righting reflex, walking initiation, learning and memory performance in the Morris Water Maze, and social choice (see Table 1). The study would be one of the first to use a full gestational exposure model. It was hypothesized that the pups born would be underweight, have developmental delays, and experience long-term deficits in social and cognitive abilities due to the lithium reducing the progression of neurodevelopment Three female mice were designated for the experimental group, with the expectation that three litters (approximately 30 – 40 pups) would be born to the Li-exposed dams. Attempts to breed the mice while on the lithium chow were unsuccessful compared to the controls, therefore this original study had to be abandoned

Within days of the Li-treated females reaching 100% lithium chow, close observation revealed that some of the lithium females became underweight, showed erratic behaviors, and had rough and/or missing fur (as well as one death for unknown reasons). Four of the most affected females were excluded from further use, and did not begin the breeding protocol. None of the three control females were excluded from the study. Specifically, lithium-fed females experienced very erratic behaviors, never habituated to being handled, and showed abnormal locomotor movement. For the lithium-fed females that remained in the study, the breeding protocol involved removal of the lithium chow, and the introduction of one male into the female cages every night, with removal of the male and return of the chow in the morning (12h later). After several months of attempts at breeding, there were no successful pregnancies. Interestingly, the females would occasionally gain weight, potentially indicating a pregnancy, and within a few

days drop down by upwards of 20 grams. After months of no successful pregnancies that resulted in litters of pups, this experiment was terminated and two new studies were developed, as follows.

Study II: The effect of lithium on the health and behavior of adult female mice

Because of the observed differences in overall health and behavior of lithium-fed mice compared to normal controls, a study was designed to evaluate sensorimotor, cognitive, and social behaviors in the two groups of females from Study I. Motor coordination, cognitive performance, and social behaviors were examined using various testing methods described in Table 1.

Careful consideration was given to the sequence of tests and the ages at which they were conducted to minimize "carry-over" and other detrimental effects that may affect performance across measures. The tests were performed in a specific manner to test the specific potential function deficit at the appropriate age where the function should be full developed. It was hypothesized that the lithium-fed adult females would demonstrate impaired performance on the sensorimotor, cognitive, and social choice tests compared to normal, unexposed female controls. The adult lithium-fed females were expected to have reduced functionality on the rotarod and watermaze but not dramatically compared to the control adult females as the majority of their development occurred prior to the lithium ingestion. The social choice apparatus was expected to see the most difference with the lithium-fed females spending more time in the familiar zone while the control females would spend more time exploring with stranger zone.

Study III: A study of the short- and long-term effects of 3rd trimester exposure to lithium

In order to study the effect of gestational exposure to lithium during the third trimester of pregnancy, an alternative study was designed that involved injecting neonatal mice with lithium during the first week of life, or postnatal days zero to seven (P0-P7). The chow was not used because of the uncertainty in the in study I, we were not sure if the neonates would survive to end of testing. The injection also gave an even dose that was tailored to their specific bodyweight. This postnatal period corresponds to the critical and sensitive period of brain growth that occurs in the 3rd trimester in the human fetus. We used a lithium exposure protocol that produces blood concentrations at the upper level of the therapeutic (non-toxic) range for adult humans undergoing treatment for bipolar disorder (22). Mouse pups from one litter were randomly assigned to receive lithium (subcutaneous injection lithium dissolved into saline) or normal saline (10 ul/g volume). The lithium pups were dosed by subcutaneous injection (3 mEq/kg) once daily on postnatal days 4-7 (P4, P5, P6, P7). The pups were removed for injections, weighed, marked, and then returned

to the home cage with the dam in standard mouse cages and housing conditions as previously described. The lithium-exposed and control pups were reared by their natural mother until weaning (P21), when they were then separated by sex and housed 5 per cage. At various ages post-weaning (see table below), growth and development was monitored for all pups to determine whether third trimester lithium exposure causes short-term developmental delays and/or long-term behavioral teratogenicity. Previous research on the neurobehavioral effects of neurotoxic drugs has focused mainly on locomotor and cognitive behaviors. However, recently Satomoto et al. (2009) found that neonatal mice exposed to sevoflurane showed learning deficits and abnormal social behaviors resembling autism spectrum disorder (24). Crawley (2007) has suggested certain tests be used in mouse models of autism that incorporate analogies to core symptoms of the disorder including abnormal social interactions, deficits in communications, and high levels of repetitive behaviors (25). Thus, the social choice test was included to evaluate social approach and social preference (familiar vs. novel conspecific), of social investigatory behaviors. The experimental endpoint was at or around P54 because that was when all specified behavioral tests were completed. Animals were euthanized by carbon dioxide exposure at the end of testing. It was hypothesized that the Li-exposed pups would show decreased body weight compared to controls, and altered righting reflex. We also expected that the lithium-treated pups would show learning and memory deficits compared to normal controls because of delays in neurodevelopment.

Evaluation of neurodevelopment and behavior

The information provided in Table 1 lists the behavioral tests, the functions these tests evaluated, and the ages when the tests were conducted. For adult Li-treated females and controls, body weights were taken daily for the duration of the experiment. For the Li-treated pups and controls, body weight data was recorded from P4-P21 (weaning).

Table 1. Behavioral testing schedule.

BEHAVIORAL TEST	FUNCTIONS	TEST AGES (postnatal day)
Sensorimotor/Learning and memory measures		
Righting Reflex (26)	Neurodevelopment	P7 - 10

Walking Initiation (26)	Locomotor/Exploratory Behaviors	P11 - 17
Rotarod (26,12)	Strength, Balance, Coordination	P35 - 39
Morris Water Maze (26)	Reference Memory/Cognition	P40 - 54
Emotionality/Social Measures		
Social Investigation (27,28,29)	Social Interaction	Adult

Behavioral Testing Protocols

Sensorimotor Battery. Balance, strength and coordination were evaluated by testing the mice on a battery of sensorimotor measures. A 60 second walking initiation test was performed by measuring the time it took a mouse to move out of a small square (21 × 21 cm) outlined on a black tabletop (26). The dependent measure is the time to move all four paws out of the designated square. This test demonstrates normal exploratory behaviors and overall development of motor control in mouse pups. A righting reflex test was also performed in order to measure motor coordination at early developmental age (26). Inability to right signifies significant sensorimotor delays. The mice were placed on their backs with two fingers gently placed on their abdomen. Once the pressure was removed, the mice were given a total of 60 seconds to turn over or 'right'. Each day the mice should have been righting faster and without any difficulty.

A Rotarod test (Rotamex-5, Columbus Instruments) was used to assess strength, balance, and coordination (26, 12). The rod where the mice were placed is 3.0cm x 9.5cm with a fall height of 44.5cm. The tests consisted of two trials per day spanning four days, beginning with a habituation period where the rod does not move. The second day was at a steady speed of 10 rpm. Consecutive tests for the final two days used an accelerating rod (20 and 40 rpm). The Rotarod tests agility, balance and movement by recording the running duration and the speed of the Rotarod at the time of fall. The Rotarod test was performed on all pups, experimental and control, as well as the adult females.

Morris Water Maze. Spatial learning and memory capabilities of rodents were assessed using the Morris water maze (MWM) protocol and a computerized tracking system (ANY-maze; Stoelting, Inc.) (26). The protocol included conducting cued (visible platform), place (submerged and hidden platform), and probe (platform removed) trials in a round pool of water. Mice were first trained on

the cued condition to determine whether non-associative factors were likely to affect acquisition performance during subsequent place trials. A typical water maze procedure was used in which mice experience four consecutive days of cued trials with a visible platform. After completing the cued trials, the mice were trained on the “place” condition to learn the location of a submerged platform. The place trials were conducted for nine consecutive days. During the place trials, the mice were trained using four consecutive trials, 60 second maximum for a trial, with each mouse being released from the same quadrant for each trial. The trials were performed two in the morning with a 3-4-hour break and the final two trials for the day occurred in the afternoon. Overall distance traveled was analyzed as a performance variable for the cued and place trials. A probe trial is administered for the final two days (four trials per day) after completion of the place trials to evaluate retention of the platform location. During the 60 second probe trial, the escape platform was removed and a mouse was placed in the quadrant diagonally opposite from the platform location where they had been placed during all trials. The analysis for the probe trial was based off time spent in quadrant four (where the platform was originally placed) rather than time as all trials ran for the entire period due to the system still having a zone designated for the platform.

