EXAMINING THE EFFECTS OF SOCIAL REINTRODUCTION ON NEUROPATHOLOGY DEVELOPED IN 129 MICE ON ACCOUNT OF SOCIAL ISOLATION

by

Michael Lamarche
A Thesis
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Arts
Psychology

Committee:

_____ Director

_____

_____

_____ Department Chairperson

_____ Dean, College of Humanities and Social Sciences

Date: _____ Fall Semester 2022
George Mason University
Fairfax, VA

Examining The Effects of Social Reintroduction on Neuropathology Developed in 129 Mice on Account of Social Isolation

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Arts at George Mason University

by

Michael Lamarche
Bachelor of Arts
George Mason University, 2021

Director: Jane Flinn, Director of Cognitive and Behavioral Neuroscience Program
Principal Investigator

Fall Semester 2022
George Mason University
Fairfax, VA

DEDICATION

I dedicate this work to my family, whose support I owe my perseverance to, my friends, who uplifted me throughout this entire process, and Ginger, who lives forever in our hearts.

ACKNOWLEDGEMENTS

I wish to thank my advisor, Dr. Jane Flinn, for her guidance and support throughout this process. I also want to thank the members of my committee for their invaluable input and infinite patience: Dr. Erin Murdoch and Dr. Jennifer Brielmaier.

I would like to thank my friends and colleagues, Lindsay Allen and Nelly Zabala for not only assisting me in the process of this research but for uplifting my spirits and providing me with their unwavering support and companionship. I would also like to acknowledge my research assistants, Matthew Charrters, Alec Gamboa, and Zahid Khan for their immense reliability, dedication, and selflessness throughout our entire protocol.

Finally, my work would not have been possible without the consistent assistance from every member of our generous Cognitive Behavior Neuroscience lab. I will forever be grateful for the bonds that our research has been able to foster.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Elevated Zero Maze ..... EZM
Morris Water Maze……MWM
Open Field……OF
Circadian Rhythm……CR

ABSTRACT

EXAMINING THE EFFECTS OF SOCIAL REINTRODUCTION ON NEUROPATHOLOGY DEVELOPED IN 129 MICE ON ACCOUNT OF SOCIAL ISOLATION

Michael Lamarche, M.A.

George Mason University, 2022

Dissertation Director: Dr. Jane Flinn

The presence of socialization amongst several species has proven to be not only be prevalent in the daily workings of neurological and behavioral processes, but also crucial. Both humans and mice alike are identified as social species due to their proclivity towards collective intraspecies involvement. Conversely, the upheaval of this social engagement has been suggested to result in detriments towards proper behavioral and thus neurological performance. This study investigated the neurobehavioral effects of different social conditions in mice models, consisting of continually isolated, continually group-housed, and socially reintroduced mice groups. Mice in these different conditions were tested by means of thorough behavioral analyses with histological analyses conducted at the study's close to determine the presence of any subsequent neuropathology developed on account of social isolation, and/or any potential alleviation provided by social reintroduction. The majority of the behavioral results in this study did

not yield statistical significance, though mice in the social conditions (both group-housed and socially reintroduced), on average, were observed to perform better than their socially isolated counterparts. Additionally, the results which did prove statistically significant at the 12-week period, namely the Open Field, and Circadian Rhythm tests, were in line with our previous isolation study results suggesting that isolation leads to heightened anxiety and disturbed circadian activity. The significant results from the Elevated Zero Maze at the 20-week period may suggest that the provision of social opportunity at least enables higher protections against deficits in anxiety-related behaviors. Significant results for sex in the EZM test may also suggest a sex-based influence in regards to the aforementioned behaviors.

Introduction:

A social species is characterized by the formation of associative, organizational, and at times, even competitive, relational mechanisms between the members of its populations (Cacioppo, 2011). Rodents and humans alike are considered innately social organisms, observable not only by their natural social involvement, but by the consequences of their social haltering as well. Social isolation is considered a threat to increase mortality rates, as it may perpetuate negative behaviors and/or emotions contributing to faults from increased blood pressure, to heightened suicidal ideations. Several studies have also recognized this detriment in the form of cell/ protein loss following periods of prolonged isolation. As outlined in a study conducted by Ikram et al., mice subjects who were isolated expressed elevated cortisol levels as well as heightened inflammation and subsequent hippocampal atrophy, collective characteristics which contribute to the overall depletion of proper neurological functioning (Ikram, et. al, 2008). These effects were not mimicked in populations which were socially opportune. Furthermore, in a similar study conducted by Huang et al., transgenic mice which were also individually housed experienced pathophysiological changes in the forms of hippocampal atrophy, protein loss, neuroinflammation, and decreased memory functionality (Huang & Wang, 2018). In our lab's previous experiments looking at the effects of social isolation in mice, we observed increased levels of anxiety, disturbed circadian activity, and even moderate cell loss (Barkey & Kraven, 2021). Considering the connectivity between these findings, there is undoubtedly a strong pattern along this

sequence of social isolation and neurological degeneration, however, there remains a concern behind the longevity of the damage imposed by this isolation. That is, does the damage persist even in the presence of social remediation, and to what extent? This study seeks to answer these questions, and to determine whether the neurological and social deficits seen following isolation can be remediated following the reintroduction of social engagement (group housing).

Neurological Impairment and Social Isolation

Psychological disorders often lead to isolative behaviors, ultimately increasing rates of harmful symptomology. According to the Center for Disease Control and Prevention, approximately a fourth of elders aged 65 and older are considered to be socially isolated (Center for Disease Control and Prevention,2021). This detriment extends further into marginalized communities, including those belonging to LGBTQIA+ and immigrant demographics as well. The extent of these impairments may be so exacerbated by the effects of social isolation that there have even been reports of subsequent psychotic-like symptomologies. In a study conducted by Lapiz et al. (2003), isolated-reared mice were observed to display significantly reduced presynaptic noradrenergic hippocampal functioning as well as enhanced presynaptic dopamine functioning within the amygdala. These imbalances were seen to result in behavioral responses which mimicked those seen in human cases of schizophrenia, such as exaggerated responses to otherwise novel stimuli, or to stimuli predicative of danger (Lapiz, 2003). On the contrary, social opportunity has been popularly known to be correlated with an array of psychological and physiological benefits, including rewarding developments within cognitive and emotional processing, as well as aiding in the proper development of our neurological networks and structures. In a study conducted by Vanderschuren et. al (2016), social play alone was seen to foster neurological development amongst rodent populations, illustrating a stark contrast between the outcomes of isolative versus socially opportune conditions (Vanderschuren, 2016).

Further significance for this experiment lies in this fact that social isolation is associated with increased mortality rates and the potential onset of further neuropsychological disorders, so exploring ways to both combat and reverse the threat of this issue pose prospective alleviations in risks within these often-isolated communities (Lapiz et al., 2003). We aim to observe these patterns between the scopes of social isolation/ reintroduction in order to establish a stronger understanding behind the mechanisms of this evident association between social isolation and behavioral/ neurological detriment. Additionally, these aforementioned studies do not include a socially reintroduced group, so presenting this unique experimental condition allows for an entirely new perspective when observing the effects of socialization.

Physiological Impairment and Social Isolation

As stated previously, the primary value of this study lies in its goal to examine the effects of social reintroduction on previously isolated (mice) populations to see if there are potential improvements or reversals in neuropathology and/or behavioral functionality. Social isolation is a notorious threat against neurological functioning, with its detriment only worsening upon the onset of Alzheimer's disease, TBIs, and other neurological pathologies. This unfortunately common dichotomy between social isolation and neurological impairment has also been viewed to contribute to alarming increased rates of developing further deficits, such as inactivity, depression, and heart failure (Center for Disease Control and Prevention, 2021). According to a report from the National Academies of Sciences, Engineering, and Medicine, social isolation is even associated with a 50% increased risk of developing dementia for Alzheimer's patients (National Academies of Sciences, Engineering, and Medicine, 2020). These extreme consequences are often combated with social interventions such as in the study proposed by Tong et al., where group intervention activities were seen to promote stronger social support for isolated elders (Tong et al., 2021). However, there remains a stronger need to assess the longevity of the damage done onto the brain by this social isolation and just how observable reversals in this damage may be, i.e., is subsequent cell/ protein loss reversable, are the behavioral detriments able to be overturned back into normal function, etc. These are questions which may only be answered through our involvement with

experimental animal models. These goals may be applicable not only to communities which are already prone to the plights of social isolation, but to the general public as well, as they would potentially provide further support for the beneficial effects of sociability on memory and behavioral improvements.

Furthermore, drawing influence from our laboratory's previous study regarding the observations of TBI effects within social isolation, results showed that isolated mice experienced deficits in circadian activity and behavioral disinhibition (Barkey & Kraven, 2021). Looking at the effects of social isolation without the rmTBI measure and instead, a reintroduction condition allows for us to broaden the previous findings by specifying the potential effect of socialization in behavioral/ neurological impairment. This study also allows for these observations to be made through manipulation of a less aggressive mouse strain (129s1). This may introduce comparatively interesting findings when analyzing the results between this prospective study, and those which we have completed prior. Additionally, regarding brain analysis, observing the significant changes within levels of cell density may provide for valuable findings concerning the role that social isolation has on the functionality of central processing, and if this potential disruption may be rectified following subsequent social engagement (Fliers, 1985). This may then highlight several generalizable benefactors to social interventions, which may then be applicable to other circumstances exhibiting similar disruptions within the functions of these neuropeptides, such as in Alzheimer's or TBI-like conditions.

Experimental Groups

In order to accurately observe the behavioral and neurological differences between social isolation and social reintroduction, subjects for this experiment were divided into three distinct groups, being those who are to be continually group housed, those who are to be continually isolated, and those who are to be socially reintroduced following an 8-week period of prolonged isolation. Loosely following the timeline outlined in our aforementioned isolation study, mice in the experimental conditions for this trial were isolated for a period of 8 weeks in order to display a significantly observable rate of cognitive impairment, as a 2-week isolation period was not shown to be effective enough (Barkey & Kraven, 2021). Levels of cognitive and behavioral impairment were measurable by means of observed burrowing, nesting, and circadian activities as well as several additional behavioral measurement examinations, including the open field test, elevated zero maze, and Morris Water Maze. There were two periods of behavioral testing and histological analyses, conducted once for the subjects belonging to the continually group housed and socially isolated conditions, and once for those in the socially reintroduced category. Both males and females were included in the study. In short, 2 x 2 (sex x housing condition) ANOVAs were conducted to analyze the behavioral data for the first two groups at the first testing period (at approximately 12 weeks). 2 x 3 (sex x housing condition) ANOVAs were conducted to compare the groups (at approximately 20 weeks). Mice in the reintroduced condition were isolated for 8 weeks and then reintroduced following another 8 weeks.

In short, a factorial ANOVA accounting for both housing condition and sex differences were conducted for the first two groups at the first testing period (at approximately 12 weeks) and additional factorial ANOVA now accounting for the three subsequent groups for the second round of testing were conducted following the second round of testing (at approximately 20 weeks). Mice in the reintroduced condition were isolated for 8 weeks and then reintroduced following another 8 weeks.

Hypotheses

In sum, the proposed study sought to evaluate the efficacy of social reintroduction following periods of social isolation in reversing subsequent behavioral and neurological impairment within 129s1 mice subjects.

The hypotheses were as follows:

1) Mice who were socially isolated were expected to display deficits in nest building behaviors compared to mice who were not socially isolated. For mice who were socially reintroduced, these deficits were expected to diminish and thus, show better improvements nest building behaviors compared to isolated mice.
2) Mice who were socially isolated were expected to display deficits in burrowing behaviors. For mice who were socially reintroduced, these impairments were expected to reduce, and burrowing behavior would thus be better compared to socially isolated mice.
3) Mice who were socially isolated were expected to demonstrate deficits in long-term learning and spatial memory capabilities as observed in their Morris water maze performances. For mice who were socially reintroduced, these impairments were expected to diminish, and long-term learning and spatial memory performance would thus be better compared to socially isolated mice.

4) The circadian activities of mice who were to be socially isolated were expected to be disrupted on account of the prolonged isolation. Mice who were socially reintroduced were expected to display improvements and thus perform better than isolated mice in circadian activities.
5) Exploratory and anxiety-related behaviors as examined by means of open field behavioral tests were expected to worsen for socially isolated mice. Mice who were to be socially reintroduced were expected to display improvements and thus perform better than isolated mice in these aspects.
6) Mice who were socially isolated were expected to show increased anxiety and behavioral disinhibition following elevated zero maze conduction. Mice who were to be socially reintroduced were expected to display improvements and thus perform better than isolated mice in these behaviors.

Method

Subjects

Considering that this experiment involves the reintroduction of mice following a period of social isolation, as well as prolonged group-housing circumstances, the mice strain was selected based on what was least likely to exacerbate instances of cage aggression between the animals. In a study which examined the rates of aggression between various strains of group housed mice, 129s were among some of the lowest recorded rates of aggressive or instigative behaviors (Lidster, 2019). Animals were bred at George Mason University's Krasnow Institute for Advanced Study Animal Facility. One male mouse was paired with two female mice for a period of two weeks. After two weeks, mother mice were separated and housed individually until the pups were weaned. Weaning occurred between 21 and 28 days after birth. Following weaning, mice were housed according to sex. Males were housed with 2-4 per cage and females were housed with 2-6 per cage. Mice were provided with enrichment in their home cages through the duration of the study, which included an "igloo", a running wheel, a NylaBone for chewing, and nesting twists. Food and water was provided regularly, and lights were maintained on a 12 hour light/dark cycle.

Our first round of breeding resulted in 58 mice, shy of our desired experimental 60. We then initiated a second round of breeding which yielded 5 more mice. Due to the

importance of time period on behavioral assessment, the mice were experimented on separately in accordance with their respective birthdates, in order to ensure that their first rounds of testing occurred at the accurate 12-week and 20-week periods. Results of behavioral assessments, however, were collectively considered. Ultimately, results of breeding yielded 22 isolated mice, 21 reintroduced mice, and 20 group housed mice.

Table 1 – *Number of subjects in each experimental condition, Continually Group Housed Mice (n=20), Continually Isolated (n=21), Socially Reintroduced (n=22) at start of experiment*

Continually Group Housed Mice	**Continually Isolated Mice**	**Socially Reintroduced Isolated Mice**
Group Housed Since Weaning	Isolated Starting at 4 weeks	Isolated since 4 Weeks, Reintroduced after 8 Weeks Isolation
n = 20	n = 22	n= 21
Total: 20	Total: 22	Total: 21

Total: Experimental Mice: 63

Table 2 – *Number of mice belonging to each sex and housing condition at 12-week testing period*

Females	32
Males	31
Isolated	43
Group Housed	20

Table 3- *Number of mice belonging to each sex and housing condition at 20-week testing period*

Females	21
Males	19
Isolated	13
Group Housed	13
Reintroduced	14

Procedures

Due to our breeding results, two cohorts were run, one consisting of the original 58 mice, and the other, for the remaining 5. For the first cohort**,** the experiment started with two groups, consisting of 20 mice who were group housed since weaning, and 38 mice who were socially isolated from 4 weeks of age. Mice in these conditions were tested at 12 weeks, after being assigned to housing conditions. Following this period of testing, 18 brains across groups were taken. Since mice who were considered socially reintroduced were only isolated at this point, this made for a total of 9 group housed brains and 9 isolated brains collected. The remaining 40 mice were then sorted into three categories, being either the continually group housed mice, continually socially isolated mice (half from previously isolated condition) and mice who were socially reintroduced via block party cages after their isolation period (the other half from the previously isolated condition). Mice were socially reintroduced gradually, by means of block party cages in order to limit their levels of subsequent aggression. At the 20-week period, behavioral testing was to be conducted, and the remaining brains collected for further histological analysis.

Regarding the cohort of 5, the mice were identified as 3 isolates and 2 prospectively reintroduced. This raised the total experimental samples to 22 isolated mice, 21 reintroduced mice, and 20 group housed mice (Table 1). No brains were taken at the 12-

week period for the smaller cohort as the previously collected brains had already been accounted for, so brain extraction did not occur until the end of the 20-week behavioral assessment.

In sum, all groups were tested behaviorally at 12 weeks and subsequently tested at 20 weeks. There was a 24-hour break period between each behavioral test. Mice did not undergo more than one test per day (see procedures listed below).

Mice who fell within the socially isolated housing conditions were isolated by means of individual housing units (one standard mouse cage per isolated mouse). Mice who were continuously group housed were housed 3-4 mice per cage unit. The mice who were initially socially isolated, and then socially reintroduced started their housing in the same individual housing design as the mice in the continually socially isolated condition, and then were reintroduced by means of block party cages. According to the UCI Office of Research, the average acclimation period for research mice to adapt to new environments is typically 48 hours (UCI, 2021). Taking this into consideration, along with concerns in minimizing aggressive opportunities, this reintroduction condition was done so gradually over the course of a few days, which included heightened enrichment, (e.g., nestlets, Nyla Bones, Love Mash supplements) and monitoring, in order to promote efficient and safe acclimation.

Following the conclusion of their isolation period, mice which were previously isolated were introduced into the blockparty systems. They remained housed in these cages and subjected to increased monitorization. Bedding from subjects in this group was pulled, mixed, and redistributed so as to ensure sufficient scent exposure. They remained in this design for the remainder of the study, during which any attempts at aggressive behaviors were observed. The blockparty cages were situated on a standard cage rack and fixed to the standard water/ food supply system in room L026. When particularly concerning cases of cage-mate aggression were observed, the subjects were separated from their cage mates and individually housed to prevent animals from any further harm. This occurred twice in the study, both within group housed conditions, ultimately resulting in permanent separation in both circumstances. Following evaluation by our laboratory's vet and care staff, the subjects were then reintroduced if their wellbeing was not deemed to be threatened, or they were separated permanently.

The following behavioral tests were performed, with statistical analyses conducted at their ends. For these analyses, a value indicating $p < .05$ was considered statistically significant. Biochemical analyses were conducted following the conclusion of behavioral testing and subsequent brain extraction. All three groups were tested behaviorally at 12 and 20 weeks. For mice who were group housed since weaning and mice who were continually socially isolated ten brains from each group were extracted for histological analysis at 12 weeks. All mice were behaviorally assessed following

another 8-week period, at 20 weeks, where the remaining brains were collected for histological analysis.

In sum, behavioral testing was run at 12 weeks and 20 weeks. All three groups were tested at both 12 weeks, and at 20 weeks.

