DIFFERENCES IN COGNITION AND PHYSIOLOGY IN CALORICALLY RESTRICTED MICE DURING AGING

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Differences in Cognition and Physiology in Calorically Restricted Mice During Aging

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Debilitating age-related cognitive decline is a major health concern today and is associated with annual health care costs of billions of dollars. Although extensive research has focused on cognitive impairment and drug treatments, no therapies are currently available. Recent studies have enumerated the beneficial effects of caloric restriction and exercise on overall health during aging. This project investigated whether intermittent fasting, a form of caloric restriction, affects cognition across aging. We utilized four weeks of alternate day feeding of *ad libitum* food intake and no food in the experimental group as a model of caloric restriction. The main project includes a combination of experiments conducted to evaluate cognitive abilities (Barnes maze, a spatial learning task), and to measure synaptic transmission (hippocampal field potentials) in a mouse model of aging. The scope of my thesis includes aiding in developing the model for fasting as well as feeding and weighing the mice. Further, I was able to observe and practice hippocampal slice recordings, and became proficient in behavioral testing. Together, this and future data will compare the levels of impairment between young and aged mice, and the effect of intermittent feeding. The importance of learning about

the effects of caloric restriction across aging can provide direction for how to investigate mechanisms to reduce cognitive impairments.

ACKNOWLEDGEMENTS

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PART I

INTRODUCTION

Mouse models have been used in biomedical research for over a century, because the physiological makeup of mice is translational to that of humans, making mouse models favorable for researching many diseases, including neurodegenerative diseases which have proven resistant to new treatments (Mendsaikhan et al., 2019). In research settings, a one-year-old mouse corresponds to a thirty-year-old human, giving them clear advantages in the study of the aging mammalian brain (Why the Mouse?, n.d.).

While a variety of health problems persist in the human population, the greatest risk factor for developing neurodegenerative diseases, such as Alzheimer's disease, is advanced age (Alzheimer's Association, 2019). Alzheimer's disease and other types of dementia are of tremendous importance to today's society, as they affect "5.5 million people age 65 and older" (Alzheimer's disease - Questions and Answers, 2019). Many therapies have been developed to alleviate the negative effects of aging with little success, but there is potential therapeutic value in using caloric restriction to alleviate negative symptoms associated with aging. Caloric restriction is a growing area of interest in the field of aging research. Studying the effects of caloric restriction across aging can provide direction for how to investigate mechanisms to reduce cognitive impairments, given that caloric restriction has potential to result in "less age-related chronic disease (including cardiovascular diseases, cancer, and dementia), and longer lifespan" (Willcox et al., 2014).

Caloric restriction experiments have been a topic of growing interest in recent years as restricted calorie intake has been shown to have positive effects in aging and cognition. A study

with elderly adults found a significant increase in verbal memory scores following caloric restriction (Witte et al., 2009). At its core, caloric restriction alters metabolism and cellular stress response systems in ways that protect neurons against genetic and environmental factors to which they would otherwise succumb during aging (Bronwen et al., 2006).

Caloric restriction is the only strategy that reliably extends health span in mammals (Madeo et al., 2019). Multiple animal models have been developed because the effects of caloric restriction can be difficult to measure in humans. In a study involving rhesus monkeys, it was found that caloric restriction has the power to "extend lifespan and delay the onset of age-related disorders" and is suggested to be replicable in humans (Mattison, et al., 2017). Similarly, a study involving grey mouse lemurs found that both chronic caloric restriction and resveratrol, a caloric restriction mimetic, led to improvements in spontaneous locomotor activity and working memory, with specific increases in spatial memory for lemurs treated with the mimetic (Dal-Pan et al., 2011). Caloric restriction has also been studied in connection with gene expression. A study of calorically-restricted C. elegans revealed that a particular gene, skn-1, functions in specific neurons to extend lifespan in response to dietary restriction (Bishop & Guarente, 2007). Moreover, consistent with the notion that cell death is a major factor associated with aging, a study found that with caloric restriction "SIRT1 deacetylates the DNA repair factor Ku70, thereby inhibiting stress-induced apoptotic cell death", demonstrating the ability to somewhat halt aging in yeast, rats and human cells (Sohal & Weindruch, 1996).

These remarkable studies have proven insightful to the potential health benefits that caloric restriction has to offer while raising questions of how the therapy might be used to enhance cognition in aging. As caloric restriction research continues to be of interest,

supplemental animal research on caloric restriction may lead to conclusions not yet drawn about its usefulness in helping cognitive decline during aging, in order to one day be applied to help humans combat age-related cognitive decline.

