A Comprehensive Assessment of Animal Welfare in Regards to Cage Size for Trio-Bred C57BL/6 Mice

A Thesis

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By

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ABSTRACT

The current edition of the *Guide for the Care and Use of Laboratory Animals* makes recommendations on minimum floor space requirements for housing female mice with litters. As a result, these recommendations preclude trio breeding of mice in standard "shoebox" cages, which impacts institutions that historically have permitted trio breeding of mice. The intent of this study is to assess the effects of cage size and paired versus trio breeding on the welfare of breeding mice and their litters, as assessed by various parameters including reproductive efficacy, weight gain, behavioral assessment, biological stress markers, microenvironment, the histopathology of respiratory tissues, and genetic expression. The 2x2 design of the study utilized C57BL/6 mice housed in either a pair- and trio-breeding scheme within either a 67 in^2 (170 cm^2) standard "shoebox" mouse individually ventilated cage (IVC) cage or a 132 in^2 (335 cm^2) standard rat IVC. No significant differences among these four groups were noted in any of the measured parameters with the exception of (1) a significant effect of cage size on microenvironment (specifically, elevated ammonia levels in smaller cages irrespective of pair or trio housing) and (2) two marginally significant behavioral affects that are not internally corroborated. Despite the increased ammonia concentrations observed in smaller cages, there were no significant gross or histologic changes noted in respiratory tissues of the trio bred mice in standard cages compared to the other groups. We conclude that a larger floor space can reduce intra-cage microenvironment parameter levels, but there are no differences in any other welfare parameter assessed in this study when comparing rat-cage sized housing to standard mouse cages. Moreover, there is no evidence of reduced welfare based on trio housing as opposed to pair housing irrespective of cage size.

BIOGRAPHICAL SKETCH

The eldest son of parents of Hindu decent, Rohit Rajoria was born in Somerville, New Jersey and has lived in the tri-state region prior to college. He received his Bachelor of Science in 2005 from Ursinus College with a major in Biochemistry/ Molecular Biology. He worked as a research associate for the U.S.D.A., Centocor Inc., and GlaxoSmithKline prior to his veterinary school matriculation. After obtaining his Doctorate of Veterinary Medicine from St. George's University in 2011, he completed a Laboratory Animal Medicine Residency at Cornell University working for the Cornell Center for Animal Resources and Education. During his residency training he was accepted into the Cornell Graduate School in the Field of Comparative Biomedical Sciences from which he will be receiving a Master of Science in May 2015.

This is dedicated to all husbandry staff and technicians who are committed to providing the highest quality of service and care for the rodents utilized in comparative research.

ACKNOWLEDGMENTS

I would like to thank Dr. David Lin for allowing me to participate in several studies in his laboratory while educating me in the field of neurobiology. Without his collaborative efforts and patience, I would not have been able to complete the goals I had in mind. In addition, I would also like to thank Dr. Bhupinder Singh (DACLAM) for providing me the opportunity to complete a project that can directly impact the standards set forth as it pertains to rodent breeding. His extensive knowledge base in this field and proactive ability to optimize the function of our project was crucial in developing a study that can provide as much information to the laboratory animal medicine community. I would also like to thank my other collaborators: Dr. Bryant Blank, Dr. Erin Daugherity (DACLAM), Dr. Thom Cleland and Mathew Einhorn (Dept. of Psychology), Dr. Teresa Southard (DACVP), Denny Totman (RLATG), Scott Soprano (RLATG), Cheri Jackmin (RLATG), Dustin O'Hara (Cornell EH&S), Brenda Collins (RLATG), and the Weill Conventional Vivarium staff (Tim Van Deusen, Stephanie Martin, Sid Reeves, Dave Diettrich). This project would not have been achieved without the departmental support from the Cornell Center for Animal Resources and Education (CARE) as well as funding through the NIH R25 grant and Office of Vice Provost for Research (OVPR). I want to thank Dr. Philippe Baneux (DECLAM), Dr. Todd Pavek (DACLAM), and Dr. Kirk Maurer (DACLAM) for their review and assistance with data and grant approval.

Most importantly, I want to thank my parents (Gunkeshi & Dalbir Rajoria) for being my role models. Their sacrifice, work ethic, dedication, love, and drive for a better living in the U.S. is a constant reminder for me to strive for greatness while remaining humble. Thanks to my brother, Gaurav, and everyone else who made an impact in my life

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CHAPTER ONE:

INTRODUCTION

The eighth edition (2011) of the *Guide for the Care and Use of Laboratory Animals* (*Guide*) describes new housing space requirements for laboratory mice in different breeding configurations, and lists a floor area of 51 in^2 (330 cm^2) for "Female + litter". This new guideline recommends a floor area of at least 117 in^2 for a trio breeding paradigm that could potentially include two females, two litters, and a male.[43] Since many institutions utilize industry standard shoebox cages of 67-74 in^2 floor area, the historical configuration for trio breeding mice would not be permitted under the *Guide's* recommendations.

The premise behind the *Guide's* recommendations is based on the potential negative consequences that might result from trio breeding, based on overcrowding. The rationale is valid in that larger numbers of pups generate more waste accumulation and can potentially lead to high microenvironment levels; [11, 14, 47] increasing space requirements is expected to dilute the intra-cage microenvironment and thereby improve welfare. However, the value of extra floor space has been contraindicated by several studies that have reported higher morbidity and reduced weight gain in pups born into larger floor spaces, which has led to conflicted opinions on floor space recommendations for rodents.[15, 29, 68-69] Each of these pros and cons of trio breeding in shoebox cages should be considered when designing a breeding configuration that is both functional and acceptable from an animal welfare perspective.

To date, it has been difficult to make an overall assessment on the *Guide's* recommendations since there has been a lack of uniformity in the designs of different published studies evaluating housing density. Attempts at tabulating multiple studies' data have been performed in order to detect trends in floor space sizes and their effects on the health and welfare of rodents. In a pair of 2004 studies by Smith

and colleagues, several parameters of adult rodent health and welfare associated with different floor space allocations were formatted in a table to compare the findings from each study. The results from these multiple studies revealed mixed outcomes of mouse weights, physiological stress, and microenvironment differences amongst the various space allocations for adult mice. [60-61] A similar databank was also formulated in a 2012 study by Whitaker and colleagues in which measures of adult rodents' health were assessed in various housing densities, and compared amongst similar studies.[75] A majority of those studies revealed no negative health effects from housing densities that did not comply with the *Guide* as they pertain to adult rodent housing. Although conflicting outcomes are apparent, the strategy to compile as much information as possible and detect trends from the results of related studies can prove beneficial in our expanding understanding of animal health and welfare. While the aforementioned publications provided substantial information in assessing adult rodent housing densities, the comprehensive studies directly evaluating the relationship between rodent breeding configurations and floor space are limited.

With the scientific literature failing to consistently support the new space recommendations for breeding rodents and their litters, the *Guide* allows the utilization of performance indices such as health, reproduction, growth rate, behavior, activity, and use of space to assess adequacy of housing. [40, 43] In this study, we measure a broad range of welfare-associated parameters in order to obtain a comprehensive assessment of animal health and welfare as it pertains to mouse breeding and their space requirements. Many of the welfare parameters assessed in this study revolved around determining sub-clinical measures of physiological chronic stress which may not be detected by observation alone. The specific objective is to determine whether trio breeding of C57BL/6 mice can be performed in standard shoebox cage (67 in^2) without any adverse effect on the overall health and welfare of animals, compared with pair breeding, larger cage sizes, or both. The hypothesis for our study: (1) Trio breeding in a standard shoebox cage does not impact the health and welfare of the mice in these cages despite the expected higher microenvironment levels.

CHAPTER TWO:

METHODS AND MATERIALS

Animals.

Female (n= 30) and male (n=20) C57BL/6J mice were obtained from a commercial vendor (Jackson Laboratory, Bar Harbor, ME) and housed in an AAALAC-accredited rodent vivarium. All mice utilized as breeders were 8 weeks old and obtained from colonies that were routinely monitored and tested negative for all pathogens screened routinely by commercial rodent suppliers in the U.S.A (Jackson Laboratory, Bar Harbor, ME). The cages contained autoclaved ¼ in size corn cob bedding (Harlan Teklad), ad libitum irradiated pelleted food (Harlan Teklad 8640), and ad libitum access to acidified water through lixits. The housing room dedicated for this study did not house any other rodents. The room was maintained at 71 ± 2°F temperature, 40-60% relative humidity, and 12 room air changes per hour (ACH). The IVCs were consistently maintained at 59-61 ACH for both IVC sizes. The animal room was kept on a 14:10-hr reverse light-dark cycle. The daily health observations, cage changes, and sampling were performed during the light phase (dormant phase) whereas the behavior tests were performed during the dark period (active phase). All mice were euthanized with CO_2 gas followed by cervical dislocation at the end of the study.

With the exception of space recommendations, all research complied with the recommendations set forth in the Eighth Edition of the *Guide*. This study was approved by Cornell University's Institutional Animal Care and Use Committee.

Experimental Design.

The mice were housed in breeding configurations either as a pair (1 male, 1 female) or trio (1 male, 2 females), and in one of two differently sized polycarbonate IVCs: either a standard "shoebox" mouse cage [170 cm^2 (67 in^2) floor area; 12.7 cm (5 in) height] or a rat cage [335 cm^2 (132 in^2) floor area; 20.3 cm (8 in) height] (Allentown Inc., Allentown, NJ). These two independent factors resulted in four different testing groups: (1) Trio-bred Mouse Cage-housed [TMC]; (2) Pair-bred Mouse Cage-housed [PMC]; (3) Trio-bred Rat Cage-housed [TRC]; (4) Pair-bred Rat Cage-housed [PRC]. A total of 20 autoclaved cages (5 per group) were used with a consistent amount of bedding supplied to yield a floor depth of 1.5 cm for each cage (107.8 g for mouse cages and 215.6 g for rat cages). Autoclaved breeding enrichment content within each cage was consistently provided: specifically, a cardboard hut, crinkle paper, and a square nestlet. Modified J-feeders, with lengths of 15 cm, were utilized in the rat cages to ensure that the mice had access to food at a similar height above the bedding floor (4.3 cm) as in the cages using wire top feeders (Figure 1). A series of environmental, physiological, and behavioral tests then were performed according to a standard protocol (Figure 2).

Reproductive Performance.

Reproductive efficacy, as a measure of health and well-being, was assessed in breeding mice by measuring (i) average litter size; (ii) inter-birth interval; (iii) the ratio between the number of pups weaned and the number of pups born within a given litter (*Wean: Born ratio*). During the light phase, all cages were visually checked daily between 9 and 10 am for any new litters born and/or any pup mortality. The litter sizes and dates of birth were recorded. The inter-birth interval corresponded to the number of days in between consecutive litters born from a single female. The study was continued until all female breeder mice in all four groups had produced and weaned three litters.

Morbidity & Weight Gain.

During the daily cage observations, any mice or pups in any group displaying clinical signs of illness were documented and managed appropriately at the discretion of the facilities' veterinary staff. Pups that were born in any testing group were weighed when they reached ages P7, P14 and P21, during daily observations. Adult breeders were weighed before being placed into one of the four testing groups, but only the males were weighed at the end of the study since the pregnancy status of females could not be exactly determined when their 3rd litter reached weaning age.

Microenvironment.

On the morning of the weekly cage change, the microenvironment of each cage was evaluated for the following parameters: temperature (°F), relative humidity (% RH), carbon dioxide (CO_2), and ammonia (NH_3). In order to minimize disturbance to the mice, all cages were modified with a built-in sampling port (Sage Supply, Johnson City, NY) on the front side of the cage to collect microenvironment samples without opening the cage. The port was the same length (6.99 cm) and diameter (2.54 cm) in all cages with a rubber seal and screw cap to prevent air leakage from the cage (Figure 1). For sampling, a TSI Indoor Air Quality Meter (Model 7525; Cole-Parmer) was first wiped clean with Spor-Klenz disinfectant followed by water. Once dried, it was inserted into the sampling port for 1 minute in order to achieve a steady-state reading of temperature, RH, and CO_2 levels. The height at which the inserted probe was positioned was 2.0 cm above the floor for mice cages and 5.7 cm above the floor for rat cages (Figure 1B). Immediately following the TSI reading, the ammonia level was measured using a Drager CMS Chip-Measurement System (Model 64 05 060; Chip # 64 06 130) with a rubber tube for air sampling. This rubber tube was cleaned in a similar manner to the TSI meter prior to each cage reading, which was performed 1 inch above the cage floor for one minute duration. If any cages contained ammonia levels above 25 ppm, a second reading was obtained in similar fashion, but using a Drager chip that could read up to 150 ppm (Chip # 64 06 020).

After microenvironment sampling, health observations and weight measurements were performed, followed by the cage change. A duplicate set of 20 autoclaved, modified cages were used to replace the dirty cages during this weekly cage change. The breeders and pups in cages containing pups born within 72 hours before cage change were transferred normally, along with their enrichment material; however, if these pups were found dead within 24 hours post-cage change, they were not included in pup mortality or morbidity data. The rationale for this strategy was that iatrogenic manipulation of pups or their environment leads to cannibalism which would not have normally occurred if cage changes were not performed.[7, 31, 55] This variable is preventable in routine breeding configurations, but was unavoidable in our study due to an established day of the week for cage change and microenvironment measurement. In order to correlate the microenvironment readings with the estimated total mass of the pups in a given cage during weekly sampling, a Biomass Pup Score (PS) was established as shown (Table 1).

Figure 1a (left) & 1b (right). Standard mouse shoebox and rat IVCs used in study with modified sampling ports and identical breeding enrichment material (hut, nestlet, and crinkle paper).

Table 1. Biomass Pup Score (PS) calculation table to approximate population/size of pups in a given cage during microenvironment sampling.

Age of pups in cage	**Biomass Pup Score (n=number of pups) for cage**
Ages P0 - P7	n x 1
Ages P8 - P14	n x 2
Ages P15 - P21	n x 3

Fecal Corticosterone Assay.

Within 2.5 hours of transferring mice into clean cages, fresh adult fecal pellets were collected from each cage and flash frozen in liquid nitrogen for corticosterone level analysis. Since the study lasted 13 weeks, a total of 13 fecal corticosterone readings were compiled for each cage. The gastrointestinal transit time for CM in mice has been determined to be between 4 and 12 h on the basis of radioactive corticosterone injection studies. We restricted the feces collection period to a maximum of 2.5 hours to reduce the confounding influence of stress from the collection method and animal handling on the CM measurements. Fecal corticosterone metabolites (CM) were quantified using a commercially available enzyme immunoassay (EIA) kit (Arbor Assays, Ann Arbor, MI).[36, 49, 57-58, 67-68] Specifically, the collected fecal samples were analyzed for immunoreactive CM using a 5α-pregnane-3β,11β,21-triol-20-one EIA, based on a double-antibody technique using microtiter plates coated with anti-rabbit IgG. The assay was performed according to manufacturer's instructions. Briefly, the fecal samples were homogenized and aliquots of 0.05 g were extracted with 1 mL of 80% methanol. The standards (range, 0.8 to 200 pg/well) and samples were incubated overnight at 4 °C with steroid antibody and biotinylated label, followed by plates being emptied, washed, and blotted dry before a streptavidin–horseradish peroxidase conjugate was added. After 45 min incubation, plates were emptied, washed, and blotted dry. The substrate (tetramethylbenzidine) was added and incubated for another 45 min at 4 °C before the enzymatic reaction was stopped with 2 mol/L sulfuric acid. Then, the optical density (at 450 nm) was recorded by using an automatic plate reader, and the hormone concentrations were calculated.