Nesting

Nests are an essential component of heat conservation for rodent populations as well as instruments for shelter and reproduction (Gaskill, 2013). Nests often measure the rodent's daily living activities and have been implemented into our lab in the form of shredded paper. For this experiment's nesting protocol, mice were housed individually for the socially isolated conditions of the trial. Corncob bedding (Teklad laboratory) was put down in the bottom of each cage to eliminate the potential for mice to construct nests from their bedding. Nest material was consistent of 3.5 grams of non-inked shredded paper which was autoclaved and scattered evenly amongst the bottom of the cage. All subjects had access to a food (un-autoclaved standard 7012 food) and water supply placed at the top of the cage and refilled regularly. In order to comply with colony conditions, the housing room was maintained on a standard of a 12-hour light/12-hour dark cycle. Researchers from our lab were instructed to take photographs of the nest both at 2 hours and 18 hours after the subject was added to their corresponding cage. Upon the second check at the 18-hour mark, the mice were returned to their housing cages. Nests

were scored on a 1-5 scale accordingly: 1: the shredded paper appears untouched; 2: there was some evident attempt to construct a nest, but the majority of the shreds remain scattered throughout the cage; 3: a nest was constructed using a majority of the shreds, however, several pieces of shredded paper remain around the cage; 4: a nest was constructed with very few shreds left remaining; 5: all of the paper has been successfully used in the construction of the nest. Blind raters, undergraduate students who were unfamiliar to the protocol and thus, free of preconceived biases, were employed to score the nests upon completion of both trials. It is notable to mention that our blind raters did not come into any contact with the mice while under evaluation. This procedure was set to account for any daily living deficits developed as effect of the subject's isolation, as well as to provide demonstration of potential behavioral improvement if it were to be observed in higher capability for the socially reintroduced condition. Ultimately, the measures to be obtained here concerned the animal subject's nesting score. Mice were given the nesting material at 5 pm and the nests were be assessed at 2 hours and 18 hours later. Each time point was analyzed separately.

Burrowing

Burrowing testing occurred following the conclusion of the nesting assessment. Rodents burrow as a means of defense and refuge as well as for food storage and the sheltering of their offspring (Deacon et al., 2006). Burrowing evaluations are designed to assess behaviors which suggest neurological impairments by observing potential deficits

within daily living activities. Mentioned in a burrowing study proposed by Deacon et al., observing burrowing behavior holds potentials to indicate the presence of hippocampal damage or defect (Deacon). This behavioral measurement is conducted through use of controlled burrowing conditions, and due to their mention to yield success in nearly all rodent burrowing conditions, pea shingles (collections of small rocks/ gravels) was identified as the selected burrowing substrate. The room for this procedure was operated within a 12-hour light/12-hour dark cycle in order to comply with the colony's circadian conditions. This procedure was initiated approximately three hours before the identified lights off cycle so as to provide a preliminary assessment of burrowing behavior before the lights went out within the facility. A canister with an open end and a closed end was filled with 250 grams of the burrowing substrate (pea shingles) and then weighed after 2 hours of the mouse's interaction with it. Additionally, the tube was weighed a second time the following morning. To conduct this evaluation, subjects were individually housed between the times of 6pm-10pm with their prepared tubes. An hour before the lights out phase, each subject's canister was weighed in order to measure how much material had been burrowed. Afterwards, the canisters were returned without any refills where they were weighed again the next morning. Following this second weighting, the subjects were returned to their nesting cages so that their nesting behavior could be evaluated. The burrowing substrate and its canister were the only enrichment items provided during this protocol so as to ensure accuracy and prevent extraneous intervention from any outside sources. Furthermore, subjects were specifically limited to individual housing during this process to omit similar error; if multiple mice were to be

housed together during this process, it may have interfered with the ability to gauge each individual's burrowing behavior/ activity. As provided in aforementioned protocols, all subjects were given access to both food (un-autoclaved standard 7012 food) and water, fixed at the top of their cages in where they could partake in freely. These resources were checked and replenished accordingly.

If potential deficits within burrowing, and thus, daily living functions were to be observed, it would indicate probable neurological impairment developed on account of the social isolation, so if improvements in these measures were observed following social reintroduction testing, then it would suggest an alleviation on account of social remediation. Measurements to be obtained here concerned the weight of the pea shingles removed from the subject's PVC pipe, which were taken at 2 hours and 18 hours after the mice were added to their cages.

Morris Water Maze (MWM)

Morris testing began a day after the completion of burrowing assessment. The Morris water maze task is used to measure long-term learning and spatial memory functionality. A pool with a platform is presented to the subject, who is trained initially to reach the platform with no obstruction (Huang et al., 2018). However, after a short period, the platform is then hidden and moved to the opposite quadrant to test the subjects escape latency, swim distance/speed, and travel patterns. A probe trial is often employed where the

platform is removed and the subject swims freely for approximately 60 seconds. Testing occurred throughout an 8-day period, with 3 consecutive trials the first 7 days, and only 2 for the concluding. Subjects were introduced to the testing room where they acclimated to the environment for at least 10 minutes prior to testing. A metal tub was filled with clouded water by use of nontoxic white paint, so that the platform was well hidden. The surrounding perimeters of the tub were set with visual cues and the whole of the testing site was shrouded by a white curtain to obstruct outside distractors. Animals conducted their trials consecutively and one at a time by being placed in the tub. Though the hidden platform remains at a fixed location, the starting point of the mouse was alternated following entry into each trail. This prompts the subject to rely on visual cues set around the tub to recall the platform's location. 60 seconds were allotted for each trial, during which the mouse was tracked by a camera, fixed to the ceiling which utilizes Videotrack tracking systems. The time spent in each quadrant and crossing patterns across the original position were then calculated, as well as notable thigmotaxis, a measurement which evaluates how much time the mouse swims in the outer 10% of the pool's perimeter. According to Brody & Holtzman (2006), this may be an alternate search strategy to memory recall, so it is necessary to note so as to promote higher accuracy. If the mouse had not found the platform in the allotted 60 seconds, then it was gently directed to the platform where it was then held for 10 seconds before removal. The mouse was then dried with an absorbent towel and then placed under a heating lamp for a resting period of 45 seconds before the next trial. Evaluation was finished upon completion of all 3 trials. As previously mentioned, probe trials, in which the platform is removed, was conducted on days 2,4, and 6 of evaluation, taking place of the

third trial. Once the 60 seconds had surpassed, the animal was again guided to the now raised platform as it was in the following trials. The purpose of the probe conduction is to evaluate the time spent by the mouse in each quadrant as an indicator of spatial recall. The more time spent in the quadrant where the platform would be indicates that they recall its proper location. On day 7 of evaluation, only the probe trial was conducted in order to assess long-term memory. Additionally, the 8th day of evaluation consisted of only 2 trials which used a visual platform to gauge the presence of visible abnormalities, of which there were none.

A total of 8 days was dedicated for this behavioral procedure, which was conducted twice in the duration of the study, accounting for 16 testing days total. The purpose of this practice was to observe potential long-term learning and spatial memory function of the animals to compare their results before and after social reintroduction. Poor Morris Water Maze performance was intended to indicate learning/memory-based deficits developed on account of social isolation. Significant improvements collected after the reintroduction period would then demonstrate a positive effect of social reintroduction and thus, a potential reversal in adverse functionality following periods of prolonged isolation. Measures to be obtained here concerned the animal subject's latency to find the platform, time spent in the proper target quadrant, number of platform crossings during their probe trials and their rates of apparent thigmotaxicity, which concerns the animal's time spent in the outer ten percent of the maze's pool.

Elevated Zero

The EZM assessment was conducted the day following completion of the MWM assessment. The elevated zero maze is used in order to observe measurements of potential anxiety. This device is designed as circular platform raised above ground and divided into two sections composed of 31cm-high walls surrounding its edges (100cm x 70cm) and two open sections (100cm x 70cm) absent of any walls. The mice were transported to the same testing room as the previously described conditions and acclimated to the environment for ten minutes before testing. Each subject performed a single trial. Trials began by placing the subject in an open portion of the apparatus facing a closed portion. Camera software fixed to the ceiling was employed to collect data for five minutes during this trial in which the total distance traveled time spent in the closed arms, time spent in the open arms, and total number of entries between each arm was measured (TopScan, Clever Sys., Inc., Reston, VA). In order to eliminate scent exposure between subject trials, the maze was cleansed with 70% ethanol between trails conductions.

If subjects appeared to be lacking in their elevated zero exploratory behaviors, it may demonstrate the onset of anxiety-related behaviors developed on account of the social isolation, so if improvements in these measures were observed following social reintroduction testing, then it would suggest an alleviation on account of social remediation. Measures to be observed here concerned the animal subject's total traveled distance, time spent in the closed arms, time spent in the open arms, and number of

entries per each arm. The subject's number of head dips over the edge of the maze was also recorded.

Open Field

The open field test was conducted following the completion of the EZM assessment. The open field test is an evaluation of exploratory and anxiety-related behaviors (Gould et al., 2009). The subject was situated in an environment similar to the Morris Water Maze and acclimated for 10 minutes before testing. The subject was placed individually within an 18 inch by 18 inch by 9.5-inch-tall box constructed out of blue plexiglass (Clever Sys., Inc., Reston, VA). Upon their entry into the apparatus, their distance traveled throughout the box was recorded over a 5-minute interval by use of the Videotrack tracking system. The tracking system measured the total distance traveled by the mouse as a representation of its general motor activity. The subject’s time spent in the center of the open field indicates anxiety-like behavior and was also recorded. Following conduction between subjects, the apparatus was sanitized with quatricide to eliminate scent exposure between mice.

If subjects appeared to be lacking in their open field exploratory behaviors, it may demonstrate the onset of anxiety-related behaviors developed on account of the social isolation, specifically if more time is spent in the center portion of the box. Distance

traveled during the assessment may be an indicator for exploratory performance so if improvements in these measures were observed following social reintroduction testing, then it would suggest an alleviation on account of social remediation. Measures to be obtained here concerned the animal subject's activity in the environment, including their traveled distance and time spent in the center versus surround regions of the box.

Circadian Activity

Circadian testing began the day following the completion of OF testing. Due to the commonality of social isolation to disrupt patterns within neurological function, circadian running wheel activity was measured in order to assess any disturbances amongst these patterns (Zulley, 2000). This was evaluated by use of the Actiview Biological Rhythm Analysis software (Minimitter Co.). The mice were individually housed for 6 days, totaling 144 hours, in cages equipped with running wheels attached to computer monitors. Computers monitored the wheel's rotations per minute. Mimicking conditions evident in normal circadian functionality, the testing room was fixed to a 12-hour light/12-hour dark cycle consistent with the colony's conditions. Mice were not to be housed together during this test as they may have interfered with each other's patterns of activity. Furthermore, mice had regular access to both food and water throughout their housing. Food (un-autoclaved standard 7012 food) and water were fixed to the tops of each cage. In order to ensure their health, the mice underwent regular health checks throughout their housing as well.

If potential deficits within circadian activity, and thus, neurological functioning were to be observed, it would indicate probable neurological impairment developed on account of the social isolation, so if improvements in these measures were subsequently observed following social reintroduction testing, then it would suggest an alleviation on account of social remediation. Measurements to be obtained here concerned the animal subject's number of rotations on the running wheel as well as the time at which the activity was taking place. The specific amount of activity was measured, and data was grouped into light and dark periods.

Biochemical Measures

Following the completion of the behavioral tests, mice were euthanized, and their brains were extracted for further analysis. Mice were euthanized using gradual CO_2 asphyxiation as a means of anesthesia. Animals were anesthetized using gradual CO_2 exposure, then euthanized by rapid decapitation using a guillotine. Brains were flash frozen using dry ice and then stored at -80 °C until further use.

Statistical Analyses

As aforementioned, behavioral analysis occurred twice, once at the 12-week period and once more at the 20-week period. For the EZM, the percentage of time spent in the open arms, entries into the open arms, and the number of head dips over the side of the platform were measured, again compared by means of a two-way (sex x housing condition) ANOVA. For the Open Field test, the distance traveled, and the percentage of time spent in the center vs. the surround was analyzed and compared by means of a two-way ANOVA. A two-way ANOVA was run on the nesting scores at both 2 and 18 hours. For burrowing, the amount of pea shingles removed at 2 and 18 hours was recorded, and a two-way ANOVA was too run.

As for the MWM, both the subject's latency to find the platform and periods of thigmotaxicity were intended to be analyzed, with the results from the three groups' performances to be compared. However, due to technical complications, only latency to find the platform was analyzed, and done so by a 2 (sex) x 3 (housing condition) x 6 (day) repeated measures ANOVA. This test was employed in order to measure the differences in each of the groups within and between days which ultimately allowed for the analysis of short-term and long-term learning.

In the circadian wheel running paradigm, the number of sessions of activity were run as a 2 (sex) x 3 (housing condition) x 12 (hour) ANOVA. The amount of activity per hour was collected and an ANOVA was run twice, once for the experimental period's light cycle and once for its dark cycle, to determine if there were differences in levels of activity and for when that activity occurred across experimental hours.

Results

Results for 12-Week Analysis:

Nesting 12-Weeks

2-Hour

A two-way ANOVA was run to determine if there were differences in nesting behaviors between sex (male and female) and housing conditions (group housed and socially isolated mice) at the two-hour measurement mark. Regarding Levene's interpretation for sex, we have met the assumption that there is homogeneity (.375), p = .140. Sex is of equal variances. For housing condition, there are also equal variances (.375), p = .623. There was not a significant effect for sex, $F(1, 59) = .576$, $p = .451$. There was also not a significant effect for housing condition, $F(1, 59) = 1.48$, $p = .228$. There showed no significant interaction between sex and housing condition at the 2-hour measurement, $F(1, 59)$, 1.15, $p = .228$ (Figure 5 in Appendix).

18-Hour

A two-way ANOVA was run to determine if there were differences in nesting behaviors between sex and housing conditions at the 18-hour measurement mark. Regarding Levene's interpretation for Sex, we have met the assumption that there is homogeneity (.375), p = .140. Sex is of equal variances. For housing condition, there are also equal variances (.375), p is .623. There was not a significant effect for sex, $F(1, 59)$

= .083, p = .775. There was also not a significant effect for housing condition, $F(1, 59)$ = .132, p = .7.18. There showed no significant interaction between sex and housing condition at the 18-hour measurement, $F(1, 59)$, .842, p = .363. (Figure 6 in Appendix).

Burrowing 12-Weeks

2-Hour

A two-way ANOVA was run to determine if there were differences in nesting behaviors between sex and housing conditions at the two-hour measurement mark. Regarding Levene's interpretation for Homogeneity of Variances, we have met the assumption that there is homogeneity for the 2-hour measurements (3,59) = .2.84, p = .045. There was not a significant effect for sex, F (1, 59) .050, p = .825. There was also not a significant effect for housing condition, F (1, 59) 1.09, p = .301. There showed no significant interaction between sex and housing condition at the 2-hour measurement, $F(1, 59)$ .220, p =.641 (Figure 7 in Appendix).

18-Hour

A two-way ANOVA was run to determine if there were differences in nesting behaviors between sex and housing conditions. For the 18-hour measurement, homogeneity of variances was violated, assessed by Levene's Test for Equality of Variances (p = .045), so an F test for unequal variances was used. There was not a significant effect for sex, F (1, 59) .025, p = .874. There was also not a significant effect

for housing condition, F (1, 59) .880, p = .352. There showed no significant interaction between sex and housing condition at the 18-hour measurement, F(1, 59) 2.42, p = .125 (Figure 8 in Appendix).

Elevated Zero Maze 12-Weeks:

A two-way ANOVA was run to determine if there were differences in Elevated Zero Maze related behaviors between sex and housing conditions at the 12-week period. Regarding Levene's interpretation for Homogeneity of Variances, we have met the assumption that there is homogeneity for head dips, F(3, 59) = 2.30, p = .087. There is homogeneity for entries into arms, F(3, 59) = .789, p =.096. There is also homogeneity for percentage of time spent in open arms, F(3, 59) = 1.58, p is .203.

For head dip scores, there was not a significant effect for sex, F(1, 59) = 1.56, p =.217. There also was not a significant effect for housing condition, F(1, 59) = 2.58, p = .114. There showed no significant interaction between sex and housing condition at the 12-week period for head dips, F(1, 59) = .017, p = .898 (Figure 1a).

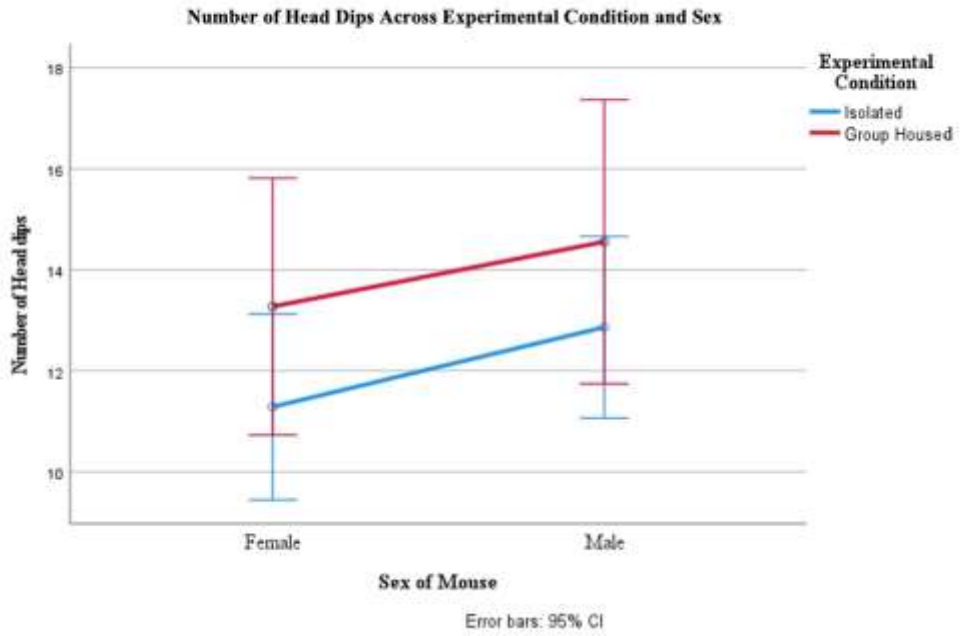

Figure 1a: Elevated Zero Maze Results for Head Dips at 12-Weeks Testing: Figure 1a shows results of the head dip count within the EZM assessment at 12-week testing period, there were no significant differences.

For entries into open arms, there was not a significant effect for sex, $F(1, 59) = 1.24$, $p = .270$. There also was not a significant effect for housing condition, $F(1, 59) = 1.77$, $p = .188$. There showed no significant interaction between sex and housing condition at the 12- week period for entries into open arms, $F(1, 59) = 3.08$, $p = .085$ (Figure 1b).