The present study utilized an intermittent fasting (subtype of caloric restriction) mouse model followed by a series of experiments to investigate the effect of caloric restriction on cognition and physiology during aging. Because an intermittent fasting diet may be performed for a short duration of time, unlike lifelong caloric restriction, intermittent fasting (feeding of the experimental group every other day) was selected for the present study. Mice consumed a fortified diet consisting of additional vitamins and minerals to ensure they would not become deficient as caloric intake was reduced.

Following intermittent fasting, cognition was measured by a Barnes maze behavioral test of spatial memory. The Barnes maze task evaluates each mouse's ability to remember the location of a rewarding stimulus following training, and involves the main brain region responsible for processing information: the hippocampus (Healy & Jozet-Alves, 2010). Changes in synaptic transmission were measured with hippocampal electric field potentials after behavioral testing, but recordings are ongoing and data are not yet finalized. Electric field potentials were conducted to observe synaptic transmission in the hippocampus, in which synchronous output of cooperating cells displayed transient network oscillations, which can be recorded and analyzed (Buzaki et al., 1992).

Because the present study is an ongoing experiment, having a well-established baseline for control values of past Barnes maze cohorts is of great importance. Thus, control data from previous cohorts, prior to the fasting experiments, were analyzed for consistency across cohorts over time. This data will determine how intermittent fasting mice compare to

control mice and variability between past litters. Overall, present and future data will be compiled to determine how caloric restriction might be a viable therapy in aging.

Keywords: intermittent fasting, Barnes maze, hippocampus, caloric restriction, cognition, aging

PART II

METHODS

To begin, the average intake of food was determined for *ad libitum* mice, and from that value, a feeding schedule for intermittent fasting was developed. Following monitored food intake, behavioral and synaptic transmission experiments were conducted. The present study is ongoing and multiple future cohorts will follow.

Intermittent Fasting

The first cohort (n=12 males; 4 young control, 4 aged control & 4 aged intermittent fasting experimental mice) began a feeding schedule which lasted for four weeks, during which all animals were weighed daily, and the intermittent fasting experimental group received food every other day (Table 1). The first cohort's weight data served as a baseline which established the likely weight fluctuations that result from a four week caloric restriction time period. To create homogeneity across groups, all mice were fed a special diet, NIH-31, which has 60% of typical pellet calories and is fortified with extra vitamins and minerals, consistent with the finding that composition of diets didn't significantly affect parameters, but the difference in feeding time period did have a significant effect (Blackwell et al., 1995). Future cohorts may be subject to longer caloric restriction feeding schedules. For the first cohort, mice were individually housed in a self-ventilated cages in a temperature and humidity-controlled environment. Mice were weighed and fed consistently at the same time each day to minimize confounds.

Table 1. Sample weekly feeding schedule.

	Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
	Food?	Food?	Food?	Food?	Food?	Food?	Food?
Young Control	Y	Y	Y	Y	Y	Y	Y
Aged Control	Y	Y	Y	Y	Y	Y	Y
IF Aged	Y	N	Y	N	Y	N	Y

Barnes Maze Task

Following monitored food intake of future cohorts, a Barnes maze behavioral test of spatial memory and learning will be conducted. The experimental timeline is comprised of five major phases: habituation, learning, learning probe, reversal, reversal probe and cue testing (Figure 1). Due to project time constraints, the long-term probe test shown in Figure 1 will not be included. Barnes maze data is video-recorded using Noldus Ethovision XT and is analyzed using the statistics program StatView.

The first phase of the Barnes maze experiment is habituation, during which mice acclimate to the table and light apparatus (Figure 2). During habituation, mice are placed under a clear beaker on the Barnes maze table under bright light for one minute. For subsequent phases of the experiment, room lights are off and bright lights are on.

Following exposure to the light and the table, mice are gently guided to the target escape hole and taught that locating the escape hole leads to the stressful bright light being turned off. For each experimental trial, mice spend exactly one minute in the dark, dry, cool escape hole to reinforce the behavior and are then removed and placed back in their cages. The second phase is the learning phase, during which mice are placed on the table under bright light for four trials a day at about 20 minute inter-trial intervals for four days as they learn where escape hole is. Each trial will last three minutes or until the mouse locates the escape hole, immediately after which the bright light is turned off. Next, a three minute learning probe test (no escape hole present)

measures spatial memory of escape hole location, coupled with simultaneous extinction of escape hole location memory, as no reward (finding and entering escape hole) is reaped by mice. The probe test is essential for transitioning mice to the reversal phase of learning, in which the escape hole is shifted across the Barnes maze table (180^{o}), challenging mice's cognitive flexibility (Figure 3). Reversal learning is performed just as the learning phase. Following reversal, another probe test measuring spatial memory is conducted. Finally, cue testing is performed to measure the sensory abilities of mice. A striped flag is attached to the escape hole and mice are expected to investigate the area and then locate the escape hole. Mice with difficulty finding the escape hole with a visual cue might be excluded from datasets due to potential blindness. Barnes maze task data for past cohorts of mice (N = 34) was analyzed (Table 2). Speed (cm/s) and distance traveled (cm) were acquired and compared across days (by blocks, which are averages of all trials in that day) between cohorts. This data was analyzed by a repeated measures ANOVA test. Distance traveled was compared by an ANOVA test for training probe and reversal probe trials.

