

EFFECTS OF ENRICHMENT ON BEHAVIORAL ASPECTS OF HTAU MICE
MODELING ALZHEIMER'S DISEASE

by

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Committee:

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Effects of Enrichment on Behavioral Aspects of hTau Mice Modeling Alzheimer's
Disease

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of
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by

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DEDICATION

I dedicate this work to all the animals used in this study and throughout the history of research. They are the silent heroes who paid the ultimate sacrifice in pursuit of advancing scientific knowledge, and whose enduring spirit will forever foster a sense of curiosity and compassion.

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LIST OF ABBREVIATIONS AND SYMBOLS

“censored”.....	✕
Activity of daily living.....	ADL
Alpha.....	α
Alzheimer’s disease	AD
Amyloid precursor protein.....	APP
Amyloid precursor protein intracellular domain.....	AICD
Apolipoprotein E.....	APOE
Average.....	<i>M</i>
Beta	β
Beta-amyloid.....	A β
Calcium/calmodulin-dependent kinase II	CAMKII
Chi-square.....	χ^2
Gamma.....	γ
Glycogen synthase kinase 3-beta.....	GSK3 β
Human tau.....	hTau
Krasnow Animal Facility.....	KAF
Microtubule associated protein tau	MAPT
p-value.....	<i>p</i>
Presenilin.....	PSEN
Repeated-measures analysis of variance.....	rmANOVA
Soluble amyloid precursor protein-alpha.....	sAPP α
Soluble amyloid precursor protein-beta.....	sAPP β
Standard deviation	<i>SD</i>
t-score.....	<i>t</i>
Tau neurofibrillary tangles.....	NFT
Tetracycline-controlled transactivator protein.....	tTA

ABSTRACT

EFFECTS OF ENRICHMENT ON BEHAVIORAL ASPECTS OF HTAU MICE MODELING ALZHEIMER'S DISEASE

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George Mason University, 2024

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This thesis investigated the effects of repeated exposure to a nesting task and different forms of enrichment on Alzheimer's disease (AD)-modeling mice. Using hTau-expressing AD mice from strain rTg4510, the nesting study examined if continual exposure to a nestlet could prevent the decay in nesting ability over time. Alternatively, the aggression study explored the efficacy of cardboard tubes and nestlets in attenuating aggression in the noncarrier/noncarrier mice from strain rTg4510. Behavioral assays and survival analyses were conducted to assess nesting ability and aggression levels, respectively. Two-way repeated-measures ANOVAs revealed no statistically significant effect of treatment in the nesting study but there was a significant effect of time; Chi-squares revealed aggression levels varied across the aggression intervention groups with some significant effects of, and driven by, sex. Implications for animal welfare and future research are discussed, highlighting the need for further investigation into enrichment protocols and their behavioral impacts on laboratory animals.

CHAPTER ONE

Alzheimer's disease (AD) is a progressive neurodegenerative disorder often characterized by the decline in neurocognitive abilities, along with the appearance of two main pathologies: β -amyloid ($A\beta$) plaques and tau neurofibrillary tangles (NFTs). AD is further categorized into familial or "early-onset Alzheimer's" (occurring before the age of 65; is often inherited) and "late-onset Alzheimer's" (occurring after the age of 65; is often acquired later in life). It is also the most common form of dementia, with an estimated 6.7 million elderly Americans suffering from Alzheimer's disease as of 2023 (alz.org, 2023). There is currently no known cure for this ultimately fatal disease.

β -AMYLOID PLAQUES

Two of the most characterized aspects of AD are the pathologies responsible for the disease. Of the two pathologies, $A\beta$ plaques develop first. In the nonamyloidogenic pathway (i.e., the "normal" pathway that does not produce toxic pathology), amyloid precursor protein (APP) is cleaved first by the enzyme γ -secretase to produce soluble α -APP (sAPP α) fragments, then is further cleaved by α -secretase in a binding site that prevents $A\beta$ peptides from forming (de Paula et al., 2009). In the amyloidogenic pathway, APP is abnormally cleaved first by β -secretase to produce soluble β -APP (sAPP β) fragments, which is then further cleaved by γ -secretase to produce APP intracellular domain (AICD) and $A\beta$ peptides (de Paula et al., 2009). These cleavages are

often attributed to various mutations that affect the APP gene, with the location of the mutation influencing where the secretases will bind and cleave, which will ultimately regulate how much A β is generated. Currently, the genes with known mutation variants attributed to AD are APP found on chromosome 21, apolipoprotein E (APOE) alleles on chromosome 19, presenilin-1 (PSEN1) on chromosome 14, and presenilin-2 (PSEN2) on chromosome 1 (nia.nih.gov, 2023). APP mutations include variants such as the Swedish APPSW and London APPLON mutations, with different variants resulting in different rates of A β proliferation and varying levels of different A β peptides, including the Swedish mutation causing a threefold increase of A β ₁₋₄₀ and A β ₁₋₄₂ (TCW & Goate, 2017). APOE is the gene most closely associated with late-onset AD and occurs in three alleles: APOE e2, APOE e3, and APOE e4. The e2 allele may offer protective effects against AD; the e3 allele is the most common APOE allele and is thought to have a neutral effect on AD; the e4 allele is the most dangerous variant and significantly increases the risk of developing AD. PSEN1 and PSEN2 mutations regulate γ -secretase cleavages and shift where γ -secretase binds to the APP peptides, resulting in an increased accumulation of different A β species like A β ₁₋₄₀ or A β ₁₋₄₂. Along with APP mutations, the presenilin mutations are most closely associated with familial AD (Ashford, 2004). After cleavage, the A β species will then continue to aggregate and fibrilize until they form plaques that cause interference in synaptic signaling which results in neurons that are no longer able to function normally, resulting in neurodegeneration and neuroinflammation (Ittner & Götz, 2011). Generally, A β plaques first aggregate in the precuneus, posterior cingulate cortex, and orbitofrontal cortex before accumulating in various regions in the

cerebral cortex, including the hippocampus (Palmqvist et al., 2017). Not only do A β plaques cause extracellular damage, A β_{1-42} fibrils, a soluble and highly toxic A β species, have also been linked to the hyperphosphorylation of tau proteins (Götz et al., 2001). When A β_{1-42} was injected into the brains of tau modeling mice, the number of tau tangles found in neurons that projected to the sites of injection were increased by five times, indicating the presence of A β_{1-42} fibrils accelerate the formation of NFTs and are involved in the phosphorylation of tau (Götz et al., 2001).

TAU NEUROFIBRILLARY TANGLES

In the context of this study, understanding the underlying mechanisms behind tau hyperphosphorylation and NFT production is critical for identifying if external enrichments can affect change in or mitigate tauopathy and associated impairments in AD. Hyperphosphorylated microtubule associated tau proteins, or tau, are the second pathology associated with AD. In a healthy brain, tau appears to function as a phosphoprotein that interacts with and polymerizes tubulin into long chains of microtubules, and then helps to stabilize the microtubule structure to form a major component of cell cytoskeleton (Iqbal et al., 2010). In an abnormal brain, neurofibrillary tangles are the product of microtubule-associated tau proteins that have undergone abnormal hyperphosphorylation, which is when the phosphorylation sites on tau protein become fully saturated (Tiwari et al., 2019). This abnormal tau hyperphosphorylation seems to be mediated by the amyloidogenic pathway, as the polymerization of A β fibrils activate kinases, such as glycogen synthase kinase 3 β (GSK3 β) or calcium calmodulin-kinase II (CaMKII), that trigger hyperphosphorylation (Tiwari et al., 2019). As the tau

proteins continue to become hyperphosphorylated, the structural microtubule networks oligomerize into tubulin that lack the necessary support features needed to remain functional, and as a result, become destabilized and form large clumps of tau filament (Tiwari et al., 2019). As more clumps of destabilized tau proteins aggregate and form neurofibrillary tangles within a neuron, these tangles disrupt the regular cellular processes required to receive, process, and transmit signals between other neurons. Tau pathology generally first appears in the transentorhinal cortex and spreads throughout the hippocampus to the rest of the cerebral cortex (Voss et al., 2011). If a neuron is unable to process signals and successfully communicate with other neurons, it will undergo cell death. When many neurons localized to the same area undergo tau hyperphosphorylation and expire, this mass cell death contributes to AD advancement and furthers dementia (Tiwari et al., 2019).

BEHAVIORAL AND COGNITIVE IMPAIRMENTS IN AD

Behaviorally and cognitively, Alzheimer's disease typically presents with deficits in episodic memory, semantic memory and language abilities, executive functions, activities of daily living (ADLs), working memory, and visuospatial abilities (Weintraub et al., 2012). These deficits do not appear simultaneously, but instead develop as the neurodegeneration spreads throughout the cerebrum. Often one of the first areas affected, the hippocampus and surrounding cortices are critical in supporting episodic function, so as the hippocampus deteriorates, it becomes difficult to learn and retain new information, which is likely due to inadequate memory consolidation and storage of new information (Weintraub et al., 2012). As the degeneration spreads towards the association cortices

found in temporal, frontal, and parietal lobes, deficits in semantic memory become prominent (Weintraub et al., 2012). Activities of daily living also show marked decline as global degeneration occurs. ADLs are associated with many different areas of the brain, so as degeneration spreads, specific ADLs may be more impaired than others as that associated area of the brain becomes damaged (Marshall et al., 2012).