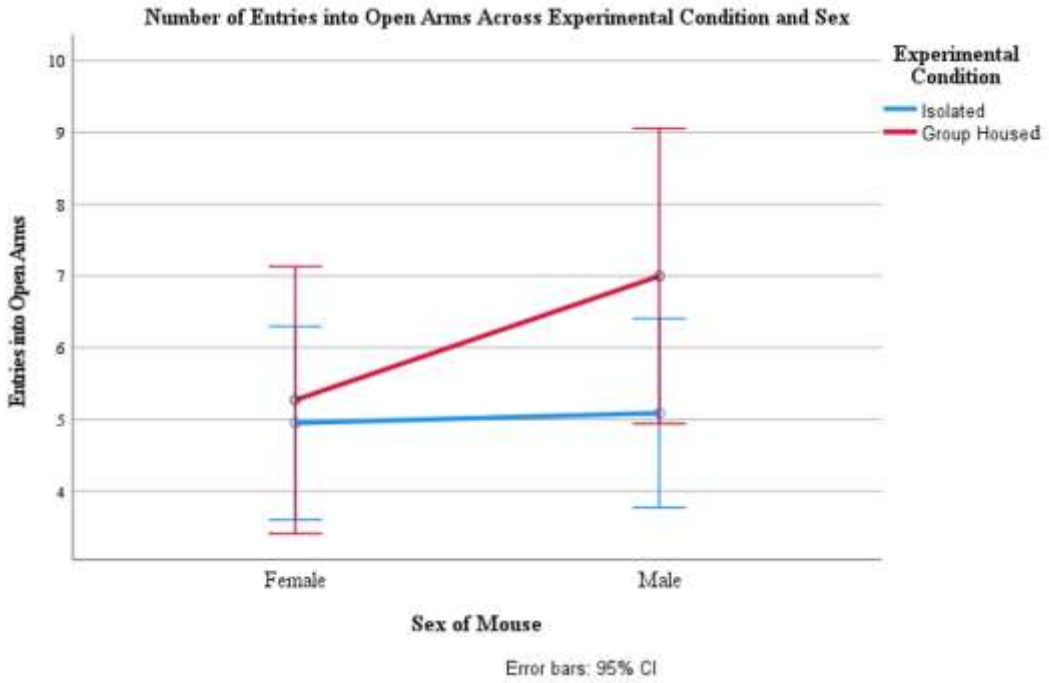

Figure 1b: Elevated Zero Maze Results for Number of Entries into Open Arms at 12-Weeks Testing:
Figure 1b shows results for the number of entries into open arms within the EZM assessment at 12-week period, there were no significant differences.

For percentage of time spent in open arms, there was not a significant effect for sex, $F(1, 59) = .007$, $p = .935$. There also was not a significant effect for housing condition, $F(1, 59) = 3.08$, $p = .084$. There showed no significant interaction between sex and housing condition at the 12- week period for percentage of time spent in open arms, $F(1, 59) = .127$, $p = .723$ (Figure 1c).

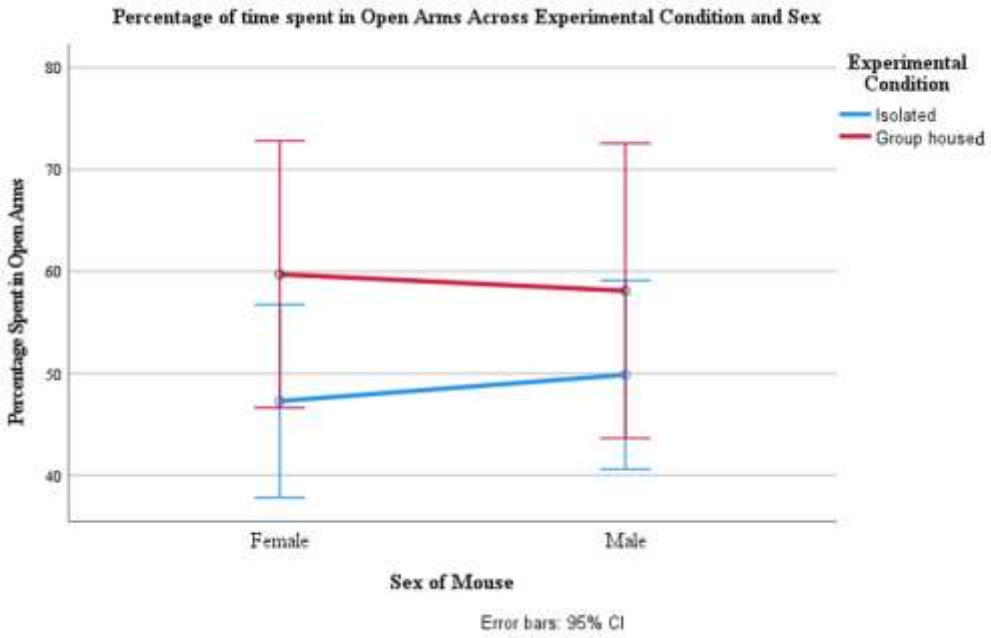

Figure 1c: Elevated Zero Maze Results for Time Spent in Open Arms at 12-Weeks Testing:
Figure 1c shows results of the percentage of time spent in open arms within the EZM assessment at 12-week period, there were no significant differences.

Circadian 12-Weeks:

A two-way repeated measure ANOVA was run to determine if there were differences in circadian rhythm light cycle behaviors between sex and housing conditions at the 12-week experimental point. Regarding Levene's interpretation, Homogeneity of Variances was violated (p= .014), so F test for unequal variances was used. There was a significant effect for sex with males (Figure 2a) performing significantly better than females, F (1, 59) 15.03, p = .000 (Figure 2b).

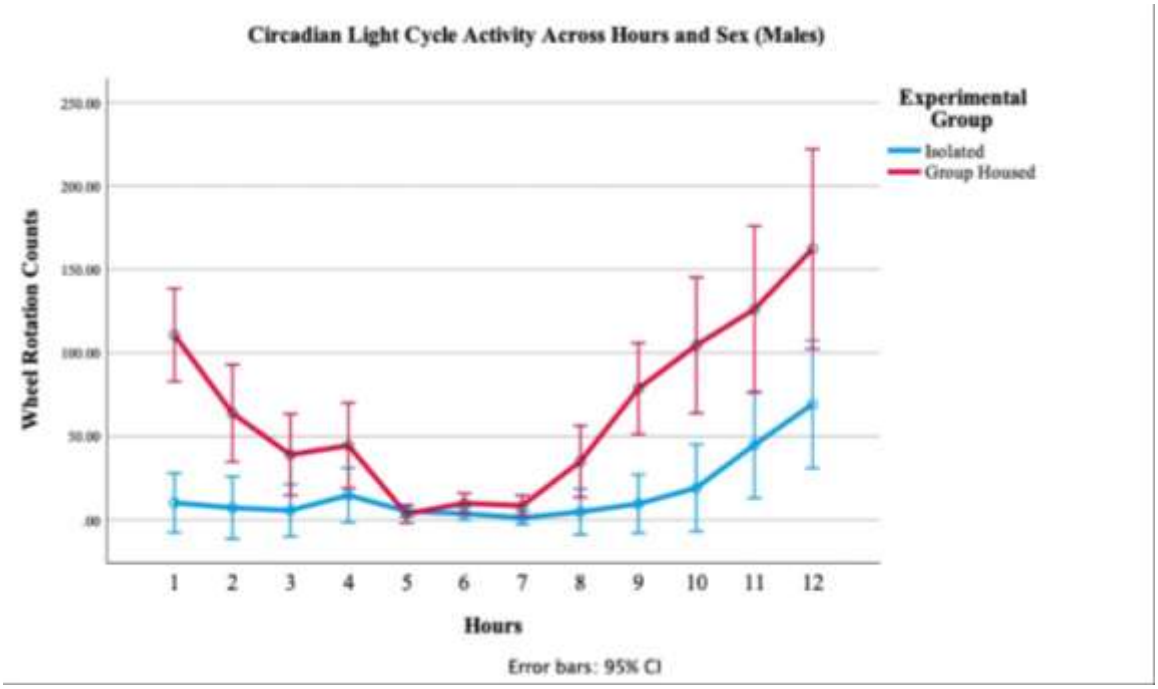

Figure 2a: Circadian Light Cycle Activity Across Hours and Sex for Males
Figure 2a shows Circadian activity across hour and sex, males specifically, during the light cycle at 12-week period. Males performed significantly better than females, F (1, 59) 15.03, p = .000.

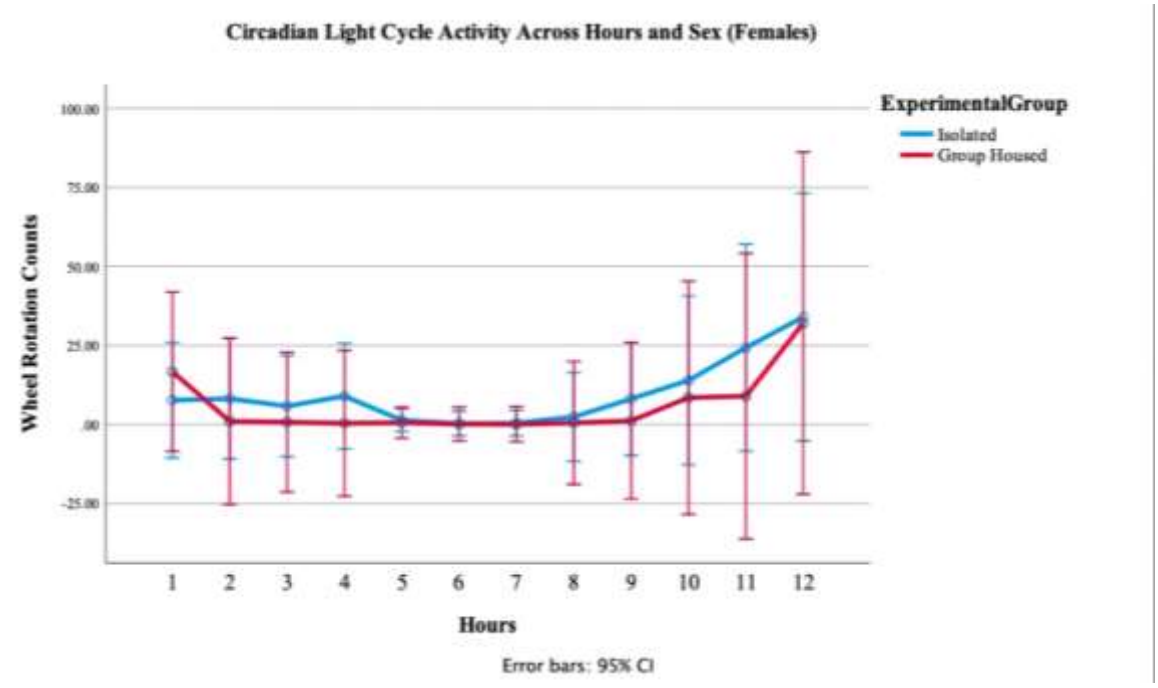

Figure 2b: Circadian Light Cycle Activity Across Hours and Sex for Females at 12-Weeks Testing
Figure 2b shows Circadian activity across and sex, females specifically, during the light cycle at the 12-week period. There were no significant housing effects.

There was a significant effect for housing condition as well, with group housed mice performing better than isolated mice, $F(1, 59)$ 7.10, $p = .010$ (Figure 2c). A full 12-hour data figure is shown in Appendix. There showed a significant interaction of sex and housing condition at the 12-week period for circadian activity in the light cycle, $F(1, 59)$ 9.62, $p = .003$ (Figure 2d).

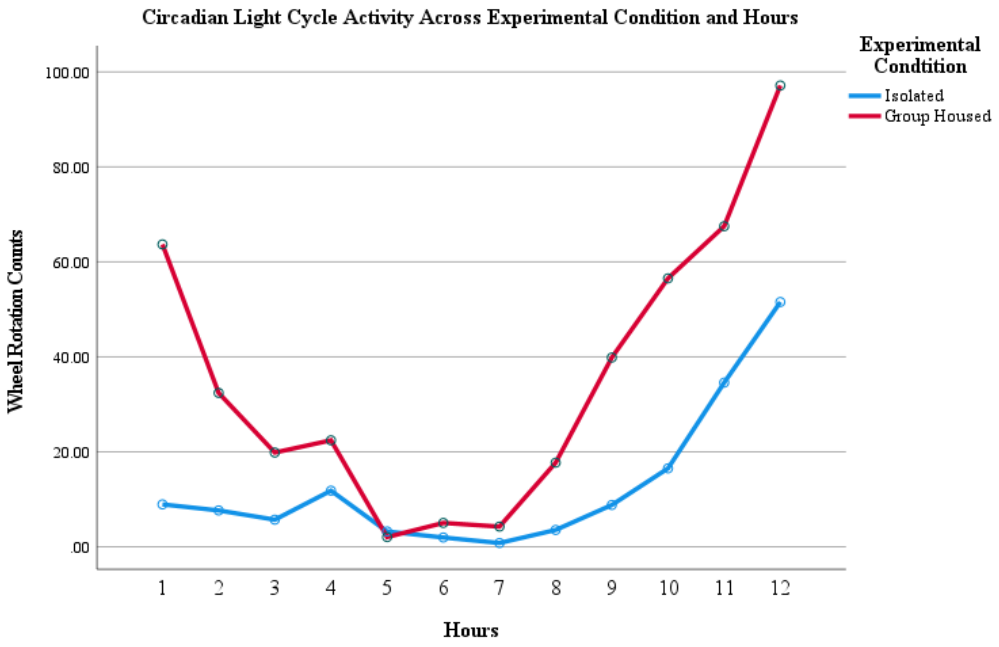

Figure 2c: Circadian Light Cycle Activity Across Experimental Condition and Hours at 12-Weeks Testing
Figure 2c shows Circadian activity across hours and experimental condition during the light cycle at the 12-weeks period. Group housed mice performed significantly better than isolated mice, $F(1, 59)$ 7.10, $p = .010$.

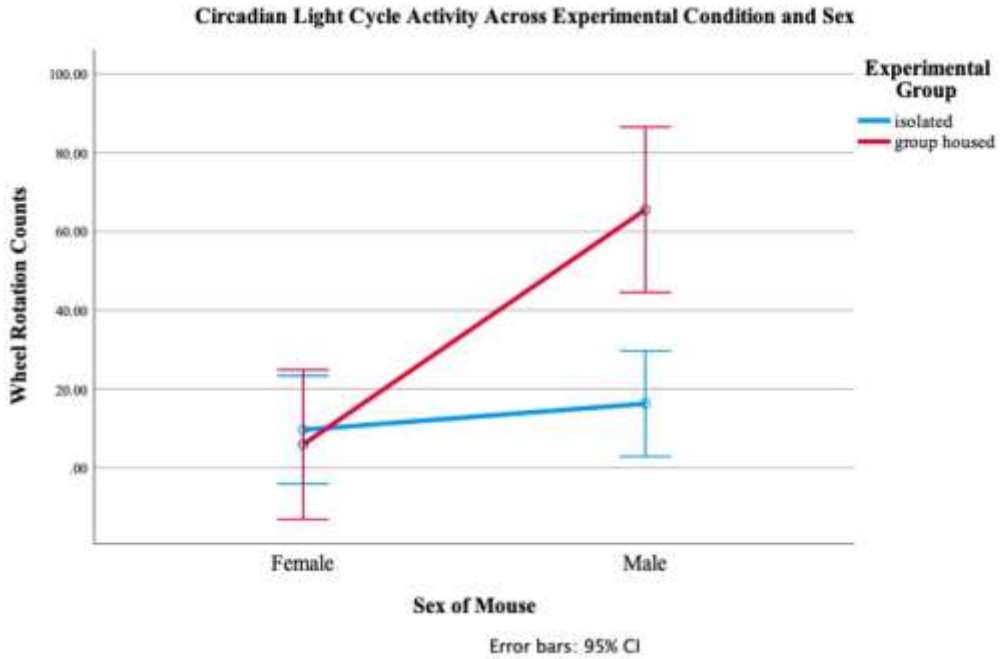

Figure 2d: Circadian Light Cycle Results for 12-Weeks Testing – Experimental Effect

Figure 2d shows results for circadian light cycle activity across housing condition and sex. Group housed mice performed better than isolated mice, F(1, 59) 7.10, p = .010. There was also a significant interaction of sex and housing condition at the 12-week period for circadian activity in the light cycle, F(1, 59) 9.62, p = .003.

Dark Cycle:

A two-way repeated measure ANOVA was run to determine if there were differences in circadian rhythm dark cycle behaviors between sex and housing conditions at the 12-week experimental point. Regarding Levene's interpretation, Homogeneity of Variances was violated (p= .004), so an F test for unequal variances was used. There was not a significant effect for sex, F(1, 59) .005, p = .946. There was not significant effect for housing condition, F(1, 59) 1.142, p = .331. There showed no significant interaction

of sex and housing condition at the 12-week period for circadian activity in the dark cycle, $F(1, 59)$ .110, $p = .896$. Dark cycle graphs are provided in Appendix, see Figures 10-13.

Open Field 12-Weeks:

A two-way ANOVA was run to determine if there were differences in Open Field performance between sex and housing conditions at the 12-week period. Regarding Levene's interpretation for percentage of time spent in the surround zone we have met the assumption that there is homogeneity, $F(3, 59) = 1.28$, $p = .289$. For percentage of time spent in center zone, there is also homogeneity, $F(3, 59) = 1.28$, $p = .290$. For distance traveled however, homogeneity of variances was violated, so separate variances were used, $F(3, 59) = 3.18$, $p = .030$.

For percentage of time spent in the surround zone, there was not a significant effect for sex, $F(1, 59) = .010$, $p = .922$. There was also not a significant effect for housing condition, $F(1, 59) = .029$, $p = .866$ (Figure 14 in Appendix).

For percentage of time spent in the center zone, there was not a significant effect for sex, $F(1, 59) = .010$, $p = .922$. There was also not a significant effect for housing condition, $F(1, 59) = .029$, $p = .866$ (Figure 15 in Appendix).

There was not a significant effect of sex on distance traveled, $F(1, 59) = .735$, $p = .395$. There was, however, a significant effect of housing condition on distance traveled, $F(1, 59) = 4.35$, $p = .041$. Group housed mice (M= 2769.92, SD = 2005.33) traveled the furthest, compared to isolated mice (M= 1766.92, SD = 1688.99) (Figure 3).

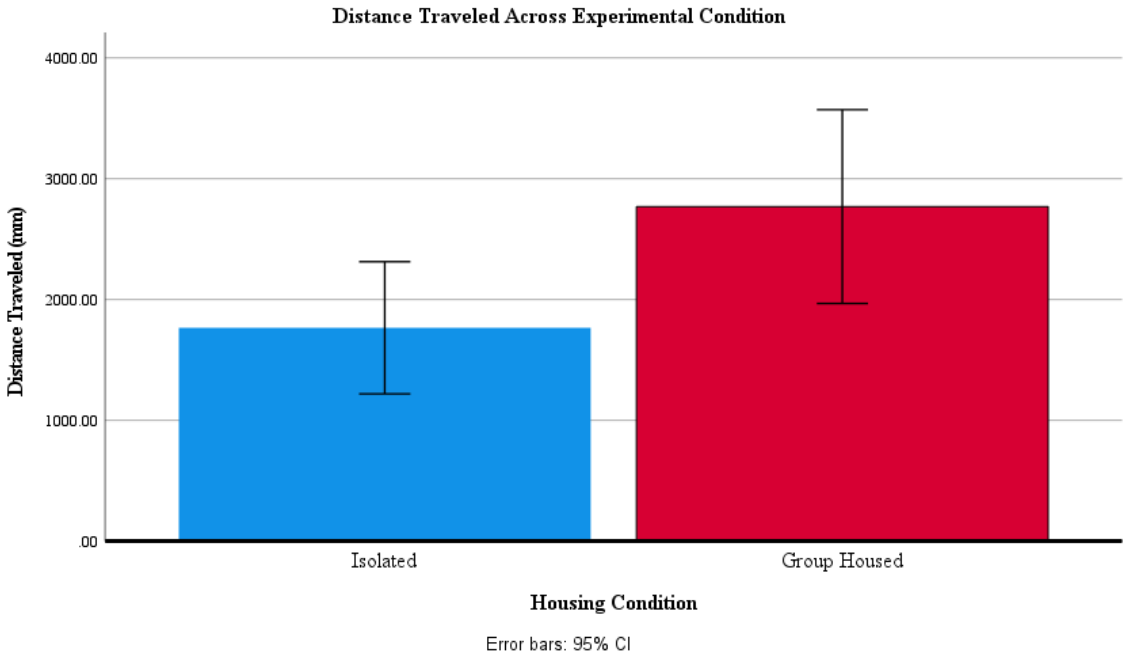

Figure 3: Distance Traveled by Mice throughout Open Field at 12-Weeks Testing: Figure 3 shows the total distance traveled (in mm) by mice throughout the Open Field assessment at the 12-week period. Group housed mice (M= 2769.92, SD = 2005.33) traveled the furthest, compared to isolated mice (M= 1766.92, SD = 1688.99)

There showed no significant interaction between sex and housing condition on percentage of time spent in surround zone, $F(1, 59) = .424$, $p = .517$. There showed no significant interaction between sex and housing condition on percentage of time spent in

the center zone, $F(1, 59) = .421$, $p = .519$. There showed no significant interaction between sex and housing condition on distance traveled, $F(1,59) = 1.04$, p = .312.