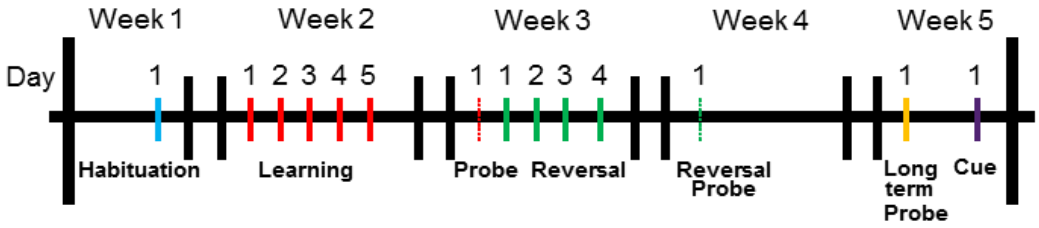


Figure 1. Experimental Barnes maze timeline.

Table 2. N values for past cohorts.

Cohort	N (Males)	N (Females)
1	4	7
2	4	6
3	9	0
4	2	6

Figure 2. Barnes maze apparatus for mice.

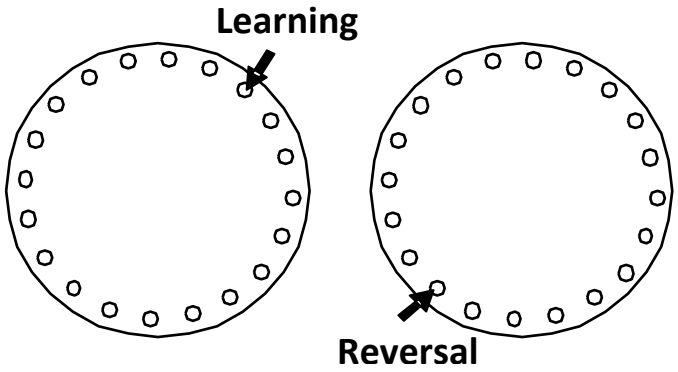


Figure 3. Example of target positions.

Hippocampal Field Potentials

Following behavioral testing, mice will be sacrificed via anesthetization with isoflurane followed by decapitation. After quick extraction and slicing of the hippocampus, extracellular electrophysiology (electric field potentials) are recorded and analyzed (Figure 4). Prior to cutting, a prepared recording bath is set up (Figure 5) and connected to multiple apparatuses, including a Digidata acquisition system, a Dagan voltage amplifier, an oscilloscope to display waveforms, and the computer program Clampex 9.2 for data acquisition and analysis.

After being cut, brain tissue is kept alive in prepared Krebs slice solution which mimics cerebrospinal fluid, and contained in a holding chamber containing a scintillated glass bubbler (Figure 7). The hippocampus is sliced thinly (300 μm) and evenly using a McIlwain tissue chopper. Slices are mounted in the recording bath and probed by one stimulating and one

recording electrode (Figure 6). The CA1 layer of the hippocampus is kept as parallel as possible to the recording electrode so as to minimize damaging tissue and maximizing surface area for collecting a response. When a response is detected by the recording electrode, analyses may be performed using the computer analysis program Clampex 9.2. This data is currently being collected and are not added to this manuscript. An example of what a typical trace from recording would look like is added to the results section.

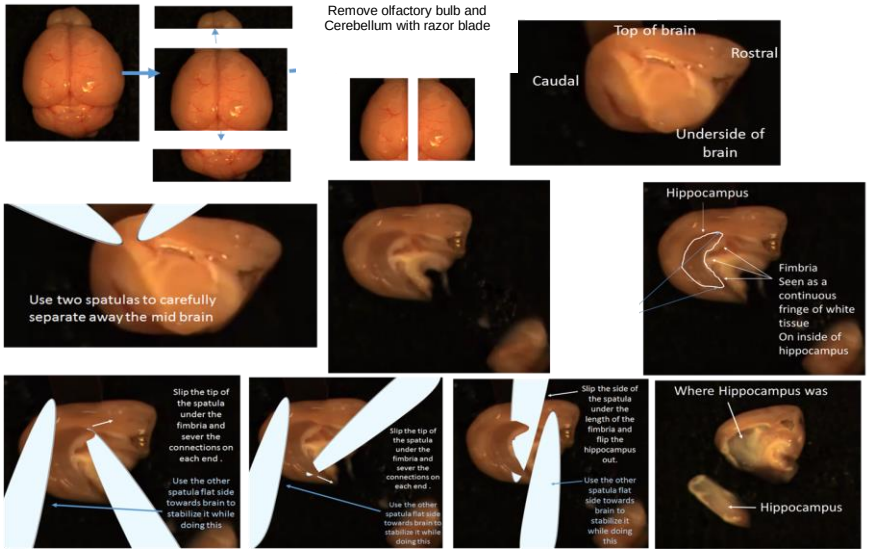


Figure 4. Hippocampal dissection protocol (from www.youtube.com).