Social Investigation. The protocol was adapted from methods previously described (27, 28, 29) and involved quantifying sociability [tendency to initiate social contact with a novel conspecific (stimulus mouse) or cage-mate] and preference for social novelty (tendency to initiate social contact with a novel vs a familiar stimulus mouse) (29, 30, 31). The apparatus used was a rectangular 3-chambered Plexiglas box (each chamber measuring 19.5 cm × 39 cm × 22 cm) containing Plexiglas dividing walls with rectangular openings (5 × 8 cm) covered by sliding Plexiglas doors. A small stainless-steel withholding cage (10 cm h × 10 cm diameter; Galaxy Pencil/Utility Cup, Spectrum Diversified Designs) was used to sequester a stimulus mouse. The withholding cage consisted of vertical bars, which allowed for minimal contact between mice but prevents fighting, and one is located in each outer chamber. A digital video camera connected to a PC loaded with a tracking software program (ANY-maze, Stoelting) recorded the movement of the mouse within the apparatus and quantified time spent in each chamber and investigation zone surrounding the withholding cages. The test ran once for a total of twenty minutes. The test mouse was placed in the middle of the box for a habituation period of five minutes without the doors in place. For the next fifteen minutes, the test mouse was allowed to explore the apparatus and investigate the two mice [one familiar (cage-mate) and one unfamiliar (non-specific)] contained in the withholding cages. The familiar mouse was a cage mate of the same sex while the non-specific mouse was not a cage-mate of the same sex. The number of interactions with each

chamber, in and out of each chamber and the overall time spent was assessed. Laboratory staff were required to leave the room for the duration of the test as to not influence the behavior of the test subject.

RESULTS

Data analysis was completed using mixed-model and between-subjects ANOVAs conducted to reveal differences between treatment groups vs. controls. For the ANOVA tests, the Wilks-Lambda adjustment was used. However, if the probability for significance for sphericity assumption was greater than 0.05, the Greenhouse-Geisser adjustment was applied. For designs comparing two groups, standard t-tests were performed. The water maze data was analyzed as three separate tests (cued/platform visible, hidden platform, and probe/platform removed). The alpha level for statistical significance was determined *a prio*ri to be set at $\alpha<0.05$. A trend was defined as $0.1>p>0.05$.

Study I: Full gestational exposure

There was no data analysis to report for the first study as there were no viable lithium pregnancies and this experimental design was abandoned. The results for data collected on the adult female mice exposed to lithium vs. control chow are reported below in Study II. Four female mice ingesting the lithium chow had to be removed from the study as one died unexpectedly from unknown complications and three other females experienced erratic behavior and overall depleted health. The three-remaining lithium fed females produced no viable pregnancies while the three control female mice experienced two viable pregnancies. The lithium fed females experienced much more difficulty in becoming pregnant and remaining pregnant while the control females were impregnated quite quickly into breeding and continued to carry healthy pups to full term.

Study II: The effect of lithium on the health and behavior of female mice

Weights. There was no statistical significance between the control adult females and the lithium adult female mice over the course of the data retrieval ($F(1,14) = 3.741$, $p=0.074$). The lack of statistical significance could be related to the small sample size in this study. Figure 1 shows the average weights for the adult females. A non-significant trend was identified as the lithium-fed females were heavier than controls.

Rotarod. For the adult females, there was no statistically significant difference between Li-fed and controls for any testing days [t(6) = -0.173, p=0.868; t(6) = -0.009, p=0.993; t(6) = -0.212, p=0.839; t(6) = -1.345, p=0.227, respectively]. Figure 2 shows the average run time from days 1 through 4.

Water Maze. The water maze data was broken up into the habituation (D1-D4) and learning/memory (D5-D13) and probe (D14-D15) trials. The first set of water maze learning data, for the adult females (D1-D4) showed a statistically significant within-subjects effect across days (F (3,18) = 5.329, p=0.008). Over the four days analyzed, there was a significant decrease in the time for the mice to find the platform. The between-groups analysis of distance traveled revealed no statistically significant difference between the lithium-fed and control groups (F (1,6) = 0.520, p=0.498). The two groups traveled similar distances to find the platform. Figure 3 represents the mean distances per day by group.

The next set of water maze trials (D5-D13) data on memory for platform location) was modified due to a degrees of freedom error (too many days of testing compared to sample size). Therefore, data for every other day of testing was analyzed for this set. There was no statistically significant effect across days (F (4,24) = 0.797, p=0.539) or between groups for distance traveled by the lithium-fed vs. control groups (F (1,6) = 0.884, p=0.383). Figure 4 represents the average distance traveled per group over the days 5 - 9 of the water maze. An interaction was not identified for the hidden platform trials, but there was a trend towards an interaction between days of testing and the groups. Although there was no statistical significance, the graph does show a non-significant trend for lithium fed mice traveling a further distance to find the platform. A larger sample size may allow for significant findings.

The last portion of the water maze probe data on reference memory for platform location (D14-D15) showed no statistically significant effect for either within days or between the lithium and control groups [t(6) = -0.412, p=0.695; t(6) = -0.345, p=0.742, respectively]. The number of times the lithium fed group and the control group entered the quadrant where the platform was located during learning and memory trials showed similar mean distances. Figure 5 shows the average number of times the two groups entered into the quadrant where the platform was located for days 1-13 (and then removed during probe trials on days 14-15).

Social Choice. The social choice test evaluated social interaction with a cage-mate and a non-specific mouse. There was a statistically significant effect identified between the time spent in the neutral, cage-mate, and non-specific zones (F (2,8) = 9.443, p=0.010), but no statistically

significant difference was identified between the control and lithium females according to zone (F (1,4) = 0.003, p=0.962). The least amount of time for both control and experimental groups was in the neutral zone while the experimental females spent more time in the stranger zone and the controls spending more time in the familiar zone. Figure 6 shows the average times spent in each quadrant for the Lithium-fed adult females vs. controls.

Study III: A study of the short- and long-term effects of 3rd trimester exposure to lithium

Weights. The weight data comparing the lithium pups vs. control pups were analyzed across days P4-P21. A non-significant trend was identified (F (16,256) = 2.979, p=0.102) between the control and experimental groups over the course of development. Overall, there was a statistically significant difference in body weight between the control pups and pups exposed to lithium in the 3rd trimester (F (1,16) =5.775, p=0.029). Figure 7 shows the average weights of the lithium pups and the control pups.

Righting Reflex. Analysis of the righting reflex data showed a statistically significant effect across days of testing (F (3,51) = 15.828, p<0.005) and a statistically significant difference between the control and experimental groups of pups (F (1,17) = 27.719, p<0.005). Figure 8 shows the average righting time for the lithium vs control pups.

Walking initiation. Data analysis for walking initiation showed a statistically significant effect across days of testing (F (6,102) = 10.261, p<0.005), but there was no overall statistically significant difference between groups (F (1,17) = 1.271, p=0.275). Figure 9 shows the average times of exploratory behaviors for each experimental group.

Rotarod. The analysis of the Rotarod data showed no statistically significant difference between the experimental and control groups [t(17) = 0.566, p=0.579; t(17) = -1.519, p=0.147; t(17) = -0.058, p=0.955; t(17) = -345, p=0.735, respectively]. Figure 10 shows the average run time for days 1-4.

Water Maze. The data analysis for this study was conducted similar to the analyses conducted on the water maze data for the adult female mice. The first set of water maze learning data for the pups (D1-D4) is represented in figure 11, showing the average distance traveled according to group. There was no statistically significant effect across days (F (3,48) = 1.968, p=0.167) and

there was no significant interaction. The lithium pups traveled more each day, but there was only a statistical trend identified between the experimental groups ($F(1,16) = 3.656$, $p=0.074$).