MWM 12-Weeks:

A two-way repeated measures ANOVA was run to determine if there were differences between sex and housing condition on subjects' performance in the Morris Water Maze at the 12-week experiment period. Regarding Levene's interpretation, homogeneity of variances was assumed, $F(3, 59) = 2.164$, $p = .102$. There was not a significant effect for sex, $F(1, 59) = 2.04$, $p = .158$. There was also not a significant effect for housing condition, $F(1, 59) = 1.34$, $p = .252$. There showed no significant interaction of sex on housing condition, $F(1, 59) = .062$, $p = .804$ (Figure 16).

Results for 20-Week Analysis:

Nesting 20-Weeks:

2-Hour

A two-way ANOVA was run to determine if there were differences in nesting related behaviors between sex and housing conditions (isolated, group housed, and socially reintroduced mice) at the 20-week period. Regarding Levene's interpretation for Homogeneity of Variances, we have met the assumption that there is homogeneity for the 2-hour score averages, $F(5, 34) = .547$, $p = .739$. There was not a significant effect for

sex, $F(1, 34) = .127$, $p = 724$. There was also not a significant effect for housing condition, $F(2, 34) = .683$, $p = .512$. There showed no significant interaction between sex and housing condition at the 2-hour measurement, $F(2, 34)$, .501, $p = .610$ (Figure 20 in Appendix).

18-Hour

A two-way ANOVA was run to determine if there were differences in nesting related behaviors between sex and housing conditions at the 20-week period. Regarding Levene's interpretation for Homogeneity of Variances, we have met the assumption that there is homogeneity for the 18-hour score averages, $F(5, 34) = .1.07$, $p = .391$. There was not a significant effect for sex, $F(1, 34) = .193$, $p = .663$. There was also not a significant effect for housing condition, $F(2, 34) = 1.12$, $p = .337$. There showed no significant interaction between sex and housing condition at the 18-hour measurement, $F(1, 34)$, 2.25, $p = .120$ (Figure 21 in Appendix).

Burrowing 20-Weeks:

2-Hour

A two-way ANOVA was run to determine if there were differences in burrowing related behaviors between sex and experimental, at the 20-week period. Regarding Levene's interpretation for Homogeneity of Variances, we have met the assumption that there is homogeneity for the 2-hour measurements, F (5, 34) = .1.77, $p = .146$. There was not a significant effect for sex, $F(1, 34)$ .016, $p = .901$. There was also not a significant

effect for housing condition, $F(2, 34)$ .213, $p = .810$. There showed no significant interaction between sex and housing condition at the 2-hour measurement $F(2, 34)$ 2.10, $p = .138$ (Figure 18 in Appendix).

18-Hour

A two-way ANOVA was run to determine if there were differences in burrowing related behaviors between sex and housing conditions, at the 20-week period. Regarding Levene's interpretation for Homogeneity of Variances, we have met the assumption that there is homogeneity for the 18-hour measurements $F(5, 34) = 1.82$, $p = .134$. There was not a significant effect for sex, $F(1, 34)$ .018, $p = .894$. There was also not a significant effect for housing condition, F (2, 34) .265, $p = .769$. There showed no significant interaction between sex and housing condition at the 18-hour measurement $F(2, 34)$ 1.17, p = .322 (Figure 19 in Appendix).

Elevated Zero Maze 20-Weeks:

A two-way ANOVA was run to determine if there were differences in Elevated Zero Maze related behaviors between sex and housing conditions at the 20-week period. Regarding Levene's interpretation for Homogeneity of Variances, we have met the assumption that there is homogeneity for head dips $F(5, 34) = 1.42$, $p = .244$. We have not met the assumption that there is homogeneity for entries into arms $F(5, 34) = 2.56$, p

=.046, so an F test for unequal variances was used. We have not met the assumption that there is homogeneity for percentage of time spent in open arms either, $F(5, 34) = 6.22$, $p < .001$.

For head dip scores, there was not a significant effect for sex, $F(1,34) = .471$, $p = .497$. There was a significant effect of housing condition, $F(2,34) = 4.25$, $p = .023$. Reintroduced mice performed the best (M = 8.64, SD = 2.84) with isolated mice performing the worst (M= 4.38, SD = 3.47) (Figure 4a). There showed no significant interaction of sex and housing condition at the 20-week period for head dips, $F(2, 34) = 1.14$, $p = .331$.

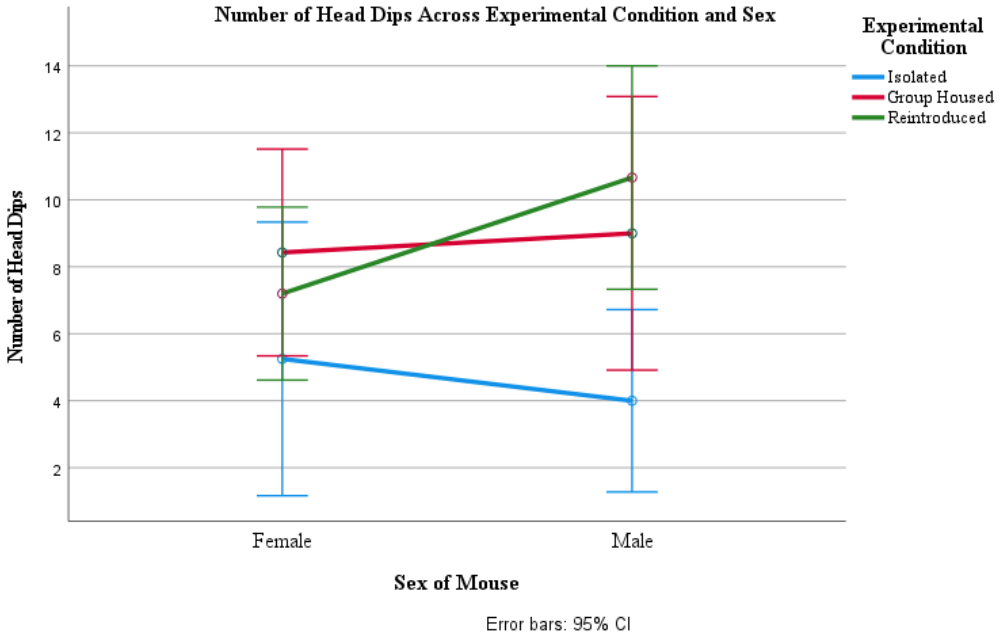

Figure 4a: Elevated Zero Maze Results for Head Dips at 20-Weeks Testing:

Figure 4a shows results of the head dip count within the EZM assessment at 20-week testing period. There was a significant effect of housing where reintroduced mice performed the best (M = 8.64, SD = 2.84) and isolates performed the worst (M= 4.38, SD = 3.47).

For entries into open arms, there was not a significant effect for sex, $F(2, 34) = .056$, p =.814. There was a significant effect of housing condition, $F(2, 34) = 5.47$, $p = .009$. Reintroduced mice performed the best (M = 7.67, SD= 3.37) with isolated mice performing the worst (M= 3.92, SD = 1.61) (Figure 4b). There was not a significant interaction of sex and housing condition at the 20-week period for entries into open arms, $F(2, 34) = .420$, $p = .660$.

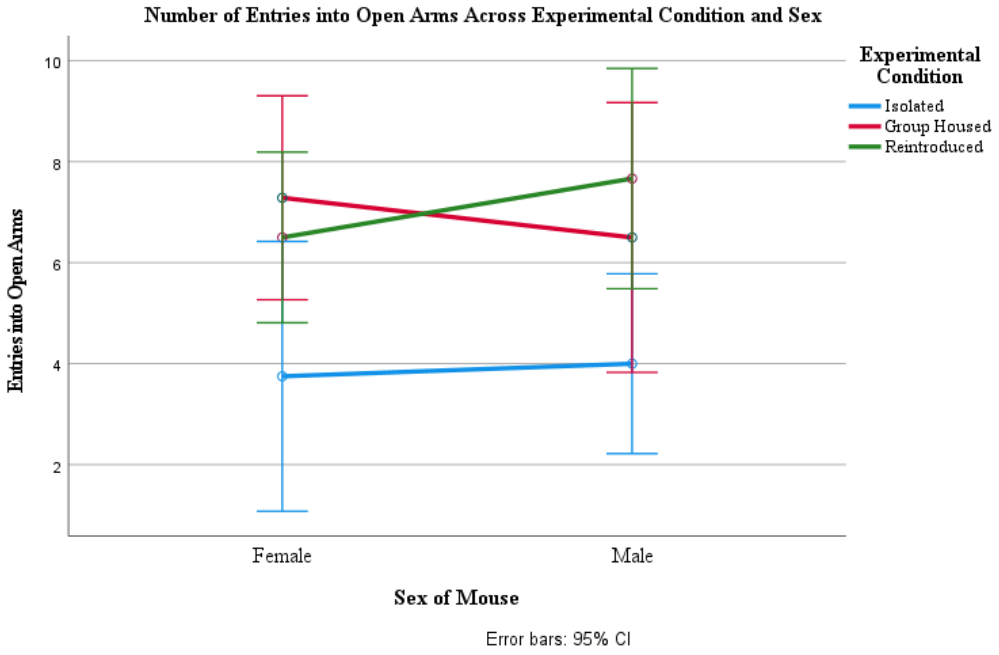

Figure 4b: Elevated Zero Maze Results for Entries into Open Arms at 20-Weeks Testing:

Figure 4b shows results of the entries into open arms within the EZM assessment at 20-week period. There was a significant effect of housing condition where reintroduced mice performed the best (M = 7.67, SD= 3.37) and isolates performed the worst (M= 3.93, SD = 1.61).

For percentage of time spent in open arms, there was a significant effect for sex such that males (M= 61.50, SD = 32.17) spent more time in open arms than females (M=19.43, SD= 8.92), F (1, 34) = 5.54, p = .024. There was not a significant effect of housing condition, F (2, 34) = 5.47, p = .122). There showed a significant interaction of sex and housing condition at the 20-week period for percentage of time spent in open arms, F(2, 34) = 7.09, p = .003 (Figure 4c).

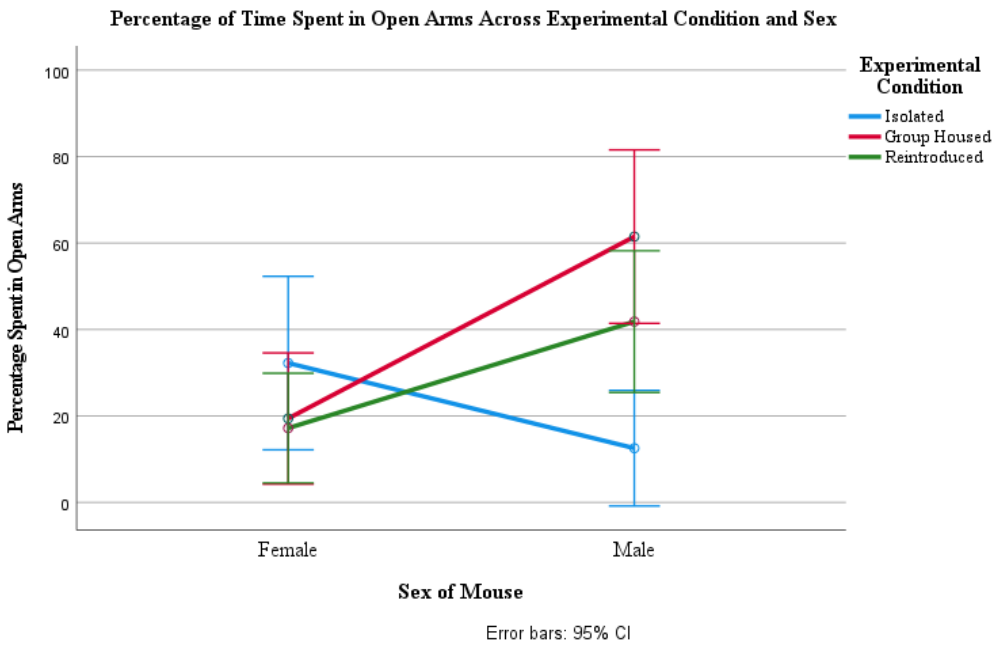

Figure 4c: Elevated Zero Maze Results for Time Spent in Open Arms at 20-Weeks Testing:

Figure 4c shows results of the percentage of time spent in open arms within the EZM assessment at 20-week period. There was a significant interaction of sex and housing condition present at the 20-week period, $F(2, 34) = 7.09$, $p = .003$.

Post hoc comparisons for head dips using the Tukey HSD test indicated that the mean score for isolated groups was significantly different than that of the group housed, ($p = .037$, 95% C.I. = -8.29, -.22). The mean score for isolated groups was also significantly different than reintroduced scores, ($p = .026$, 95% C.I. = -7.79, -.44). There was no statistically significant difference between the mean scores of group housed and reintroduced categories for head dips, ($p = .996$, 95% C.I. = - 3.72, 3.99).

Post hoc comparisons for entries into open arms using the Tukey HSD test indicated that the mean score for isolated groups was significantly different than that of the group housed, ($p = .019$, 95% C.I. = -5.72, -.44). The mean score for isolated groups was also significantly different than reintroduced scores, ($p = .011$, 95% C.I. = -5.42, -.61). There was no statistically significant difference between the mean scores of group housed and reintroduced categories for open entries, ($p = .998$, 95% C.I. = -2.46, 2.59).

Post hoc comparisons for percentage of time spent in open arms using the Tukey HSD test indicated that the mean score for isolated groups was not significantly different than that of the group housed, ($p = .130$, 95% C.I. = -3.72, 35.94). The mean score for isolated groups was also not significantly different than reintroduced scores, ($p = .889$, 95% C.I. = -26.40, 10.75). There was also no statistically significant difference between

the mean scores of group housed and reintroduced categories for percentage of time spent in open arms, (p = .875, 95% C.I. = -11.19, 27.77).

Circadian 20-Weeks:

A two-way repeated measure ANOVA was run to determine if there were differences in circadian rhythm light cycle behaviors between sex and housing conditions at the 20-week experimental point. Regarding Levene's interpretation, Homogeneity of Variances was violated (p=.014), so an F test for unequal variances was used. There was not a significant effect for sex, F (1, 34) .005, p = .946. There was also not a significant effect for housing condition, F (2, 34) .331, p = .331. There showed no significant interaction of sex and housing condition at the 20-week period for circadian activity in the light cycle, F (2, 34) .110, p = .896. Light cycle graphs are provided in Appendix, see Figures 25-27.

A two-way repeated measure ANOVA was run to determine if there were differences in circadian rhythm dark cycle behaviors between sex and housing conditions at the 20-week experimental point. Regarding Levene's interpretation, Homogeneity of Variances was violated (p =.019), so an F test for unequal variances was used. There was not a significant effect for sex, F (1, 34), .005, p = .946. There was also not a significant effect for housing condition, F (2, 34), 1.142, p = .331. There showed no significant interaction of sex and housing condition at the 20-week period for circadian activity in

the dark cycle, F (2,34) .110, p = .896. Dark cycle graphs are provided in Appendix, see Figures 28-31.

Open Field 20-Weeks:

A two-way ANOVA was run to determine if there were differences in Open Field performance between sex and housing conditions at the 20-week period. Regarding Levene's interpretation for percentage of time spent in the surround zone we have not met the assumption that there is homogeneity, so F test for unequal variances was used, $F(5, 34) = 3.42$, $p = .013$. For percentage of time spent in center zone, homogeneity was also violated an F test for unequal variances was used, F (5, 34) = .3.37, p = .014. For distance traveled however, there was homogeneity, $F(5, 34) = .876$, $p = .507$.

For percentage of time spent in the surround zone, there was not a significant effect for sex, F (2, 34) = 1.64, p = .209. There was also not a significant effect for housing condition, F (2, 34) = .723, p = .493 (Figure 22 in Appendix).

For percentage of time spent in the center zone, there was not a significant effect for sex, $F(2, 34) = 1.63$, $p = .210$. There was also not a significant effect for housing condition, F (2, 34) = .714, p = .497 (Figure 23 in Appendix).

There were no significant effects for sex on distance traveled, F (2, 34) = .497, p = .486. There were also no significant effects for housing condition on distance traveled, F (2, 34) = .037, p = .963 (Figure 24 in Appendix).

There were no significant main effects observed for the interaction of sex and housing condition on the percentage of time spent in the surround zone, F (2, 34) = .456, p = .637. There were no significant main effects observed for the interaction of sex and experiment on the percentage of time spent in the center zone, F(2,34) = .448, p = .642. There were no significant main effects observed for the interaction of sex and experiment on the amount distance traveled, F(2, 34) = 1.52, p = .229.

MWM 20 Weeks:

A two-way repeated measures ANOVA was run to determine if there were differences between sex and housing condition on subjects' performance in the Morris Water Maze at the 20-week experiment period. Regarding Levene's interpretation, Homogeneity of variances was assumed, F(5,34) = 1.132, p = .362. There was not a significant effect for sex, F (1, 34) = .833, p = .368. There was also not a significant effect for housing condition, F (2, 34) = .758, p = .476. There showed no significant interaction of sex on housing condition, F (2, 34) = .142, p = .868 (Figures 32-33 in Appendix).

Conclusions

Compiling the results of behavioral analyses across both 12-week and 20-week experimental periods, three behavioral tests yielded results that were statistically significant. At the 20-week period, housing condition proved a significant factor on the subjects' level of subsequent anxiety developed on account of prolonged isolation. This was evident by reintroduced mice performing higher levels of both head dips and entries into open maze arms during the EZM assessment. In both testing periods for this assessment, it was isolated mice which performed the poorest, indicated by less entries into the open arms and less recorded head dips, when compared to group housed and reintroduced conditions. This may indicate a potential deficit in neurological functioning developed on account of prolonged isolation and manifested in the form of increased anxiety levels, with social reintroduction a potential means of reversing this previous damage. There was also a significant interaction of sex and housing condition at this period as well, specifically for time spent in open arms, indicating that there are differences in the influence of sex and housing condition at their respective levels for this particular measure.