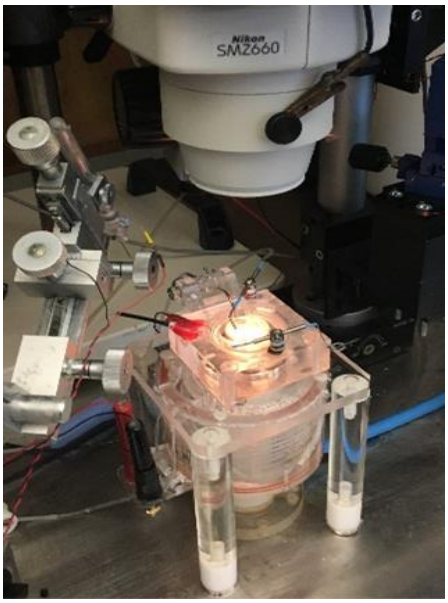


Figure 5. Recording bath.

Figure 6. Slice in solution.

Figure 7. Holding chamber containing Krebs slice solution and bubbled by carbogen gas.

PART III

RESULTS

Intermittent Fasting

Weight data for the first cohort showed that intermittent fasting mice's weight fluctuated in accordance with intermittent feeding and had a general decline in body weight over days (Figure 8A). Percent change in daily body weight from initial weight is shown below (Figure 8B). Additionally, the intermittent fasting mice retained 80% of control body weight, shown by the percent change in body weight across days (Figure 8C).