Behavior Analysis.

Two behavioral assays measuring activity and anxiety-related parameters were performed during the dark period in both weaned pups (P21) and adult breeder mice (after the weaning of their third litter). The mice were acclimated to the testing room for at least 15 minutes before behavioral testing began.

The behavioral testing room was illuminated with six 32-W fluorescent overhead lights (2,800 lumens each) to enable adequate video-tracking and to provide sufficient and consistent illumination to render the open areas of the behavioral arenas as mildly aversive. The behavioral tests described below were performed sequentially for each mouse with an inter-test interval of at least 30 minutes. Upon completion of the behavior tests, the mice were euthanized and tissue samples were collected.

Open Field: Open field testing was performed in a square arena (18.5 in x 18.5 in area x 17.8 in wall height). The arena was wiped clean with Spor-Klenz disinfectant, followed by water, and dried after each use in order to remove excretory material and odors that could influence the behavior of the next mouse tested. A digital video camera was mounted 121 cm off the floor so that it overlooked the open field apparatus; video-tracking software (Any-Maze, Stoelting Co., Wood Dale, IL) was used to track mouse movements within the open field for 5 minutes (following 30 seconds of non-analyzed time during which the investigator left the testing cubicle after placing the mouse into the center of the open field). The field was divided (in software) into a uniform 4x4 grid, with the central four squares (totaling 9.25" x 9.25") defined as the "center" region. Four parameters were analyzed: mean traveling speed (cm/sec), total time spent immobile (sec), the cumulative time spent in the center region (sec), and the number of entries into the center region from the periphery. Time spent immobile corresponds to fear or uncertainty, whereas time spent in the brightly lit center region is an indicator of lower anxiety levels (e.g., it is typically increased by administration of anxiolytic drugs). The number of center entries couples this measure with overall activity, and disambiguates time spent occupying the central region with time spent transitioning across it.

Elevated Plus Maze (EPM): The elevated plus maze was constructed of stainless steel and painted matte black, and consisted of four arms, each 12 in long and 2 in wide. Two opposing arms were enclosed by 6 in high walls (*closed* arms), while the other two were *open*, without walls.

The entire apparatus rested on four legs such that the maze was elevated 20 in off the floor. The floor of the maze was lined with quarter-inch thick gray rubber mats in order to prevent deposition of excreta on the apparatus and to eliminate the lip surrounding the open arms. The EPM apparatus was cleaned after each use similarly to the open field arena. For testing, each mouse was placed in the center of the maze facing an open arm and tracked by video for 5 minutes (following 30 seconds of non-analyzed time during which the investigator placed the mouse and left the testing cubicle). Four parameters were analyzed for each arm type (closed and open): the number of entries into that type of arm, the cumulative time spent on that type of arm (sec), the distance traveled while on that type of arm (m), and the durations of individual visits to that type of arm (sec). (Cumulative time measurements for closed and open arms do not add exactly to 300 seconds because time in the central crossing area of the EPM was not scored). Like the center of the open field, longer times spent on the open arm is an indicator of lower anxiety levels, whereas the other three factors couple this indicator with activity levels (greater exploration also indicative of lower anxiety levels; for a given level of movement, movement between arms can be thought of as more exploratory while more movement around a single arm is more perseverative). Batteries of partially redundant behavioral tests protect against false positives while also enabling a more detailed interpretation of genuinely positive effects.

Brain-Derived Neurotrophic Factor (BDNF).

Immediately following euthanasia (of pups or adults), the skin on the skull was removed and the skull was cleaned with 70% ethanol. A midline sagittal section was made through the skull, separating the two hemispheres. The brain was removed and the cortical telencephalon was dissected, placed into a 2.0 mL microcentrifuge tube, flash frozen in liquid nitrogen, and stored in a -80°F freezer until processing. BDNF protein concentrations in cortical lysates were determined using the BDNF Emax immunoassay system (Promega Corporation, Madison, WI) with recombinant mature BDNF as a

standard. This ELISA is based on an antibody binding to the carboxy terminal region of BDNF; hence, it also recognizes proBDNF. The hippocampus was dissected out of each hemisphere. Specifically, cortical samples were lysed with TNE Lysis buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, and 1% NP-40) using a dounce homogenizer. The lysates were diluted 1:1 in sample buffer (provided in kit). Triplicate samples were taken from each test group (100 mg cortical tissue per sample for adults, *n*=3-5 animals; 50 mg from weaned third-litter pups; *n*=24 animals). All samples were run in triplicate on the same plate. Further details regarding tissue preparation are available in the manufacturers' protocol.[4, 33, 79]

DNA Microarray.

Cortices were dissected from three pairs of the adult male TMC and TRC breeder mice. After RNA isolation, one microgram of total RNA was linearly amplified using a T7-based *in vitro* transcription reaction and labeled as described (Lin et al. 2004). Briefly, total RNA was reverse transcribed using a modified oligodT primer containing a T7 promoter sequence. After generation of second strand cDNA, the entire cDNA pool was amplified by performing a T7 based *in vitro* transcription reaction. Five micrograms of purified, amplified RNA was then reverse transcribed in the presence of 0.2 micrograms of random hexamers and a dNTP mixture containing a 2:1 ratio of amino allyl dUTP:dTTP. Five micrograms of TMC (Cy3) and TRC (Cy5) aRNA were combined and hybridized to custom microarrays generated in house. These microarrays comprise approximately 33,000 clones derived from the National Institute Aging (NIA) 15k, 7.5k and Brain Mouse Anatomy Project (BMAP) clone sets. Three biological replicates were performed using RNA obtained from three TMC and three TRC cortices. Slides were scanned on an Axon 4000B scanner. Raw data from the three replicates were analyzed using the genepix automated processor (GPAP v. 3.2; http://darwin.biochem.okstate.edu/gpap), with no background correction, global loess normalization, and scale interarray normalization.

Quantitative Reverse Transcription PCR.

Primers for qRTPCR were designed using the primer bank algorithm (http://pga.mgh.harvard.edu/primerbank/). Sequences for each gene are as follows:

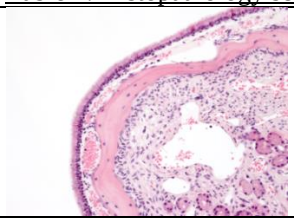
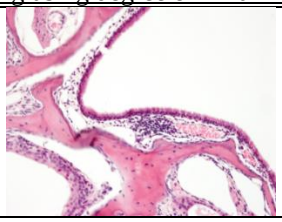
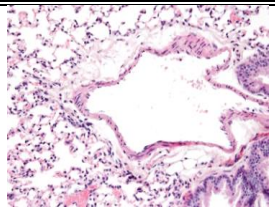
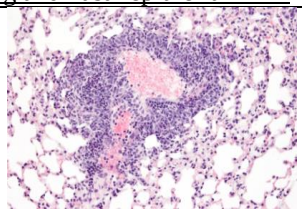
Brain derived neurotrophic factor	TTACCTGGATGCCGCAAACAT	TGACCCACTCGCTAATACTGTC
Nerve growth factor	ACTGGACTAAACTTCAGCATTCC	GGGCAGCTATTGGTGCAGTA
Glucocorticoid Receptor	AGCTCCCCCTGGTAGAGAC	GGTGAAGACGCAGAAACCTTG
Colony stimulating factor 2 receptor, alpha, low-affinity	TGCTCTTCTCCACGCTACTG	GGGGTCGAAGGTCAGGTTG
Interleukin 1 receptor, type 1	CATTGCCGGGGATGGAAGTC	CACTGGACCTCGGGTAACTC
Ribosomal protein L19	ATGAGTATGCTCAGGCTACAGA	GCATTGGCGATTTCATTGGTC

RNA from the same TMC and TRC cortices used for the microarray analysis were also used for the qRTPCR. One microgram of total RNA was reverse transcribed with Superscript III in the presence of 0.5 micrograms of random hexamers, as per manufacturer's instructions (Life Technologies). QRTPCR reactions were performed with Fast Start SYBR green Master mix (Roche) using an ABI7300 machine (Life Technologies). Three technical replicates per gene were performed for each biological sample. Relative quantitation was performed with the $2^{-\Delta\Delta Ct}$ approach using ribosomal protein L19 to normalize expression between samples. A one-way ANOVA was used to determine statistical significance.

Histologic Evaluation of Nasal and Lung Tissue.

A histologic evaluation of the nasal tract and lungs was performed to evaluate any correlation between microenvironment and respiratory tissue pathology. The skull and the respiratory tract were collected from adult breeders. The skull was decalcified with hydrochloric acid, after which three 7 uM transverse sections were made across the nasal passages at the levels of the incisors, the first molar, and the base of the ears. These nasal cross sections and the inflated lungs were fixed with 10% formalin paraffin-embedded, sectioned at 5μM, stained with hematoxylin and eosin, and evaluated by a board certified veterinary pathologist (TS) who was blinded to the study groups. Nasal sections were scored as follows: 0, normal; 1, mild infiltrates of lymphocytes and plasma cells in the nasal submucosa; 2, moderate infiltrates of lymphocytes and plasma cells; 3, severe inflammation with turbinate atrophy; 4, bilateral rhinitis with ulceration and bone necrosis (Table 2). A similar scoring system was utilized for sections of the lungs based on perivascular inflammatory infiltrates, fibrosis, and necrosis (Table 2). To evaluate the effects on older breeders housed for longer periods of time, we repeated these histological evaluations with a separate cohort of retired breeders, not otherwise investigated in this study, that had been either pair-housed or trio-housed in standard (67 sq in) IVC mouse cages (C57BL/6, 9-11 months old, both sexes, never used for research purposes other than breeding; *n*=11 for TMC, *n*=9 for PMC). These data were analyzed separately.

Table 2: Histopathology scoring using degree of inflammation, ulceration, necrosis in lung and nasal epithelium

			
Nasal Score = 0 out of 4	Nasal Score = 2 out of 4	Lung Score = 1 out of 4	Lung Score = 3 out of 4

Hair Corticosterone Assay.

This assay was performed only on adult breeders because a 80-100 mg hair sample was required. Corticosterone metabolite (CM) extraction from fur was performed according to Meyer's methodology (developed in rats).[28, 34] Briefly, the protocol steps include: [i] washing fur, [ii] extracting CM from fur homogenized with a Retsch ball mill at 25 Hz, [iii] purifying CM extract using Supelco Supel-Select HLB solid-phase extraction columns at 30mg/mL, [iv] running an EIA CM Assay (Arbor Assays) as previously described for fecal CM. To estimate the concentration of CM in fur, we used the following formula:

$$\frac{((C) \times 1\text{mL}/10^3\mu\text{L}) \times (S) \times (P / Q) \times (R)}{Y}$$

C = concentration (pg/mL), S = the volume of assay buffer used in assay (uL), P = the volume of methanol used for extraction (uL), Q = amount of methanol after drying down (uL); R = amount of assay buffer used to reconstitute dried samples (uL); Y = weight of powdered fur used in methanol extraction (mg);

Data Analysis.

The criterion for statistical significance was set at $\alpha = 0.05$ for all tests. For physiological and biochemical tests, results from the four testing groups were generally analyzed using analyses of variance (ANOVA); an analysis of covariance (ANCOVA) was used when comparing microenvironment parameters for each group and their biomass PS. Pairwise analyses were performed using unpaired t-tests without multiple comparisons correction (to increase the reliability of our negative results by ensuring that these results were not rendered insignificant solely by reducing the experiment wise alpha criterion). Statistical analyses of DNA microarray data between the TMC and TRC groups was performed using an unpaired T-test.

Behavioral analyses of open field and elevated plus maze data, both in weaned pups (3-4 weeks old) and adult breeders (after their third litters), were performed using multivariate analyses of variance (MANOVA) with four dependent variables (*open field*: mean speed, time spent immobile, time spent in center region, number of entries into center region; *plus maze*: number of open arm entries, total time spent on open arms, distance traveled on open arms, durations of individual visits to open arms). Initial MANOVAs were performed using three factors: cage size (mouse/rat), breeding configuration (pair/trio), and sex. In preliminary analyses, among the pups, there was no significant effect of sex and no significant interactions between sex and either of the other factors, whereas among adult breeders, there were no significant interactions between sex and either of the other factors. There was a significant effect in adult breeders of sex alone on the time spent in the center of the open field, but this effect was not corroborated by other anxiety-related behavioral results. Accordingly, the sexes were combined in all behavioral analyses in order to increase statistical power; i.e., two-factor MANOVAs were performed.

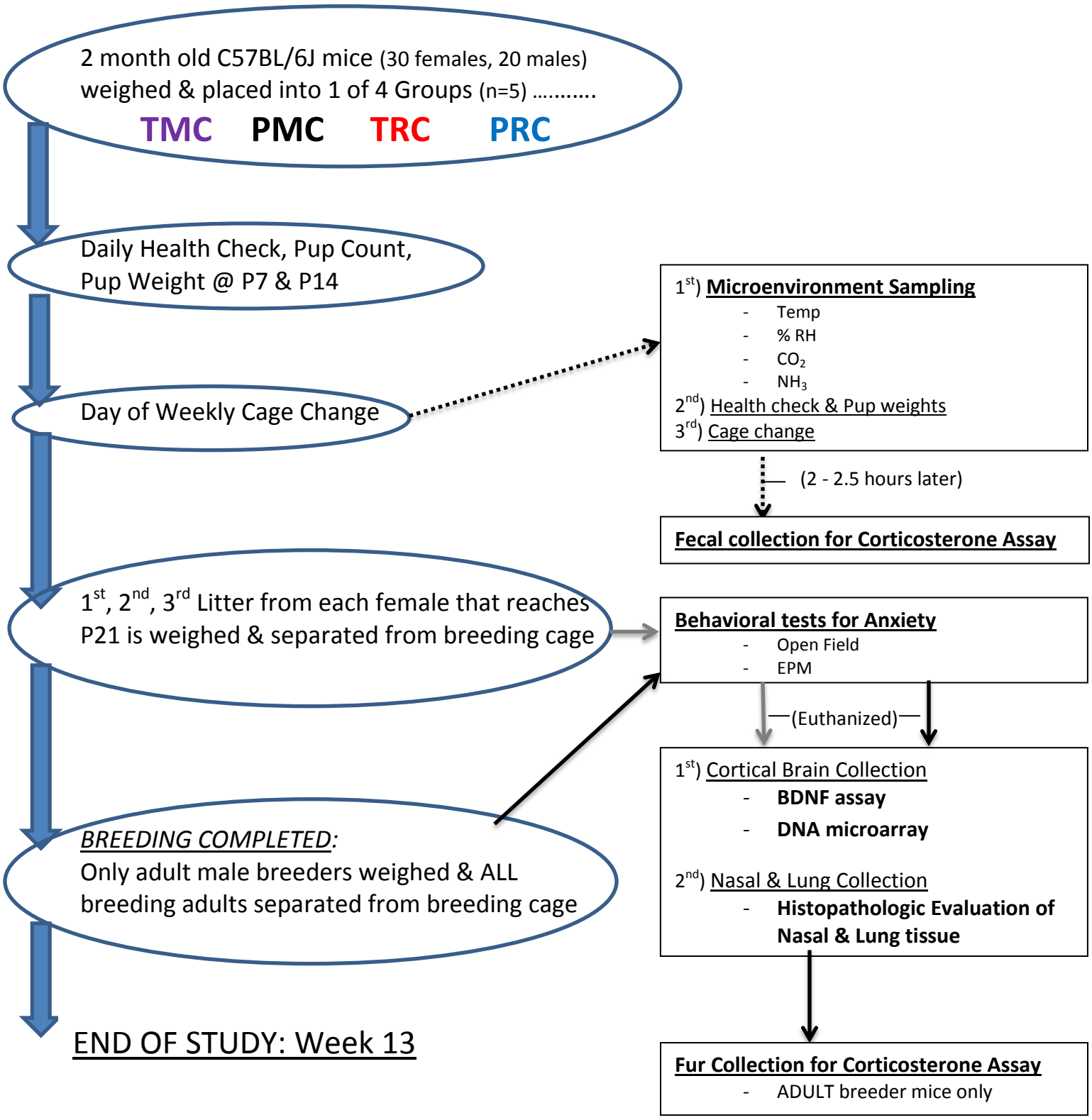


Illustration 2. Chronologic timeline of the study design

CHAPTER THREE:

RESULTS

Reproductive Performance.