Nesting

Innate activities include different instinctive behaviors performed by all species that are genetically inherited and do not need to be learned or practiced (Damalas & Koutroubas, 2018). In mice, nesting is an example of an innate behavior and ADL that is found in both males and females. Nesting is done to fulfil needs for shelter, reproductive purposes, temperature regulation, and often displays the animal's state of wellbeing and quality of life. The inability of a mouse to adequately construct a nest generally indicates their wellbeing is hindered, which may be attributed to factors such as pain, injury, and distress (Gaskill et al., 2013). Lesions to the hippocampus can also cause deficits in nesting abilities, as identified by Deacon et al. in 2002. Female C57Bl/6J mice were lesioned to destroy the dentate gyrus and dorsal region of their hippocampus while sparing other cortical, thalamic, posterior ventral, and subicula areas. They were then run through a battery of tests, including nesting, which determined the lesioned mice struggled with impaired spatial working memory and spatial reference memory. The lesioned mice also demonstrated nesting deficits that were attributed to the hippocampal lesions, as there were no impairments to gross motor skills that would indicate a locomotive problem (Deacon et al., 2002).

Nesting deficits can also be widely attributed to AD pathology. Filali & Lalonde (2009) conducted a study aimed at identifying cognitive decline and impaired nesting ability in transgenic mice modeling the APPSW/PSEN1 mutation. The APPSW mouse model is useful as the human APPSW mutation results in five to ten-fold more soluble and insoluble $A\beta_{1-40}$ than $A\beta_{1-42}$ in the hippocampus and neocortex; cells harboring the mutation secrete high levels of sAPP β that influence where β -secretase and γ -secretase will cleave the APP and markedly affect behavioral abilities (Thinakaran et al., 1996). The human presenilin mutation was included in this strain to accelerate the rate of amyloidogenesis and make the pathology more pronounced. The experimental mice ranged between three months and 12 months of age and were run through a battery of behavioral tests. The nesting test demonstrated that the APPSW mice aged between six and 12 months experienced lower nesting activity than the control mice, but there were no significant differences between the two groups at ages earlier than six months. This is expected, as the APPSW mutation aggregates plaques in the hippocampus as early as six months and become abundant by nine months (alzforum.org). The relationship between $A\beta$ and nesting was further examined by Neely et al. in 2019 using a transgenic mouse model containing a humanized APOE e4 allele. Mice with the e4 allele built worse nests at six-, nine-, and 12-months when compared to the healthy controls; several of the e4 mice failed to interact with the nesting materials and failed to make a nest (Neely et al., 2019).

As a well-documented occurrence, AD pathology is known to cause severe damage to the hippocampus, which is a region that appears to be significantly involved

with nesting behavior. Lin et al. (2007) identified hippocampal neurons called nest cells in wildtype B6BCA/J mice that would selectively fire or cease to fire only during interactions with nests, however, nest building as a skill was neither examined nor measured during the study. The nests provided to the mice were prebuilt and made of various materials in various shapes and colors, a design choice used to determine what parameters a mouse considers when perceiving a nest. Changes to the nests in shape, style, material, color, or odor did not change the reactions of the nest cells when exposed to the stimuli, indicating that these hippocampal cells represent an encoded, conceptual understanding gained from episodic experience regarding nests and nesting. Using large-scale ensemble recording techniques and electrodes, Lin et al. (2007) measured neural activity in area CA1 of the hippocampus while the mice interacted with the nests, which revealed a very small number of cells that significantly responded to the nests. Based on the spike-discharge patterns, the researchers identified three groups of nest cells: transient-on type, persistent-on type, and persistent-off type. Transient-on type cells would significantly increase their firing transiently while the mice encountered the nests but remained nearly silent when the mice were not facing and perceiving the nests. Persistent-on type cells would significantly increase their firing the moment the mice entered the nests and would maintain increased activity until the mice left the nest; these cells would remain nearly silent the moment the mice left the nests until they returned. Persistent-off type cells would significantly increase their firing the moment the mice would leave the nests and would cease firing immediately upon the animals' heads crossing the threshold into the nests. To test for response selectivity, the mice were also

exposed to various objects to determine if the nest cells would respond to shapes similar and dissimilar (such as a drinking water cup or floor pad, respectively) to nests. Lin et al. (2017) did not find any significant change in nest cell reactivity to any of the non-nest objects, as encountering objects like food pellets and toys or partaking in behaviors like food and water consumption did not alter the nest cell firing patterns, further showing these cells exist to react specifically to nests. Other encoding features were examined, such as if the size of the nest affected the firing rate. Standard home nests (7.5cm diameter) and medium sized nests (twice the diameter of a standard nest, 15cm) elicited significant nest cell activity, but the super-sized nests (four times the diameter of a standard nest, 30cm) did not generate any nest cell activity, indicating a spatial and abstract understanding of what constitutes as a nest through functionality-based conceptualization (Lin et al., 2007). As nest cells make up a very small percentage of the hippocampus (Lin et al., 2007 identified only eight single units in seven mice), it is understandable that damage to the hippocampus, and thusly damage to nest cells, would result in deficits in nesting behavior.

Even though nesting is an innate and vital task for mice to complete, Alzheimer's-modeling mice often demonstrate impairments in completing the task. However, it is possible that repeated exposure to a certain task or behavior, or a type of repetition priming technique, may reduce the decline in ability to perform. Priming is a technique where the subject is exposed to a stimulus with the goal of influencing how the subject will react to a subsequent similar stimulus (apa.org). In 1994, Ostergaard utilized a long-term priming procedure through different word identification tasks. Participants had

either been diagnosed with Alzheimer's disease, an amnesic disorder, or were healthy controls. Participants were asked to work under various experimental conditions in which words were visually occluded but became more visible over time, providing a single exposure to the word; and a perceptual identification threshold task in which a word would be tachistoscopically presented with exposure duration increasing over time, providing multiple exposures to the word; then after a specified delay, word recall was tested. Ostergaard (1994) found that AD patients experienced compromised priming after a 10-minute delay from the single-exposure occlusion task, but there was a significant priming effect seen in recall after a 10-minute delay from the repeated-exposure tachistoscopic task. However, this significant priming effect after repeated exposure was only seen in the condition where the target words were presented four times, and in no other experimental condition. This demonstrates that repeated exposure during an acquisition phase, such as learning target words, can facilitate stronger memories and better recall in AD patients under the correct conditions. In the case of nesting impairments in transgenic Alzheimer's-modeling mice, it is possible that repeated exposure to a nesting task could prevent against cognitive decline.

Aggression

An issue faced by most researchers working with laboratory mice is dealing with aggression in their mouse colonies. Aggression between mice often results in pain or physical injury to an animal, and in extreme cases, mortality. In-cage fighting between group-housed mice affects not only the variables and monetary costs of a study, but the social and emotional wellbeing of the animals is also greatly affected, which in turn may

also affect study validity (Weber et al., 2017). While in the wild, an intrusive or submissive mouse may flee to safety if aggressed by another mouse, laboratory mice are typically confined to home cages with no means of escape if they encounter aggressive situations. Because of this, cases of aggression may result in the isolation of mice by removing an aggressor from the cage or separating an aggressed mouse from further harm; however, as mice are highly social animals, isolation is often emotionally distressing for the animal and reduces their wellbeing. Along with animal deaths inappropriately increasing attrition, the distress experienced from physical harm, social stressors, or isolation all can compromise a mouse's ability to perform in a study, which ultimately affects a study's validity (Weber et al., 2017).

Events are considered as a case of aggression if a mouse partakes in a behavior that elicits defensive or retaliatory responses from another mouse (Lidster et al., 2019). Aggressive behaviors can also be further categorized by the context of the interaction (such as fighting over territory versus organizing social structures) or the outcome of the interaction (such as mediation or escalation) (Theil et al., 2020). Regarding the outcome of aggressive interactions, mediation includes behaviors that successfully mitigate violence, such as submitting to the dominant mouse or fleeing; and escalation includes behaviors that result in violence or injury, and in extreme cases, death. There are many different reasons for why mice aggress each other. In males, fighting may occur as means to establish social hierarchies and exert dominance over submissive males; to protect territory from intruding males; to guard resources such as enrichment, toys, food, or mates; or as an expression of pain or frustration (Weber et al., 2017). Displays of

aggression in females is far less common but may also occur as means of establishing social hierarchies and exerting dominance over submissive females, or as an expression of pain or frustration (Williamson et al., 2019).

Despite understanding the causes of aggression, there are few solutions that can be implemented to reduce the amount of aggression experienced by male and female mice. Isolation is the most typical response to aggression, as removing the aggressor mouse from the home cage removes the primary threat, and removing the aggressed mouse protects them from further harm and gives them a safe place to recover. Unfortunately, once an animal is isolated, it is unlikely it will ever return to a group-housed environment and will remain in solitude (if not otherwise euthanized). However, isolation is not a proactive solution; it is a reactive solution that is only utilized after aggressive behavior is discovered. Singly housing mice can result in isolation syndrome, which may present as worsening physiologic changes to immune defense and pathologies, and behaviorally as stereotypies (ritualistic or repetitive actions), restlessness, or anxiety (Lockworth et al., 2015); all of which will affect the outcomes of a study. Isolation should be used only as a last resort if it has been determined that the rewards outweigh the risks. For example, while the emotional detriment of a mice from isolation is not ideal, it outweighs the attrition effect of an aggression-induced death.