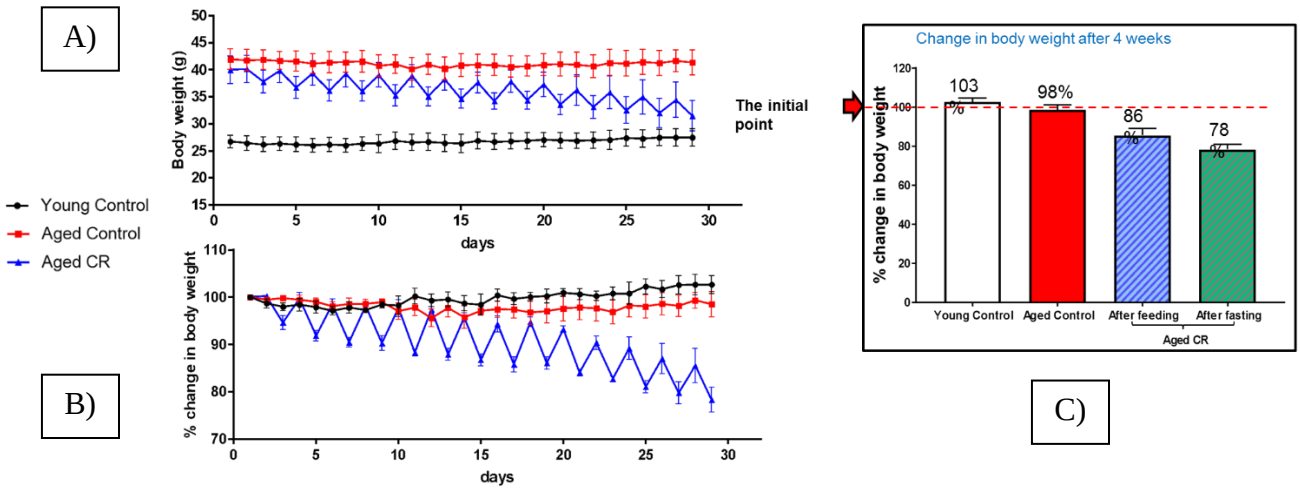
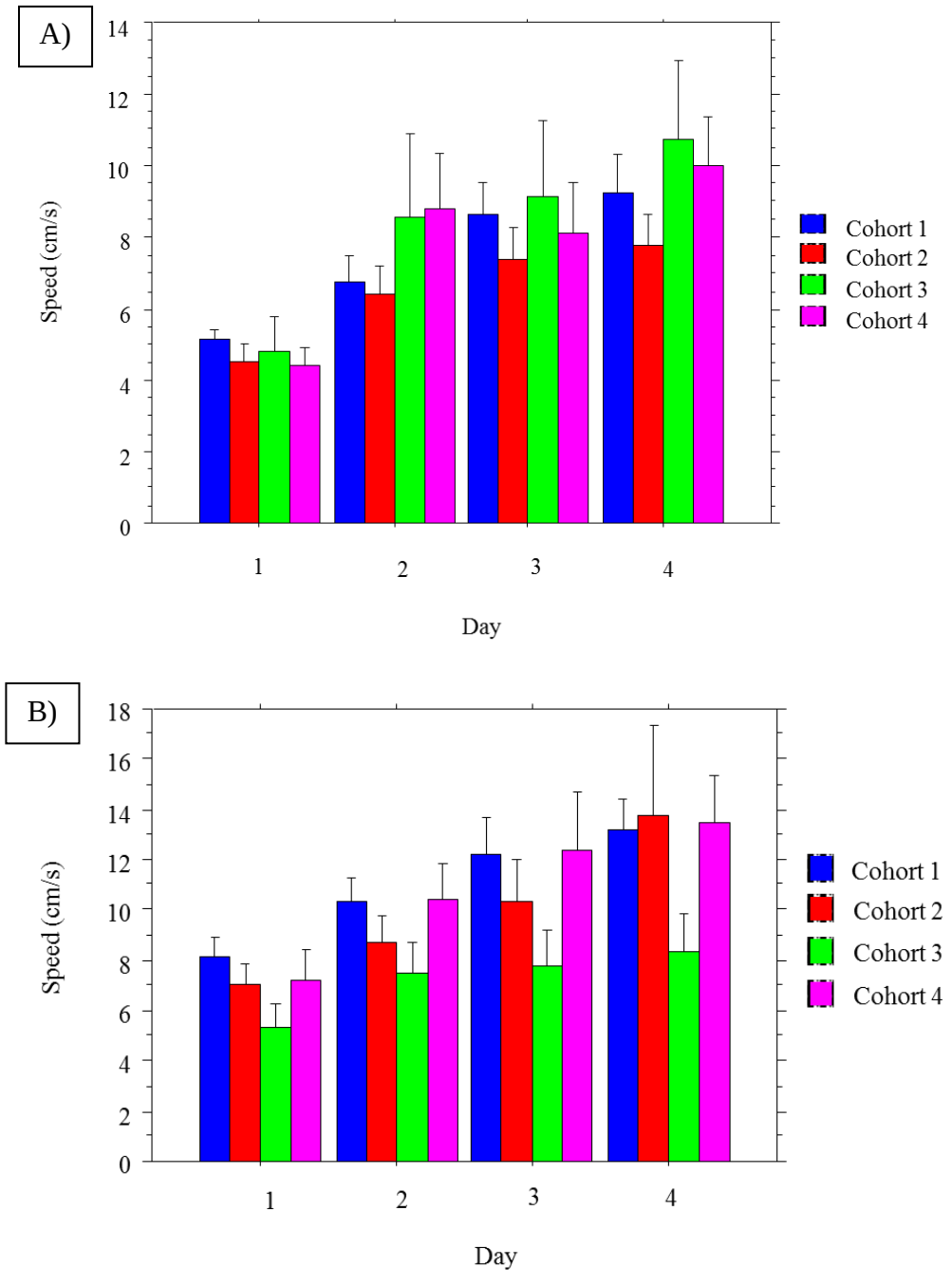


Figure 8. A) Normalized weight data across days. Young cohort (black) shows lower body weight in grams over time. B) Aged controls (red) maintained consistent higher weight values than aged that were in the intermittent fasting schedule. C) Percent change in body weight across days.

Barnes Maze

Results and analyses that will be performed in the intermittent fasting cohorts will be analogous to the analysis performed on prior non-fasting cohorts in this section.

A repeated measures ANOVA test for speed (cm/sec) traveled by mice across four cohorts for learning training ($F(9, 34) = 1.04$, $p = .412$), reversal training ($F(9, 34) = 0.592$, $p = .801$), and cue testing ($F(9, 26) = 1.145$, $p = .342$) showed no significant differences or interactions (Figure 9).