Average Litter Size

Only PMC was found to be significantly less ($P<0.05$) in average litter size compared to TMC ($P=0.0078$) with the mean values (TMC= 7.3; PMC= 5.0; TRC= 7.5; PRC= 6.5) depicted (Fig. i).

Inter-birth Interval

No significant difference was noted when comparing inter-birth interval duration amongst the four groups with the mean values (TMC=29.1; PMC=31.0; TRC=28.9; PRC=27.1) depicted (Fig. ii).

Wean : Born ratio

No significant difference was noted in wean to born ratio amongst all four groups with the mean values (TMC=0.74; PMC=0.53; TRC=0.60; PRC=0.63) depicted (Fig. iii).

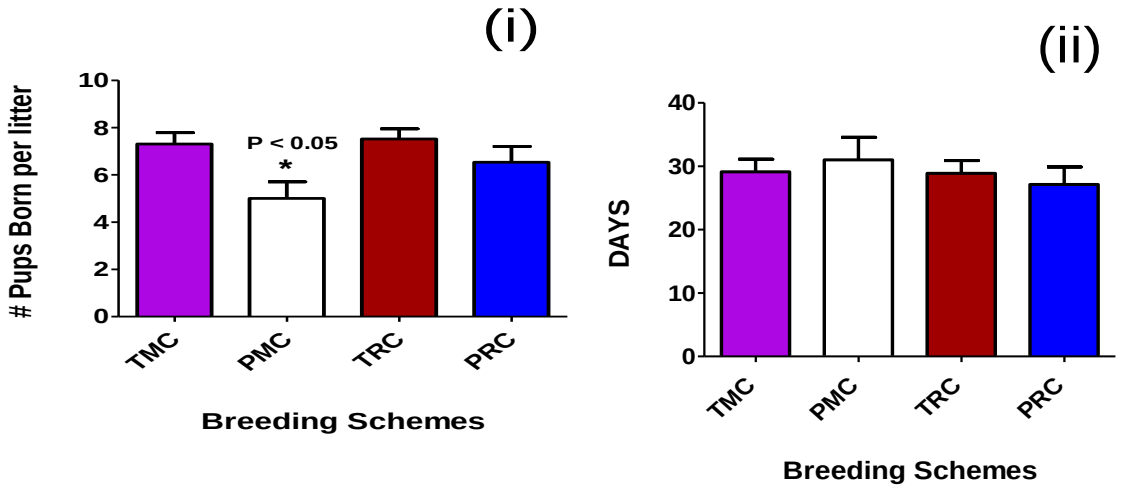


Figure (i): Average Litter Size (mean ± SE) with only PMC showing a significantly less mean value (*P<0.05). [PMC & PRC: n=15] & [TMC & TRC: n=25. **Figure (ii):** Inter-birth interval (mean ± SE) in days between consecutive litters born with no significant difference amongst the four groups. [PMC & PRC: n=15] & [TMC & TRC: n=26].

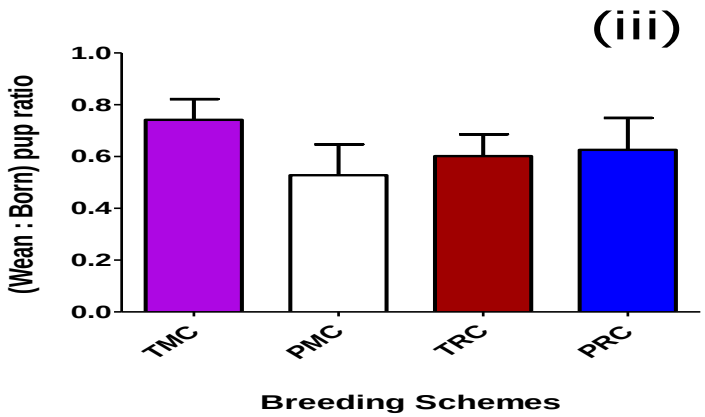


Figure (iii): Wean: Born pup ratio (mean ± SE) revealed no significant difference amongst the four groups. [PMC & PRC: n=15] & [TMC & TRC: n=26].

Pup/Adult Morbidity & Weight Gain.

Morbidity

There were three mortalities related to dystocia which equated to10% incident in TMC & TRC, and 20% incident in PRC. The cages in which the females were found dead were no longer used for the study. A total of 14 cases of focal alopecia and barbering were also observed in 10 pups (1.5% of all TMC & TRC pups; 3% of all PMC & PRC pups) as well as 2 adult males [TMC & PRC] which self-resolved. The lesions in the pups self-resolved (65% of cases) or did not progress to a state of pain or distress, therefore these animals were maintained for the entire duration of the study.

Pup Weight Gain

No significant differences in weight gain were noted amongst the four groups at ages P7, P14, and P21 as depicted. The number of pups per litter at the designated weight checks varied from 0-11 pups, and those individual weights were totaled to find the mean weight (±SE) per litter. That mean value for one litter at a given age (ex: P7, cage 1 of PMC) was then compiled with mean values from cages within the same age and testing group (ex: P7, cages 2,3,4,5 of PMC) to represent an overall mean weight as seen in figure (v).

Adult Male Weight Gain

Only PMC was significantly less ($p<0.05$) in terms of weight gain (mean ±SE) of adult male breeders, but there were no difference appreciated in the other groups including TMC as seen in figure (vi) after confirmation via two-factor ANOVA with Turkey HSD post-hoc comparison analysis.

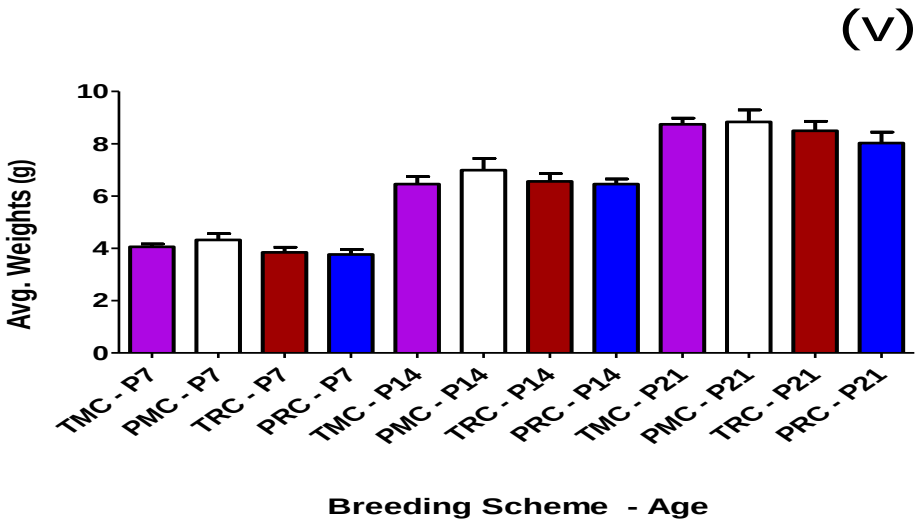


Figure (v): Average pup weight gain at ages P7, P14, P21 for each group. [PMC & PRC: n=9-10] & [TMC & TRC: n= 18-22].

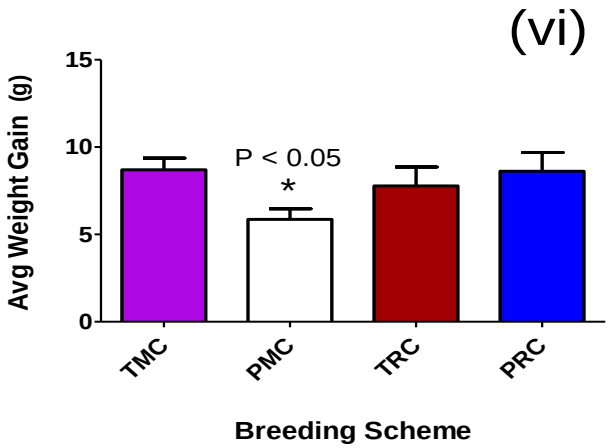


Figure (vi): Average weight gain of adult male breeders from start to end of study. [n=4-5 per group]. Only PMC revealed a significantly lower average weight gain (*P< 0.05).

Microenvironment.

Carbon dioxide

Intra-cage mean CO_2 levels increased significantly ($P < 0.05$) with increase in biomass PS in PMC & TMC. The ANOVA model was significant when comparing the mice cage groups to the rat cage groups at PS=0, 1-5, and 16-30 ($F=65.43$, $P<0.0001$; $R^2=0.8015$) after exclusion of the PS>30 groups. The mean CO_2 values were also statistically significant ($P < 0.05$) when comparing TMC and PMC at the aforementioned PS ranges as seen in figure vii.

Ammonia

NH_3 concentrations (mean ± SE) increased significantly ($P < 0.05$) with each increase in biomass PS in all four testing groups, but more drastically in both TMC and PMC. The overall ANOVA model was significant when comparing TMC & PMC to all other groups ($F=4.53$, $P=0.0009$; $R^2=0.1807$) as seen in figure viii.

Humidity

Percent relative humidity (mean ± SE) increased significantly ($P < 0.05$) with each increase in biomass PS in all four testing groups, but slightly higher in both TMC and PMC. The overall ANOVA model was significant when comparing TMC & PMC to all other groups ($F=25.61$, $P<0.0001$; $R^2=0.6086$) as depicted in figure ix.

Temperature

Temperature (mean ± SE) increased significantly ($P < 0.05$) with each increase in biomass PS for TMC compared to the other three testing groups as seen in figure x. The overall ANOVA model was significant when comparing TMC to all other groups ($F=12.17$, $P<0.0001$; $R^2=0.4259$).

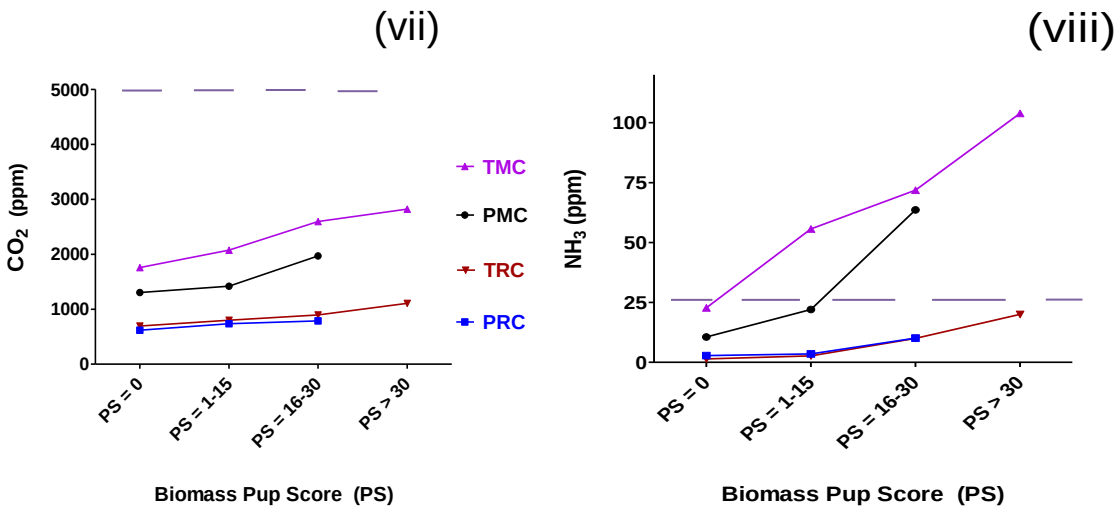


Figure (vii): CO_2 measurements amongst the four groups under various Biomass Pup Score (PS) ranges. **Figure (viii):** NH_3 measurements amongst the four groups under various Biomass PS ranges. The horizontal dotted line in figures (vii) & (viii) reflect the maximum permissible exposure limit (PEL) under Occupational Safety and Health Administration (OSHA) standards for human workplace exposure during an 8-h day of a 5 day work week.

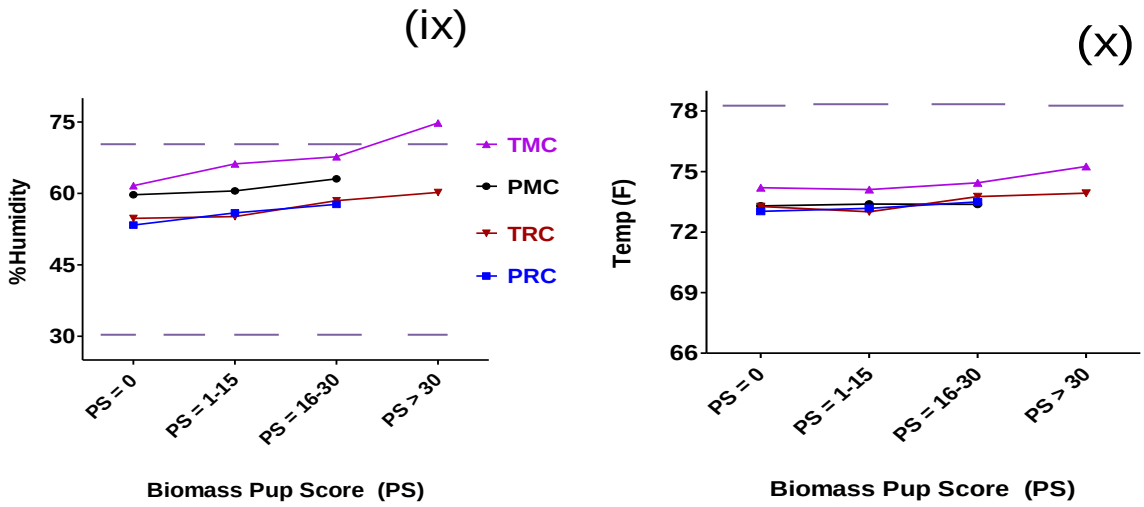


Figure (ix): Relative Humidity (%) measurements amongst the four groups under various Biomass PS ranges. The horizontal dotted lines reflect the recommended range for relative humidity which is 30-70% according to the *Guide*. Figure (x): Temperature (°F) measurements amongst the four groups under various Biomass PS ranges. The horizontal dotted lines in figure (x) reflects the recommended range for temperature which is 64°F-78.8°F (18°C-26°C) according to the Guide for the Care and Use of Laboratory Animals. Due to the spontaneous outcomes of breeding and rearing, the number of samples per PS range was various for each testing group during the 13 week sampling period (PS=0: n=16-26; PS=1-15: n=13-24; PS=16-30: n=5-18; PS>30: n=4-7). In addition, the limited pup production of the paired breeding groups compared to trio breeding groups prevented PMC and PRC to achieve PS > 30.