While isolation is used reactively for aggression, there are very few, if any, preemptive or proactive measures that can be taken to prevent aggression from occurring in the first place. One such measure is the introduction of foraging crumbles into the bedding of the mice's home cages. A study recently conducted at George Mason

University was able to successfully decrease cases of aggression in male noncarrier/noncarrier Alzheimer's disease-modeling mice through the utilization of foraging crumbles by periodically sprinkling a small amount of the crumbles into the bedding of the cages. Because mice have such strong olfactory abilities and are driven by the need to forage for sustenance, the mice that received crumbles tended to be more focused on foraging throughout the cage and spent less time aggressing each other. These preliminary results indicate that there are in fact measures that can be taken to proactively defend against aggression, and that reactively isolating mice may not always be necessary. It is also important to note that the previously conducted study on foraging crumbles to mitigate aggression utilized male "wild type"-genotype mice from The Jackson Laboratory transgenic strain rTg4510, which is a model often used to study tauopathy in AD and frontotemporal dementia .Wild type (noncarrier for any abnormal genes) mice from strain rTg4510 are anecdotally known to be especially aggressive, particularly males housed together. As these noncarrier wild type mice are more aggressive than other mouse models, high instances of in-group fighting between mice can be detrimental to study designs utilizing this strain and behavioral outcomes .

CHAPTER TWO

This chapter delves into the reasoning and methodology behind the current study to build upon existing research to employ novel approaches in examining nesting behaviors and mitigating aggression in Alzheimer's-modeling mice. Through a combination of behavioral assays and enrichment strategies, this study aimed to better understand the mechanisms behind behavior and the implications for potential therapeutic interventions.

CURRENT STUDY

It has been demonstrated throughout several studies that nesting, an innate task, is often hindered in AD-modeling mice, likely due to damage of specific cells in the hippocampus. Previous studies have also shown the detriments of aggression and highlighted how the current reactive approach of isolation for aggression can also be detrimental, and with the lack of current literature discussing proactive means for mitigating aggression. Based on the findings from the literature, we proposed two separate studies.

The first was a study aimed at identifying if encouraging and engaging in nesting behaviors over time decreases the severity of nesting deficits in AD hTau mice when assessed during a nesting assay. To encourage nesting behaviors, laboratory mice are often provided a nestlet, which is a cloth square that can be shredded and torn to create

soft fibers that can be built into a nest. On the basis on nesting, we proposed that if hTau mice were continually provided a nestlet and were continually engaged in nesting behaviors, these actions would have a protective effect against degeneration of cells specific to nesting, which would prevent against deficits in performing the nesting task. As there is currently no literature that discusses if repeated exposure to an innate task in hTau-expressing mice can rescue or prevent impairments in performing the task as they age, implications include a better understanding of priming and how it may positively affect change in global behavioral abilities across different populations of AD, as well as general improvement in quality of life if mice successfully perform this instinctive task.

To determine if repeated exposure to a nestlet prevented against a decline in nesting ability over time, this study used a behavioral assay, nesting, to assess the behavioral ability of the experimental mice and identify any behavioral differences within the groups.

The proposed second study focused on the effects of different enrichment tools or interventions for reducing cases of aggression in a particular mouse model. On the basis of aggression, the proposed study aimed to identify if providing additional enrichment in the form of either cardboard tubes or nestlets were capable of attenuating levels of aggression in noncarrier/noncarrier hTau mice. Noncarrier hTau mice tend to have higher levels of aggression than typical laboratory mice, so it is vital to adopt solutions that are effective at limiting or preventing aggressive actions when using mice with who aggression is a known risk. Cardboard tubes and cloth nestlets were selected as enrichment variables because anecdotal experiences regarding cardboard tubes have

successfully lessened aggression cases in various strains of laboratory mice, and nestlets are commonly provided to the animals as an outlet for fulfilling an innate desire which in-and-of-itself may act as a redirection of behavior. Had this study been effective in successfully affecting a change in cases of aggression in hTau mice, implications may have included initiating better forms of enrichment and updating current protocols to reduce levels of aggression while maintaining study integrity by not needing to separate any laboratory animals. Results from these mice will be combined with a similar study that aimed to identify the effects of a specific enrichment, foraging crumbles, on cases of aggression in this specific strain of mice.

For the nesting study, it was hypothesized that (1) AD hTau mice that continually receive a nestlet would perform better during a nesting assay than AD hTau mice that did not receive a nestlet. For the aggression study, it was hypothesized that (1) noncarrier hTau mice that received a cardboard tube would demonstrate fewer cases of aggression than noncarrier hTau mice that did not receive additional enrichment. It was hypothesized that (2) noncarrier hTau mice that received a nestlet would demonstrate fewer cases of aggression than noncarrier hTau mice that did not receive additional enrichment.

METHOD

Animal Models

The experimental mice used in the aggression and nesting studies were bred from hTau-expressing mice from strain rTg4510. Mice from strain rTg4510 have been genetically modified to express MAPT P301L and CAMKIIa-tTA genes, of which both are required to develop and accumulate tau neurofibrillary tangles. The MAPT P301L

gene is the primary gene for expressing human Microtubule Associated Protein Tau (MAPT), which must be activated by the CAMKIIa-tTA for tau tangles to form. Female breeders were noncarriers for both genes, while male breeders were carriers for both genes. Using noncarrier female breeders decreased the likelihood of pup cannibalization or abandonment. This genetic pairing of breeders produced three types of offspring: heterozygotic pups, fully transgenic pups, and noncarrier/noncarrier pups. Heterozygotic offspring carried either the MAPT P301L gene or the CAMKIIa-tTA gene, but not both. As these offspring were not carriers of both genes, they were not used in the experiment. Any heterozygotic offspring were either humanely euthanized or transferred to a training protocol. The transgenic (AD) offspring were carriers of both the MAPT P301L gene and the CAMKIIa-tTA gene and developed tau neurofibrillary tangles as they aged. Pretangles could be seen by two-and-a-half months; tangles were expected to be forming in the hippocampus at around four months. These transgenic offspring were the focus of the proposed nesting study. The final group of offspring, the noncarrier/noncarrier (“noncarrier”) pups, would carry neither gene nor develop any AD pathology as they aged. These noncarrier offspring were the focus of the proposed aggression study.

To breed for the aggression and nesting studies, transgenic rTg4510 males and noncarrier rTg4510 females were ordered from the Jackson Laboratory or transferred from a separate protocol to be used in breeding. Male breeder mice arrived between four-weeks and five-weeks-old; six-month-old female breeders were transferred from a separate protocol. After arrival to the Krasnow Animal Facility (KAF), breeder mice

were housed for one week to allow for acclimation. Males were singly housed and females were housed in groups of two. After one week had passed, a handful of bedding from each cage was placed into cages of the opposite sex to allow for familiarization of scent to aid in breeding and induce estrus in the females. Bedding swaps occurred daily for one week. After one week passed, harem breeding was conducted, with two females per one male. Harem breeding occurred for 14 days, after which all animals were separated and singly housed. This was to prevent potential pup cannibalization, unintentional fostering, and overcrowding of pups. Female breeders were checked daily for signs of a vaginal plug (gestational day 0 to 0.5). As not all females plug, other signs of pregnancy included a pear-shaped appearance and enlarged nipples.

After the breeder females gave birth, all pups were ear-punched and genotyped between 17 and 28 days after birth. Ear-punching allowed for identification of individual animals. Genotyping was done by reserving the ear punch tissue and sending these samples to Transnetyx for genotype analysis to determine which of the pups would be used as experimental animals. Pups were weaned 21 days after birth. Weaned experimental offspring were sexed and housed with littermates of the same sex in rat-sized cages. Animals were housed in KAF room L025, which was on a 12-hour light/dark cycle. Each cage had *ad libitum* access to a hopper filled with unautoclaved 7012 feed, water from a lixit attached to the cage rack water system, igloos, and a running wheel, providing enrichment consistent with experiments done in our lab. If a mouse singly housed, it received the same enrichment and was provided a Nyla bone.

Breeder males and females were used to produce more than one litter, as needed. After the pups from the first litters were weaned after 21 days of birth, the female breeders remained singly housed for seven days. After seven days, they underwent harem breeding under the same conditions. After the pups were born, they underwent the same timeline and procedures for ear-punching, genotyping, weaning, and the experimental conditions.

Nesting Study

For this nesting study, only the P301L-CAMKII carrying AD offspring were used. Once genotype was confirmed, the experimental AD mice were randomly assigned to either receive a nestlet, or not receive a nestlet for the duration of the study and act as the control. Only carrier AD offspring were used; no undesirable offspring acted as littermates to remove a confounding variable of heterozygotic mice not displaying tauopathy. All animals were housed in rat-sized cages with *ad libitum* access to a hopper filled with unautoclaved 7012 feed, water from a lixit attached to the cage rack water system, igloos, a running wheel, and if applicable, the nestlet. If an animal was isolated and singly housed, it continued to receive its assigned enrichment and received a Nyla bone. All experimental animals were handled by a researcher twice a week for the duration of the paradigm. This enrichment paradigm continued uninterrupted until the animals were four months old, after which they underwent a nesting behavioral assay to identify any differences in behavioral abilities. While aggression was not the focus of this study, cases of aggression and isolation were also documented.

All mice were housed with littermates of the same sex, with only the AD offspring being used. Up to four males or up to six females were housed per cage. A minimum of 8 experimental mice were assigned to receive a nestlet, 8 mice would not receive a nestlet. Nestlets were ordered from Ancare and were not autoclaved prior to use. Mice assigned to the nestlet condition received their first nestlet the day they were weaned and received replacement nestlets once their current intervention was no longer useable (e.g., the nestlet was completely shredded). After the animals turned four months, they underwent a nesting behavioral assay to assess nesting abilities across the two groups to determine if repeated exposure to a nestlet affected nesting abilities in the AD mice.

Table 1 No. of Animals Assigned to Each Nesting Intervention Group

	rTg4510 carriers AD
Nestlet	$n = 12$
Control	$n = 8$

Nesting Behavioral Assay. Once the animals in the nesting study turned four months old, the nesting paradigm ended, and they underwent a behavioral assay designed to measure a specific behavioral ability: nesting. During the nesting assay, all animals were individually housed to assess each animal's behavioral abilities. Animals could not be group-housed during this assessment as the researchers would not be able to determine

the individual contributions to nesting. All animals in the nesting study participated in the nesting assay.