For the 12-week Open Field test, there were significant results of housing condition on the cumulative distance traveled by the mice throughout the task's conduction, with group housed mice traveling further than their isolate counterparts. This was not reflected in the 20-week results, though this may be subject to change following the addition of data from our prospective cohort. Albeit, this 12-week significant result

could potentially indicate a neurological impairment developed on account of isolated housing as it were the grouped conditions which yield further distances, and hence, a more thriving sense of exploratory behavior. Like the EZM assessment, poorer performance on the OF test may also reflect heightened levels of anxiety, so these results may aid in the suggestion that isolated housing imposes risks of neurological and behavioral impairment, with the reintroduction of social opportunity arising as a potential mitigation against these deficits.

For 12-week CR testing, there were significant results found for both sexes, with males again performing significantly better than females, and housing condition, with group housed mice performing better than isolated mice. There was also a significant interaction of sex and housing condition at the 12-week period for circadian activity in the light cycle, indicating that there are differences in the influence of sex and housing condition at their respective levels for circadian activity. This was illustrated by male group housed mice performing the highest levels of circadian activity across the other experimental conditions and sex.

The sex differences for both EZM and CR testing are notable findings as they seem to indicate that it is the male mice that are driving these sex differences in performance. This is interesting as our previous study did not account for sex differences, so a gap in previous findings may start to develop a close here (Barkey & Kraven, 2021). These sex results may suggest that males maintain higher protections against neurological

impairment developed specifically on account of isolation. One reason that this may be is due to the instinctually aggressive behaviors of the male mice over the females. In a study proposed by Neumaan et al. in which the effects of aggression and anxiety on mice populations were observed in social contexts, it was found that highly aggressive behaviors were likely developed on account of disturbed emotional regulation and anxiety (Neumaan & Veneema, 2010). This was proposed to be due to an overlap in brain circuitries between the neurochemical systems that regulate aggression and anxiety (Neumaan & Veneema, 2010). Taking this into consideration, if this overlap exists, it may explain why the male mice are showing better performance, as their innate aggressive behaviors may be yielding a higher influence over their levels of isolation-based anxiety.

Significant results found at the 12-week period for EZM and CR are in line with our previous isolation studies' findings in that isolation is found to produce neurological impairments in the forms of increased anxiety levels and disturbed circadian activity (Barkey & Kraven, 2021). The 20-week open field results present a promising suggestion that social reintroduction may aid in alleviating some of this subsequent anxiety, however, 20-week results for OF and CR proved nonsignificant. This is interesting as the 12-week results for these tests did yield significant results. Perhaps the creation of the reintroduced housing condition presented too much of a blur between the distinctions of isolated versus rehoused conditions, thus not producing a strong enough effect to yield significance. If this were entirely the case, however, then EZM results would likely not

have yielded significant results either, so perhaps these outcomes were simply due to socialization having its strongest impact on subsequent anxiety levels rather than circadian or exploratory affairs. Burrowing and nesting results, tests which aim to measure the subjects' performance within daily living functions, showed no significance at any point in the experiment, suggesting that isolation may not significantly impair the mice in this regard, while subsequently proving social reintroduction more or less ineffective.

While the rest of the behavioral results proved to not be statistically significant, it is worthy to consider the importance of nearly every assessment illustrating a very apparent contrast between housing condition performances. For the 12-week burrowing, EZM, MWM, OF (distance traveled only), and CR assessments, isolated conditions showed a clear detriment in the measured outcome, outperformed by their respective group housed counterparts (see Figs 1a-1c, 2a-2c, 3, and Figs 7 and 16 in Appendix). For the 20-week assessments, the same trend held true, with isolated mice performing worse on burrowing, EZM, and OF, than the group housed subjects (see Figs 4a-4c and Figs 18, 24 in Appendix). In the instance of both overnight burrowing measurements and EZM scores (head dips and entries into open arms) reintroduced mice even prevailed over both other conditions (see Figs 4a, 4b, and Fig 19 in Appendix), aiding to the implication that reintroducing subjects to social opportunity presents the potential to alleviate if not slightly improve neurological impairment or degeneration developed on account of initial isolation.

For MWM in particular, it is worth mentioning that all groups had low latency to find the platform at the 20-week period, likely due to their prior learning involvement in the test which remained unaffected. This may have inhibited any group differences, though also the results may suggest that social isolation alone does not have a negative impact in regards to hippocampal-dependent learning or memory, as the isolated mice were able to perform as well as the group housed and reintroduced conditions.

Understanding the results from this study and pulling influence from our previous isolation studies, one may postulate on how these results may have concluded had sex been a fixed factor. Perhaps a cohort dedicated solely to male mice and another for females, or one examining the effects of one sex only, could have presented results that were higher in favor of the originally presented hypotheses. Furthermore, employing a cohort of only female mice would have enabled sample size to increase, as more mice could be housed together, while also diminishing the likelihood of casemate aggression, which while very infrequent in this experiment, still resulted in temporary separation of group housed mice, ultimately presenting a potential confound in the validity of experimental outcomes. Or rather, the present data could be reanalyzed without sex included as a variable in order to see if any borderline results are pushed into significance by looking at housing condition alone.

In a study which looked at the neural circuitry effects associated with post-weaning social isolation, the researchers highlight how the differing findings of several other isolation studies, in regards to heightened levels of anxiety, may be due to several "varying adversities", one of which may include the strain of mice selected (Lukkes, 2009). Due to the 129 mice being exceptionally docile, results of this study may very well differ dramatically across other strains, and thus, a question regarding the applicability of these findings emerges. Perhaps a study analyzing the behavioral measures across these housing conditions and various strains could then be upheld in order to look at these "adversities" in higher effect.

Nonetheless, the results that were indeed significant could strengthen our argument that social isolation presents a detriment to neurological functioning and overall wellbeing, at least in regards to exploratory, circadian, and anxiety-related behaviors, with the reintroduction of social opportunity - a potential mitigation strategy for anxiety levels in particular. Considering these findings, real life implications of implementing social reintroduction may be applicable in circumstances where socialization is scarce or at least significantly affected. Examples may include the affairs of neurodivergent individuals, where social provision could be introduced in order to alleviate levels of anxiety. Additionally, with the aforementioned 25% of elders 65 and older considered socially isolated, the engagement of higher social involvement may also aid in mitigating their levels of anxiety, and thus promoting a higher sense of overall wellbeing. Lastly, looking back into the effects of socialization in animals, providing your pet with an

additional partner may help lower their levels of general anxiety and subsequently foster a better living environment for both them and their owner. The benefits that socialization may offer are arguably limitless, though hopefully this study may provide some insight behind this simple yet equally as intriguing phenomenon.

APPENDIX

Figures include in the following Appendix all failed to show statistical significance.

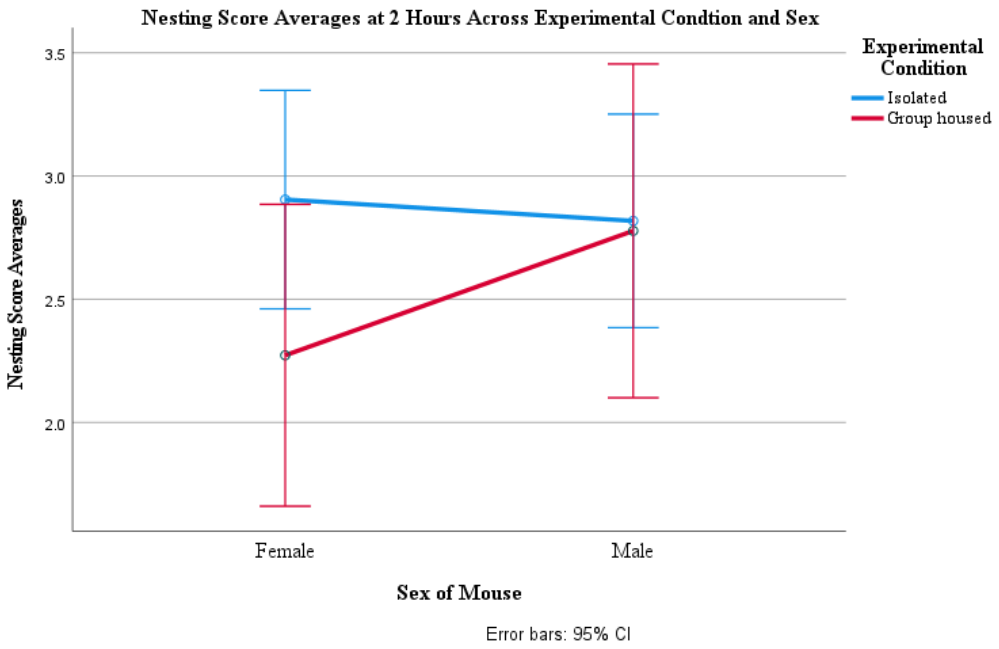

Figure 5: 2-Hour Nesting Results for 12-Weeks Testing:
Figure 5 shows results of 2-Hour Nesting assessment at 12-week testing period

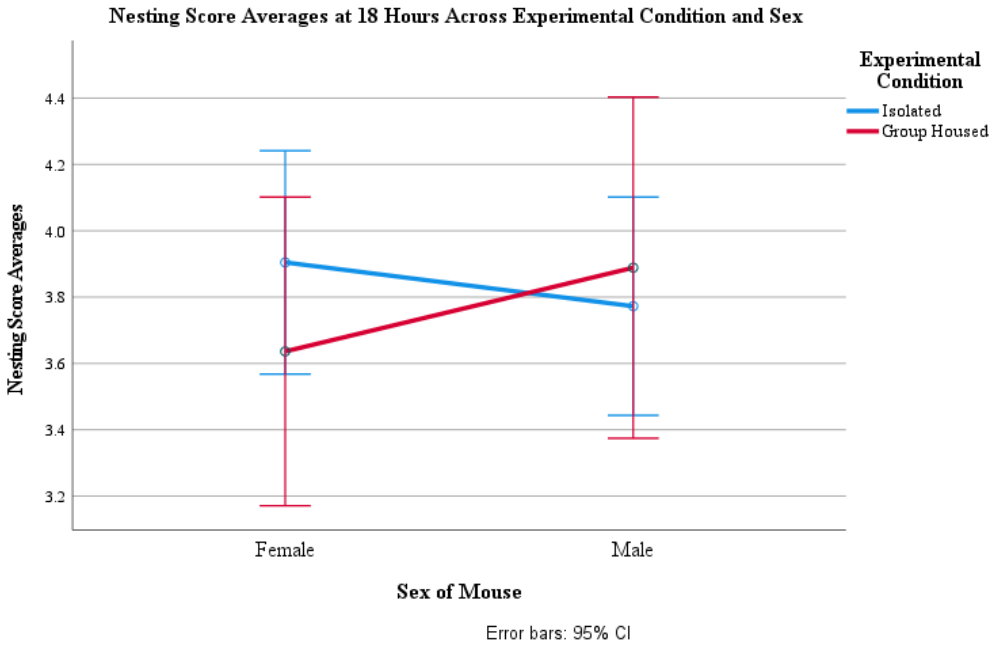

Figure 6: 18-Hour Nesting Results for 12-Weeks Testing:
Figure 6 shows results of the 18-Hour Nesting assessment at 12-week testing period

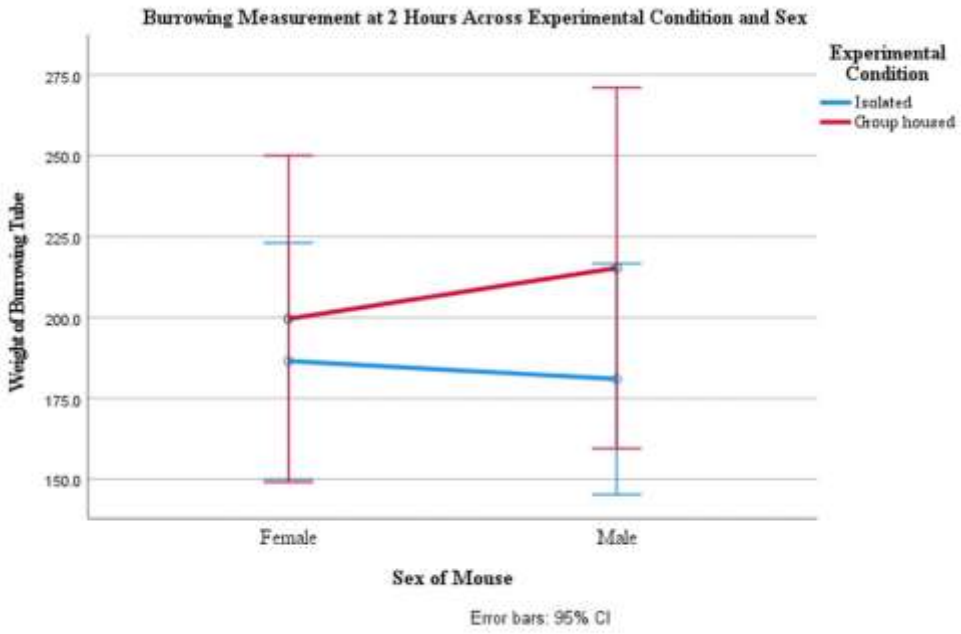

Figure 7: 2-Hour Burrowing Results for 12-Weeks Testing
Figure 7 shows results of 2-Hour Burrowing assessment at 12-week testing period.

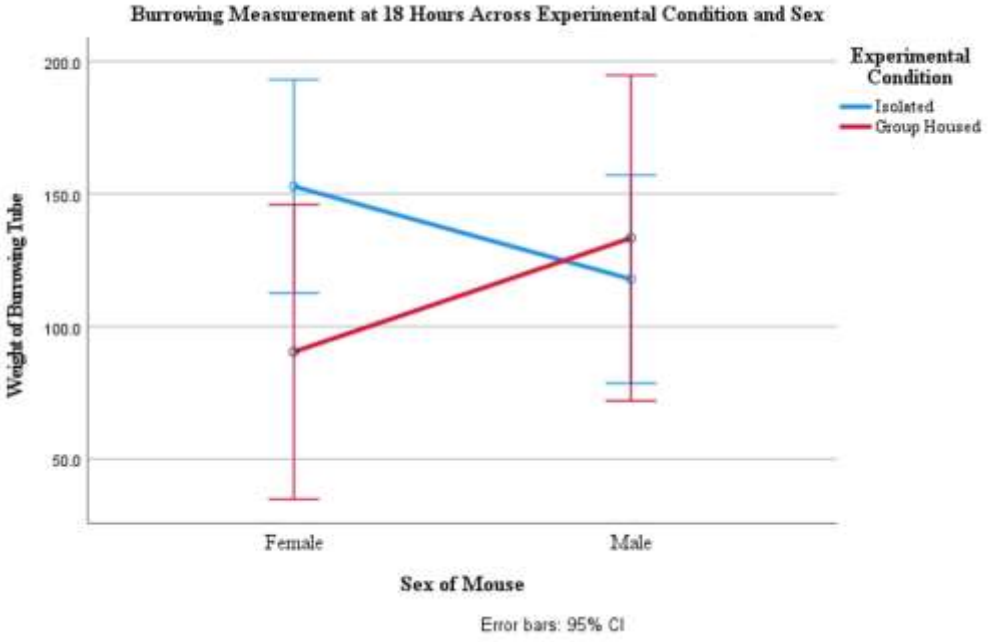

Figure 8: 18-Hour Burrowing Results for 12-Weeks Testing:
Figure 8 shows results of 18-Hour Burrowing assessment at 12-week testing period

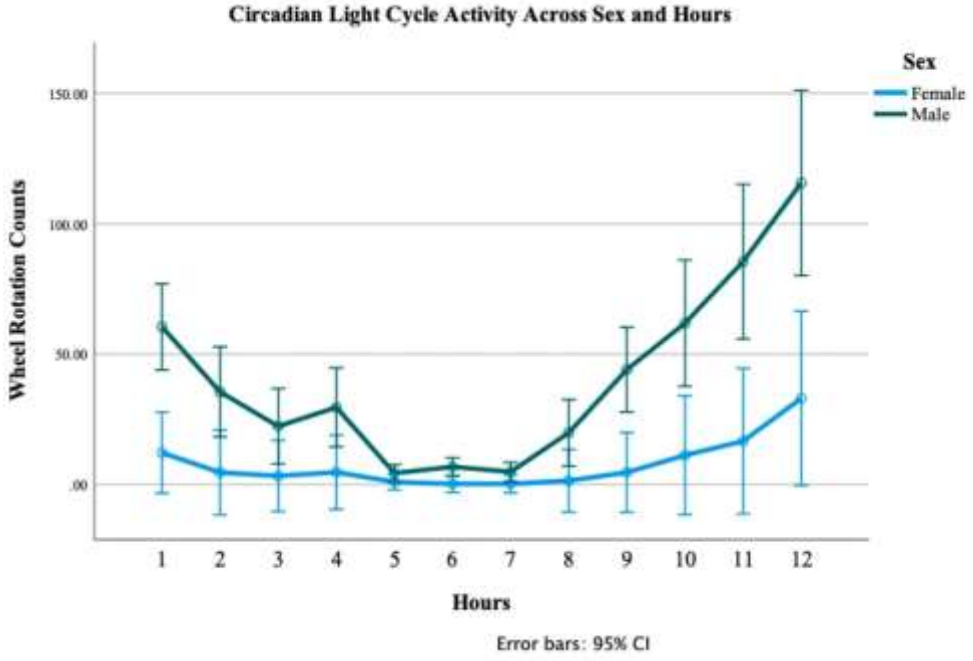

Figure 9: Circadian Light Cycle Results for 12-Weeks Testing – Sex Effect:
Figure 9 shows results for circadian light cycle activity across sex and hours. Males were shown to perform significantly better than females, *F* (1,59) 15.03, p = .000.