Fecal Corticosterone

Mean values of fecal CM were obtained by using triplicates of every fecal sample collected in order to perform a group based-time course graph. No significant difference (F=0.01; P=0.99) in expression of adult breeders' fecal CM concentrations were noted over the 13 week period as seen in figure (xi). Our baseline values are reflective of weeks 1 & 2 of breeding since the earliest time for gestation could not occur before the 14th day of breeding (no pups born).

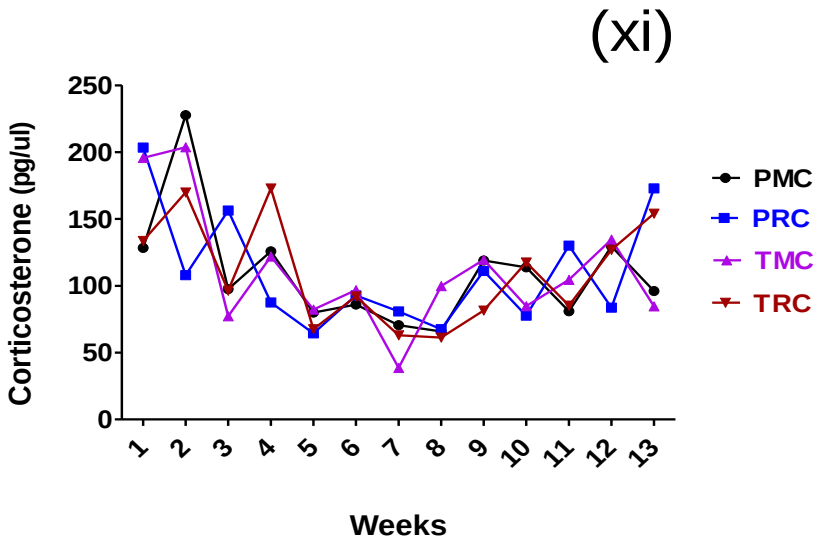
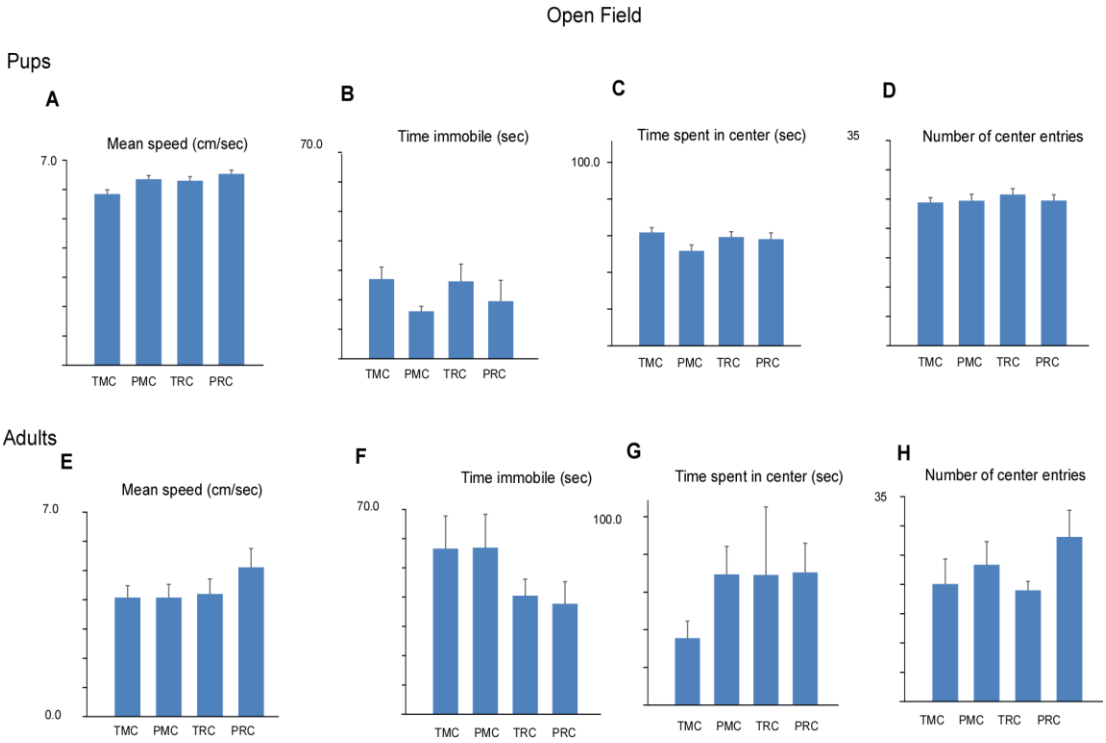


Figure (xi): Weekly adult fecal Corticosterone Metabolite (CM) concentrations(pg/µL) for each testing group (n=4-5). No significant difference (F=0.01; P=0.99) in expression of adult breeders' fecal CM concentrations were noted over the 13 week period.

Behavior analysis.

Two standard behavioral testing paradigms with partially redundant metrics were used to assess baseline anxiety and activity levels in P21 pups and adult breeders. *Open field analysis.* Among pups (Figure xii A-D), MANOVA indicated a barely significant effect of breeding configuration on open field performance, but no significant effect of cage size or of the interaction of these factors (Wilks' lambda; *cage size*, $F(4,306) = 1.277$, $p = 0.279$; *breeding configuration*, $F(4,306) = 2.473$, $p = 0.045$; *interaction*, $F(4,306) = 1.068$, $p = 0.372$). Testing of between-subject effects indicated that breeding configuration significantly affected only the mean speed ($p = 0.039$; Figure xii A), an activity measure that was not corroborated by other activity measures such as numbers of arm entries or distance traveled within the elevated plus maze. Among breeding adults (Figure xii E-H), MANOVA indicated no significant effects of cage size, breeding configuration, or their interaction on open field performance (Wilks' lambda; *cage size*, $F(4,24) = 1.099$, $p = 0.380$; *breeding configuration*, $F(4,24) = 0.842$, $p = 0.512$; *interaction*, $F(4,24) = 0.595$, $p = 0.670$). *Elevated plus maze.* Among pups (Figure xiii A-D), MANOVA indicated a significant effect of cage size on plus maze performance, but no significant effects of breeding configuration or the interaction of these factors (Wilks' lambda; *cage size*, $F(4,274) = 2.769$, $p = 0.028$; *breeding configuration*, $F(4,274) = 0.242$, $p = 0.914$; *interaction*, $F(4,274) = 0.757$, $p = 0.554$). Testing of between-subject effects indicated that cage size significantly affected only the distance traveled within the open arms ($p = 0.023$; Figure xiii C). In isolation, this could be interpreted as smaller cage sizes leading to increased activity and reduced anxiety, but the anxiety effect is not corroborated by significant differences on time spent on the open arms or time spent in the center of the open field, and the activity effect is contradicted by open field results. Among breeding adults (Figure xiii E-H), MANOVA indicated no significant effects of cage size, breeding configuration, or their interaction on elevated plus maze performance (Wilks' lambda; *cage size*, $F(4,32) = 1.517$, $p = 0.221$; *breeding configuration*, $F(4,32) = 1.588$, $p = 0.201$; *interaction*, $F(4,32) = 0.636$, $p = 0.641$).



Figures (xii): Each of the eight graphs represent parameters that represented the OF test for anxiety with time (sec) and distance (m) being measures (y-axis) to assess exploratory behavior. For P21 pups [PMC & PRC: n=100-108] & [TMC & TRC: n=195-205] while the adults (Fig xii E-H) had n=11-20 with no significant differences were observed ($P<0.05$).

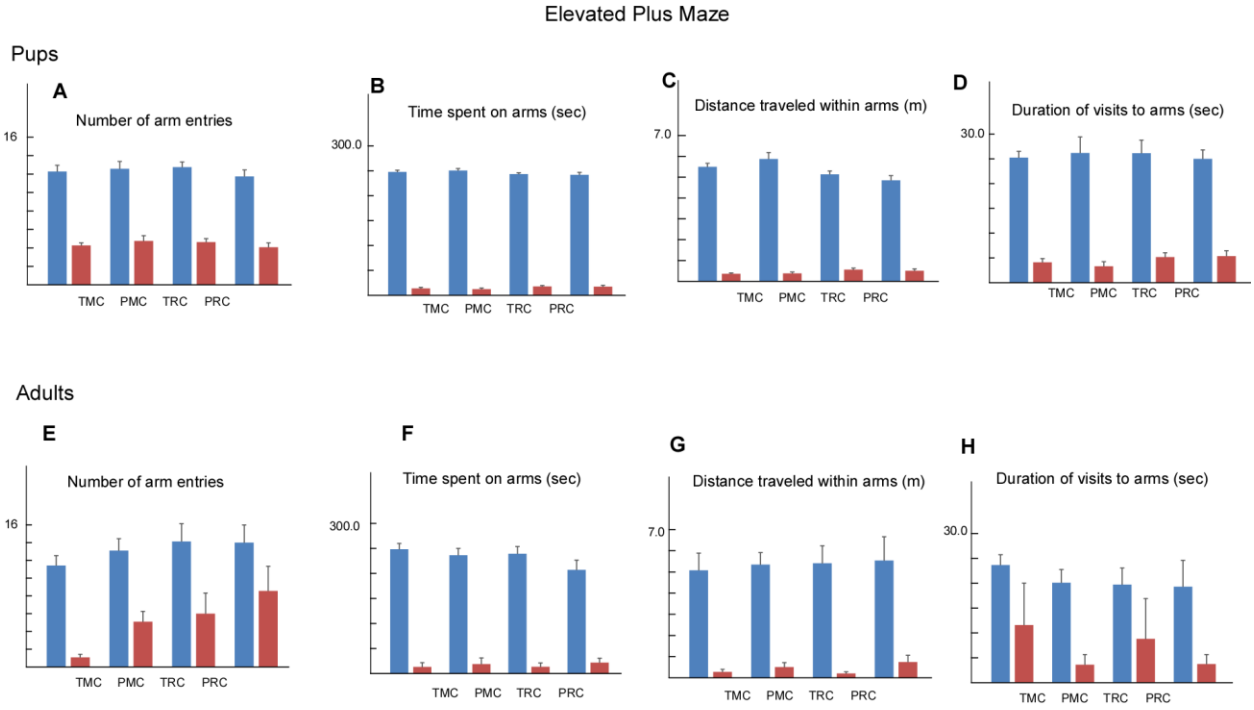


Figure (xiii): Each of the eight graphs represents the parameters assessed using EPM test for anxiety with number of entries, speed (m/sec), distance (m), and time (sec) being measures (y-axis) to assess exploratory behavior. For P21(Fig xiii A-D) pups [PMC & PRC: n=100-108] & [TMC & TRC: n=195-205] while the adults (Fig xiii E-H)) had n=11-20. No statistical significant differences were observed amongst the four groups in adults and P21 pups.

Brain-Derived Neurotrophic Factor (BDNF).

Two-way ANOVA with cage size and breeding paradigm as factors revealed no significant differences in expression of BDNF (mean ± SE) amongst the four groups tested (Fig. xiv & fig. xv). Triplicate samples performed from each test group with cortical tissue (100mg = adults; 50mg = pups) taken from adult breeders (n=3-5) and weaning 3rd litter pups (n=24).

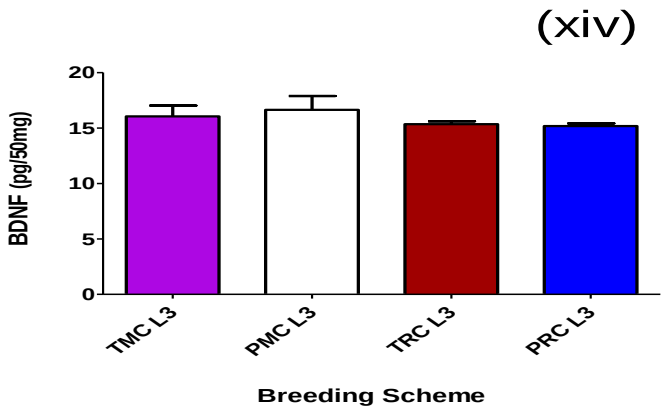


Figure (xiv): BDNF concentrations in both P21 Litter 3 pups (n=24) revealing no statistical significant difference in expression amongst the four groups.

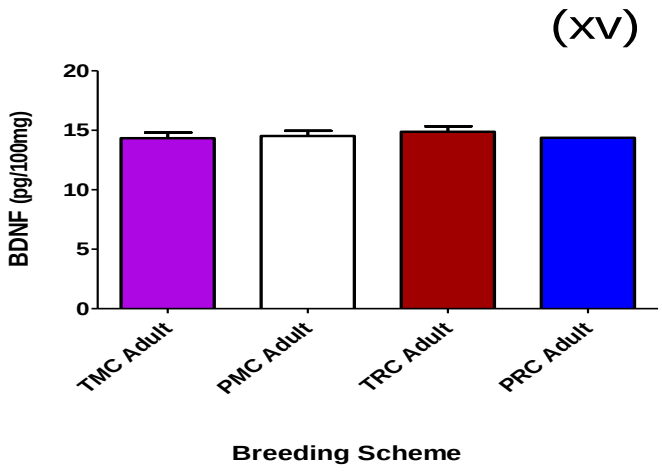


Figure (xv): Adult breeders (n=3-5) revealing no statistical significant difference in expression of BDNF amongst the four groups.

Histologic Evaluation of Nasal and Lung Tissue.

Three out of the total tissues analyzed per group were exposed to the highest biomass PS (equates to highest NH_3). These tissues were objectively analyzed for histopathologic confirmation of inflammation within the respiratory tissues based on a scoring system developed by the blinded pathologist (TC).

Adult Lung & Nasal Pathology

No significant difference (mean ± SE) in inflammation score was noted amongst the four groups in adults lungs (n=4-6), with the average inflammation score as follows: TMC= 1.5; PMC= 2.3; TRC= 2.0; PRC= 1.0. The overall ANOVA model confirmed no significant differences ($F= 2.04$, $R^2= 0.37$, $P=0.162$) as depicted (fig. xvi). No significant differences (mean ± SE) in nasal pathology were noted amongst the four groups (n=3-7) with 14 out of 15 tissues possessing a score of 0, and the one tissue (from PMC) with a score of 1 out of 5.

P21 Lung Pathology

No significant difference (mean ± SE) in inflammation score was noted amongst the four groups in P21 lungs (n=8-10), with the average inflammation score as follows: TMC= 1.3; PMC= 2.0; TRC= 1.5; PRC= 1.0. The overall ANOVA model confirmed no significant differences ($F= 1.05$, $R^2= 0.17$, $P=0.39$) as depicted (fig. xvii).

P21 Nasal Pathology

No significant difference (mean ± SE) in inflammation score was noted amongst the four groups in adults lungs, with the average inflammation score as follows: TMC= 0.28; PMC= 0.50; TRC= 0.67; PRC= 0.33. The overall ANOVA model confirmed no significant differences ($F= 0.27$, $R^2= 0.06$, $P=0.84$) as depicted (fig. xviii).