The water maze memory results (D5-D13) are represented in figure 12**.** The control pups traveled a steady distance each day while the control pups decreased their overall distance over the nine experimental days. Results showed a significance across days ($F(8,128) = 2.333$, $p=0.023$), but not between groups ($F(1,16) = 0.105$, $p=0.751$). There was also no significant interaction for the hidden platform trials.

The water maze probe data (D14-D15) showed no statistically significant effect between groups [$t(16) = -0.120$, $p=0.906$; $t(16) = 0.077$, $p=0.939$, respectively]. Figure 13 represents the number of entries into the quadrant where the platform was located on day 14. Controls entered less than lithium pups on the first day of testing, but the next day the controls entered the quadrant more than the lithium pups.

DISCUSSION

Overall, it is difficult to reach definitive conclusions about the effects of lithium during pregnancy according to the results of the three studies completed as part of this research project. Firstly, it is difficult to say whether or not lithium treatment affects fertility and reproduction. Currently, there is no research on infertility and potential reproduction issues in women. The adult lithium-treated females did not produce a litter of pups even though breeding occurred for months on a regular schedule. Future research should design studies that involve an examination of the reproductive cycle of the female mouse using vaginal cytology to measure whether lithium causes changes in the estrous cycle. There is only one animal study examining the full gestational exposure to lithium, which had females ingesting lithium through drinking water at 15 and 30 mg/kg of body weight. The ingestion of lithium occurred at day 1 of pregnancy until postnatal day 15 of delivery and once weaning occurred, the pups received regular drinking water. The experimental design was quite different than what was attempted in this paper. The findings of this paper identified lithium exposed pups were smaller compared to the controls. They also identified delayed development of body fur and eyes opening compared to their controls. Sensory motor reflexes were affected in a dose-dependent manner for those lithium pups exposed at 15 and 30 mg/kg. There are similar findings between this study and my study however, there is not enough evidence provide significant information about lithium using a full gestational model (32).

The development of a full gestational model would be highly beneficial to the research and clinical community to allow better understanding of how lithium affects fertility, gestation, and prenatally-exposed offspring. Future research should involve developing a new model of

gestational exposure, whether it be with a lower dose chow (e.g. 0.1% is available), or attempting daily intraperitoneal injections on the dams before/during breeding, and throughout pregnancy. One female on the lithium chow died, and all of the females had rough fur with missing patches, showed erratic behaviors and were underweight compared to controls - which are all side effects that can be attributed to lithium. A major limitation of this study was that we did not determine lithium blood levels in the lithium fed groups, but previous research shows that human studies monitored plasma levels with lithium doses ranging between 0.8 to 1.2 mEq/L. Plasma level values were found to range from 0.7 to 1.9 mEq/L when lithium was administered at 3mEq/kg and for blood drawn at 1, 3, 6, 12 and 24 hours. Therefore, the values at 12 hours will be higher than at 24 hours. It was also identified that lithium is cleared from the bloodstream much more rapidly in adults than in infant mice (22). This is one of the first studies of this kind using a lithium chow. We suspect that the concentration of lithium in the chow may have been too high to produce viable pregnancies. Using a lower dose of lithium chow (i.e. 0.1%) is an option, and could result in viable pregnancies and offspring.

Our study of the effects of lithium on the adult female produced results that were also difficult to interpret. The hypothesis that there would be a difference in body weight between the lithium fed females and the control females was not supported. Based on the observed poor health and altered behavior of the lithium-fed females, we also expected there may be locomotor and behavioral deficits. Even though it was expected to see a difference in weights between the experimental and control adult females, no weight difference is positive leaving little issue with gaining or losing weight excessively while on a lithium treatment regimen.

It was expected that locomotor ability may be affected – specifically that as the task difficulty increased, the effect of lithium exposure would emerge. For the adults, a change was not identified over the four days of testing as the parameters became harder, which was to be expected as major development and maturation had already occurred. There was also no significance for the pups throughout the four days of testing for the rotarod. Although there was no difference identified between the experimental and control females over the course of testing for the rotarod, the lack of difference is beneficial as the females are over the age of intense maturation thus the lithium treatment will have little to no effect on the overall development.

Results from the water maze supported the hypothesis that there would not be a statistically significant difference in distance traveled during the learning or habituation days (cued, visible platform, D1-D4) between the lithium and control groups. This is expected as there should be no difference between groups as these four days are used as habituation for the mice to get used to swimming in the water four times a day. We expected that there would be evidence

of significant deficits in learning for lithium-treated vs. adult females (D5-D13). Although there was no identifiable difference over days of testing or between experimental groups, it can be inferred that the lithium is not having any major effects on cognition and memory formation in the adult brain. However, there was no evidence to suggest that the control females completed the memory phase of the task with greater ease and accuracy compared to the adult lithium females. The final two days of the water maze assessing reference memory (probe trials, D14-D15) did not show any statistical significance. This did not support the hypothesis that lithium-fed females would exhibit cognitive impairment.

Social behavior was also not affected. It was expected that the control group would be socially inquisitive (i.e. spend more time in the unfamiliar zone) and the lithium group to be less (i.e. spend more time in the cage-mate zone), due to previous research suggesting lithium exposure may alter serotonin system functionality, which could possibly lead to depression-like, anti-social behavior. Although the control and experimental groups spent time in different zones, they both explored all three zones interacting with the cage-mate and non-specific thus it can be inferred that lithium treatment does not have any effect on social tendencies on adults.

Chronic lithium treatment seemed to have little effect on adult behavior in the female mouse. Despite observable appearances, systematic measurement shows that lithium does not appear in this study, to adversely affect female learning, memory, and social behaviors. Because some of the results were close to significant, it would be important to dedicate time and effort to future studies both in animals and humans.

Most research on the effect of lithium during pregnancy examines the effects of first trimester exposure, and focuses on teratogenic effects (i.e. heart defects). To our knowledge, there is no systematic animal research specifically designed to investigate the behavioral teratogenicity of third trimester exposure. Developmentally, lithium affected growth-body weights of exposed pups were underweight compared to the controls. Neurodevelopment was also affected, as measured by righting reflex and later neurodevelopment testing of walking initiation.

The righting reflex test is commonly used to assess basic motor coordination in early days of development. Lack of righting to delayed righting times may indicate impairments or delays in growth and motor coordination. For the righting reflex, the experimental hypothesis was that we would see differences between groups, because the ability to right relies on overall body development. If the lithium adversely affects early development, the lithium pups should take longer to right themselves. The results support the hypothesis – lithium-treated pups took significantly longer to right, indicating delayed neurodevelopment. Over the course of

development and testing, the experimental pups experienced an overall delay in righting which can be correlated to lithium exposure.

Although a difference in groups for the walking initiation was expected, the results showed no difference between the two experimental groups but there was a difference identified over the days of testing. The walking initiation looks at overall body and neurological development as well as exploratory behaviors, which are identified to be affected by lithium treatment. Figure 9 for the pup walking initiation data shows the lithium pups took longer to walk out of the square than the control pups. Although the data was not statistically significant, the test lightly supports the theory of lithium leading to developmental delays due to the lithium exposed pups explored less and took longer to walk out of the square. The lack of difference between groups does not necessarily show any major differences but the difference over the course of testing supports the idea that some changes are occurring between the experimental and control groups but not significantly enough between the two groups.

The results of the learning and memory formation phases of the water maze showed that lithium did not impair cognition. However, a statistically significant trend between the experimental groups could suggest that with a larger sample size, chronic lithium exposure may impair cognitive ability in the adult female mouse. Results did show that both groups learned the water maze task over time, but chronic lithium treatment did not significantly impair memory compared to controls.

Third trimester exposure of lithium seems to have an effect on the developing fetus and thereafter. Although all the results were not necessarily significant, or were as hypothesized, all the results of the tests are vital to our understanding of whether lithium influences the development of the fetus or not. More research is needed to improve on the gestational model, and add power (i.e. increased sample size) to the third trimester study. It would also be interesting to examining pathways that are affected by lithium, and see how those interact with neurodevelopment.