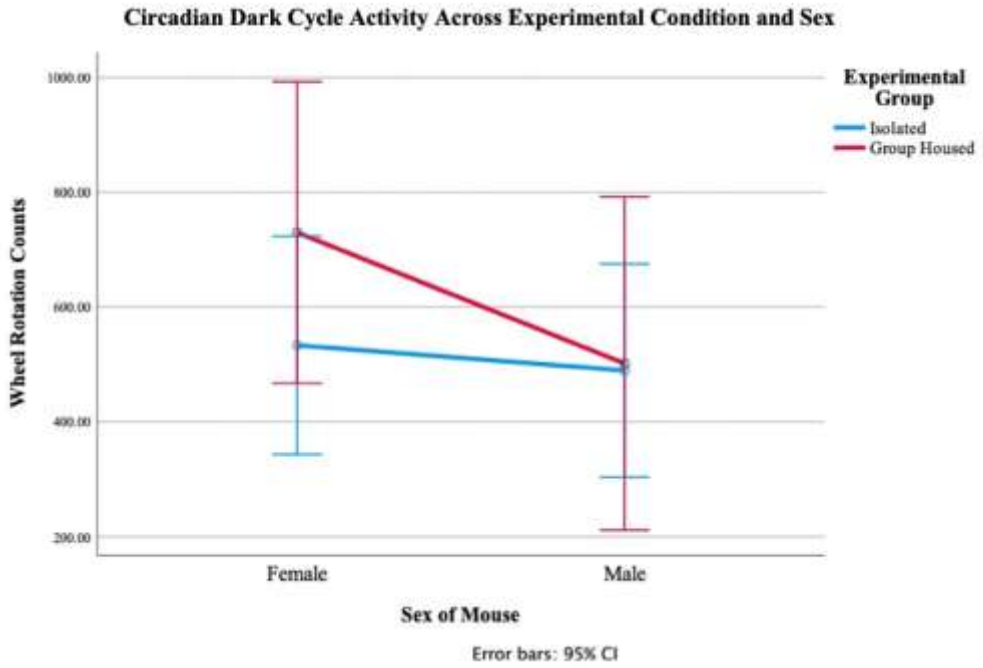

Figure 10: Circadian Dark Cycle Activity Across Experimental Condition and Sex at 12-Weeks Testing

Figure 10 shows Circadian activity across hours experimental condition during the dark cycle at the 12-weeks period

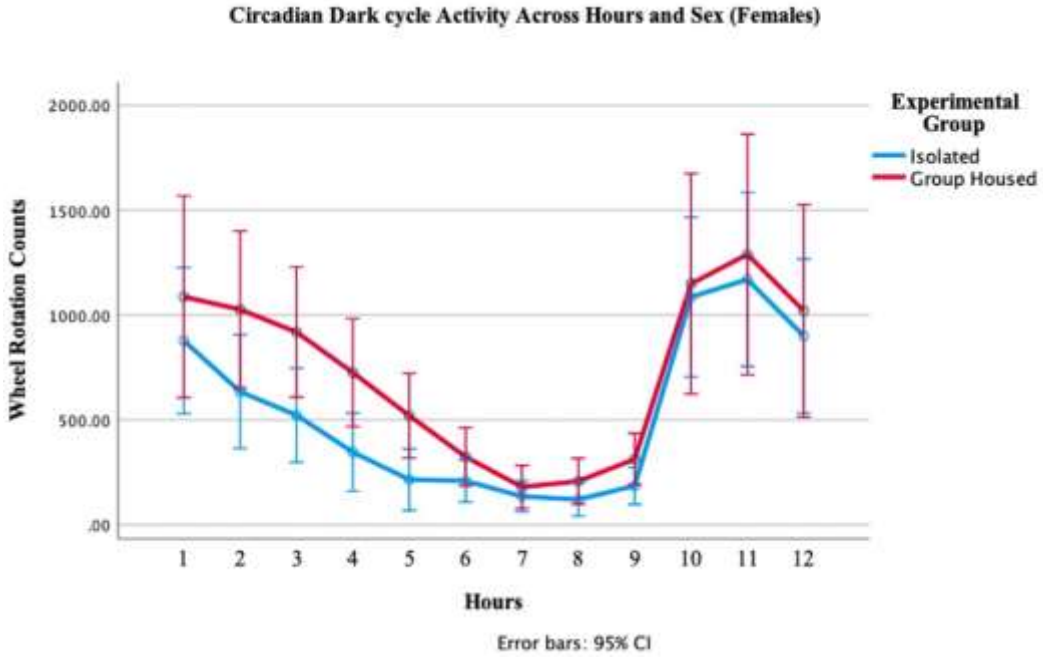

Figure 11: Circadian Dark Cycle Activity Across Hours and Sex for Females at 12-Weeks Testing

Figure 11 shows Circadian activity across hours and sex, for females specifically, during the dark cycle at the 12-week period

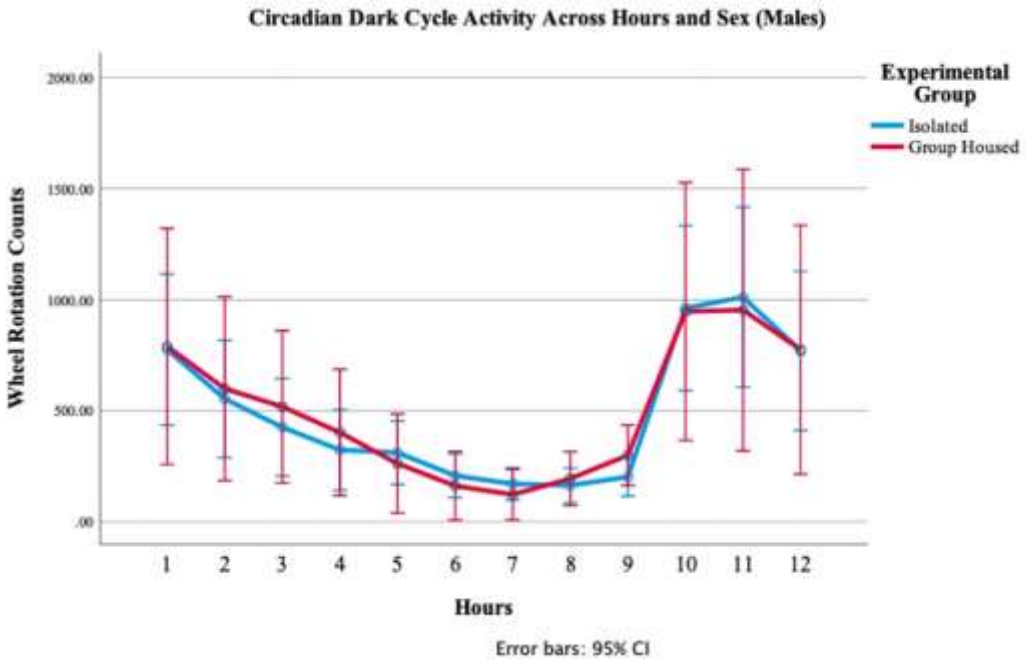

Figure 12: Circadian Dark Cycle Activity Across Hours and Sex for Males at 12-Weeks Testing

Figure 12 shows Circadian activity across hours and sex, for males specifically, during the light cycle at the 12-week period

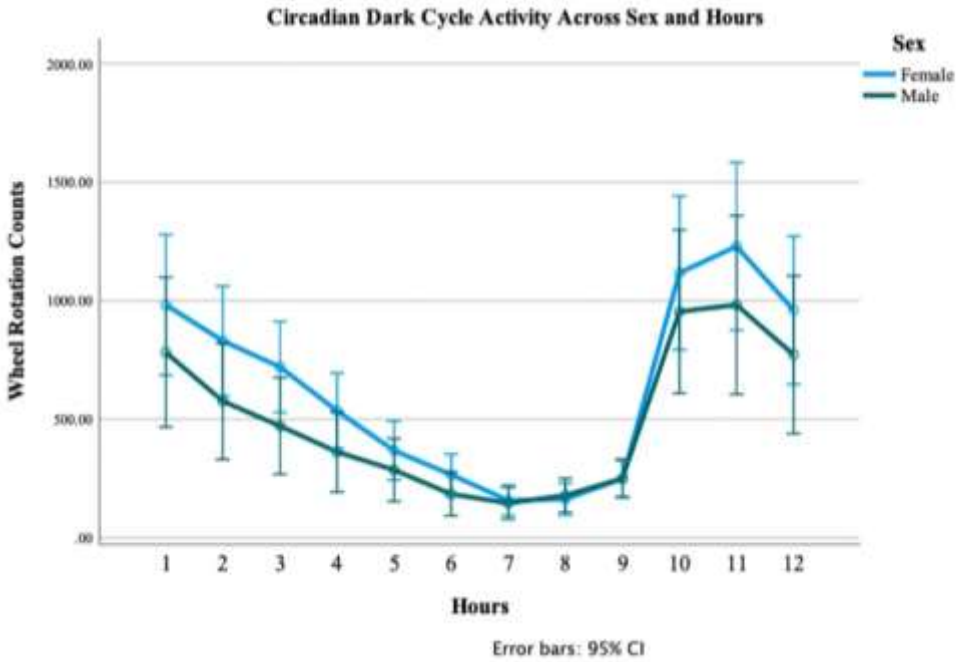

Figure 13: Circadian Dark Cycle Activity Across Sex and Hours at 12-Weeks Testing

Figure 13 shows Circadian activity across both sexes and hours at the 12-week period

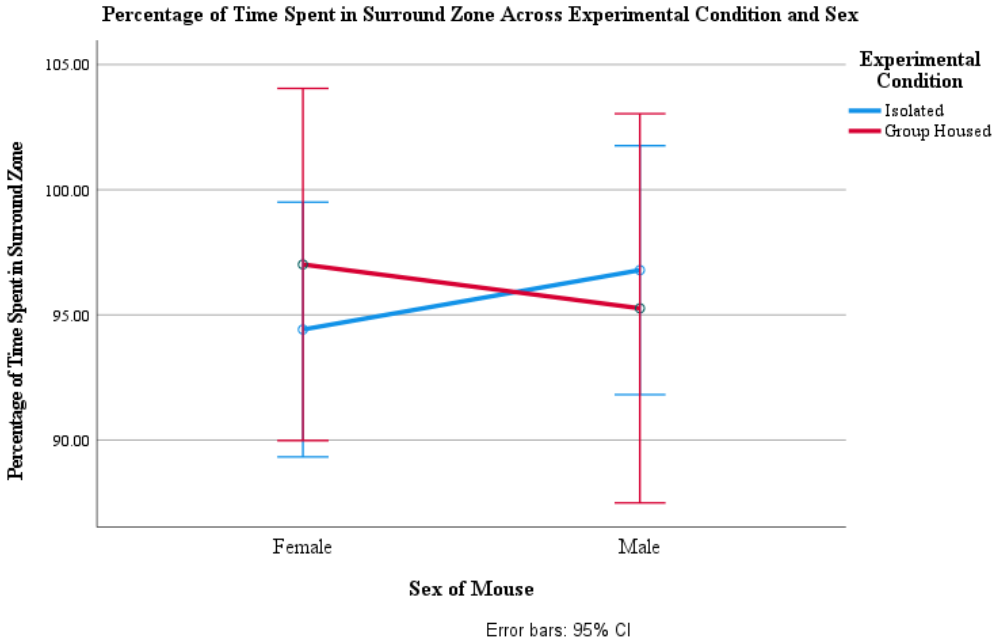

Figure 14: Time Spent in Surround Zone of Open Field Assessment at 12-Weeks Testing:

Figure 14 shows results of time spent in the surround zone for the Open Field test at the 12-week period

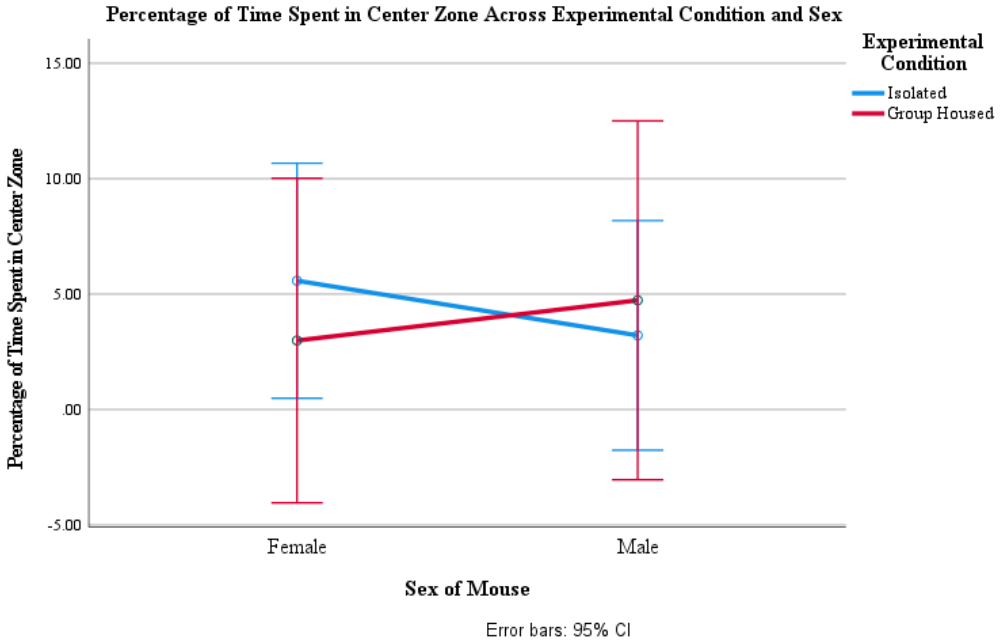

Figure 15: Time Spent in Center Zone of Open Field Assessment at 12-Weeks testing:

Figure 15 shows results of time spent in the center zone for the Open Field test at the 12-week period

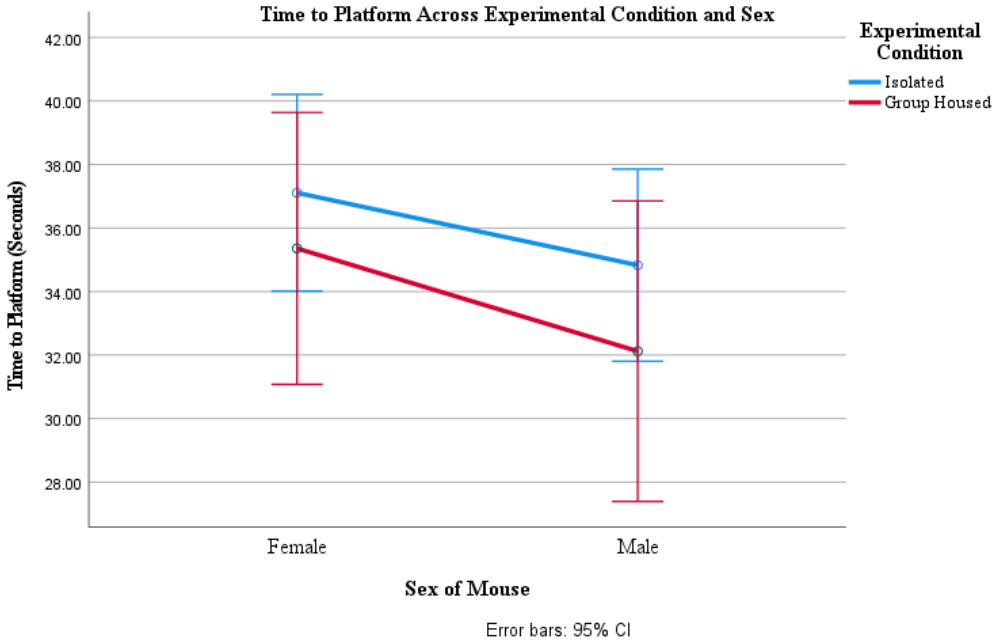

Figure 16: Duration to Reach Platform for Morris Water Maze at 12-Week Testing: Figure 16 shows the duration taken to reach the platform for the Morris Water Maze assessment at the 12-week period

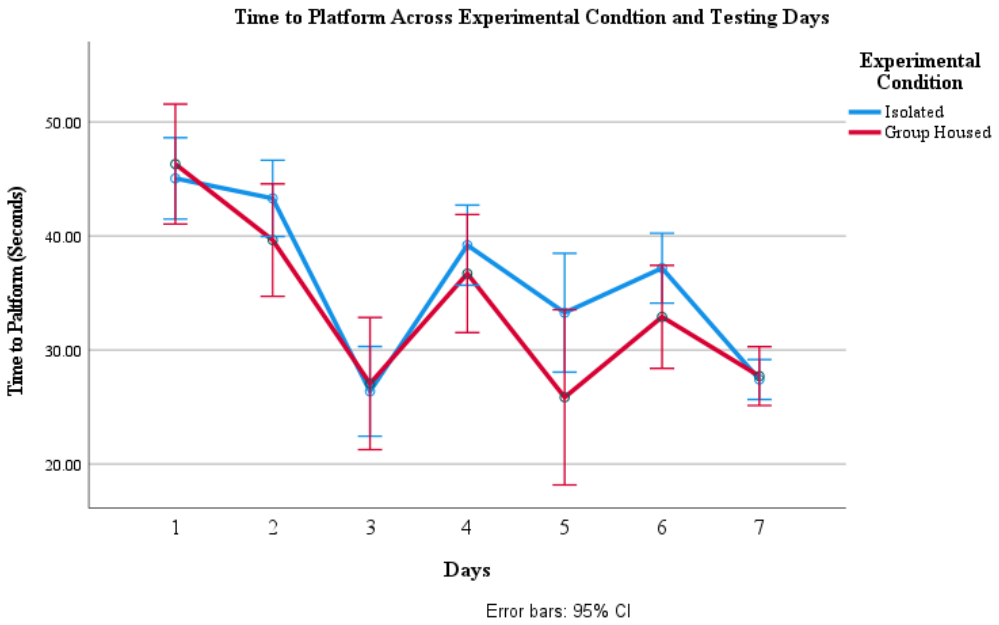

Figure 17: Duration to Reach Platform for Morris Water Maze at 12-Week Testing Across Days:

Figure 17 shows the duration taken to reach the platform for the Morris Water Maze assessment at the 12-week period across experimental days