A nesting assay is an assessment of the animal's ability to carry out an activity of daily living and innate task, and acts as a measure of wellbeing. The nesting assay was conducted in KAF L29 and followed the procedures of Deacon (2006). Mouse cages were fitted with a glass bottles holding facility water, a tray of HydroGel, a hopper holding unautoclaved 7012 feed, and filled with corncob bedding that covered the entire bottom of the cage to ensure that the animal used the provided nesting material and not the bedding to construct a nest. Each cage had exactly 3g of nestlet placed into the cage as nesting material. Each nestlet was weighed and material was trimmed as needed so each cage received the same amount. No additional enrichment items were added to the cages. The first data collection occurred two hours after the nesting assay began and consisted of taking pictures of each nest and weighing the amount of nestlet material that had been utilized (also known as touched mass). The second data collection occurred 18 hours after the nesting assay began and consisted of taking pictures of each nest and weighing the amount of used nestlet material (touched mass). The assay ended after 18 hours, and the animals were returned to their home cages to await euthanasia. "Blind" researchers visually scored the pictures of the nests on a scale of 1 – 5. A score of 1 indicated the nestlet remained untouched, and a score of 5 indicated all the nestlet was used to build an identifiable nest.

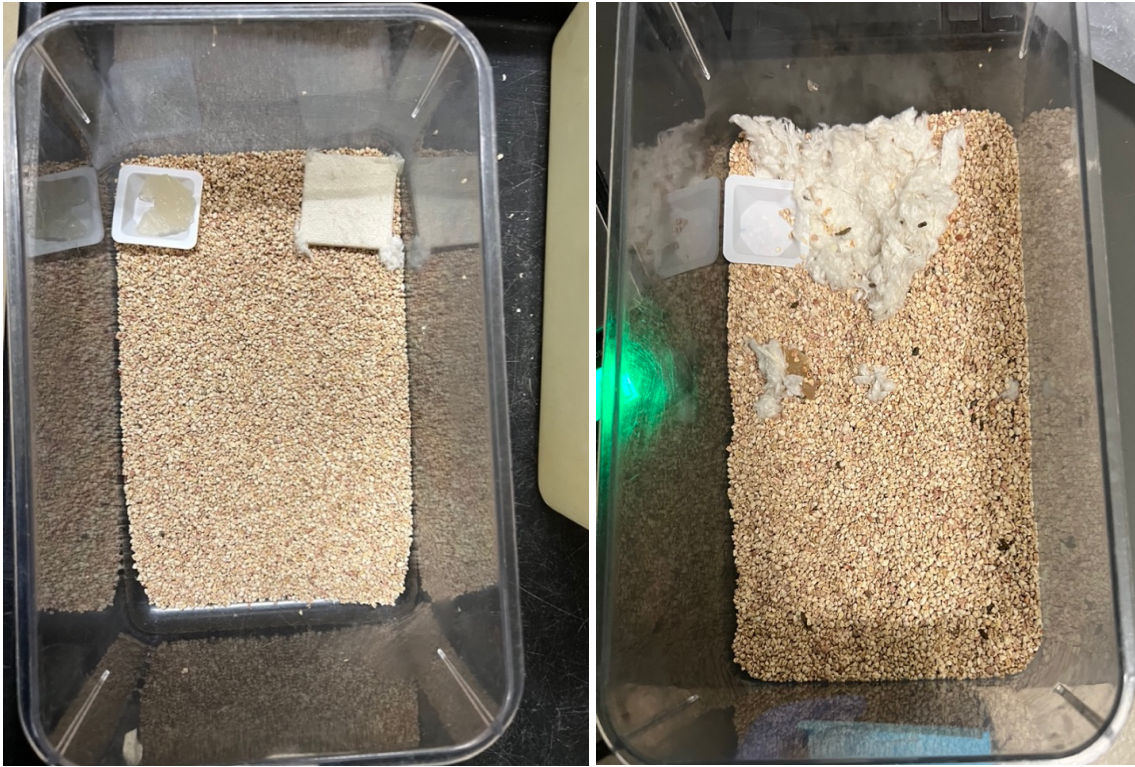


Figure 1 Example of Nesting Assay Scored as “1” and “5” Respectively

Aggression Study

For this aggression study, only the noncarrier offspring were used. Once genotype was confirmed, the experimental noncarrier animals were randomly assigned to an enrichment group. There were three different enrichment groups: mice that received a cardboard tube, mice that received a nestlet, and mice that did not receive either intervention. All animals were housed in rat-sized cages with *ad libitum* access to a hopper filled with unautoclaved 7012 feed, water from a lixit attached to the cage rack water system, igloos, and a running wheel. If an animal was isolated and singly housed, it

was subsequently humanely euthanized and the time (in days) until isolation was recorded. Once isolated, an animal could not be reintroduced to group housing. All experimental animals were handled by a researcher twice a week for the duration of the paradigm. This enrichment paradigm continued uninterrupted until the animals were at least four months old.

Cardboard tube enrichment group. The cardboard tube enrichment mice were consistently provided with cardboard tubes. All mice were housed in rat-sized cages and were housed with littermates of the same sex, with only the noncarrier offspring being used. Up to three males or up to four females were housed per cage. In cages containing two mice, one cardboard tube was provided. In cages containing three or more mice, two cardboard tubes were provided. A minimum of eight experimental mice were assigned to receive a cardboard tube. Cardboard Bio-Tunnels for Mice (tubes) were ordered from Bio-Serv and were not autoclaved prior to use. Mice in the first cohort received their first cardboard tube five days after they were weaned, and mice in the second cohort received their first cardboard tube the day they were weaned. Both cohorts received replacement tubes once their current item was no longer useable (e.g., the tube had been destroyed). Animals had *ad libitum* access to the cardboard tubes, as well as other standard enrichment, food, and water. Aggression was closely monitored.

Nestlet enrichment group. The nestlet enrichment mice were consistently provided with nestlets. A nestlet is a small square of cloth typically made of cotton fibers that can be torn and shredded to create nesting material. All mice were housed in rat-sized cages and were housed with littermates of the same sex, with only the noncarrier

offspring being used. Up to three males or up to four females were housed per cage. In cages containing two mice, one nestlet was provided. In cages containing three or more mice, two nestlets were provided. In each cohort, a minimum of eight experimental mice were assigned to receive a nestlet. Nestlets were ordered from Ancare and were not autoclaved prior to use. Mice received their first nestlet the day they were weaned and received replacement nestlets once their current item was no longer useable (e.g., the nestlet had been completely shredded). Animals had *ad libitum* access to the nestlets, as well as other standard enrichment, food, and water. Aggression was closely monitored.

No additional enrichment (control) group. The final group of experimental mice received neither a cardboard tube nor a nestlet and functioned as the control group. All mice were housed in rat-sized cages and were housed with littermates of the same sex, with only the noncarrier offspring being used. Up to three males or up to four females were housed per cage. In each cohort, a minimum of eight experimental mice were assigned to this group. Animals continued to have *ad libitum* access to the other standard enrichment, food, and water. Aggression was closely monitored.

Table 2 No. of Animals Assigned to Each Intervention Group in Cohort One

	Cardboard Tube	Nestlet	Control
rTg4510 Noncarrier/noncarrier	<i>n</i> = 10	<i>n</i> = 9	<i>n</i> = 11

Table 3 No. of Animals Assigned to Each Intervention Group in Cohort Two

	Cardboard Tube	Nestlet	Control
rTg4510 Noncarrier/noncarrier	$n = 8$	$n = 8$	$n = 9$

Table 4 No. of Animals Assigned to Each Intervention Group in Both Cohorts Combined

	Cardboard Tube	Nestlet	Control
rTg4510 Noncarrier/noncarrier	$n = 18$	$n = 17$	$n = 20$

Monitoring of aggressive behaviors. Cases of aggression were closely monitored by both the researchers and KAF animal care technicians. Each animal underwent multiple wellness checks daily by care staff and researchers for the duration of the study, where their general health and appearance were assessed and examined for evidence of injury or distress. Training was provided to ensure staff and researchers understood what behaviors or injuries to monitor. Animals were not monitored overnight but were carefully examined each morning. Cases were considered aggression if a mouse partook in a behavior that elicited defensive or retaliatory responses from another mouse (Lidster et al., 2019). Aggression was documented if more than one mouse was witnessed partaking in fighting behaviors such as mounting another mouse, displaying threatening postures, biting at another mouse (e.g., the genitals, tail, face, or ears), chasing another

mouse around the cage, vocalizing during interactive behavior, tail rattling, or submission of a subordinate mouse to the aggressor mouse. Aggression was also documented if injuries were identified on a mouse in the absence of witnessing the aggressive behaviors. Aggression-induced injuries often included hair loss or wounds on the tail, rump, lower back, or upper back. When applicable, documentation included the date aggression was witnessed, identification of the aggressor mouse, identification of the aggressed mouse, what behaviors were witnessed, and any injuries or deaths discovered. If injuries were discovered, KAF animal technician staff treated the injuries accordingly, and if necessary, consulted with the KAF veterinarian. If an aggressor mouse was found severely fighting with submissive mice, it was isolated from the main. If an animal had severe injuries, it was also isolated from the main cage. Once an animal was isolated and singly housed, it was not permitted to return to the main cage and remained in isolation for the duration of the study. As isolated animals were no longer producing data, they were humanely euthanized to minimize cage costs and undue suffering. Cases of, and time to, isolation were documented.

CHAPTER THREE

This chapter will present the outcome of the nesting and aggression studies, which investigated the effects of repeated exposure to a nesting task and different forms of enrichment on behavior in AD-modeling mice. Using a variety of measurements and statistical analyses, the impact of the behavioral interventions are better understood.