Even though performing animal studies can be difficult at times, preclinical animal models are the most effective and systematically controlled compared to studies involving pregnant women. Although the results were not what was expected, and the first study did not work out as planned, the studies we conducted did provide information on the use of lithium and its effects on the developing fetus.

Overall, it is hard to say whether the results of this study provide any positive or negative support regarding the safety of lithium has any effects on adults or infants. There are no major significant results in this study or other well-controlled animal or human studies to definitively conclude that lithium is safe *or* harmful to the mother or the developing fetus and child. More preclinical, highly controlled studies need to be established in order to support the hypothesis that

lithium may be detrimental to the behavior and development of offspring exposed during pregnancy. It is important to note that just because there is no sound support for the negative effects of lithium does not mean there are no negative effects, but that there needs to be more research. It is also important to remember that in studies testing the adverse effects of drugs, “negative” findings that do not provide evidence of adverse effects are as important as studies with “positive” results – both types of results should be reported for research of this kind where patient safety is a concern.

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APPENDIX

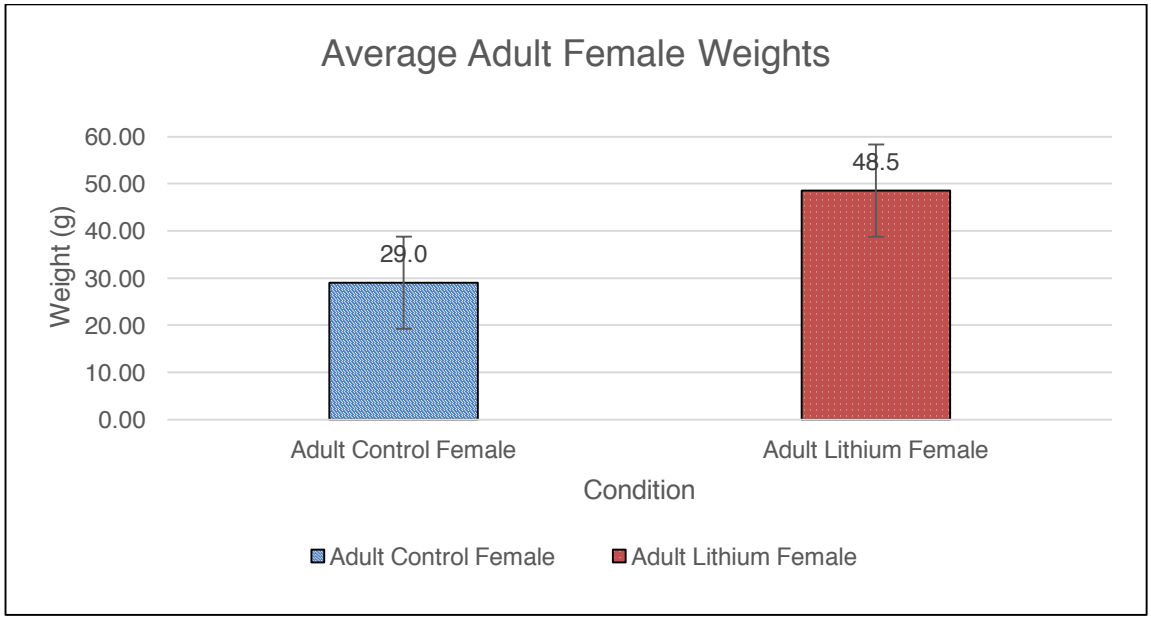

Figure 1. *Average Adult Female Weights*. The average adult control female body weight was 29.0g while the average adult lithium weight was 48.5g. Error bars represent the standard error.

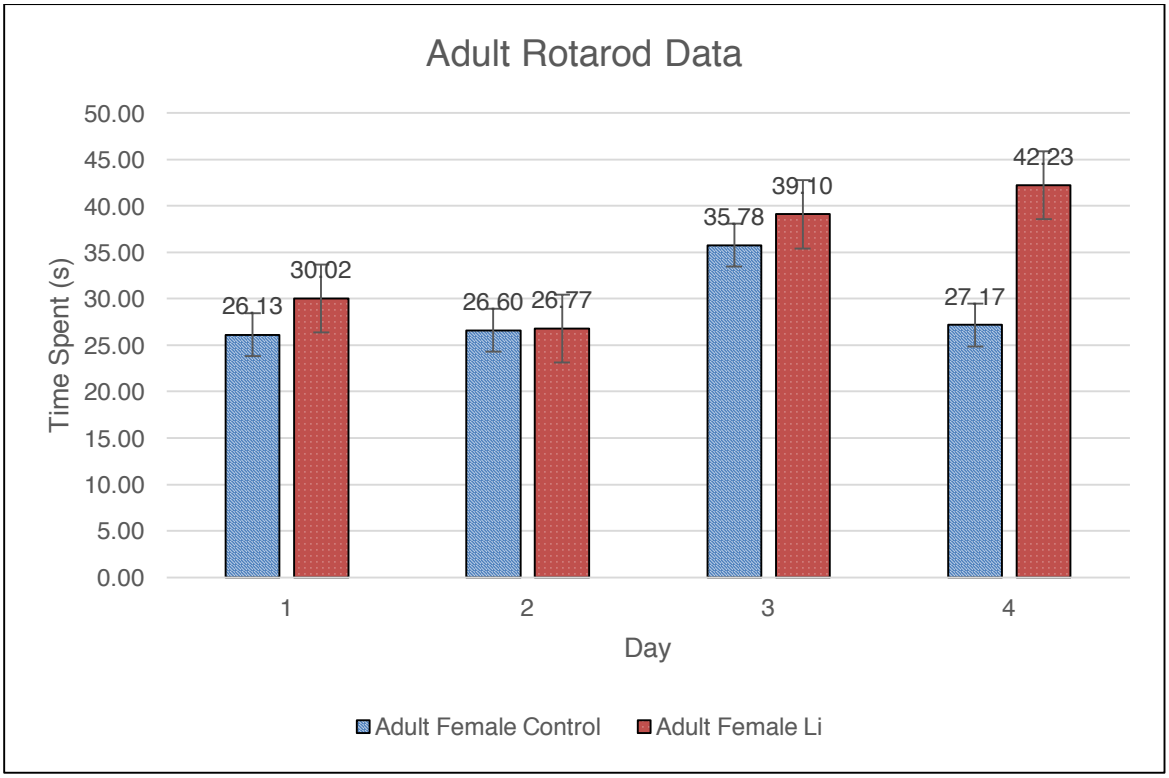

Figure 2. *Adult Rotarod Data.* The average time spent on the rod for days 1-4. Means are given above bars and standard error indicated.