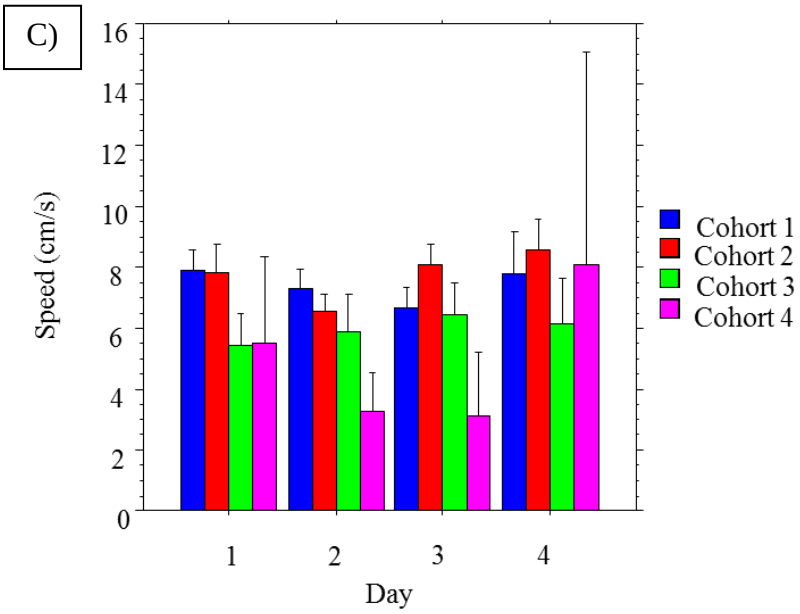
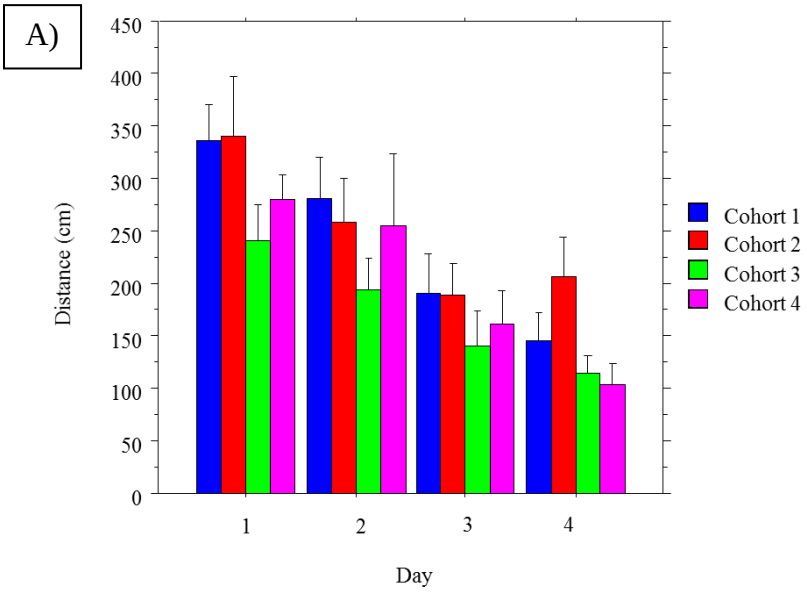


Figure 9. A) Travel speed by mice in learning training across days. B) Speed of mice in reversal training across days. C) Speed of mice in cue testing across days.

Additionally, a repeated measures ANOVA test was run for distance traveled (cm) during learning training ($F(9, 34) = 0.466$, $p = 0.895$), reversal training ($F(9, 34) = 1.003$, $p = .442$), and cue testing ($F(9, 26) = .890$, $p = .538$), with no significant differences or interactions found (Figure 10).