RESULTS

Nesting

Nesting was measured by administering a nesting behavioral assay to determine if mice who were provided repeated exposure to a nesting task would perform significantly better than mice who experienced the nesting assay as a novel task. The first method of measuring ability was through a subjective measurement of scoring the nest construction two hours after initiating the assay and 18 hours after initiating the assay. The second method of measuring nesting ability was through an objective measurement of the weights of touched nesting materials two hours after initiating the assay and 18 hours after initiating the assay. Both measurements were analyzed through a two-way repeated-measures analysis of variance (rmANOVA).

Subjective data (visual score). A two-way repeated-measures ANOVA with a Greenhouse-Geisser correction showed a statistically significant effect of time on visual nest score ($F(1, 18) = 6.278, p = .022$). The average visual nest score improved from the

two-hour measurement ($M = 1.515$, $SD = 0.569$) to the 18-hour measurement ($M = 2.210$, $SD = 1.326$) independent of the nestlet intervention (see Figure 2). An interaction effect between time and nestlet intervention did not show to be statistically significant, but there was a positively trending effect of time and intervention on visual nest score ($F(1, 18) = 3.935$, $p = .063$). Mice with repeated exposure to a nestlet improved from an average score of 1.350 ($SD = 0.432$) at two hours to an average score of 2.425 ($SD = 1.540$) at 18 hours, whereas the control mice scored on average 1.763 ($SD = 0.684$) at two hours and only improved to an average score of 1.888 ($SD = 0.922$) at 18 hours (see Figures 3 and 4).

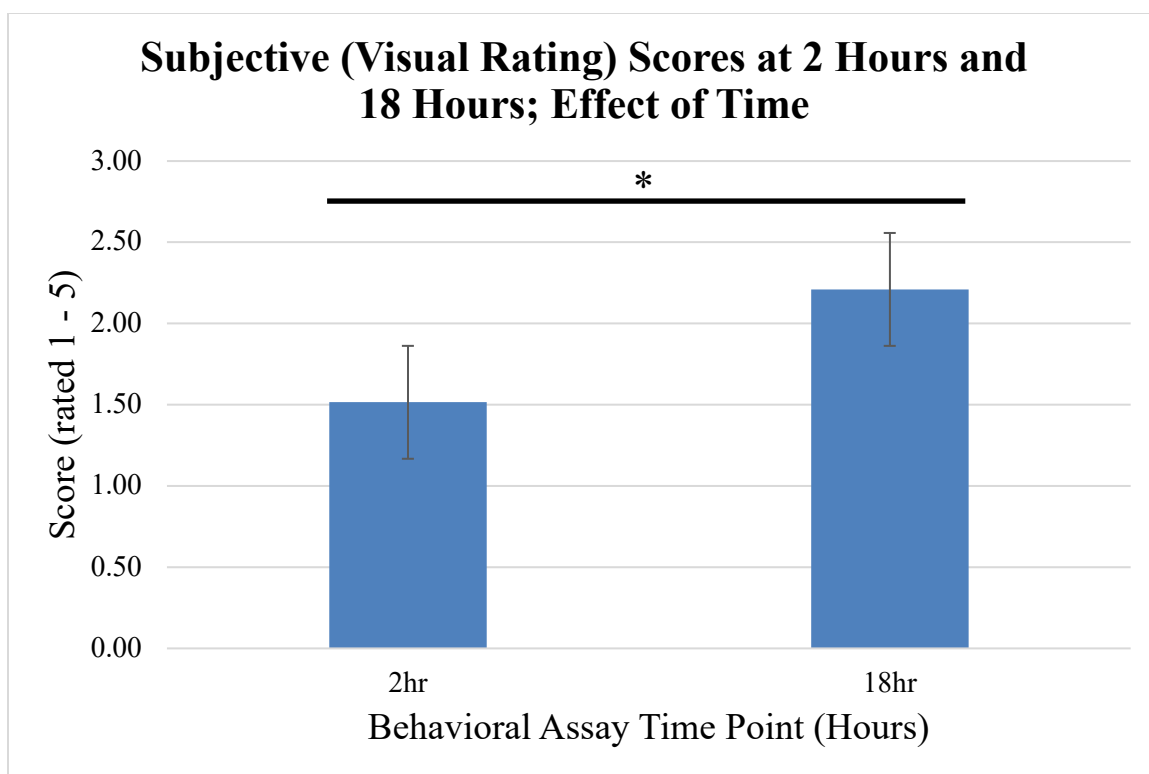


Figure 2 Overall Subjective Nesting Scores at Both Time Points

Note. * indicates significance at $p = .022$. Error bars represent standard error.

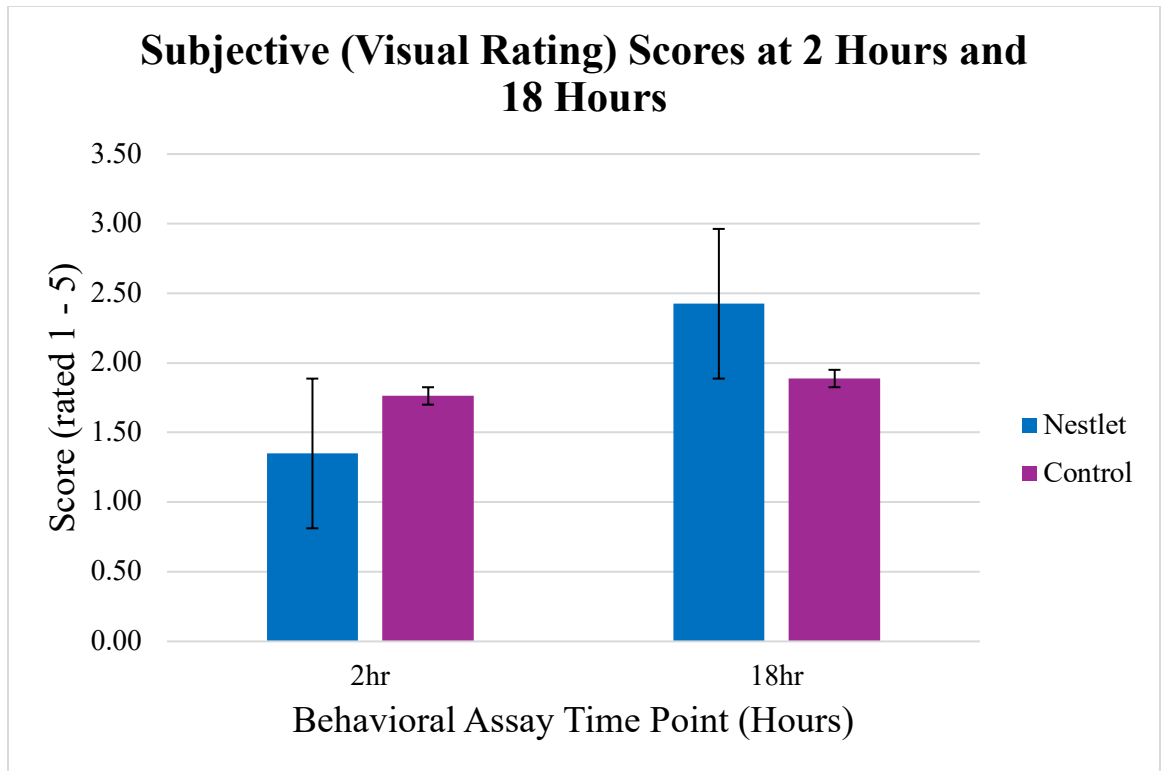


Figure 3 Subjective Nesting Scores at Both Time Points

Note. Error bars represent standard error.

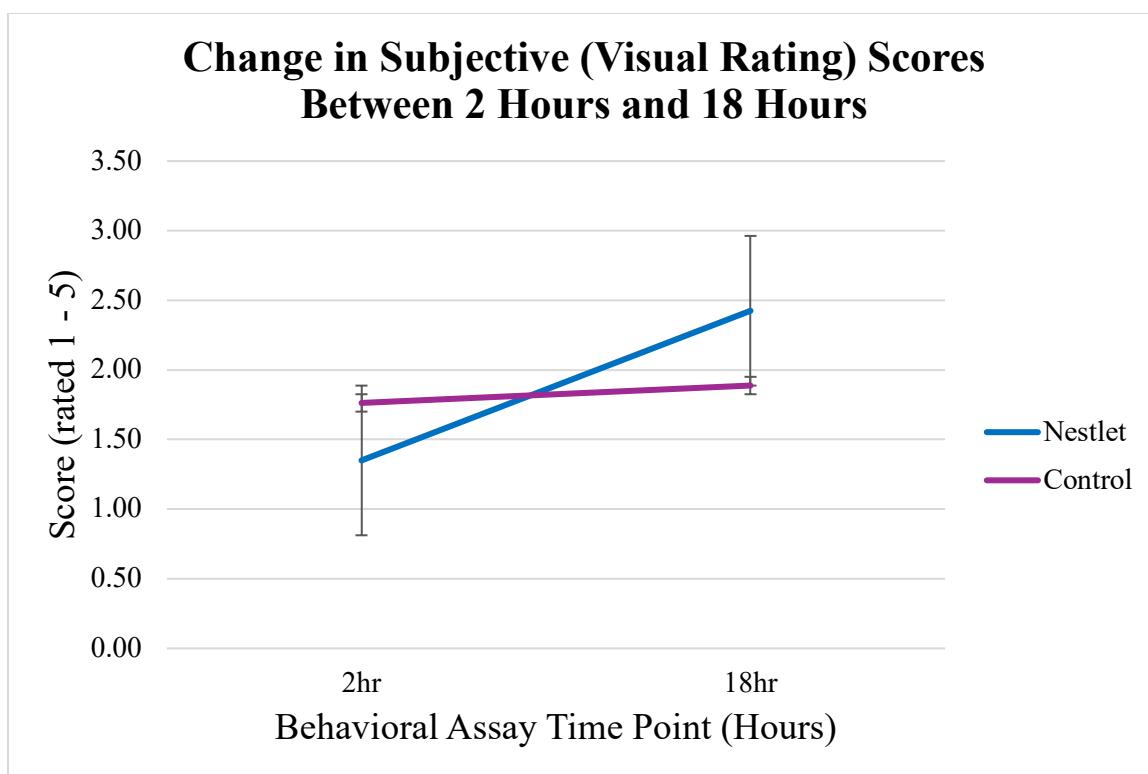


Figure 4 Subjective Nesting Score Changes Over Time

Note. Error bars represent standard error.