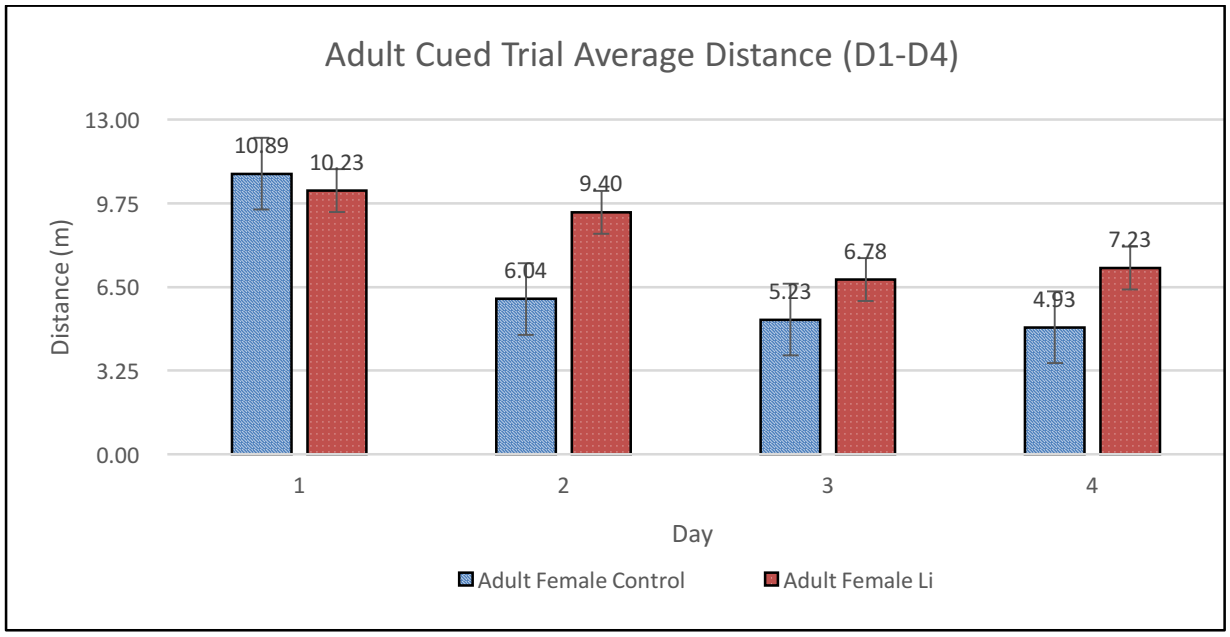

Figure 3. *Adult Cued Trial Average Distance (D1-D4).* The first four days of the Morris Water Maze is described as the habituation days. Means are given above bars and standard error indicated.

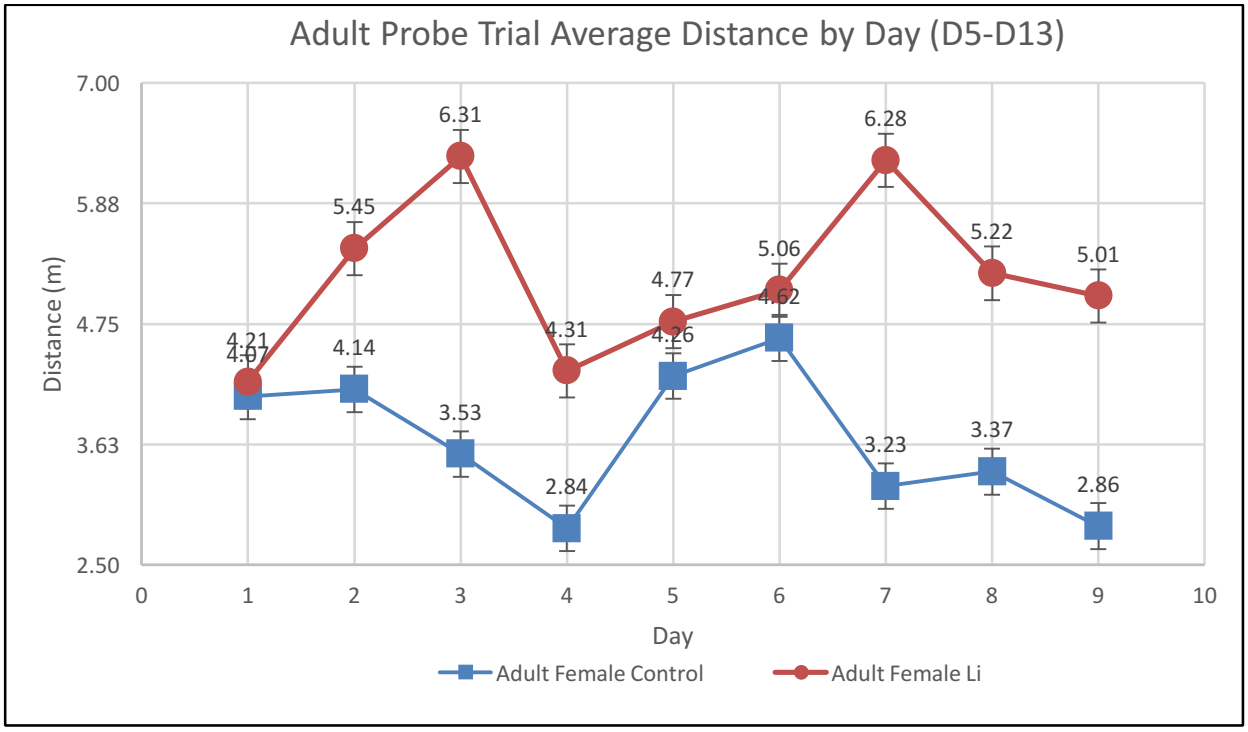

Figure 4. *Adult Probe Trial Average Distance by Day (D5-D13).* The next nine days of the Morris Water Maze was used to identify a learning curve for whether or not the mice can identify the platform sooner each day. Means are given above markers and standard error indicated.

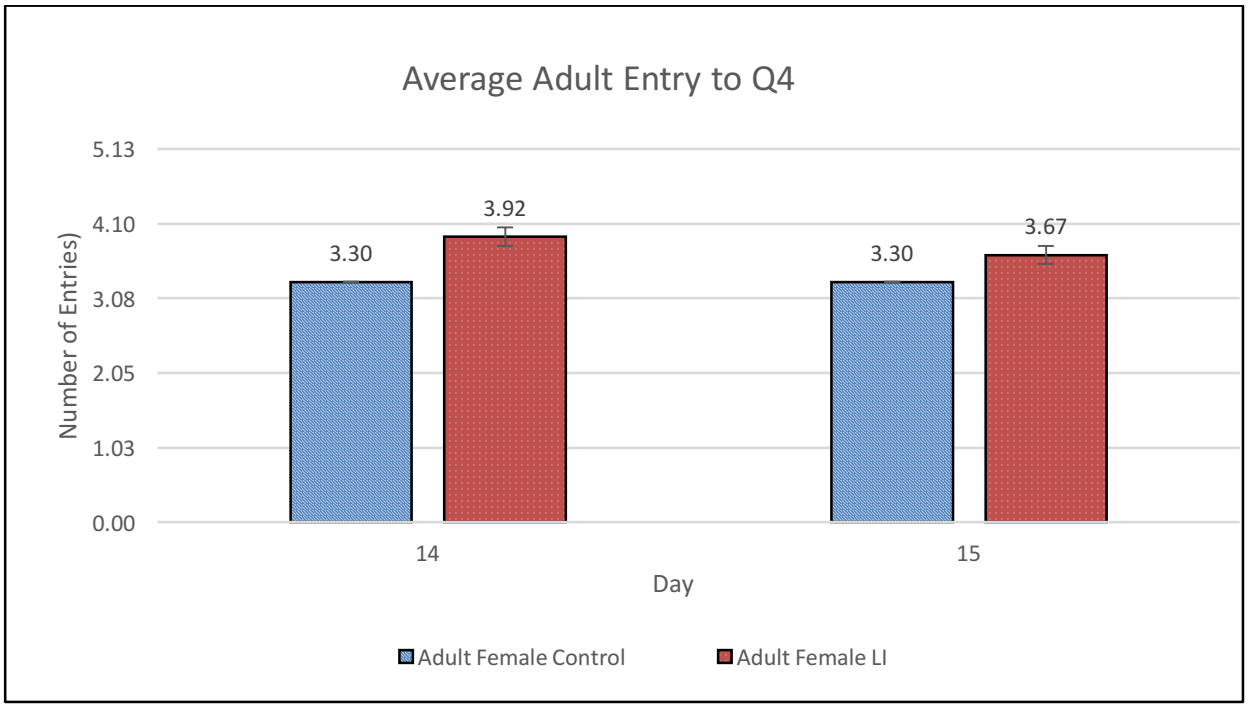

Figure 5. *Average Adult Entry to Q4.* The last two days of the Morris Water Maze was to examine whether or not the mice learned where the platform was located. Q4 is the quadrant where the platform was located for the first two Morris Water Maze trials. Means are given above bars and standard error indicated.