Table 3: Mean (± SE) NH_3 concentrations (ppm) in the four test groups under various Biomass PS conditions.

	TMC	PMC	TRC	PRC
No Pups: PS = 0	22.73 ppm	10.60 ppm	1.0 ppm	1.43 ppm
PS = 1-15	55.66 ppm	22.04 ppm	2.72 ppm	3.49 ppm
PS = 16-30	71.84 ppm	63.60 ppm	10.02 ppm	10.11 ppm
PS > 30	103.90 ppm	NA	20.03 ppm	NA

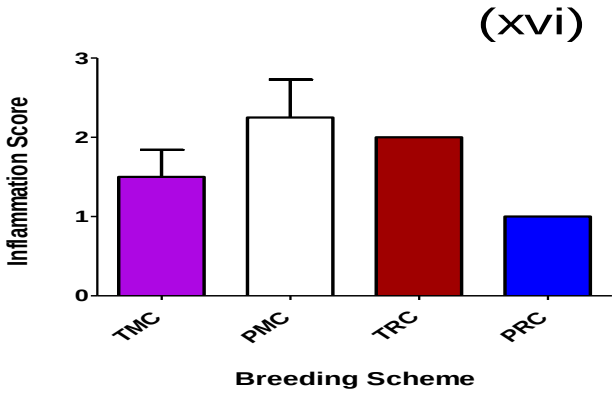


Figure (xv): Lung inflammation scores amongst adults in each group with n=4-6.

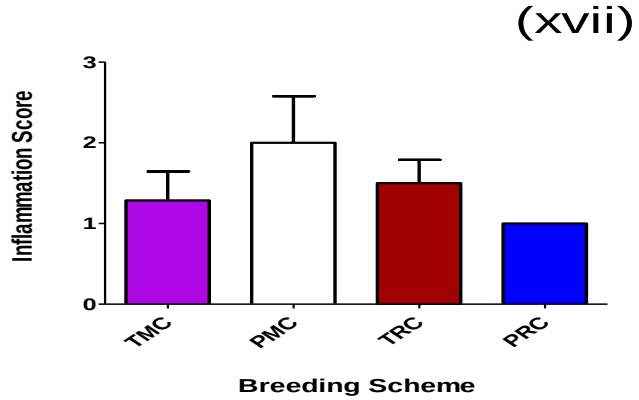


Figure (xvii): Lung inflammation scores amongst P21 pups in each group with n=8-10.

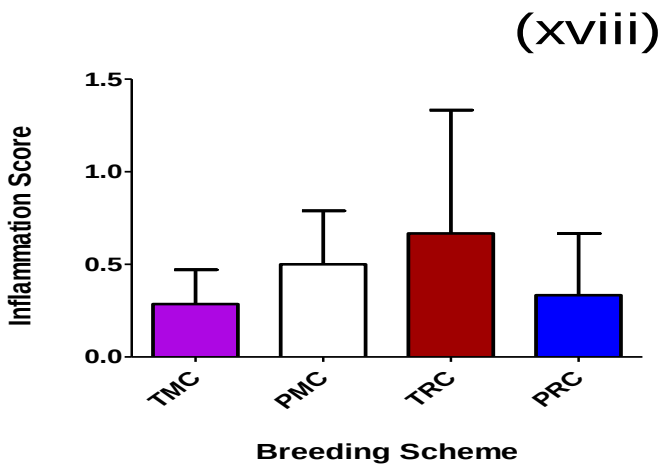
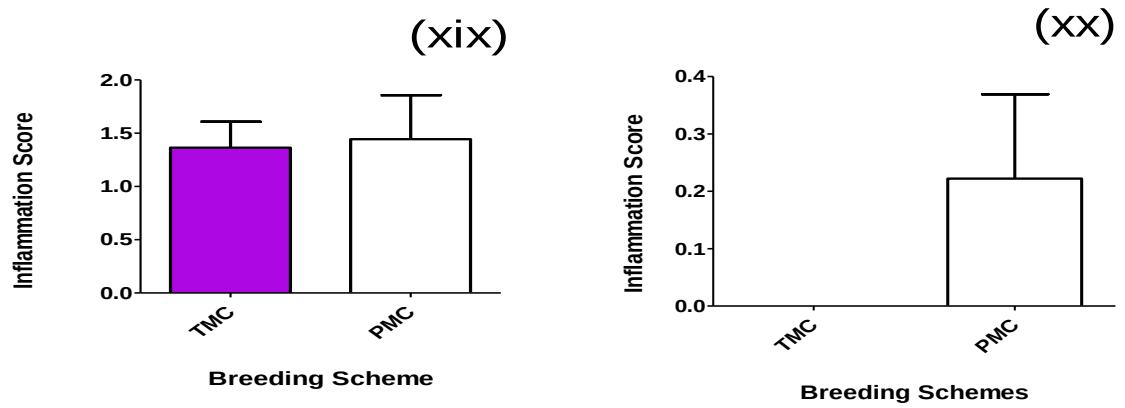


Figure (xviii): Nasal inflammation pathology scores amongst P21 pups (n=8-10).

Supplemental Data for Histologic evaluation.

Adult Lung & Nasal Pathology

No significant difference (mean ± SE) in inflammation score was noted in both tissues amongst the pair and trio bred mice. The average inflammation score in lung pathology was 1.36 for TMC and 1.44 for PMC while the average score in nasal pathology was 0.0 for TMC and 0.22 for PMC as indicated (fig. xix & fig. xx). Only two of the 20 tissues analyzed had a score of 1 out of 5 while the rest scored zero. Since these mice were utilized from other breeding populations and were not analyzed for microenvironment, the NH_3 concentration exposures could not be determined. Table 4 compares reproductive efficacy between the retired breeders and our study's breeders as a means to display how populated the cages are in relation to each other.



Figures (xix) & (xx): Lung (left) and Nasal (right) pathology scores of adult breeders in both TMC (n=11) and PMC (n=9) set-up not affiliated to our experiment. No statistical significant difference in inflammation was noted.

Table 4: Comparing average (±SE) reproductive efficacy measures to predict cage space occupancy and its correlation to respiratory pathology.

	Avg. Litter Size	**Interbirth Interval (days)**	**Wean: Born ratio**
TMC (study)	7.3	29.1	0.74
TMC (supplemental)	6.8	26	0.68
PMC (study)	**5.0**	**31**	**0.53**
PMC (supplemental)	5.3	28	0.64

Hair Corticosterone.

No difference was noted in levels of CM (mean ± SE) from the hair of adult breeder mice with the exception of PRC (which only had n=2). All other groups (TMC= 37.24; PMC= 32.62; TRC= 42.69 pg/mg) had mean values in range amongst the other 2 groups as depicted (fig. xxi).

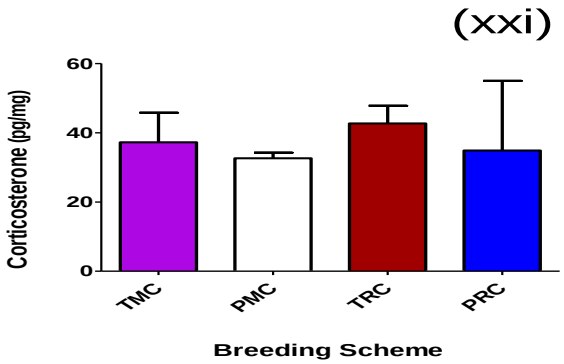


Figure (xxi): Hair corticosterone (pg/mg) amongst adult breeders in all four groups (n=3-4; PMC: n=2).

DNA Microarray and Quantitative Reverse Transcription PCR.

No significant gene expression changes were identified among three biological replicates comparing cortices derived from TMC and TRC adult male breeding mice. Since the clone sets used for the custom microarrays did not include all of the annotated genes in the mouse genome, we used quantitative reverse transcription PCR (qRTPCR) to further test a select subset of genes. BDNF, NGF, glucocorticoid receptor (GR), colony stimulating factor receptor alpha (CSF2rα), and the interleukin 1 receptor (IL1r1) have all been shown to respond to stress. [45, 59, 63] Using RNA derived from the same biological replicates used for the microarrays, we found no statistically significant differences in expression among TMC or TRC mice for any of these genes.

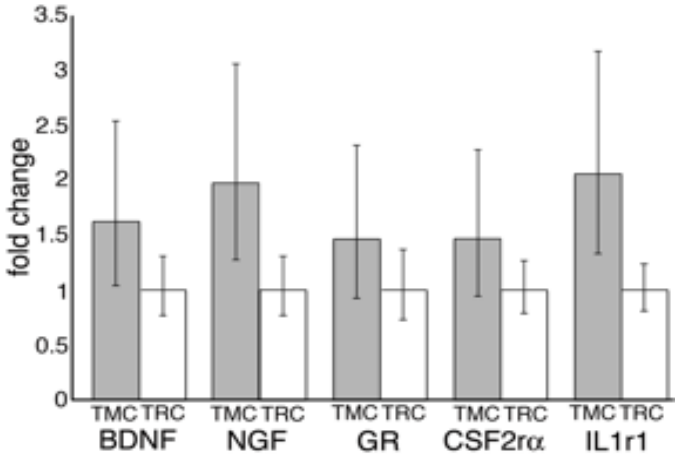


Figure (xxii): Stress responsive gene expression amongst TMC and TRC groups (n=3).

CHAPTER FOUR:

DISCUSSION

The results from our study revealed that trio-bred mice in standard cages (TMC) did not demonstrate any significant differences or negative consequences, despite the expected elevated CO_2 and NH_3, in the tested parameters of animal health and welfare. Our hypothesis was supported by comparing each parameter assessed (reproductive efficacy; hair & fecal corticosterone, BDNF, anxiety, weight gain, morbidity, microenvironment, and tissue histopathology) amongst the four testing groups.

The results from our assessment of reproductive efficacy revealed that TMC had no significant differences compared to the other groups in regards to (i) average litter size, (ii) inter-birth intervals, and (iii) wean: born ratio. These findings conflict with the speculation that higher rodent breeding populations lead to increased mortalities due to insufficient space or nutrients for each pup. Although we were assessing each female's fecundity and weaning success, the extra female in our trio-bred group supports the historical perspective that trio breeding provides improvement in breeding efficacy while conserving animal resources and sustaining limited health issues as compared to pair breeding. [21, 31, 47-48, 75-76] One of the two mice cage groups (PMC) revealed a significantly lower average litter size compared to the two rat cage groups. Since the other mouse cage group (TMC) was not different in mean litter size, it is difficult to correlate cage size or breeding configuration with this particular reproductive parameter. In a previous study, the comparison of trio-breeding of C57BL/6Tac mice in a 82 in^2 cage and a larger cage (124 in^2) showed no significant difference in reproductive parameters.[76] Although reproductive performance cannot be used solely to assess animal well-being, the findings may indicate that any potential outcomes of trio-breeding, such as perceived overcrowding, do not correlate to inferior reproductive output & pup survival.

Despite seeing a 1.5% increase in alopecia/barbering cases in the pair bred groups (PMC & PRC), our assessment of rodent health was more influenced by weight gain since the morbidity cases were skin related with minimal impact on the daily functions of the mice. In many studies that may have detected physiological or behavioral change in mice housed under various spatial dimensions, the morbidity incidents were not detectable thus indicating that certain welfare parameters are more focused on subclinical findings. [17- 18, 30, 35, 37, 39, 48, 60-61] One of those sub-clinical assessments of overall health is weight progression of reared mice and the adult breeders.

Weight gain of pups at 7, 14, and 21 day age revealed no significant difference in the weights of TMC pups (at all three ages) compared to the other three testing groups. Despite the consistency in weight gain in B6 pups amongst all four testing conditions in our study, conflicting results are well documented in prior reports. A previous study compared weight gain from trio-bred ICR dams, left with their intact litter (or) with only 6 nursing pups (compliant with *Guide's* standards). The pup growth rates of the two groups were not significantly different once the numbers were adjusted to account for the number of pups weaned between both groups. [42] The aforementioned study also discovered that there was no difference in pup weight gain between groups from the second generation.[48] A similar study design to assess pup weight progression was conducted in a 2007 study which confirmed that pups raised in large cages (50% increased space) weighed less than those in standard cages by weaning age. The authors suggested that the lower weight of pups in large cages could have been due to either less nursing time as a result of higher activity or more energy exhausted by all mice (adults and pups) in larger cages. [75-76] This trend was supported by another study discovering that inbred pups reared under communal nesting (CN) had increased growth rates versus single nesting (SN).[21] These studies suggest that CN in laboratory mice is a potential evolutionary adaptive behavior that may have beneficial consequences for laboratory mouse welfare.

For future considerations, intra-cage video monitoring of mice under these breeding configurations and spatial paradigms would be an effective way in determining if a compensatory component is present in CN groups. In addition, visual observations of increased adult and pup activity in larger cages would help in determining spatial effects on rodent health.

Our assessment of weight gain on adult male breeders revealed that TMC males had no significant difference compared to the other groups, and only PMC possessed significantly lower weight gain. Studies consistent with our findings utilized non-breeding, adult B6 and ICR mice housed in various spaces (5-30 in^2 per mouse) with no changes in weight gain over 3 months duration.[18, 30, 48] Despite the lack of visual effects on overall wellbeing of adult B6 mice, with housing densities 50-100% more than the *Guide*'s recommendations, one study did confirm an increase in weight gain under higher space densities.[39] Since it is common husbandry practice to utilize adult breeders beyond 3 months duration, obtaining trends in weight gain over 8-10 months under breeding configurations may be more tangible to current breeding practices. Our data concluded that regardless of cage size or breeding configuration, the body weight of TMC adults and pups (at all three age groups measured) were consistent compared to the pair-bred and larger cage size groups.

Our results confirmed part of our hypothesis in that smaller cages (TMC & PMC) with higher rodent populations (TMC) lead to significantly higher CO_2 and NH_3 levels compared to larger spaced cages (TRC & PRC). Since the population of pair and trio adults per group in mice cages was acceptable by the *Guide's* spatial standards, the variable of focus was the effect of litter numbers and pup growth towards the cage's microenvironment.

The biomass PS (Table 1) was used to emphasize that a higher PS equates to the increased growth of pups as they get older in age. This method is a simpler way of categorizing multiple litters of various ages and their projected spatial occupancy within a cage. This method was less specific than labeling each cage microenvironment reading with pup numbers and pups ages, but provided the same exponential increase in both PS score and NH_3 values. The PS confirmed the obvious direct relationship between population number, volume of excreta, and accumulation of NH_3. An example of how the biomass PS categorizes litter housing density can best be understood by displaying possible scenarios that can result in a PS > 14: ten P5 mice and three P10 mice; five P5 mice and five P10 mice; or just five P20 mice. As indicated from our results, once group PMC had a PS >14, their NH_3 levels would exceed Occupational Safety & Health Administration's (OSHA) human working permissible limit of 25ppm.[41, 44] Meanwhile, the TMC group had a significant increase compared to PMC with NH_3 levels above OSHA's exposure limit once their PS > 0. The current literature assessing rodent housing densities' correlation to the microenvironment revealed similar trends as our study, with both breeding adults + litters as well as housing 5 adults per cage (65-72 in^2), which lead to NH_3 levels above 25 ppm. [15-17, 52-54, 60-61, 72, 77-78] It is worth noting that even though a PS=0 will imply that no pups were present during weekly microenvironment sampling, the residual accumulation of waste from a population of pups (with a different PS) could remain within the cage in the days leading up to the sampling day.[6, 11] Although weighing each mouse in a cage after daily microenvironment sampling would be a more accurate correlation between spatial occupancy of mice and microenvironment, the increased time on personnel as well as the physical handling of mice deterred that possibility.