Objective data (touched nestlet mass). A two-way repeated-measures ANOVA with a Greenhouse-Geisser correction showed a statistically significant effect of time on the objective nest masses ($F(1, 18) = 10.143, p = .005$). The average weight of the touched nesting materials improved from the two-hour measurement ($M = 2.255g, SD = 0.302$) to the 18-hour measurement ($M = 2.885g, SD = 0.936$) independent of the nestlet intervention (see Figure 5). However, an interaction effect between time and nestlet intervention did not show to be statistically significant ($F(1, 18) = 2.941, p = .104$), though a slight positive trend is apparent. Mice with repeated exposure to a nestlet

improved from an average touched nestlet mass of 2.200g ($SD = 0.263$) at two hours to an average touched nestlet mass of 3.075g ($SD = 1.038$) at 18 hours, but control mice used on average 2.338g ($SD = 0.354$) of the nestlet at two hours and only improved to an average touched nestlet mass of 2.600g ($SD = 0.729$) at 18 hours (see Figures 6 and 7).

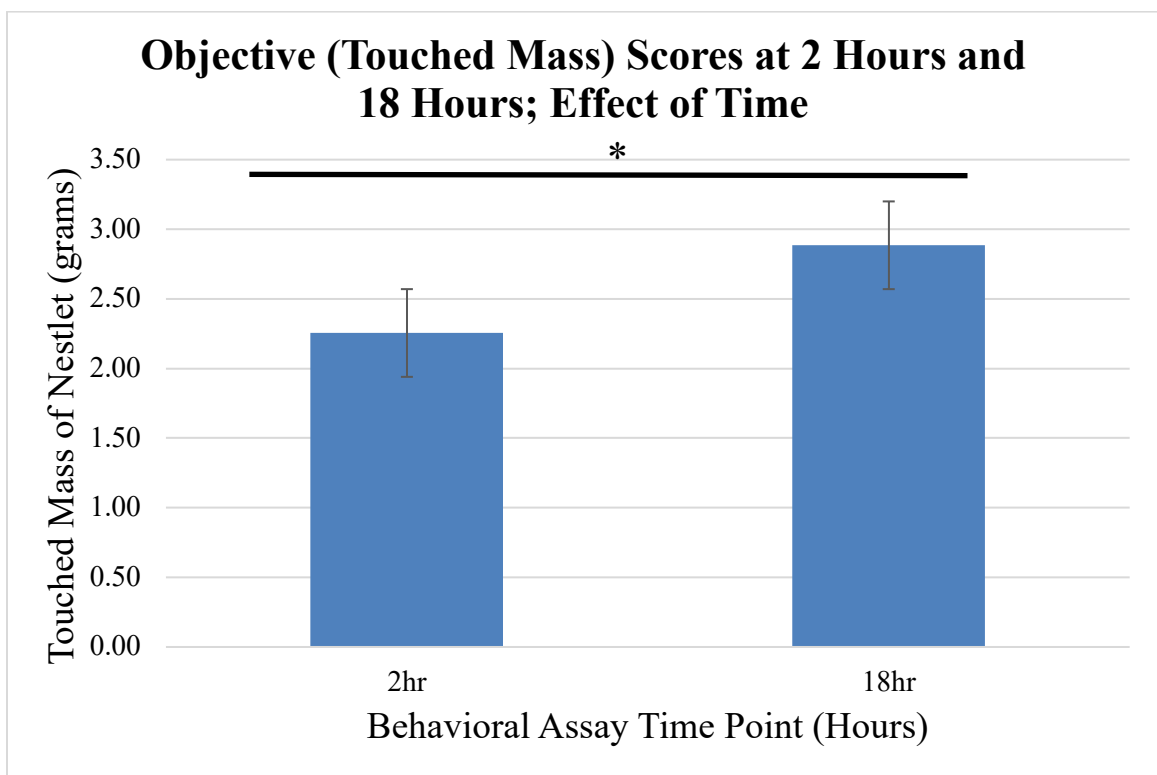


Figure 5 Overall Objective Nesting Scores at Both Time Points

Note. * indicates significance at $p = .005$. Error bars represent standard error.

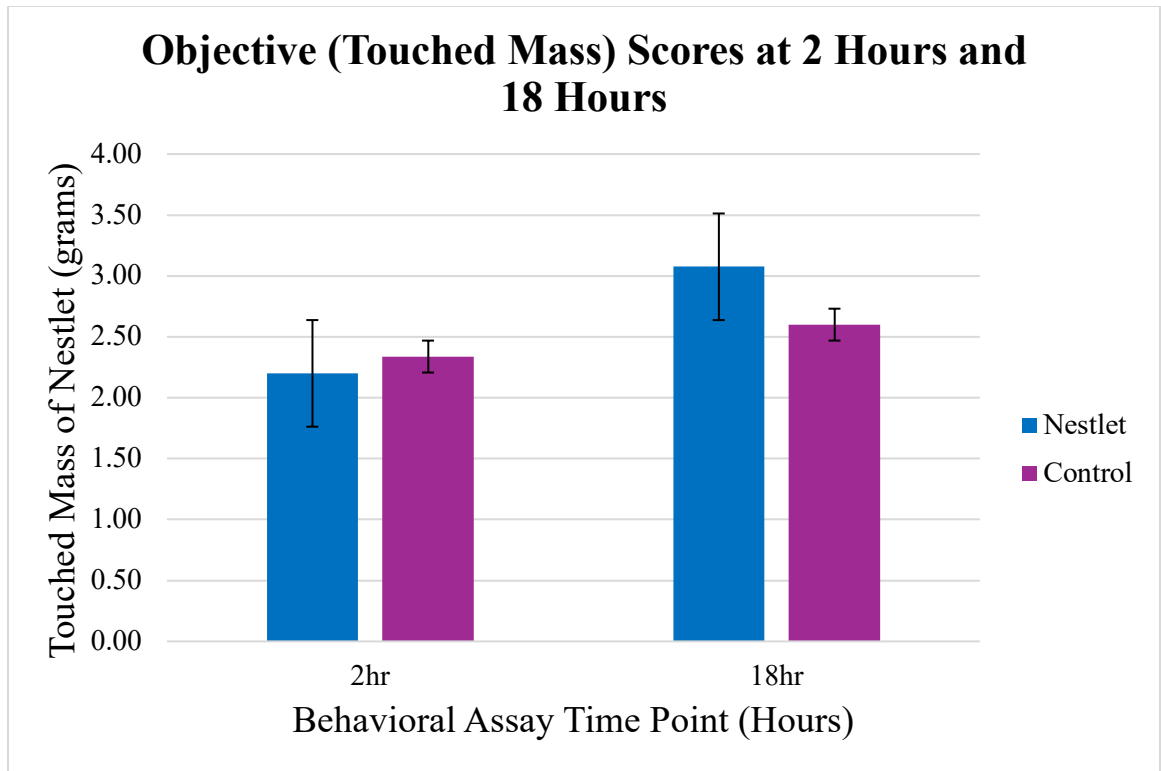


Figure 6 Objective Nest Masses at Both Time Points

Note. Error bars represent standard error.

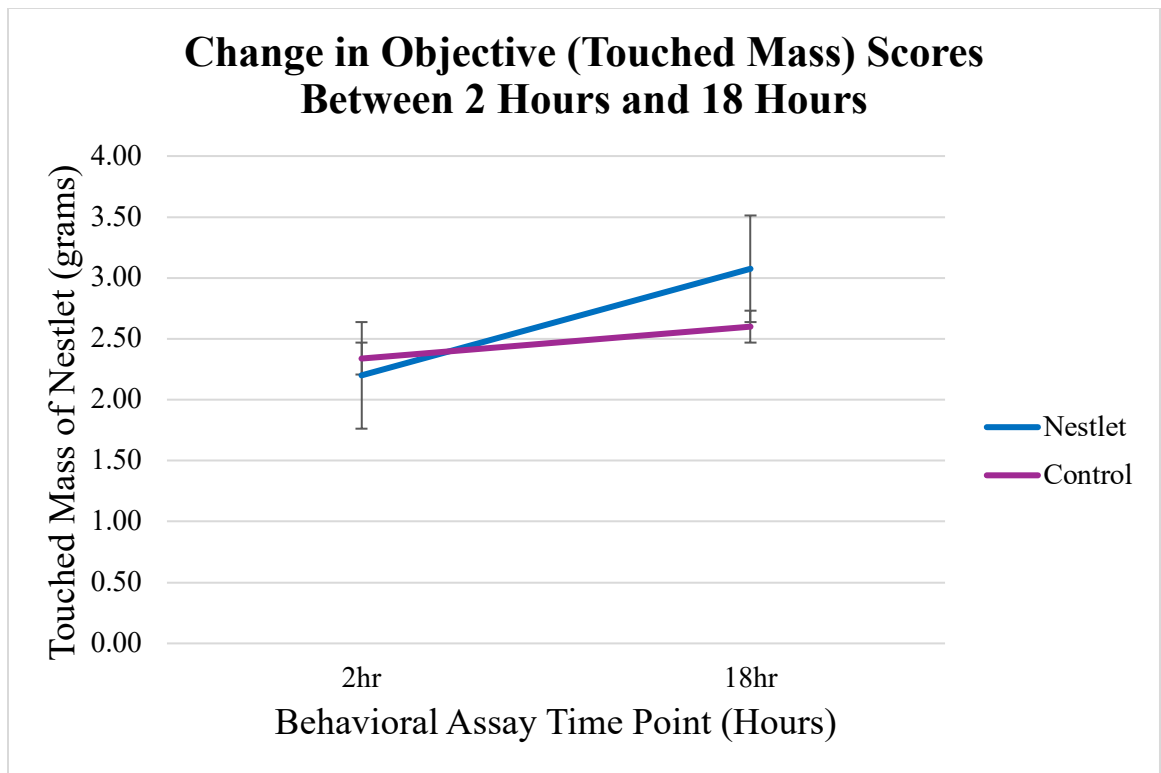


Figure 7 Objective Nest Masses Changes Over Time

Note. Error bars represent standard error.