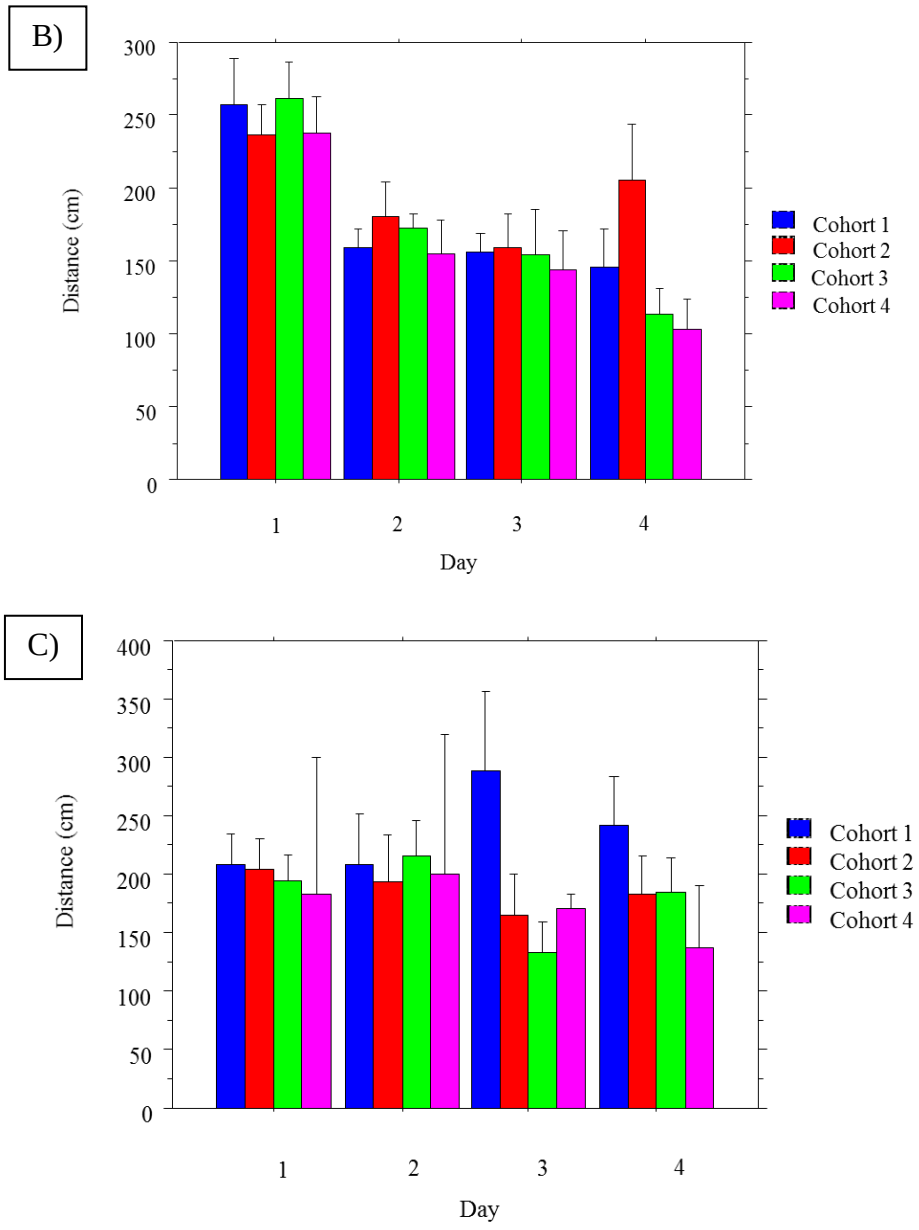


Figure 10. A) Distance traveled in learning training. B) Distance traveled in reversal training. C) Distance traveled in cue testing.

Similarly, a one-way ANOVA test showed no significant difference between cohorts for both learning probe ($F(3, 34) = 0.399$, $p = .754$) and reversal probe testing ($F(3, 34) = 0.548$, $p > .653$) (Figure 11).

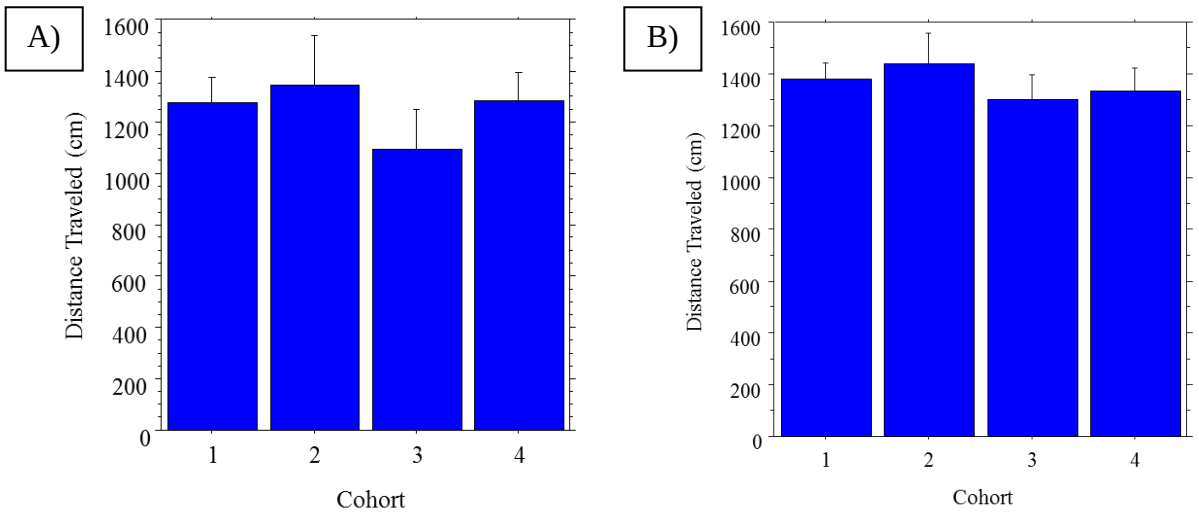


Figure 11. A) Distance traveled in learning probe across cohorts. B) Distance traveled in reversal probe across cohorts.