Despite examining the obvious parameters of spatial dimensions and rodent populations per cage, there are several other factors that can influence the microenvironment within a cage. It was important to find studies that utilize the same conditions in order to provide a more accurate comparison between study

findings. In a recent study assessing different bedding substrates' ability to control ammonia, the results concluded that IVCs require twice a week cage change for 5 adult female B6 mice with corncob bedding whereas wood pulp bedding was not sufficient to control ammonia.[17] However, another study examining pair bred BALB/c & CD-1 mice + litters revealed high ammonia levels (>25 ppm) despite weekly cage changes.[16] Although no prior studies can emulate the specific practices of our research design, the trends mentioned can help support the use of certain husbandry practices (cage change frequency, cage type, ventilation rates, strain of mice, bedding material, etc.) implemented in our study.

The results of our histopathology data did not depict a correlation between housing-breeding configuration and pathology of respiratory tissue. Even for certain cage groups (specifically TMC) reaching four times the NH_3, the pathology score did not significantly differ between any of the four groups.[44] The additional use of the PS scoring system was to determine if a gradual progression of respiratory tissue pathology ensues as the PS increases since our microenvironment data confirmed a relationship between PS, cage size, and NH_3. We wanted to ensure that tissues exposed to maximum NH_3 concentrations from each PS range were used to evaluate if a correlation between NH_3 level and tissue pathology exists. The results from our study were confirmed in both weaned P21 pups as well as adults, who were exposed to approximately four times the length (3 months) of time in those housing/breeding configurations as the pups (21 days). In order to support the histopathologic findings from our study, we also used retired C57BL/6 pair and trio-breeders (9-11 months breeding) from non-affiliated researchers at our institution to confirm our observations. Although their microenvironment could not be determined, the reproductive parameters we used for our study were calculated based on breeding records of these retired breeders. The results were analogous to our study with no significant difference in reproductive efficacy (Table 3) nor pathology of respiratory tissues (figures xix & xx).

In most instances, a lack of clinical signs encourages institutions to assess intra-cage ammonia level as it corresponds to sub-clinical, histopathology of respiratory epithelium, which has resulted in contradictory findings. A study from the Jackson Laboratories revealed that when bedding changes were reduced to once every two weeks, 60 ACH were sufficient for cages housing adult B6 males. However, 100 ACH were necessary to control ammonia levels for cages housing trio-breeders + litter.[78] The level of sufficient ACH was based on pup survival, clinical signs, and histological lesions of the upper respiratory tract. The histological lesions found in the study were random between both groups (breeding & non-breeding) indicating no strong correlation between poor health, microenvironment, and pathology of the nasopharynx. Similar findings were observed in another study where adult B6 mice living in half of the approved housing density (according to the *Guide*) revealed no pathological findings in nasal and ocular tissues despite high NH_3 levels (> 50 ppm).[60] However, there has been evidence of rhinitis and ulcerative lesions in other rodent strains housed in standard cages as well as B6 mice under different cage change/ air change rates.[15,17, 72] A 2012 study revealed rhinitis & ulcerations found in both pair and trio-breeding groups of Swiss Webster mice but no inflammation was found in the non-breeding groups.[15] As seen in our study, similar trends in elevated ammonia levels were observed (50-200 ppm) in the breeding cages of that 2012 study which performed cage changes every other week as opposed to our weekly cage change. It is apparent that even a slight difference in one husbandry practice (cage change frequency) can lead to variable results as it pertains to the relationship between ammonia and tissue pathology.

Determining why our four study groups did not reveal significant difference in tissue pathology despite the elevated ammonia levels can be attributed to the specific husbandry conditions adopted from this institution, and implemented in our study. However, another explanation could possibly be due to the evolutionary traits of wild-feral rodents inherited by laboratory rodents. A prior study showed that BALB/c, non-breeding rodents revealed no significant preference when subjected to an Environmental

Preference Chamber (EPC) during a 48 hour spatial tracking system.[19] The EPC comprised of 4 adjacent chambers with various NH_3 concentrations per chamber (4, 30, 56, 110 ppm). These results were compared to other species tested (pigs and chickens) which displayed a strong, but delayed aversion to NH_3 concentrations of 20ppm and higher.[19] The preference or aversion of mice may be related to their reliance on olfaction as the dominant sensory modality since olfactory cues are important for sexual, social, and maternal behavior.[32, 46] In particular, ammonia may be associated with urinary odors used in territorial scent marking.[19] The lack of clinical signs (respiratory distress, decreased weight) seen in this study are supported by the discovery that there was no significant difference in the sub-clinical pathology of nasal and lung tissue despite the high ammonia levels.

The premise behind the *Guide's* recommendations is based on potential consequences that could result from trio breeding (such as overcrowding), and its potential to produce more waste accumulation that can potentially lead to hazardous microenvironment levels. [15, 52-54] In addition to the potential welfare concerns, the lack of documented research measuring ammonia levels in natural habitats of feral-wild mice is why the maximum ammonia level exposure limit is set according to human conditions (25ppm) by OSHA.[44] It is important to understand the environmental and habitual patterns of feral mice that naturally dwell in closed, burrowed spaces where the microenvironment maybe different than standards set forth in a commercial setting.[19, 62, 65]

The results of our weekly fecal CM measurements revealed no significant difference in TMC expression values compared to the other three groups. Due to the fact that corticosterone needs to be cleared from the plasma by the liver before appearing in feces, the levels of CM are more representative of chronic stress values than plasma corticosterone levels, which are more prone to acute variability.[20, 25, 27, 29, 38, 49,57-58, 66-68, 74] The gastrointestinal transit time for CM in mice has been determined to be between 4

and 12 h on the basis of radioactive corticosterone injection studies.[66-68] We restricted the adult feces collection period to a maximum of 2.5 hours (post cage change) to reduce the confounding influence of stress from the collection method and animal handling on the CM measurements. Due to the limited time for collection from each cage, the frozen fecal samples represented the adults within a cage, but not a specific adult breeder.

Since no group displayed any trends in fecal CM over the 13 weeks, it was concluded that fecal CM level is not significantly influenced by cage size nor breeding configuration over a three month breeding period. It is worth noting at the 3rd week mark, the CM expression levels for all groups (except PRC) dropped 50% from their 2nd week values (figure xi). Another possible explanation could be that by the 3rd week the mice are acclimated with their designated breeding configuration especially having to be housed with the opposite sex. By this time point, primiparous rearing and subsequent breeding occurred consistently until the end of the study (13th week). Despite the drop off from week 2-3, there were no significant trends in adult fecal CM from any group tested from a time perspective to assess chronic stress. However, a more accurate capture of fecal CM levels in regards to cage densities can be achieved by determining the biomass PS for each cage evaluated during cage change/ fecal collection. Since our fecal sampling was only once a week, we did not have an appropriate sample size to compare each PS score range amongst the four testing groups. If daily fecal samples were retrieved, the data would reveal if increased PS values (implying higher NH_3) accruing from higher housing densities correlate to increased fecal CM, and consequently confirm confounding factors (lack of space & high ammonia) attributing to chronic stress. If more time were available, collecting individual fecal contents could provide differences in stress between sexes, age groups, and breeding configurations. Similar variability from our fecal CM evaluation was confirmed in prior studies assessing strain and cage density conditions.[25- 26, 77] However, in some of those same studies there was a difference in plasma

corticosterone values in densities above the Guide's recommendations in BALB/c mice but not in B6 mice. [30, 51]The authors suggested that some mice strains are prone to higher expression of acute stress when housed in novel housing which evidently self-subsides once acclimation is achieved.

In regards to hair corticosterone, our study was the first study to our knowledge, evaluating chronic stress in breeding mice using a method previously validated in other species.[5, 28, 34, 50, 56, 64] As confirmed in other studies, the hair CM was significantly correlated to cortisol in saliva with less variability over hourly sampling periods. The detection periods of hair CM have been achieved from weeks to months duration as seen in the human medical setting. [34, 56, 64] The amount of hair needed per sample to evaluate hair cortisol was 80-100 mg which limited this assay to be utilized in adult breeder mice only. In addition, since the adult breeders were exposed to their specific spatial breeding conditions for much longer duration (3 months) than weaned pups (21 days), the chronic stress was more applicable to them. The results from our study suggest that hair CM levels are not significantly different in B6 mice regardless of cage size or breeding configuration. The intent of our study was not to establish reference values for mice, but just for detection of significant trends within each group as compared to the other three groups.

As revealed in prior studies, the behavioral component of animal welfare as it pertains to stress and anxiety did not change significantly amongst breeding configurations or cage size in our study, including the TMC group. An inconsistent difference in behavior of weaned C57BL/6Tac mice was noted in a 2007 study where mice were reared under trio-breeding conditions in standard mouse cage of 82 in^2 (208 cm^2) compared to a larger cage of 120 in^2 (304 cm^2).[76] Similar behavioral assessments of rodent housing have revealed mixed results despite the notion that CN pups grow faster and develop complex social environments which decrease the anxiety of forming social structures once weaned.[3, 8-9, 80]

This argument has been refuted stating that SN pups are less dependent on their mother thus making them more likely to explore and become independent in contrast to CN pups that displayed less activity and more episodes of thigmotaxis when subjected to OF testing.[3, 4] Another component worth mentioning is strain predisposition in behavioral response to anxiety. Since C57BL/6 mice are the most commonly used background strain in biomedical research, their use for our study was justified. In addition, the B6 strain is also a preferred inbred strain for behavior tests in contrast to BALB/c, which have a tendency to exhibit neophobia.[2, 8, 70-71]This was verified in studies that observed decreased locomotor activity in BALB/c mice in both OF and EPM as well as other behavioral patterns of aggression.[9, 71]

Many of the specific parameters assessed in each behavioral test of anxiety (duration of stay, frequency of entries, nose poke, mean speed) have been extensively used to assess the effect of several factors (social, spatial, enrichment) on their behavioral patterns[1, 8-10, 12-14, 22-24, 73] In many behavioral studies, it is recommended to habituate the animal to the testing apparatus to reduce the anxiety of exposure to a novel object (neophobia). However, due to the design of the study and our focus on optimal breeding standards (minimal disturbance to cage) we chose not to perform any habituation. This variable was consistent in all testing groups since all rodents were handled in the same manner. For future considerations, more behavior tests (Holeboard Test) would help further support the current trends as well as investigate other behavioral measures such as cognition and learning (Morris Water Maze and/or Route Learning test). One could argue that some of these conditional behavioral tests require training and habituation before measuring for trends in behavior. A more thorough behavioral assessment was achieved in a 2014 study that confirmed that CN inbred pups (B6, DBA, 129 strains) did have increased growth rates compared to SN pups, but the behavior and neurobiology was not significantly different. As a result, such variable outcomes lead to the belief that behavior and neurophysiological phenotypes are heavily influenced by genetically predetermined baseline characteristics with a susceptibility to epigenetic

influences.[21] Such influences may be exposed with the use of constant intra-cage visual monitoring of breeding cages, which could help provide insight into each individuals' activity and behavioral patterns. The lack of significant behavioral changes (for both OF and EPM tests) exhibited by TMC weaned pups and adults further support our hypothesis that welfare parameters (such as anxiety) in TMC are not significantly affected when compared to the pair-bred or larger cage size (132 in^2 rat cage) groups.

The objective of the BDNF immunoassay was to detect any differences in BDNF expression among the test groups. Prior studies have correlated BDNF expression reduction from stressful scenarios such as anxiety and immobilization stress.[3] However, increased BDNF expression has also been correlated to enrichment and/or CN conditions.[4] The mechanism of BDNF expression is yet to be determined. The aforementioned studies indicate that BDNF increases under acute stressors that stimulate neural plasticity, but decreases when exposed to chronic stressors.[72] Our BDNF results were used to evaluate expression patterns, not to determine whether BDNF expression was negatively or positively influenced in our study groups. Studies have shown that the female mice housed in CN display care-giving behavior that results in higher BDNF, and the total amount of maternal behavior displayed is higher compared to females housed in pair breeding.[4] Based on the logistics of our study design, assigning one of the four testing groups as a baseline standard for normal BDNF levels is difficult to determine. With the lack of rodent breeding studies involving BDNF as a measure of health and welfare, the objective of the BDNF results was to identify differences in expression of BDNF among the test groups. The lack of a significant difference in BDNF expression for the TMC group supported the same findings that TMC possessed in regards to behavior and both modalities of corticosterone determinations?.

In addition to using cortical brain tissue to determine physiological stress via BDNF, a subset of cortical tissue samples was used to determine changes in genetic expression using microarray amongst trio-breeding adult mice in the 2 different cage sizes tested. No significant gene expression changes were identified between cortical samples comparing TMC and TRC adult male breeding mice. Psychological stress is known to stimulate the neuroendocrine, sympathetic nervous and immune systems. By analyzing mRNA expression levels in leukocytes (which express receptors for hormones, neurotransmitters, growth factors, cytokines, and other stress related signals) various degrees of stress can be adequately measured.[45, 59, 63] This data was a novel diagnostic tool that could potentially help distinguish physiological characteristics of mice under different breeding configurations or spatial parameters by focusing on down regulation of genes responding to long term stressors.

It is historically known that successful rodent breeding colonies require a level of consistency with a low frequency of disturbance and manipulation of the breeding cage. In order to perform a comprehensive assessment of animal health and welfare, specific conditions were implemented (weekly microenvironment sampling) to reduce the confounding influence of stress on breeding rodents upon other subclinical parameters, such as behavior and breeding efficacy. By reducing the frequency of intra-cage manipulation to fit the historical rodent breeding standards performed by the university, our results are more reflective of the efficacy of this institution's standards for rodent breeding and spatial requirements. Despite the lack of any significant differences in measures of health and welfare using the parameters addressed in this study, the management of intra-cage microenvironment for mice bred in standard IVCs is the only parameter that may need to be addressed from a regulatory perspective. This issue can be resolved without affecting the historical practices of rodent breeding by increasing ventilation rates and/or cage change frequencies followed by microenvironment sampling to confirm acceptable ammonia levels. The intent of this study was to contribute more information to the laboratory

animal community about methods to assess animal health and welfare as they pertain to breeding mice and their spatial requirements. The findings based on this study reveal that there is no difference in physiological stress (as well as other indicators of welfare and health) when C57BL/6 mice are trio-bred in standard "shoebox" mice IVCs with weekly cage changes.

CHAPTER FIVE

APPENDIX

Introduction: The Centers for Disease Control and Prevention (CDC) estimates 1 out of 68 children in the U.S. are diagnosed with Autism Spectrum Disorder (ASD) every year. In addition to the emotional toll and time invested by parents and caretakers, the estimated medical costs of ASD are projected at 137 billion dollars [Knapp, 2013]. However, a major problem in understanding ASD is that many genes are thought to contribute to the etiology of ASD. Recently, a breakthrough in understanding ASD came with the discovery that patients with Epilepsy in Females with Mental Retardation (EFMR), part of the ASD spectrum, is caused by a mutation of *Protocadherin 19* (*Pcdh19*) [Dibbens, 2008]. Understanding how mutations in one gene can lead to epilepsy and autism can shed light on how other forms of autism arise. However, what *Pcdh19* does during normal development, or how mutations lead to epilepsy and mental retardation, is still essentially unknown.