Aggression

Aggression was monitored daily over the duration of the study, and instances of aggression resulted in the identification and isolation of the aggressor mouse, identification and isolation of the aggressed mouse, and identification and subsequent isolation of an uninvolved mouse (if applicable). The date a cage was isolated was recorded, and analyses were performed based on how many days each mouse lasted in a group-housed environment and to test for differences between each month.

Cohort one. A Kaplan-Meier Survival Curve under a Breslow test was run to determine if there were differences in the survival distribution for the different forms of

enrichment used to attenuate aggression. In cohort one, there was no statistical significance in the survival distributions for the three enrichment groups, $\chi^2(2) = 1.957, p = .589$; Figure 8 indicates on average, 38.9% of mice provided with a nestlet survived to the end of the study ($M = 57.481$ days, $SD = 13.350$), 30% of mice provided with a cardboard tube survived to the end of the study ($M = 55.200$ days, $SD = 10.698$), and lastly, 0% of mice provided with no additional enrichment (control group) survived as they were all isolated by day 102 ($M = 50.727$ days, $SD = 12.606$). As indicated by the ✂ symbols in Figure 8, if a population decrease was due to an event unrelated to aggression, such as an unrelated health condition, or there was a lack of involvement in an aggression incident, that animal's date of isolation was marked as "censored". This can be seen in Cohort One at approximately 10 days, when an animal assigned to the Nestlet group was isolated because of an aggression incident between two other animals; the aggressed mouse was isolated for injuries, the aggressor mouse was isolated to prevent further harm from occurring, and as a result, the uninvolved third mouse was subsequently isolated and censored. Censoring is also seen in Cohort One at approximately 102 days. All animals in the Control group had been isolated around the 100-day period, ending the study, resulting in the remaining mice in the Tube and Nestlet groups censored as they never experienced an aggression incident by day 102. Figure 9 visualizes the decay of each intervention group at a weekly count.

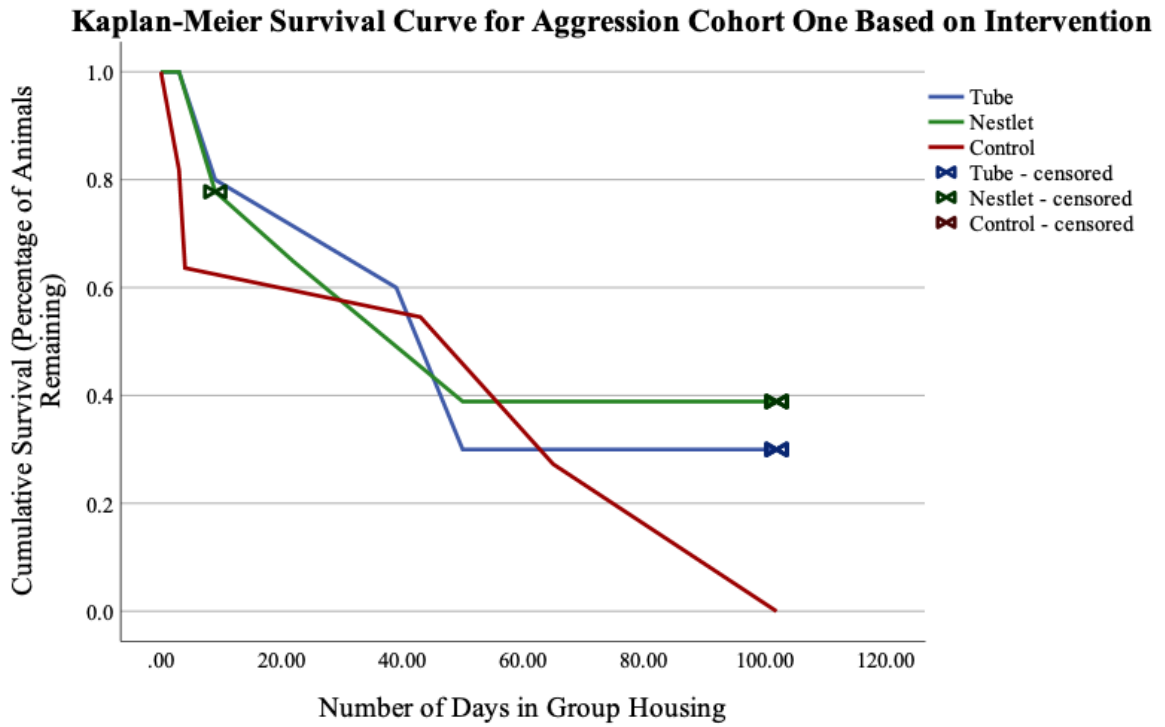


Figure 8 Cohort One Survival Curve Based on Intervention

Note. Censored (⌘) indicates an instance where an animal was removed from the study for a reason unrelated to aggression, or because the study had completed and the remaining animals never experienced aggression.

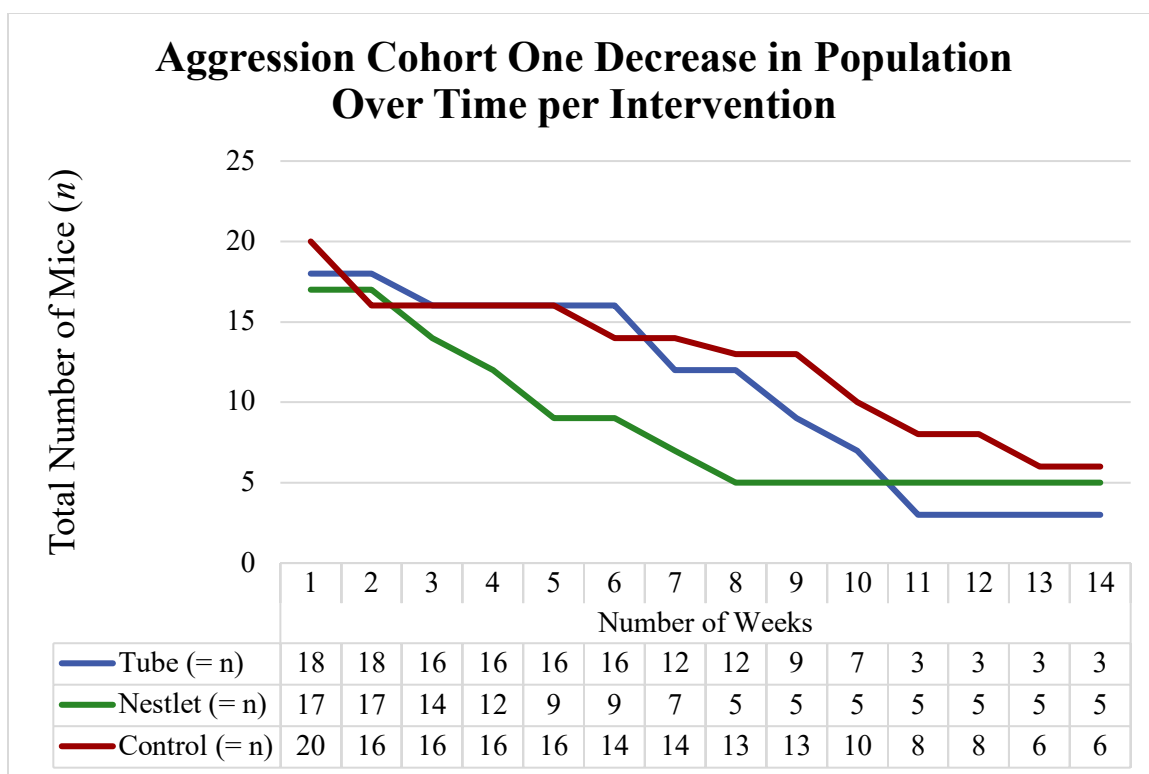


Figure 9 Cohort One Decrease in Population Over Time

Note. Table represents number of animals left per intervention group at each week.

Cohort one was also examined for any interactions with sex, to which there was statistical significance in the survival distributions for male and female mice, $\chi^2(1) = 15.049, p < .001$ (see Figure 10). On average, 60% of female mice survived to the end of the study ($M = 96.100$ days, $SD = 6.463$), but by approximately 65 days, all male mice had been isolated (see Figure 10). As indicated by the ✕ symbols in Figure 10, if a population decrease was due to an event unrelated to aggression, such as an unrelated health condition, or there was a lack of involvement in an aggression incident, that

animal’s date of isolation was marked as “censored”. This is seen at approximately day 9 as a male uninvolved with an aggressive incident was isolated as a result of removal of the aggressor and aggressed mouse, and around day 100 when all remaining female mice concluded the study. Figure 11 visualizes the decay of only the female population based on intervention at a weekly count, and Figure 12 visualizes only the male population decay.