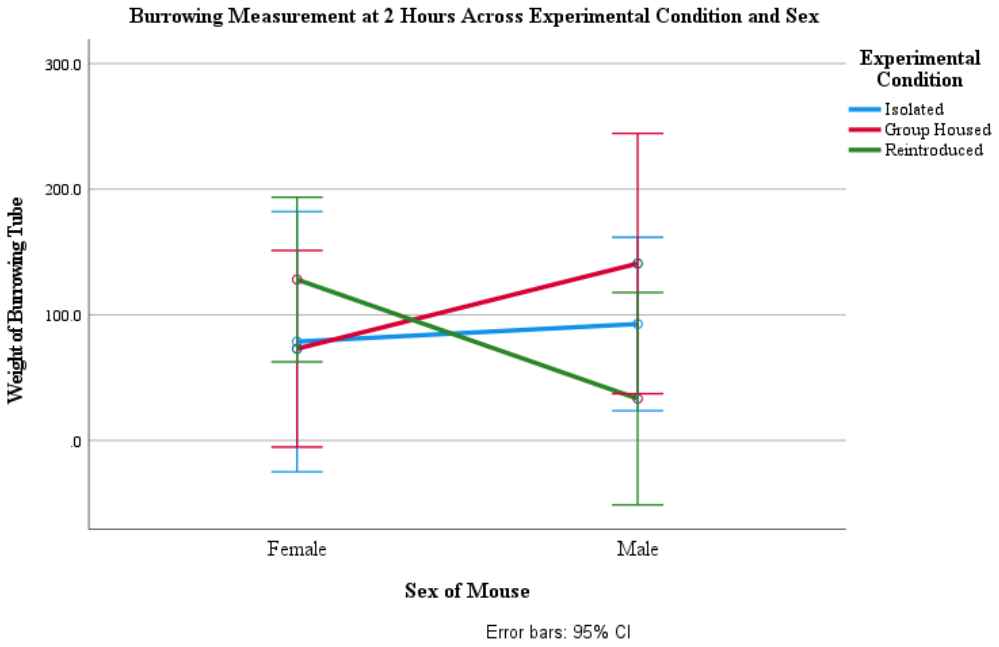

Figure 18: 2-Hour Burrowing Results for 20-Weeks Testing

Figure 18 shows results of 2-Hour Burrowing assessment at 20-week testing period

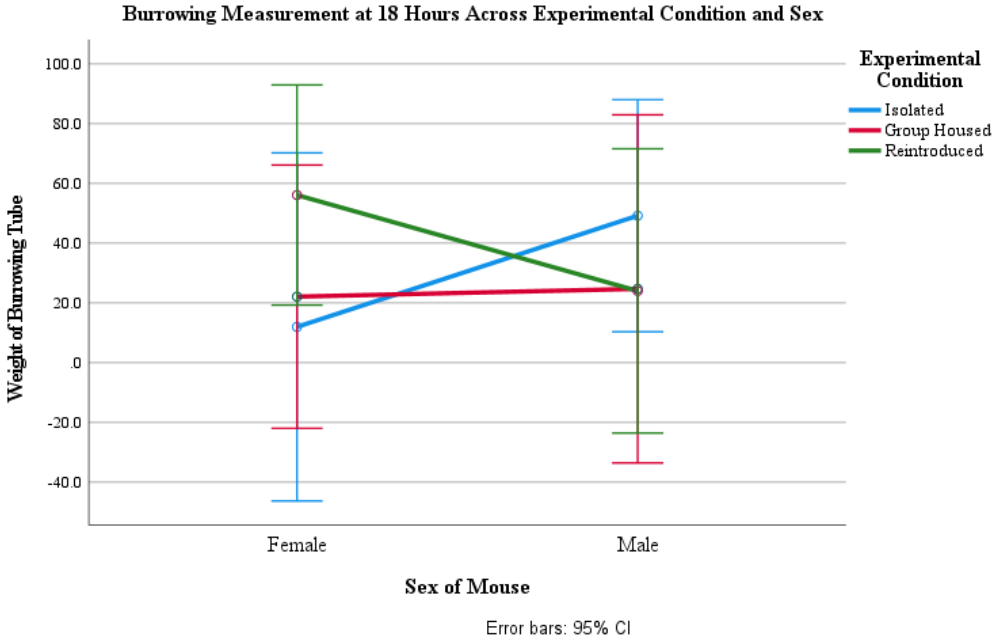

Figure 19: 18-Hour Burrowing Results for 20-Weeks Testing:
Figure 19 shows results of 18-Hour Burrowing assessment at 20-week testing period

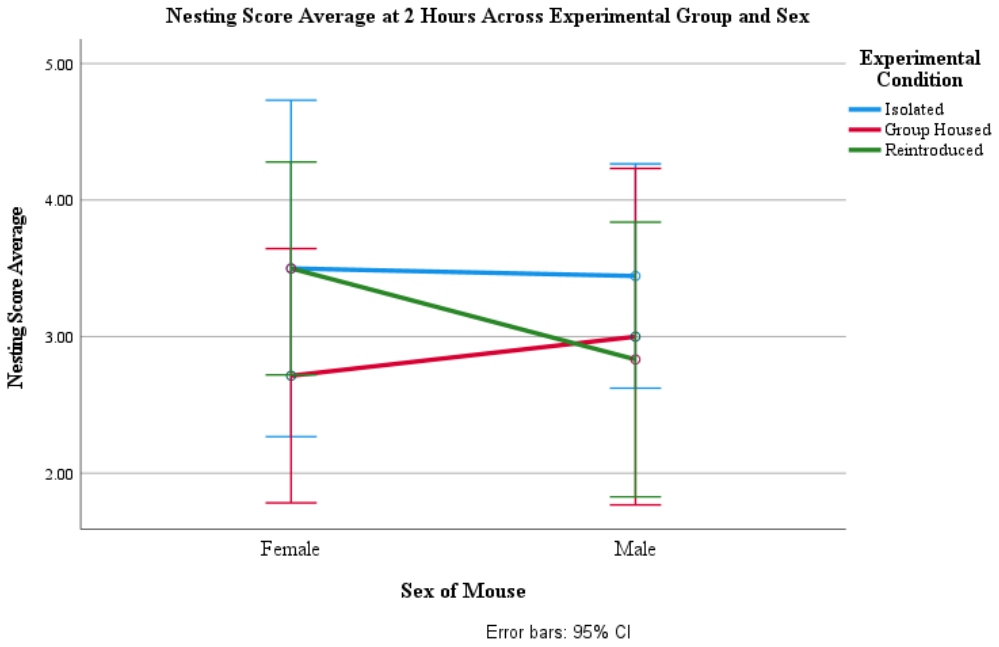

Figure 20: 2-Hour Nesting Results for 20-Weeks Testing:
Figure 20 shows results of 2-Hour Nesting assessment at 20-week testing period

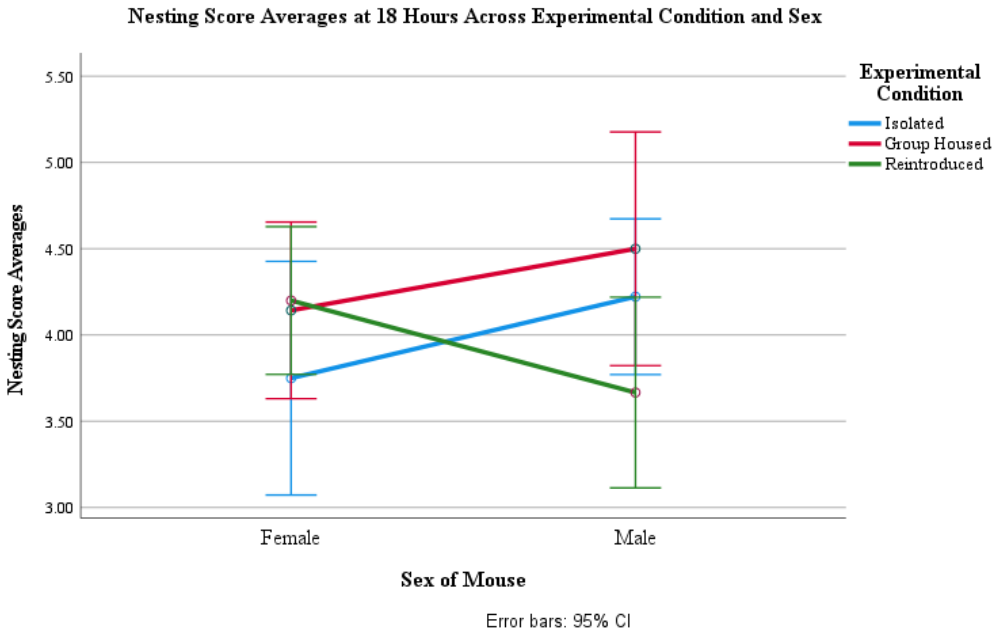

Figure 21: 18-Hour Nesting Results for 20-Weeks Testing:
Figure 21 shows results of the 18-Hour Nesting assessment at 20-week testing period

Figure 22: Time Spent in Surround Zone of Open Field Assessment at 20-Weeks Testing:

Figure 22 shows results of time spent in the surround zone for the Open Field test at the 20-week period

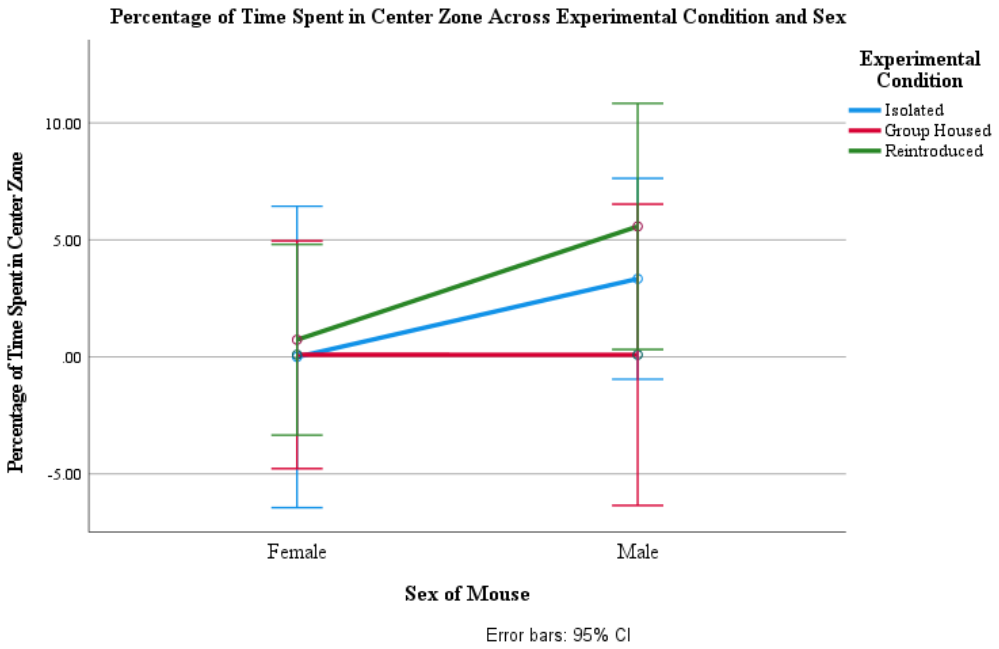

Figure 23: Time Spent in Center Zone of Open Field Assessment at 12-Weeks testing:

Figure 23 shows results of time spent in the center zone for the Open Field test at the 20-week period

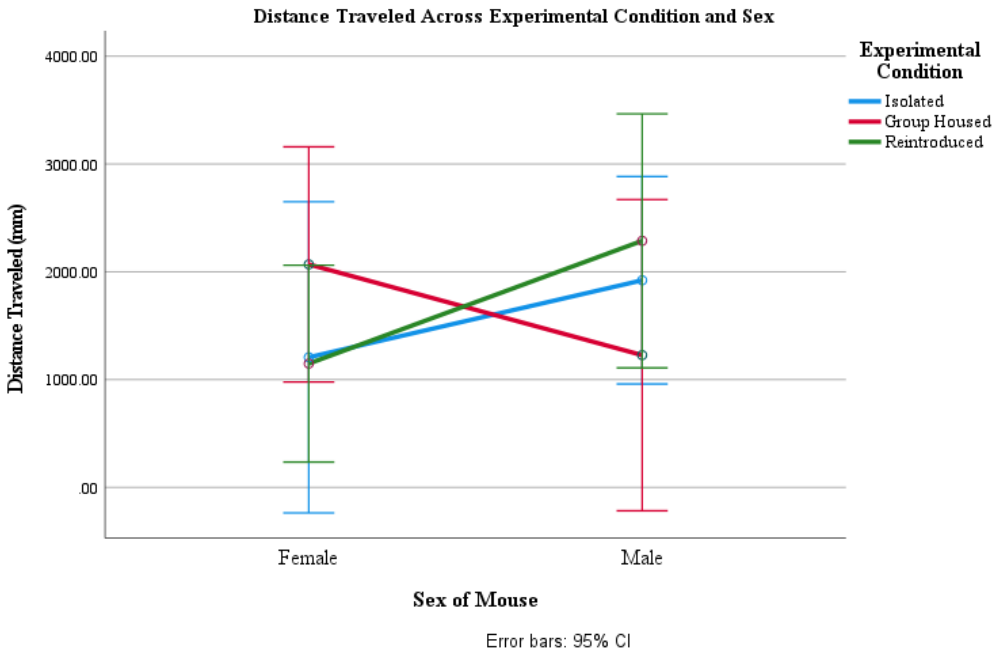

Figure 24: Distance Traveled by Mice throughout Open Field at 20-Weeks Testing: Figure 24 shows the total distance traveled (in mm) by mice throughout the Open Field assessment at the 20-week period

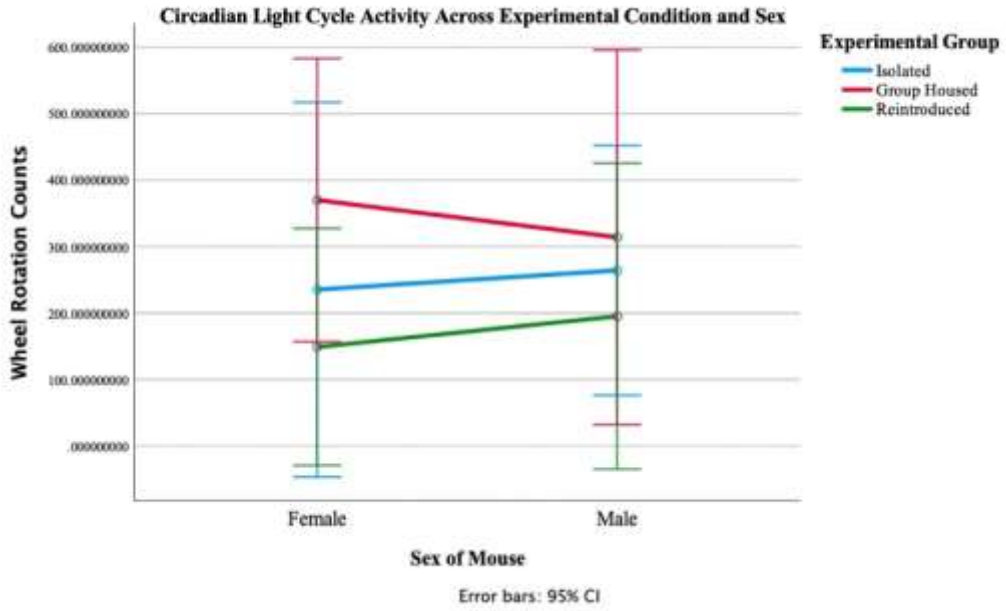

Figure 25: Circadian Light Cycle Activity Across Experimental Condition and Sex at 20-Weeks Testing
Figure 25 shows Circadian activity across hours and experimental condition during the light cycle at the 20-weeks period

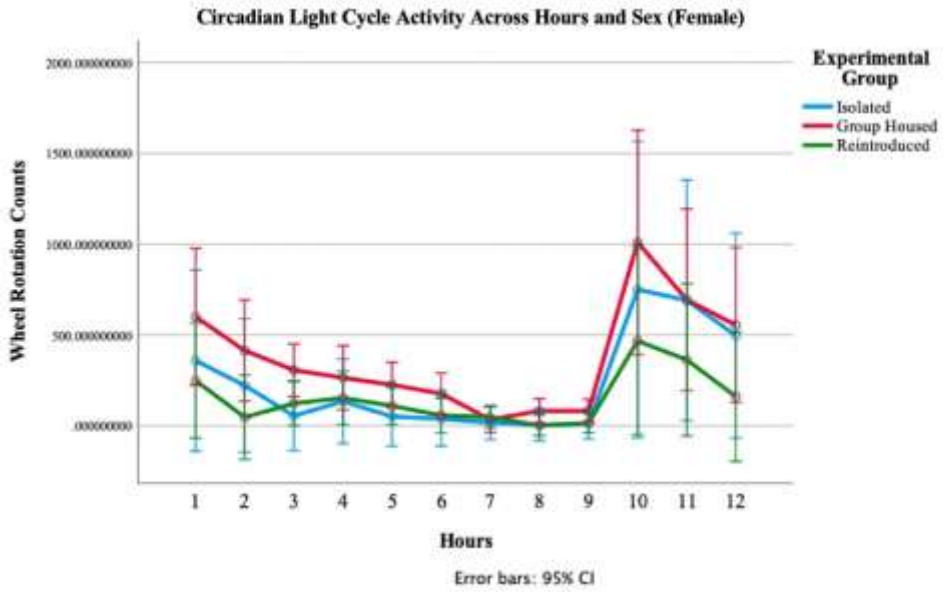

Figure 26: Circadian Light Cycle Activity Across Hours and Sex for Females at 20-Weeks Testing

Figure 26 shows Circadian activity across hours and sex, for females specifically, during the light cycle at the 20-week period

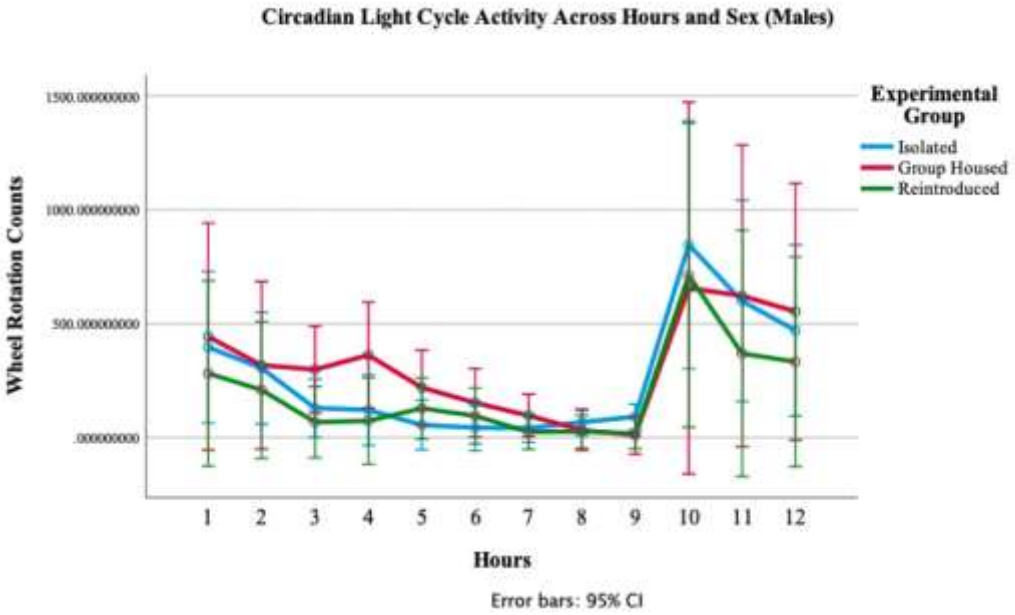

Figure 27: Circadian Light Cycle Activity Across Hours and Sex for Males at 20-Weeks Testing

Figure 27 shows Circadian activity across hours and sex, for males specifically, during the light cycle at the 20-week period

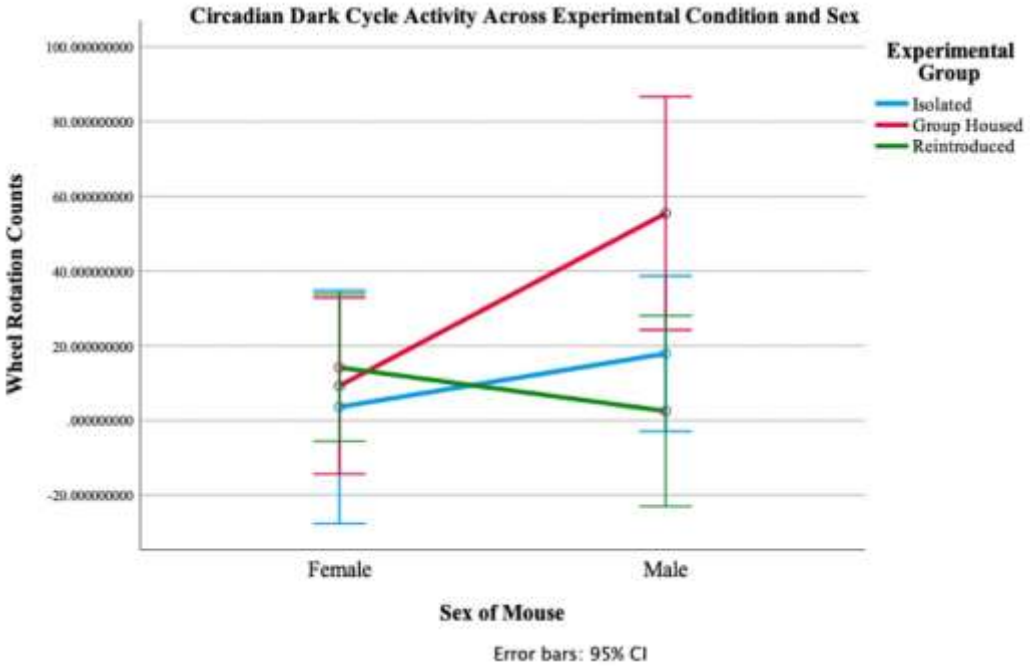

Figure 28: Circadian Dark Cycle Activity Across Experimental Condition and Sex at 20-Weeks Testing