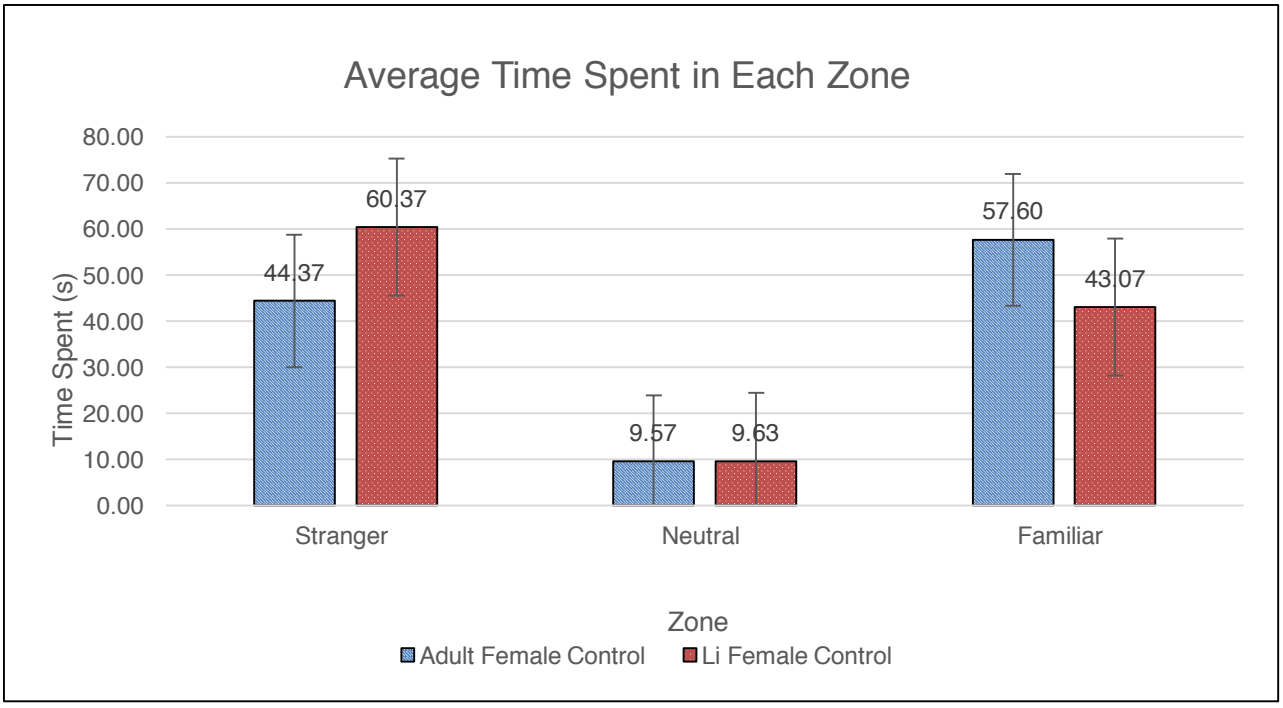

Figure 6. *Average Time Spend in Each Zone.* The figure represents the average time spent in each zone for the experimental groups. Means are given above bars and standard error indicated.

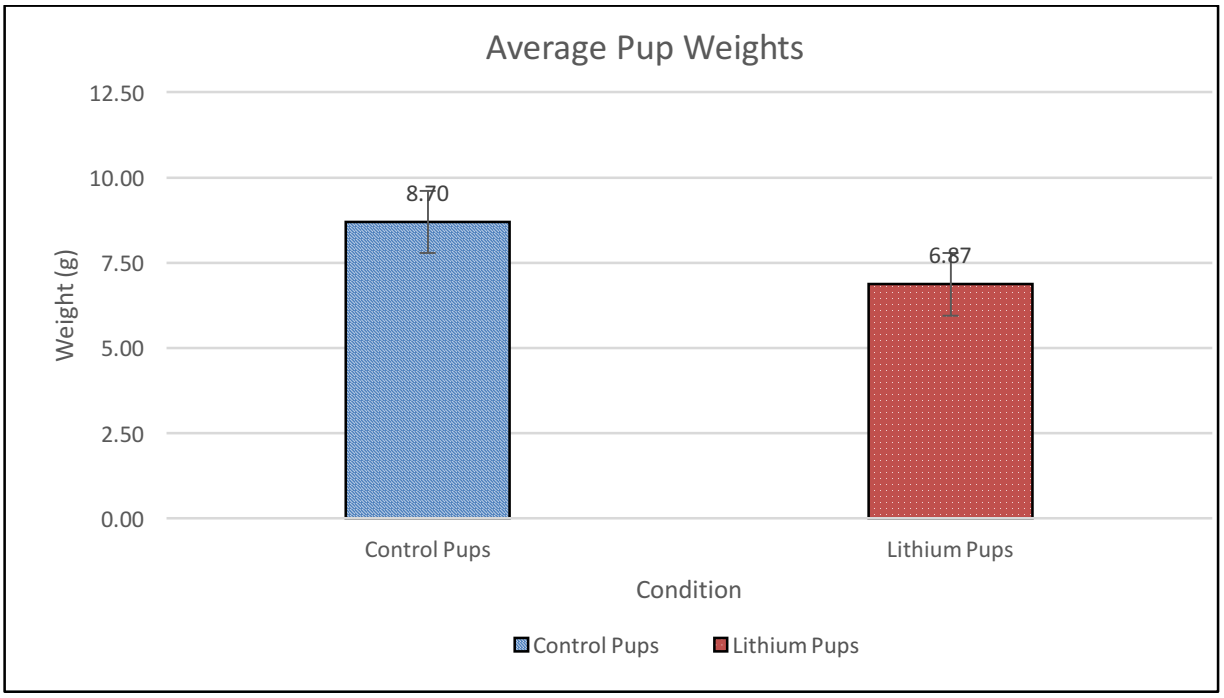

Figure 7. *Average Pup Weights.* The average control pup body weight was 8.70g while the average lithium exposed pup weight was 6.87g. The endpoint of weight collection was P21. Means are given above bars and standard error indicated.

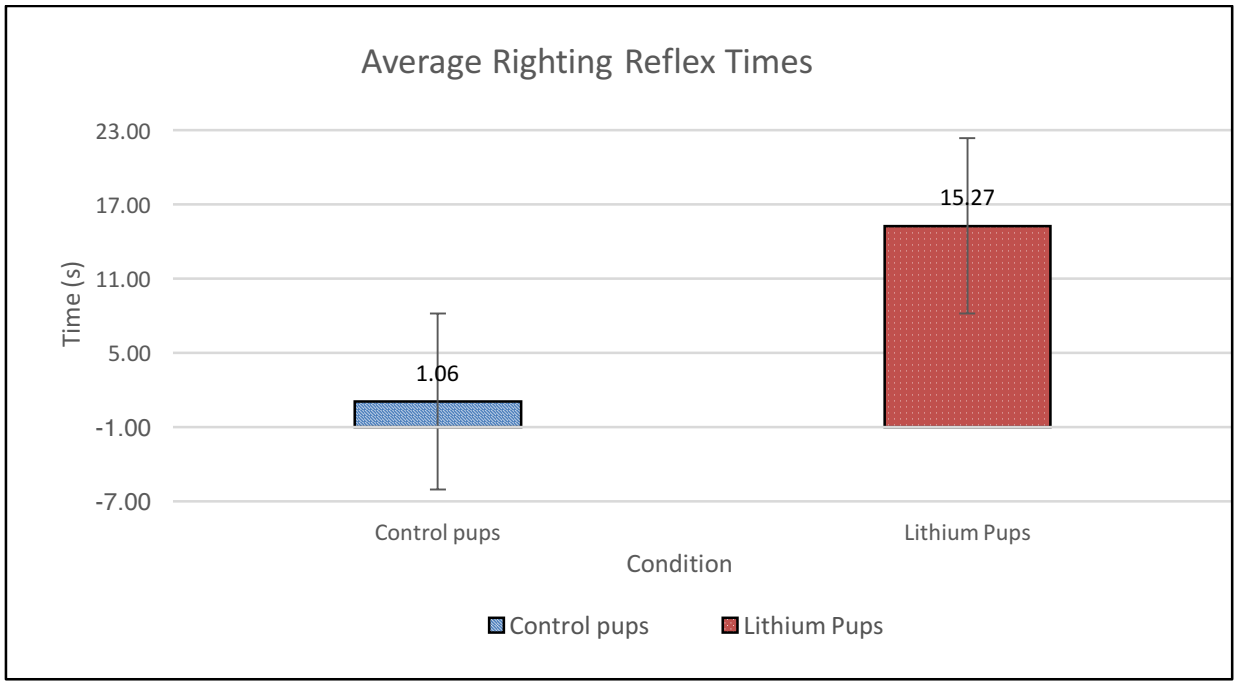

Figure 8. *Average Righting Reflex Times.* The righting reflex times reflect the theory that the lithium pups develop slower physically and neurologically. Means are given above bars and standard error indicated.