Cadherin function: During embryonic morphogenesis, adhesion molecules are required for selective cell-cell interactions. *Pcdh19* is part of the cadherin superfamily, which mediate cell-to-cell adhesive interactions. Several types of cadherin molecules, such as neural cadherin (*Ncdh),* serve as cues during axon guidance, and are important for directing growth of axonal neurites to their appropriate targets in order to ensure normal brain circuitry [Lin J, 2014]. Because *Pcdh19* is a member of the cadherin family, it is also thought to be essential for axon guidance and neural development [Yagi, 2008]. Prior research has used *Pcdh19* morpholinos in zebrafish to knock down *Pcdh19* expression, and confirm a role in brain development [Biswas,

2010]. In this study two-photon time-lapse image sequences revealed that loss of either *Pcdh19* or *Ncdh* impaired cell movements during neurulation, disrupting both the directedness of cell movements and the coherence of movements among neighboring cells [Biswas, 2010]. The potential interaction of *Pcdh19* and *Ncdh* was also investigated by using standard aggregation assays in which purified *Pcdh19* and/or *N-cadherin* protein are bound to beads either individually or in combination. Quantitative characterization of aggregate sizes revealed that *Pcdh19* alone is only weakly adhesive, but *Pcdh19-Ncdh* together exhibited robust adhesion. Direct interaction of *Pcdh19* and *Ncdh* was shown via the formation of protein-protein complexes *in vitro* and *in vivo*. Adhesion by the *Pcdh19-Ncdh* complex was unaffected by mutations that disrupt *Ncdh* homophilic binding, but was inhibited by a mutation in *Pcdh19*. An additional component of the study revealed the complex exhibited homophilic specificity, as beads coated with *Pcdh19-Ncdh* did not intermix with *Ncdh* or *Pcdh17-Ncdh*-coated beads [Emond, 2011].

These results lead to a model that argues *Pcdh19* and *Ncdh* may interact to regulate axon guidance during neurodevelopment. If this model holds true, then disruption of *Pcdh19* function in EFMR would lead to subtle defects in axon guidance caused by disrupting normal *Pcdh19* function and by disrupting any interactions *Pcdh19* may have with *Ncdh*. Appropriate neuronal migration and circuitry are essential for nervous system function. Communication between neurons is achieved by the differentiation of neurites that result in the formation of axons and dendrites. The characterization of neurite formation and migration can be pivotal in unveiling the cellular mechanisms behind certain neuropathological disorders and injuries, including autism and EFMR.

To test this model, we propose *Pcdh19* promotes neurite outgrowth of primary neurons. We further hypothesize that presenting neurons with both *Ncdh* and *Pcdh19* protein will have a synergistic effect on neurite outgrowth. In order to obtain information on *Pcdh19*'s effect on axon outgrowth, an *in-vitro* model culturing neonatal olfactory sensory neurons (OSNs) was used. This proof of concept experiment in turn provides insight into the etiology of certain ASD (such as EFMR), and may one day aid in the development of gene therapies to treat ASD.

Axon Guidance of Olfactory Sensory Neurons: Olfactory sensory neurons (OSNs) are receptor neurons designated to detect odors. The olfactory epithelium is comprised of approximately 2000 populations of OSNs with each population expressing one allele of one of 1000 odorant receptor (OR) genes. An OSN projects a single unbranched axon towards a single glomerulus, and approximately 1600-1800 glomeruli are present in the olfactory bulb. OSNs expressing the same odorant receptor project towards the same glomeruli (2 glomeruli per olfactory bulb) which equates to four total glomeruli per mouse. Extensive genetic studies show that ORs determine not only the odorant response profile of the OSN, but also the projection of its axon to a specific glomerulus [Malnic B, 2010]. Previous studies utilizing genetic mouse models have proven that OSN axon guidance is dependent upon a wide array of different guidance cues. However, the role of N*cdh* and *Pcdh19* in guidance has never been studied.

The utilization of neonatal rodent OSNs for primary culture may provide a quantitative assessment or axon (neurite) outgrowth under the influence of extracellular cell adhesion molecules such as *Ncdh* and *Pcdh19*. Since the clinical manifestations of ASDs (such as EFMR) are reflective of changes in emotional memory and social behavior, the use of OSNs is justifiable

since the olfactory bulb is a component of the limbic system [Shepherd GM, 2010]. The strong correlation between olfaction and cognitive function can best be exemplified by the fact that only two synapses separate the olfactory nerve from the amygdala, which is involved in experiencing emotional memory. In addition, only three synapses separate the olfactory nerve from the hippocampus, which is implicated in working memory and short-term memory [Herz, 1996]. A major technical difficulty in utilizing the olfactory system to study the effects of *Pcdh19* on guidance, however, is a lack of *in vitro* culture models. Primary neuronal culture has been used extensively to study the impact of axon guidance cues on neurons isolated from the hippocampus, cortex, and cerebellum. However, primary culture methodologies for OSNs are still poorly developed.

Another challenge in characterizing the effects of *Pcdh19* on OSN outgrowth is the limited parameters and tracing algorithms that have historically been used to assess neuron function and neurite growth. Previous studies have attempted to create novel, measurable parameters to help characterize neurites by using various tracing algorithms that can automatically count neurons and neurite length [Yu, 2009]. The difficulty in isolating and quantifying individual neurite outgrowth has been a continuing obstacle in accurate characterization of cultured neurons with the use of automated tracing software [Smit, 2003]. This is due in large part to the fact that hippocampal, cortical, and cerebellar neurons are multipolar. The fact that OSNs are bipolar may help to greatly simplify the analysis. Before the use of these automated computer programs, manual counting was the historical process for quantifying neuron viability. Each possesses advantages and disadvantages, but there has been scant information comparing the accuracy

between the two methodologies. By first optimizing OSN viability in cell culture, our goal was to establish reproducible, quantitative measures that can best determine the impact of *Pcdh19* on neuronal outgrowth and axon guidance.

Objective: The objective of this study is to utilize bipolar OSNs to study the effect of *Pcdh19* protein alone and in combination with N*cdh* on neurite outgrowth. An advantage of culturing these neurons is that they readily polarize, forming distinctive axons and dendrites, on a two dimensional substrate at very low densities [Gong, 2012]. It is these attributes that make them extremely useful for understanding neuronal development with a focus on the impact of axon guidance cues in regards to neurite length and outgrowth. The lack of quantifiable data to assess neurite outgrowth for *Pcdh19* has led our lab to explore other parameters that may indicate phonotypical differences in neurite outgrowth under the influence of various protein conditions (such as *Pcdh19* and *Ncdh*).

Study Design. Acid washed coverslips (18 mm Fisher Scientific) were incubated with poly-ornithine (Sigma) for 1 hour followed by overnight submersion in ddH_2O. The following day 50-200µL of diluted mammalian cell derived protein extract, containing *Pcdh19* and/or *Ncdh*, was positioned on top of the coverslip. Due to the fact that homogeneity was not achieved through protein purification, we titrated the volume of protein applied to each coverslip. The volumes used were sufficient to show an effect on neurite length, as described in Figure 2 and Table 2. The protein was then rinsed off the coverslip with 1X-Modified Eagle Medium (Life Technologies). Olfactory epithelia were dissected from P7-P11 mice and dissociated using the Worthington Biochemical Papain Dissociation protocol. The cell

suspension was adjusted to fit a range between 200,000 – 400,000 OSNs for plating each coverslip. The serum free media (MEM –d-Valine, US Biochemical) harboring the neuronal OSNs also contained the neuronal supplement NeuroCult^{TM} SM1 (Stem Cell) to help support viability of neurons in cell culture. Each coverslip was harbored in a 35mm dish and were submerged in 3mL of fresh, SFM-SM1 media, and media was replaced daily for up to 48-72 hours followed by fixation of coverslips using 4% paraformaldehyde. Primary antibody staining (type III β-tubulin (Sigma), 1:250) of fixed OSNs were performed followed by DAPI staining (Figure 1).

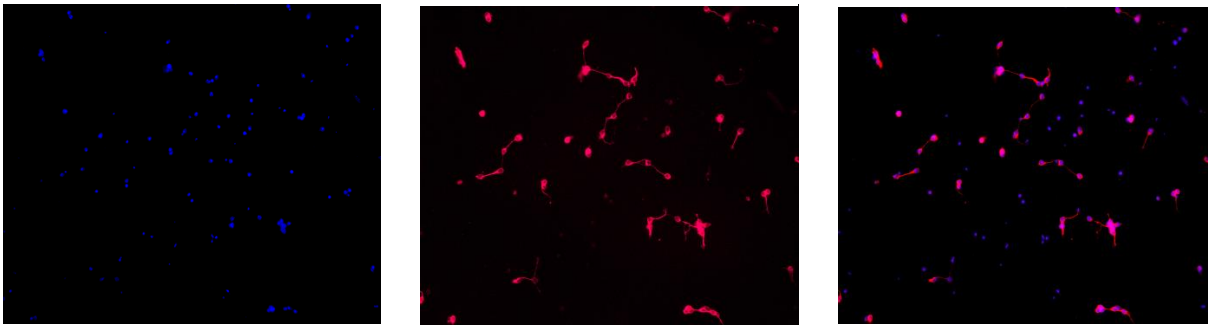


Figure 1A: DAPI stained nuclei of OSNs; B: Type III β-tubulin stain of OSN neurites; C: Merged image (20X)

Approximately 250-350 OSNs were analyzed by assessment of 9-10 antibody stained image fields per coverslip. When the two stained images are superimposed into a merged image (Figure 1), one can visually differentiate specific cellular characteristics such as neurite extensions or "branching" as depicted below (Table 1). Images were analyzed using the ImageJ NeuriteTracer Plugin (Pool, 2008) and by manual tracing, as described below.

	DAPI (blue)	**β-tubulin (red)**	**Merged (DAPI + β-tubulin)**	**"Branching" off cell body**
Non - Neuronal Cell	x			
Neuronal Cell	x	x	x	
Neurite Outgrowth of Neuronal Cell	x	x	x	x

Table 1: Visual confirmation of OSN neurite outgrowth by manual counting of DAPI and β-tubulin positive stained OSN.

Study Results. Our data revealed that coverslips pre-coated with diluted mammalian cell protein extract of *Ncdh* (5µL), and *Ncdh* (5µL) + *Pcdh19* (10µL) possessed significant increase in average neurite length per neuron relative to control (no protein) or to *Pcdh19* only protein (Figure 2). Although *Pcdh19* alone did not show an increase in neurite length, the combinatorial effects of *Ncdh* + *Pcdh19* did provide a distinguishable phenotype in neurite outgrowth length (even significantly greater than *Ncdh* alone). The data obtained do not support our first hypothesis, as we could not determine an effect of *Pcdh19* protein alone on neurite outgrowth. However, the data supports our second hypothesis as well as the prior bead aggregation studies suggesting *Pcdh19* in combination with *Ncdh* can enhance cell aggregation [Emond, 2011].

As noted previously, there are technical difficulties associated with the automated analysis of neurite outgrowth. The NeuriteTracer plugin identifies neurons based on a merged DAPI and beta-tubulin image. If the signal is weak, or is below the threshold cutoff for detection of the plugin, these neurons will be overlooked by NeuriteTracer. Furthermore, neurons sometimes clump together to form aggregates. This can confound NeuriteTracer, as the plugin cannot

accurately quantify the number of neurons in the aggregate, or potentially trace the neurite back to the appropriate cell. Both will ultimately affect the "average neurite outgrowth per cell" output from ImageJ.

We therefore performed a manual analysis of the results. We visually assessed the same images used for the automated image analysis, manually counting the number of cells present. We then divided the total neurite length (generated by ImageJ) by our manually determined neuronal number. We found this manual approach produced essentially identical results as the automated approach.

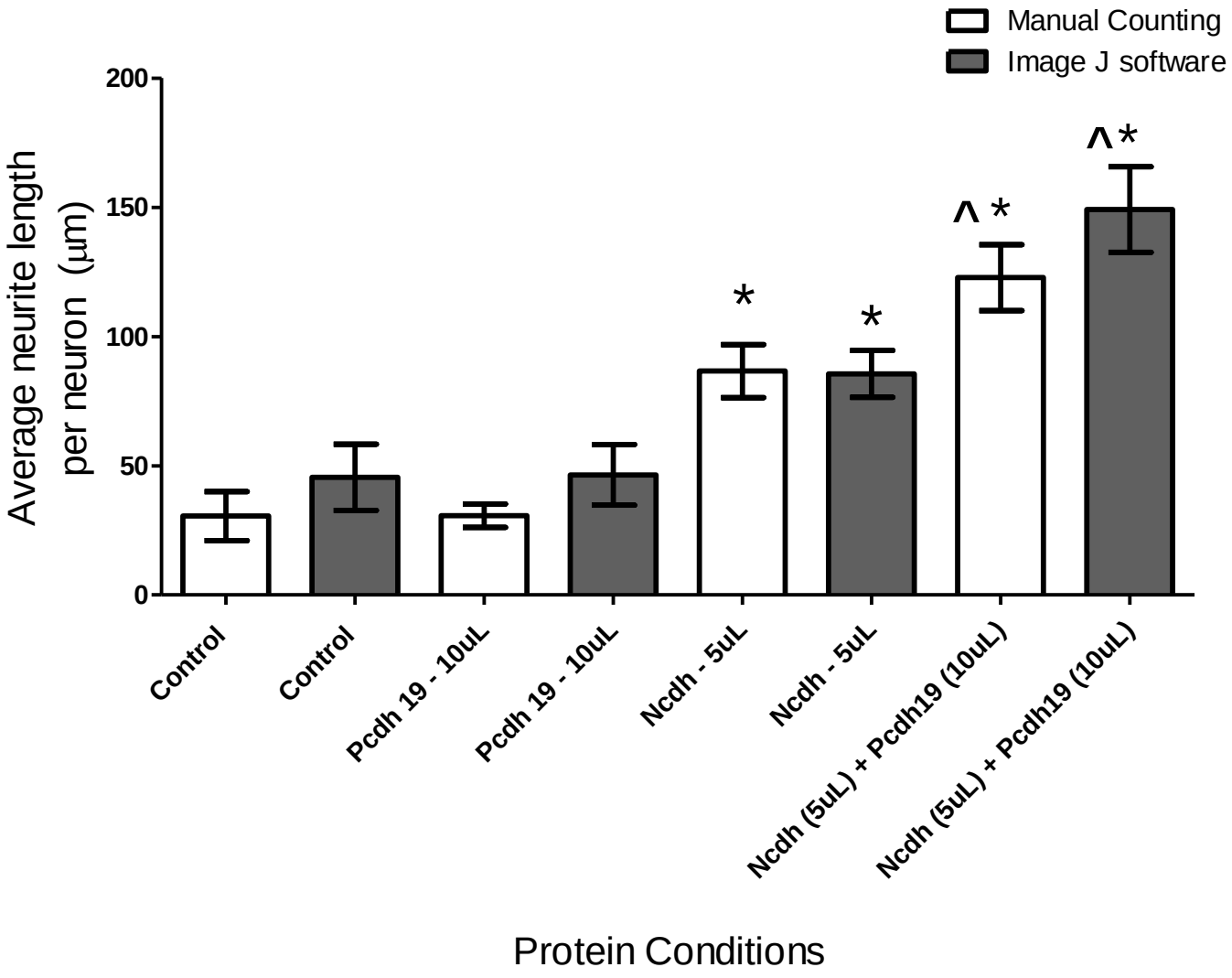


Figure 2: Average neurite length per neuron as calculated by ImageJ software and manual counting. For each OSN experiment, 9-10 antibody stained images per coverslip were assessed. Statistical significance observed in both: *Ncdh* (5uL) and *Ncdh* (5uL) + Pcdh19 (10uL) groups for manual counting and Image J. Data was analyzed by Tukey Post Hoc (ANOVA); p values are: *<0.001; ^<0.005.