Hippocampal Field Potentials

Paired-pulse facilitation, a form of short-term synaptic plasticity, is the ratio of the amplitude of the second response to that of the first, dependent on the probability of vesicular release at the synapse, making paired pulse facilitation a good measure of presynaptic cell transmitter release probability (Manita et al., 2007). It is expected that if a presynaptic cell in a slice has a low release probability, the first spike will cause a small postsynaptic response, but the build-up of calcium in the presynaptic terminal will lead to an increased release probability on the second spike. As a result, greater transmitter release, greater postsynaptic response, and a higher paired-pulse facilitation will result (Figure 12). In addition to paired-pulse facilitation, long-term potentiation of synaptic transmission may also be measured and analyzed from electric field potentials in the hippocampus. Long-term potentiation of synaptic transmission involves inducing a subtype of glutamate receptor which endows long-term potentiation with Hebbian characteristics, allowing electrical events at the postsynaptic membrane to be transduced into chemical signals which, in turn, are thought to activate both pre- and postsynaptic mechanisms to

generate a persistent increase in synaptic strength (Bliss & Collingridge, 1993). Input-output curves may be used to represent data collected from electric field potentials.

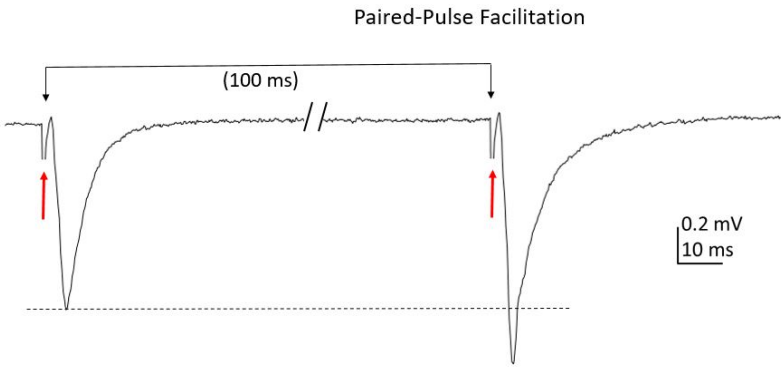


Figure 12. Paired-pulse facilitation. Example of a trace acquired from Dr. Griffith's laboratory. Red arrows represent stimulus artifact.

PART IV

CONCLUSION

The present study aims to understand the effects of intermittent fasting, a subtype of caloric restriction, on cognition and physiology during aging by building upon existing data and thus making predictions for potential findings, with the overall objective to augment existing knowledge about the usefulness of caloric restriction therapy.

The intermittent fasting mice's weight data from the first cohort establishes a feeding model that maintains a healthy weight over time. Overall, there was a general decline in weight for intermittent fasting animals while they were eating more, but mice still maintained the majority of their original body weight. Control animals showed general stability in their weight across time. In a study using a similar intermittent fasting feeding regimen to the present study, researchers found that intermittent fasting of C57BL/6 mice's overall food intake was not decreased and their body weight was maintained (Anson et al., 2003). The present feeding schedule was deemed as successful and will be replicated for subsequent cohorts as the study endures. Future cohorts might also be subject to additional weeks of intermittent fasting to examine how duration of caloric restriction can mediate its effects.

Analyses of past Barnes maze cohorts have maintained that no significant differences exist between cohorts for speed and distance traveled by mice, thereby establishing a reliable baseline for intermittent fasting study results to be compared to. Additionally, there was no difference seen between past cohorts for either the learning probe or the reversal probe tests of spatial memory, indicating that overall, cohorts learned equally well. There was variability within cohorts, but for all animals, mice increased in performance over days. Identical results

were found in a study by Rosenfeld & Ferguson, in which control subjects improved in the Barnes maze task, indicative of normal learning and memory (2014). Based on previous data, it is predicted that aged intermittent fasting mice will perform better than aged controls, and that future data from aged intermittent fasting mice will more closely resemble that of young controls than of aged controls. It is expected that caloric restriction will improve spatial memory abilities for the aged experimental mice in contrast to the aged control mice, and a clear distinction will be seen.

Hippocampal field potentials are expected to reveal differences between groups in underlying synaptic transmission occurring. Using analyses including paired-pulse facilitation and long-term potentiation, the present study hopes to determine the differences in strength of synaptic transmission between groups. It is expected that strength of synaptic transmission will be positively correlated with spatial memory ability measured by the Barnes maze task, and that there will be a clear distinction between intermittent fasting mice and aged controls.

Ongoing cohorts in the present intermittent fasting study will aid in determining the effect of caloric restriction in aging. Ultimately, research aims to discover potential therapeutic uses for caloric restriction with the overall hope to improve cognition and physiology in aging. Possible future experiments may include varying the duration of intermittent fasting and adding a young intermittent fasting group. Overall, the present study's main goals are to add to existing caloric restriction research which may ultimately contribute to finding possible therapies to aid in human aging.

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