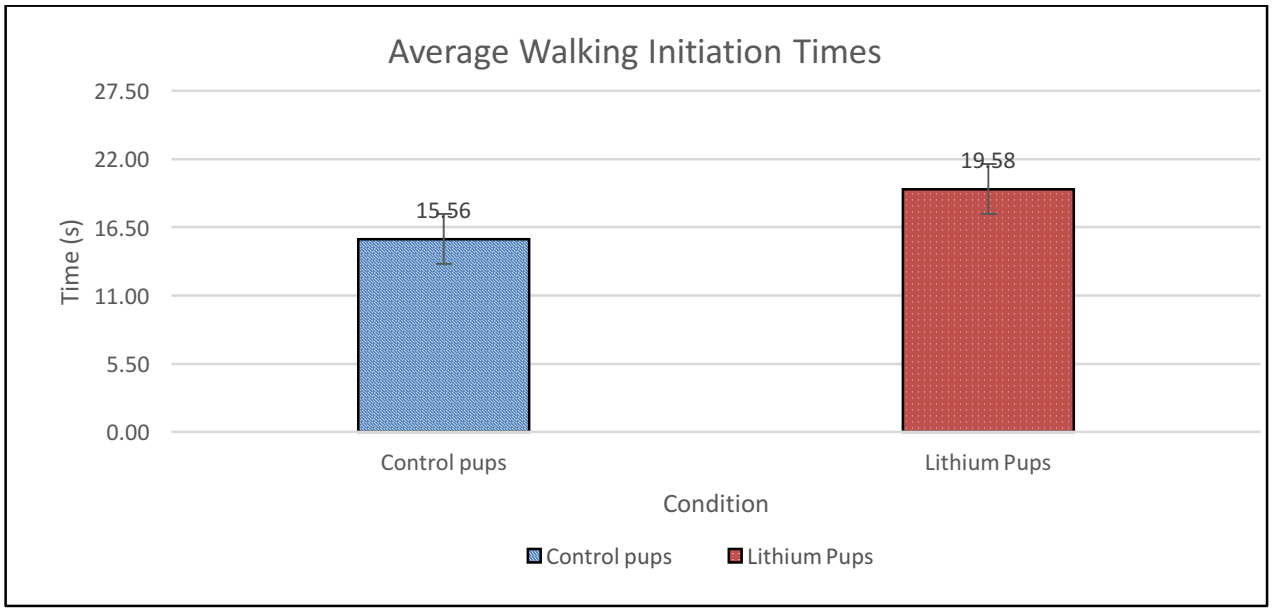

Figure 9. *Average Walking Initiation Times.* Walking initiation examines development and exploratory behaviors. Means are given above bars and standard error indicated.

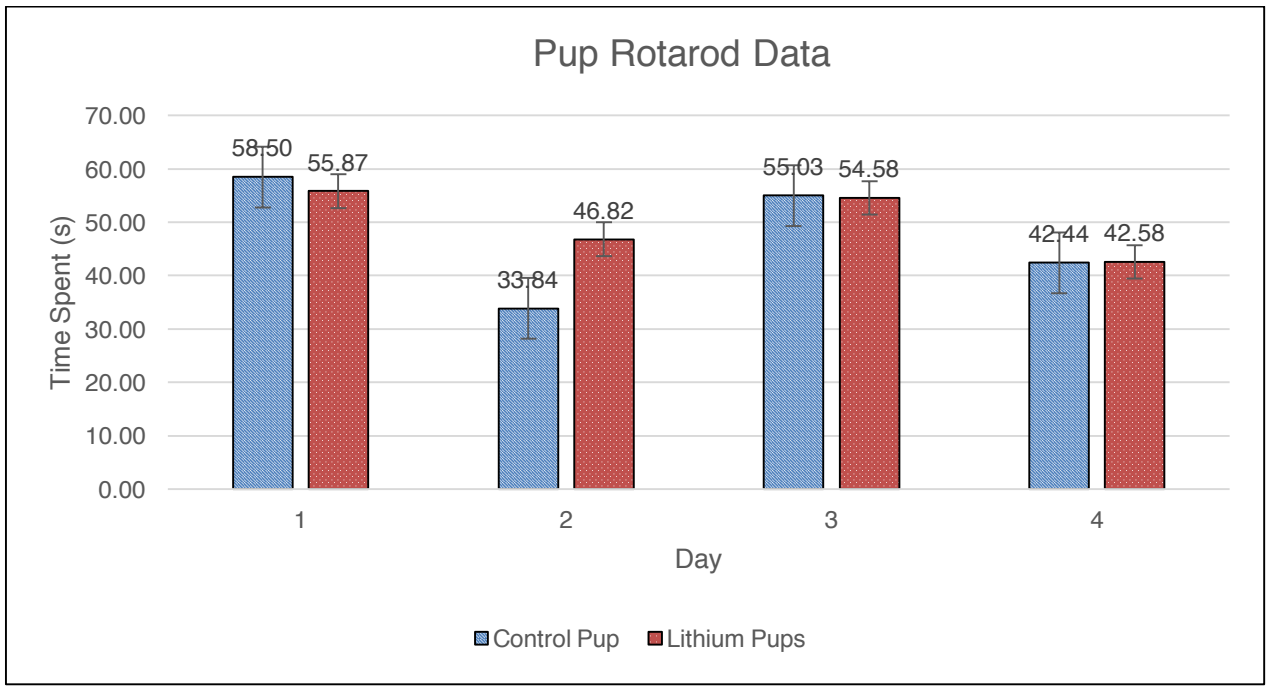

Figure 10. *Pup Rotarod Data.* Average pup rotarod data from days 1-4. Means are given above bars and standard error indicated.