We next asked whether or not the NeuriteTracer plugin can accurately distinguish neurons from non-neuronal cells. We manually counted the ratio of neuronal cells (β-tubulin + DAPI) to non-neuronal cells (DAPI alone) (figure 3). We found no difference in manual vs. automated

analysis. Furthermore, the particular protein applied to the coverslip had no significant effect.

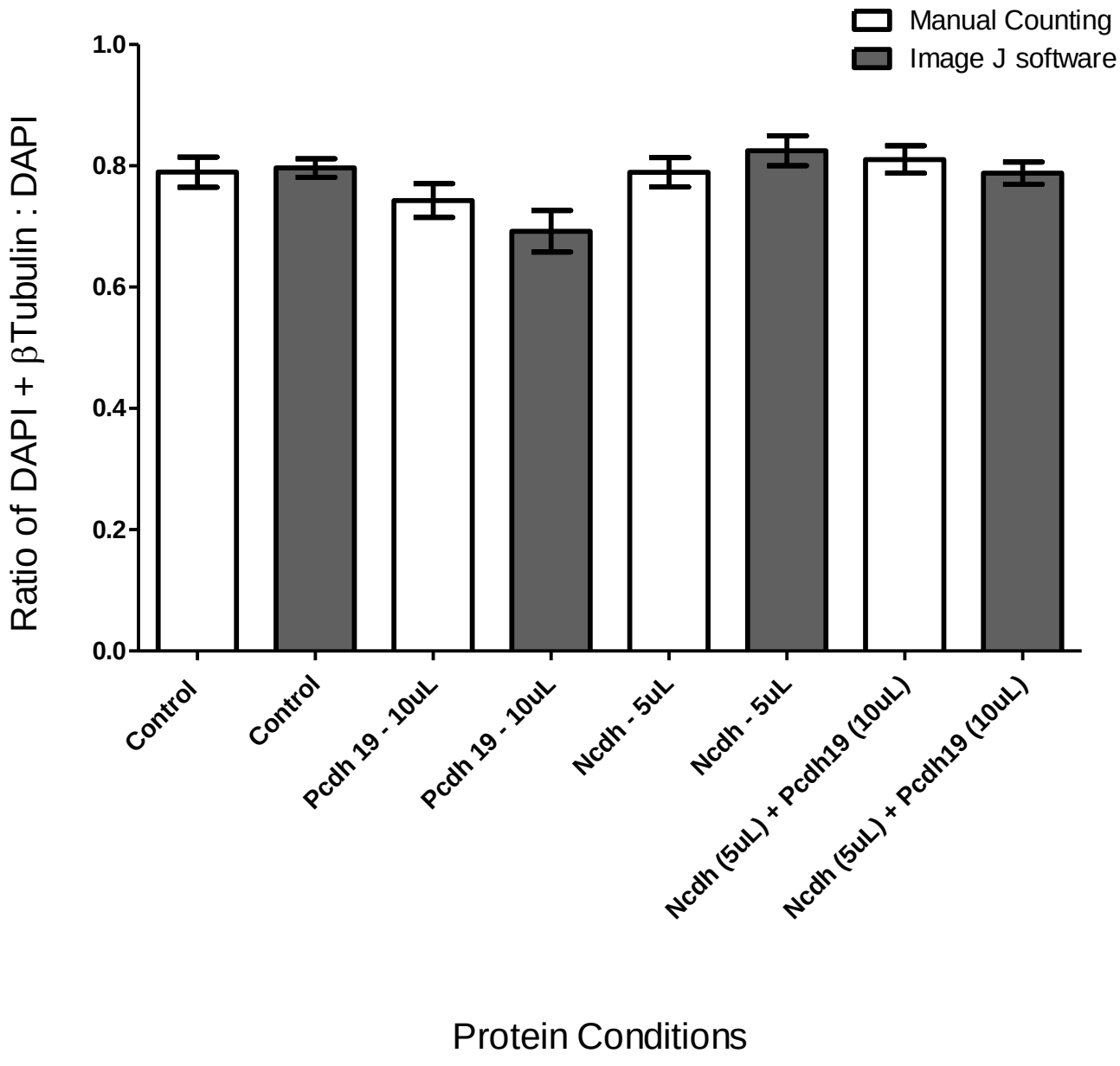


Figure 3: Ratio of cells stained positive for Beta-Tubulin & DAPI to cells only stained for DAPI. Data was analyzed by Tukey Post Hoc (ANOVA), p values are: *<0.005. No significant differences observed between manual counting and Image J as well as amongst various protein conditions.

NeuriteTracer generates an overall "average neurite length per cell" as its primary output. However, a flaw in utilizing the NeuriteTracer software is that it does not distinguish between neurons with neurite outgrowth from those that do not. *Pcdh19* might significantly influence the neurite outgrowth of a subset of cells, which would lead to increased overall neurite outgrowth per cell. However, if this occurs, it cannot be distinguished as accurately using automated software. This led us to manually count the same cells in order to compare both methods. Manual counting involved first determining the number of neurons present in an image based on DAPI+ cells that were also beta-tubulin positive. As noted previously, manual counting can distinguish cells with weak beta-tubulin signal and also help determine how many cells are present when neurons form small aggregates. Next, neurons with neurite outgrowth were visually assessed. This process involved making a judgment call regarding whether or not a neuron had a neurite or not. In most cases, this was straightforward, as many neurons had no neurites at all (e.g. figure 1). This manual data was compared against data derived from the NeuriteTracer program. For each image, NeuriteTracer determines the total number of neurons present (based on a preset threshold), and also provides the number of neurons with neurites in the image as an output. We used both of these NeuriteTracer derived values in the following equation to calculate *ImageJ Ratio of Neurons with outgrowth to Neurons with No outgrowth:*

$$\frac{\textbf{[\# Neurite Length Readings]}}{\textbf{[Total Number of Neurons counted]} - \textbf{[\# Neurite Length readings]}}$$

The results are shown in Figure 4. All of the manually counted samples for all protein conditions had significantly higher ratios (except for *Pcdh19* only) than that obtained using NeuriteTracer data.

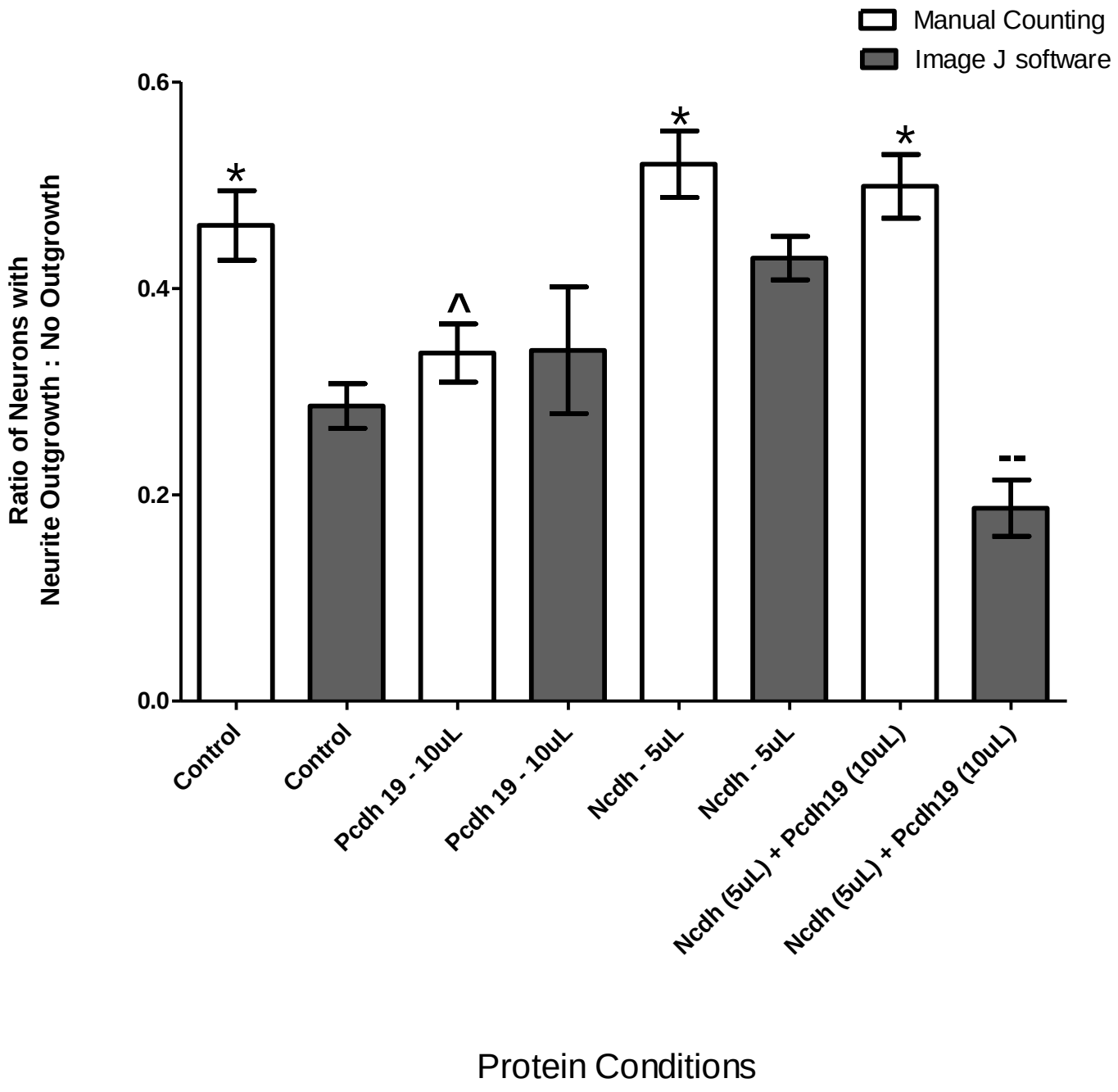


Figure 4: Ratio of Beta Tubulin positive neurons with neurite growth to Beta Tubulin positive neurons without visible neurite outgrowth. Significant differences observed amongst all protein conditions (except *Pcdh19* – 10 uL) when comparing manual counting and Image J automated software. Data was analyzed by Tukey Post Hoc (ANOVA), p values are: p values are *<0.005; ^<0.05; ---< 0.001.

Discussion: The purpose of this study was to assess the effects of *Pcdh19* and *Ncdh* on neurite outgrowth. This objective was achieved by the use of quantitative measures to assess neurite outgrowth and neuron viability. There was a significant increase in the average neurite length per neuron when OSNs were incubated with 10µL *Pcdh19* + 5µL *Ncdh* protein in combination (figure 2). Significant increase in neurite length per neuron was confirmed using both manual and automated counting of individual neurons. Although the presence of 5 µL *Ncdh* alone proved to significantly increase neurite length per neuron, the combinatorial effect of *Pcdh19* and *Ncdh* on OSN demonstrated a significant increase compared to *Ncdh* alone. The length of a neurite is dependent on axon guidance cues to assist in the navigation of an axon towards its appropriate synapse. This is the first demonstration that *Pcdh19* can affect neurite outgrowth of mammalian OSN when combined with *Ncdh*. The results from figure 2 of our study support using the quantitative measure of "average neurite length per neuron" in assessing the function of cell adhesion molecules on OSN outgrowth. The utilization of mammalian OSNs with manual and automated quantification of neurite outgrowth can be beneficial for predicting abnormal neurite outgrowth as seen in clinical manifestations of EFMR.

The ImageJ NeuriteTracer software does not distinguish between neurons with outgrowth versus neurons without outgrowth. Although manual counting is time consuming and laborious, the benefit of this method is that it does not possess a threshold limitation in determining neurite outgrowth as opposed to NeuriteTracer. Another limitation with NeuriteTracer is that it cannot automatically produce a ratio of outgrowth to no outgrowth per neuron. We wanted to determine if *Pcdh19* alone or in combination with *Ncdh* increases the proportion of neurons with outgrowth.

We compared manual evaluation of images against those analyzed with the software. Manual counting produced a higher ratio of outgrowth: no outgrowth than NeuriteTracer amongst all groups except the *Pcdh19* alone group. However, among those samples counted manually, adding *Ncdh* or *Pcdh19 + Ncdh* did not affect the ratio of neurons with outgrowth to those without. We interpret this to mean that the increased neurite outgrowth seen with these proteins appears to affect neurons equally. If a subset of neurons were strongly affected by these proteins, then we would expect there to be a difference in the ratio of neurons with outgrowth to those without in the presence of *Ncdh* or *Pcdh19 + Ncdh*.

The combination of *Pcdh19* and *Ncdh* did not significantly increase the number of viable neurons per image field (figure 3). The number of viable neurons in culture seems more dependent on performing a contamination-free preparation rather than the impact of cell adhesion molecules. The results of our data suggest that *Pcdh19*'s function as a contributor to axon guidance in combination with *Ncdh* may be solely limited to its influence on neurite length and branching as indicated in figure 2. Although the use of an *in-vivo* model of genetically modified *Pcdh19* and *Ncdh* neonatal rodents would be the ideal method to determine neurite function, the application of our *in-vitro* OSN cell preparation model can aid in determining appropriate metrics to assess axon guidance cues.

When utilizing the aforementioned OSN cell culture experiment, strong consideration must be focused on technical components that are crucial for sustaining cell viability. During the first 24 hours of OSN culture growth, there is the potential for a large amount of cell death which is why there was particular focus on the following culture conditions: temperature of culture

media, sufficient volume of culture media, volume of non-OSN cells within cell culture. The rate of survival is not correlated with the age of the pups, but the higher density of the culture the better survival rates are observed based on preliminary results. By two days *in vitro* the majority of neurons in culture were bipolar in morphology, indicative of viable neurite outgrowth. Preservation and reproducibility of the described OSN cell preparation is vital for neurite outgrowth assessment which is why an emphasis was made to provide fresh cell media of ample volume. Future considerations into quantifying cell death during the 48 hour cell culture will help determine the efficacy of the OSN dissection and cell preparation. Despite being regarded as bipolar neurons, OSNs tend to have its axons branch out towards their apices with an emphasis on branching seen in areas where neurons are close to each other. If bipolar morphology is the desired morphology for axonal outgrowth analysis, controlling the density of the culture is important in preventing cross over effect of axonal branching.

These results have provided a baseline for assessment of *Pcdh19* function on OSN outgrowth and function. The ability to study how a single gene affects neurite outgrowth will help determine the function of *Pcdh19* as it pertains to neurodevelopmental disorders such as EFMR. Future considerations must focus on the use of more highly purified *Ncdh* and *Pcdh19* protein to assess neurite outgrowth of OSNs. Future studies will involve the phenotypical assessment of OSNs under the influence of mutated *Pcdh19*, which may be beneficial in finding clues to the pathogenesis of EFMR and other autism spectrum disorders.

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