Figure 28 shows Circadian activity across hours experimental condition during the dark cycle at the 20-weeks period

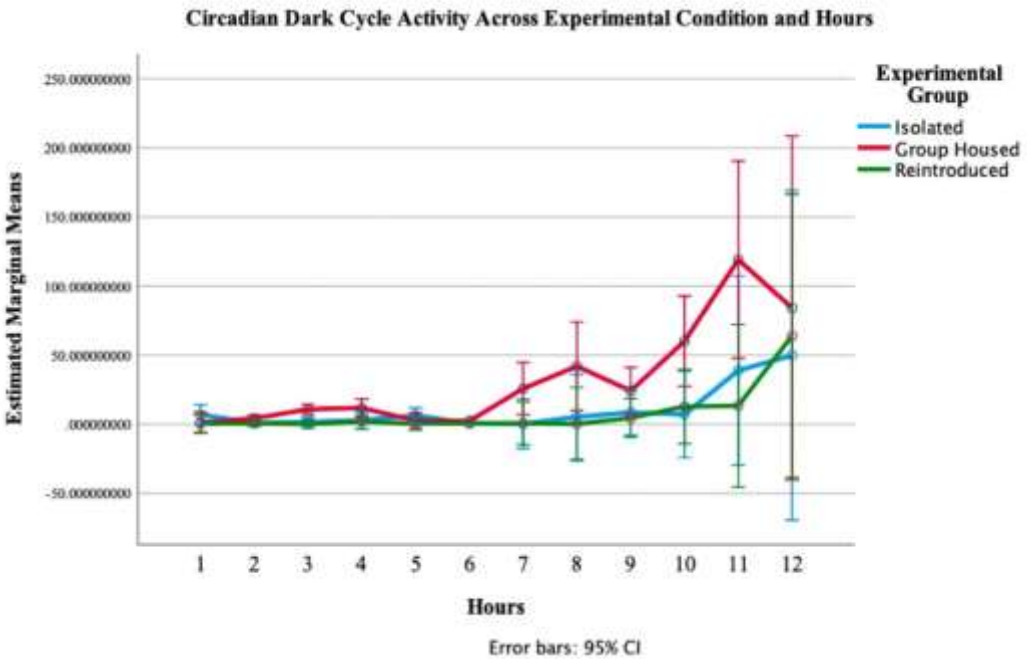

Figure 29: Circadian Dark Cycle Activity Across Experimental Condition at 20-Weeks Testing

Figure 29 shows Circadian activity across hours experimental condition during the dark cycle at the 20-weeks period

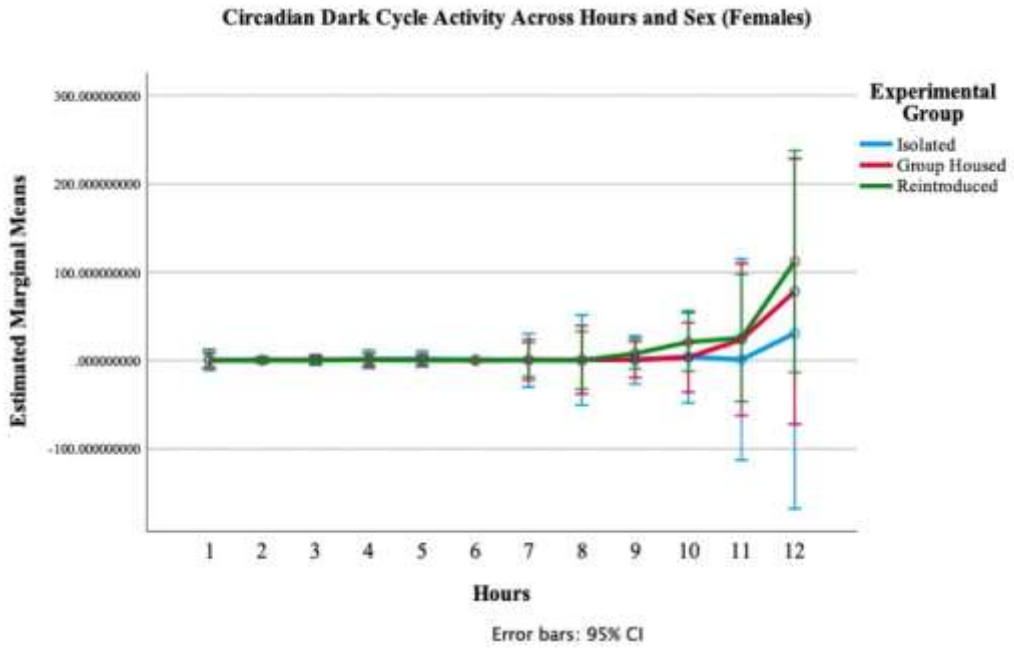

Figure 30: Circadian Dark Cycle Activity Across Hours and Sex for Females at 20-Weeks Testing

Figure 30 shows Circadian activity across hours and sex, for females specifically, during the dark cycle at the 20-week period

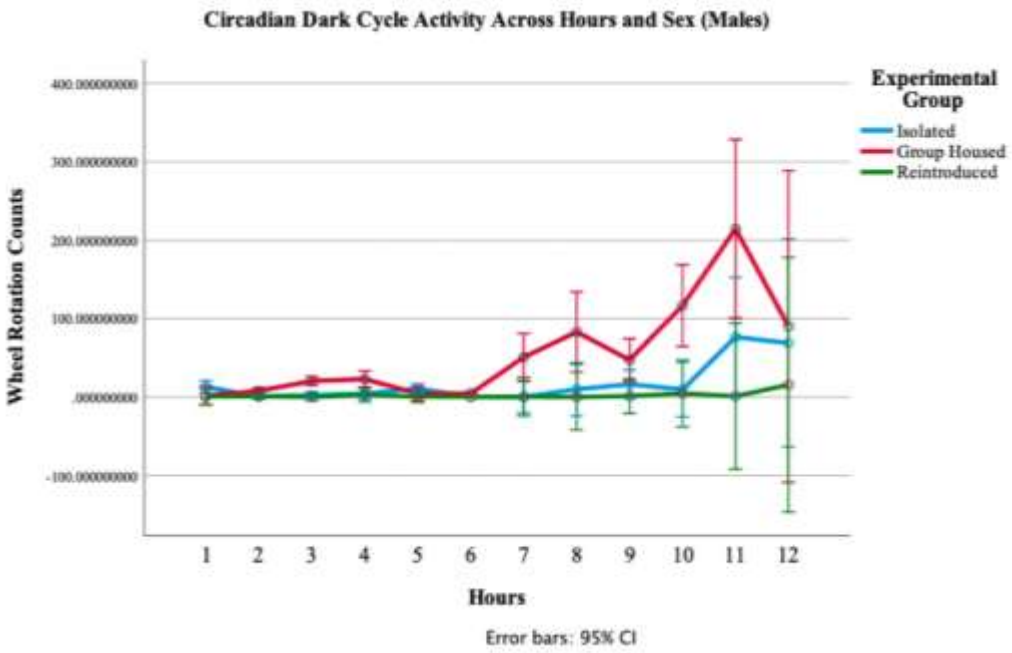

Figure 31: Circadian Dark Cycle Activity Across Hours and Sex for Males at 20-Weeks Testing

Figure 31 shows Circadian activity across hours and sex, for males specifically, during the dark cycle at the 20-week period

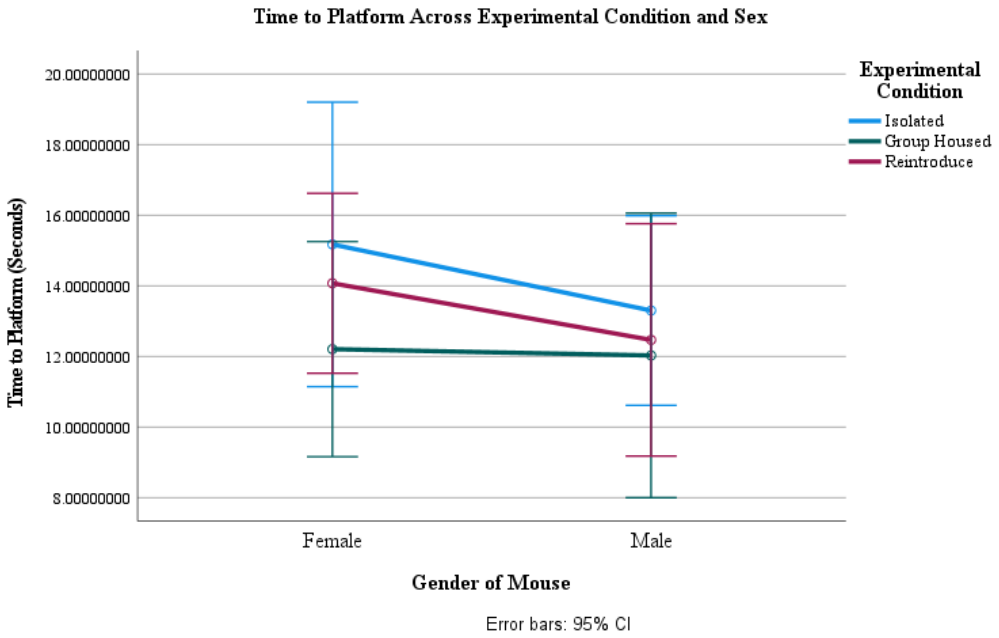

Figure 32: Duration to Reach Platform for Morris Water Maze at 20-Week Testing:
Figure 32 shows the duration taken to reach the platform for the Morris Water Maze assessment at the 20-week period

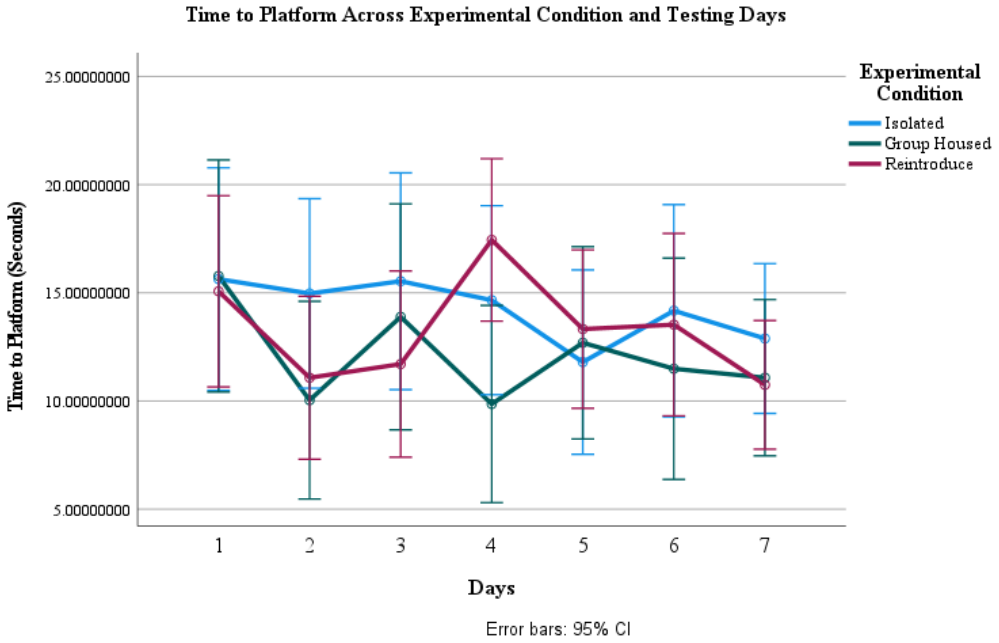

Figure 33: Duration to Reach Platform for Morris Water Maze at 20-Week Testing Across All Days:
Figure 33 shows the duration taken to reach the platform for the Morris Water Maze assessment at the 20-week period across all experimental days

REFERENCES

Ali AA, Khalil MG, Elariny HA, Abu-Elfotuh K (2017) Study on Social Isolation as a Risk Factor in Development of Alzheimer's Disease in Rats. Brain DisordTher 6: 230. doi: 10.4172/2168-975X.1000230

Barkey, r. e.1, Craven, k. m.2, Kochen, w. r.3, Cerri, d. m. 1 & Flinn, j. m. 1 1. Dept. Psychology, Cognitive/Behavioral Neuroscience, George Mason University, Fairfax, VA 2. National Institutes of Health, Bethesda, MD 3. Southeastern University, Lakeland, F

Blockparty®. How to increase floor space without buying a new mouse rack | Animal Care Systems. (n.d.). Retrieved September 28, 2021, from https://animalcaresystems.com/products/blockparty.

Center for Disease Control and Prevention. (2021, April 29). *Loneliness and social isolation linked to serious health conditions*. Centers for Disease Control and Prevention. Retrieved November 5, 2021, from https://www.cdc.gov/aging/publications/features/lonely-older-adults.html.

Fliers, E., Swaab, D. F., Pool, C. W., &Verwer, R. W. (1985). The vasopressin and oxytocin neurons in the human supraoptic and paraventricular nucleus; changes with aging and in senile dementia. Brain research, 342(1), 45–53. https://doi.org/10.1016/0006-8993(85)91351-4

Gaskill, B. N., Karas, A. Z., Garner, J. P., & Pritchett-Corning, K. R. (2013). Nest building as an indicator of health and welfare in laboratory mice. *Journal of visualized experiments :*

JoVE, (82), 51012. https://doi.org/10.3791/51012

Gould, Todd D., et al. “The Open Field Test - Researchgate.” *ResearchGate*, Mar. 2009, https://www.researchgate.net/publication/226063026_The_Open_Field_Test.

Ikram MA, Vrooman HA, Vernooij MW, den Heijer T, Hofman A, Niessen WJ, van der Lugt A, Koudstaal PJ, Breteler
MM (2010)

Ikram, M &Vrooman, Henri &Vernooij, Meike&Heijer, Tom &Hofman, Albert &Niessen, W.J. &Lugt, Aad&Koudstaal, Peter &Breteler, Monique. (2008). Brain tissue volumes in relation to cognitive function and risk of dementia. Neurobiology of aging. 31. 378-86. 10.1016/j.neurobiolaging.2008.04.008.

Kane, M. J., Angoa-Pérez, M., Briggs, D. I., Viano, D. C., Kreipke, C. W., & Kuhn, D. M. (2012). A mouse model of human repetitive mild traumatic brain injury. *Journal of neuroscience methods*, *203*(1), 41–49. https://doi.org/10.1016/j.jneumeth.2011.09.003

Lapiz, M.D.S., Fulford, A., Muchimapura, S. *et al.* Influence of Postweaning Social Isolation in the Rat on Brain Development, Conditioned Behavior, and Neurotransmission. *NeurosciBehavPhysiol* **33,** 13–29 (2003). https://doi.org/10.1023/A:1021171129766

Lidster, K., Owen, K., Browne, W.J. et al. Cage aggression in group-housed laboratory male mice: an international data crowdsourcing project. Sci Rep 9, 15211 (2019). https://doi.org/10.1038/s41598-019-51674-z

Lukkes, Jodi L, et al. “Consequences of Post-Weaning Social Isolation on Anxiety Behavior and Related Neural Circuits in Rodents.” Frontiers, Frontiers, 1 Jan. 1AD,

https://www.frontiersin.org/articles/10.3389/neuro.08.018.2009/full

Marklund N. (2016). Rodent Models of Traumatic Brain Injury: Methods and Challenges. *Methods in molecular biology (Clifton, N.J.)*, *1462*, 29–46. https://doi.org/10.1007/978-1-4939-3816-2_3

National Academies of Sciences, Engineering, and Medicine. 2020. *Social Isolation and Loneliness in Older Adults: Opportunities for the Health Care System*. Washington, DC: The National Academies Press. https://doi.org/10.17226/25663external icon.

Neumann, Inga D, et al. "Aggression and Anxiety: Social Context and Neurobiological Links." *Frontiers*, Frontiers, 1 Jan. 1AD, https://www.frontiersin.org/articles/10.3389/fnbeh.2010.00012/full.

Rowe, R. K., Harrison, J. L., Thomas, T. C., Pauly, J. R., Adelson, P. D., &Lifshitz, J. (2013). Using anesthetics and analgesics in experimental traumatic brain injury. *Lab animal*, *42*(8), 286–291. https://doi.org/10.1038/laban.257

Texas Department of State Health Services. (2021). *What is alzheimer's disease? Questions and answers*. Texas Department of State Health Services. https://dshs.texas.gov/alzheimers/qanda.shtm.

Tong, F., Yu, C., Wang, L., Chi, I., & Fu, F. (2021). Systematic Review of Efficacy of Interventions for Social Isolation of Older Adults. *Frontiers in psychology*, *12*, 554145. https://doi.org/10.3389/fpsyg.2021.554145

UCI. (n.d.). *Acclimation period*. Acclimation Period for Lab Animals. Retrieved November 1, 2021, from https://research.uci.edu/compliance/animalcare-use/research-policies-and-guidance/acclimation.html.

Vanderschuren, L. J., Achterberg, E. J., &Trezza, V. (2016). The neurobiology of social play and its rewarding value in rats. *Neuroscience and biobehavioral reviews*, *70*, 86–105. https://doi.org/10.1016/j.neubiorev.2016.07.025

Zhang, Y. P., Cai, J., Shields, L. B., Liu, N., Xu, X. M., & Shields, C. B. (2014). Traumatic brain injury using mouse models. *Translational stroke research*, *5*(4), 454–471. https://doi.org/10.1007/s12975-014-0327-0

Zidar, J., Weber, E. M., Ewaldsson, B., Tjäder, S., Lilja, J., Mount, J., Svensson, C., Svensk, E., Udén, E., &Törnqvist, A. E. (2019). Group and Single Housing of Male Mice: Collected Experiences from Research Facilities in Sweden. Animals : an open access journal from MDPI, 9(12), 1010. https://doi.org/10.3390/ani9121010

Zulley J. (2000). The influence of isolation on psychological and physiological variables. *Aviation, space, and environmental medicine*, *71*(9 Suppl), A44–A47

BIOGRAPHY

Michael Lamarche received his Bachelor of Arts in Psychology from George Mason University 2021, then continued his time at the university to receive his Master of Arts in Psychology. After receiving his Masters, he will continue to work in the field of psychology to help better the lives and wellbeing of others.