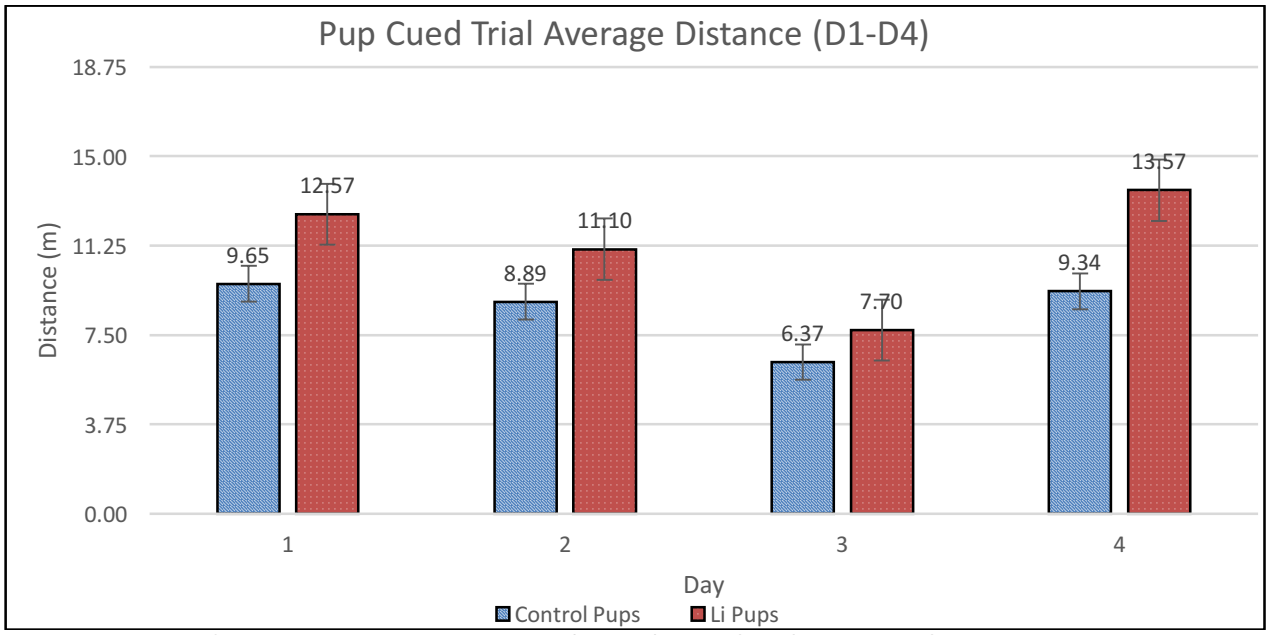

Figure 11. *Pup Cued Trial Average Distance (D1-D4).* The first four days of the Morris Water Maze is described as the habituation days. Means are given above bars and standard error indicated.

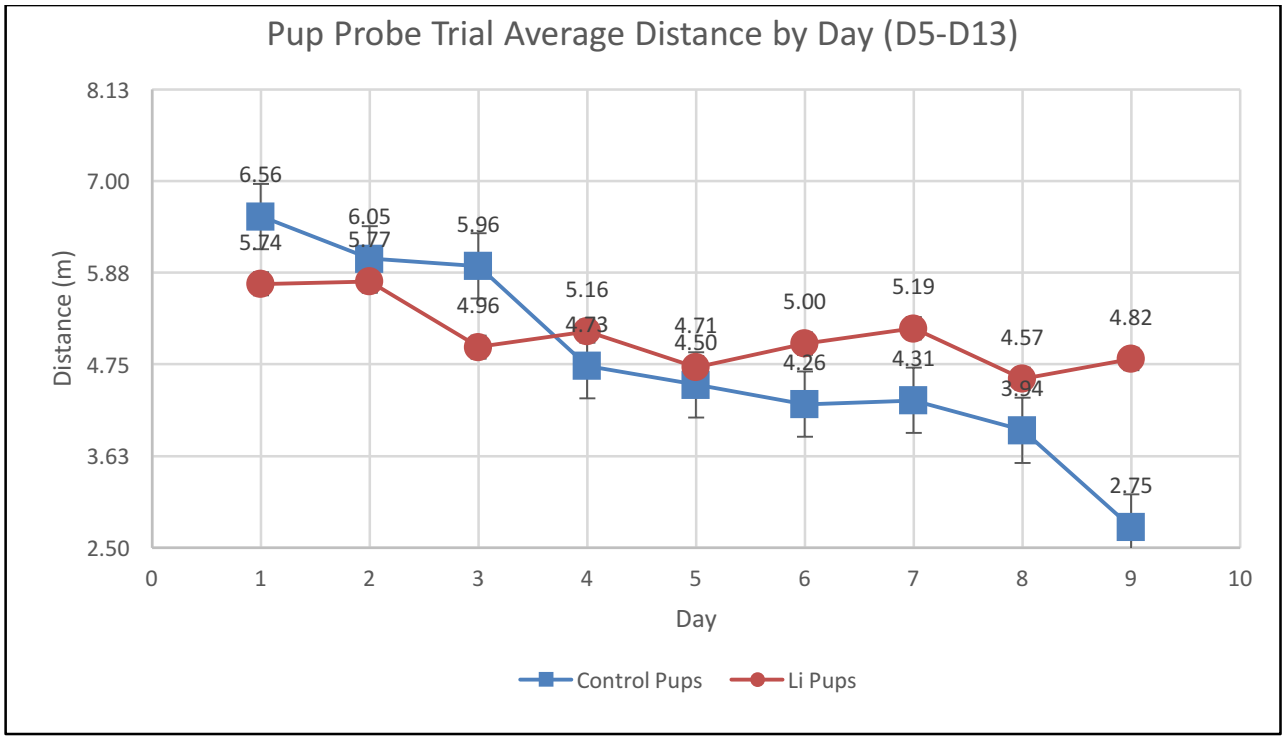

Figure 12. *Pup Probe Trial Average Distance (D5-D13).* The next nine days of the Morris Water Maze was used to identify a learning curve for whether or not the mice can identify the platform sooner each day. Means are given above markers and standard error indicated.

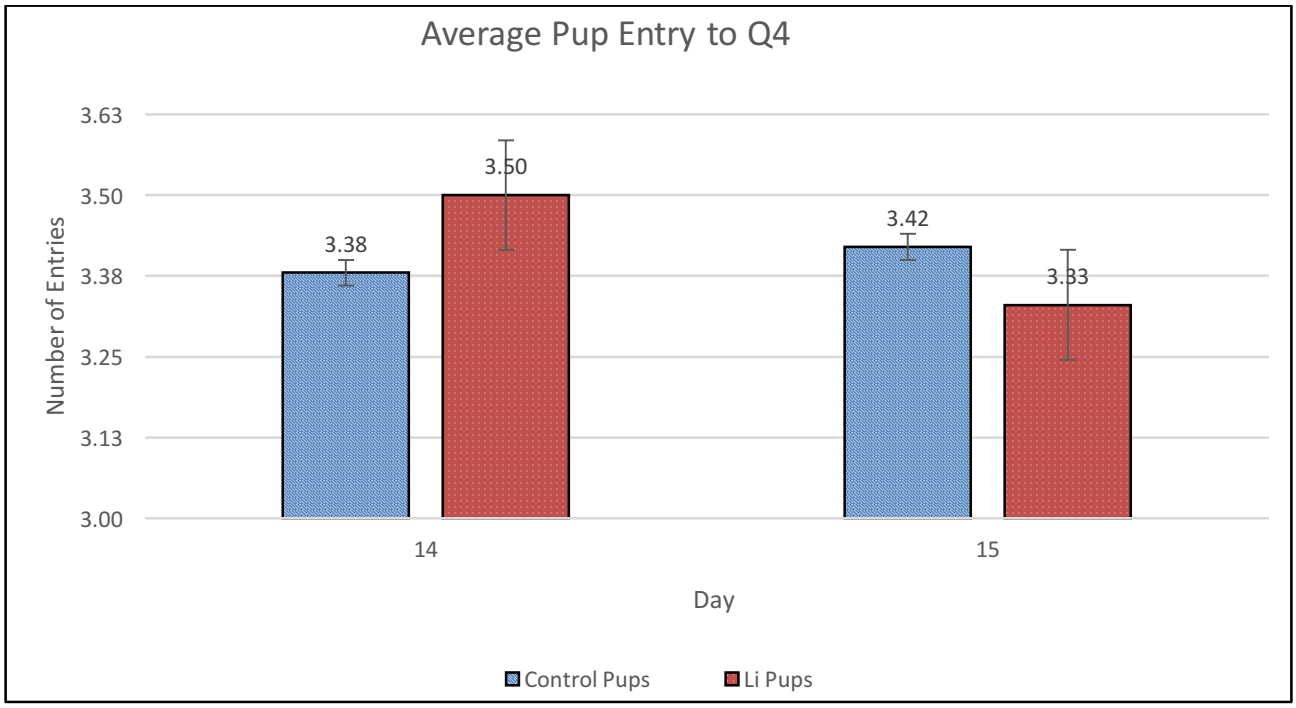

Figure 13. *Average Pup Entry to Q4.* The last two days of the Morris Water Maze was to examine whether or not the mice learned where the platform was located. Q4 is the quadrant where the platform was located for the first two trials. Means are given above bars and standard error indicated.