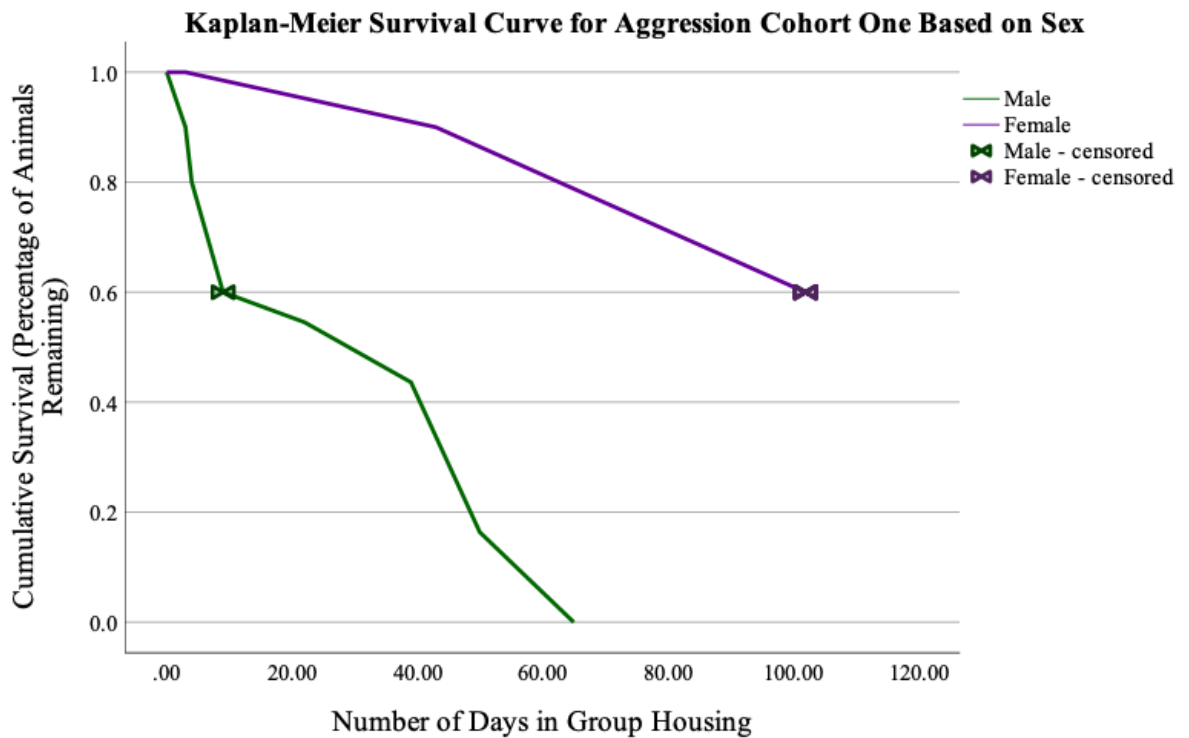


Figure 10 Cohort One Survival Curve Based on Sex

Note. Censored (☒) indicates an instance where an animal was removed from the study for a reason unrelated to aggression, or because the study had completed and the remaining animals never experienced aggression.

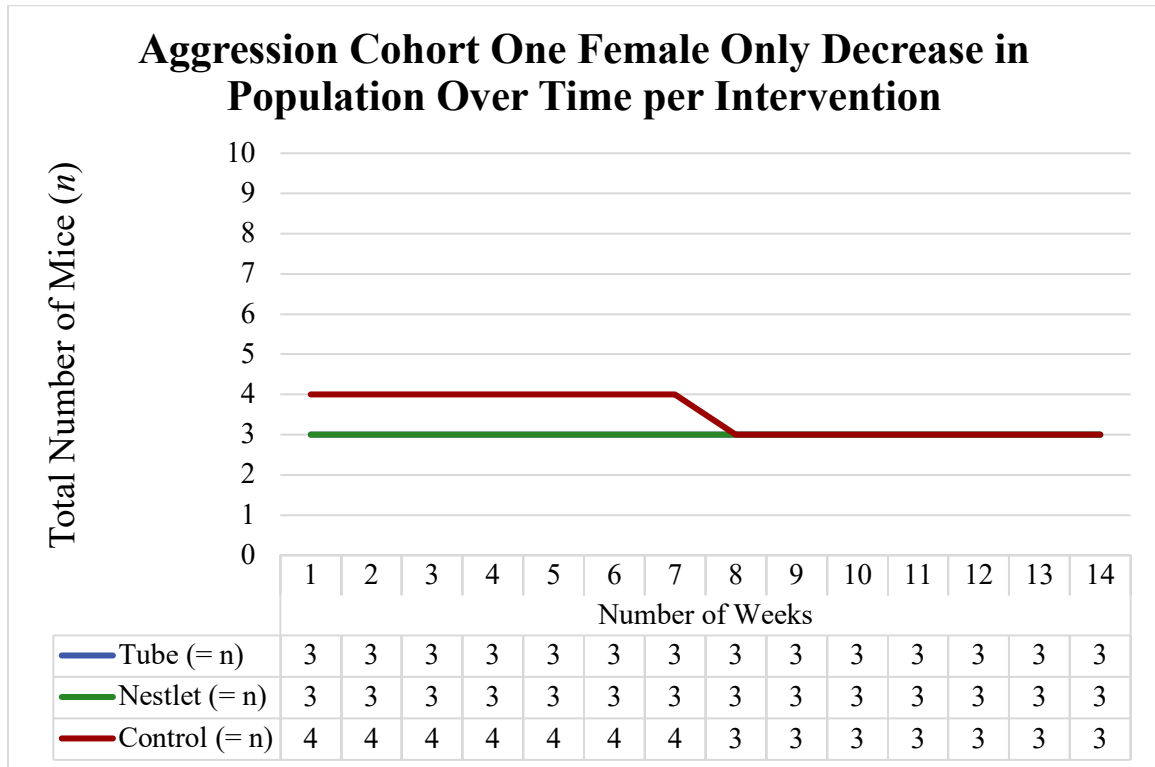


Figure 11 Cohort One Female Only Decrease in Population Over Time

Note. Table represents number of animals left per intervention group at each week. The cardboard tube condition population competes with the nestlet condition population but is present.

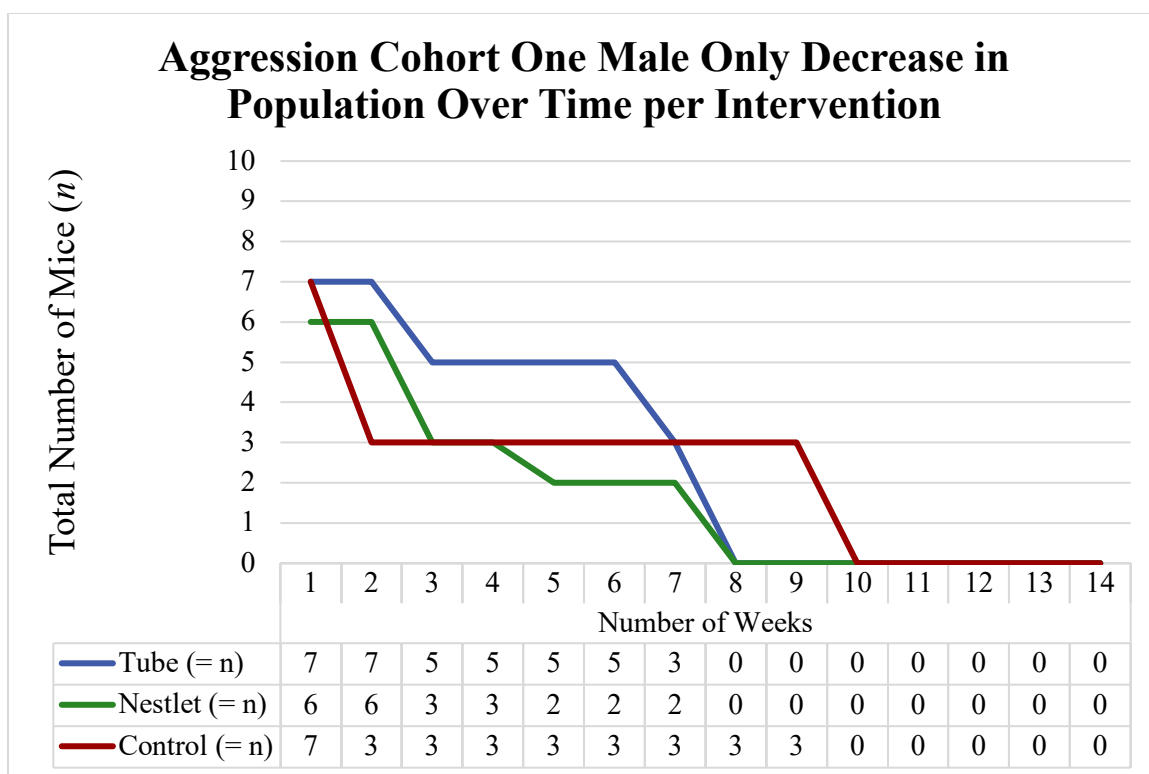


Figure 12 Cohort One Male Only Decrease in Population Over Time

Note. Table represents number of animals left per intervention group at each week.

Cohort two. In cohort two, there was no statistical significance in the survival distributions for the three enrichment groups, $\chi^2(2) = 5.238, p = .073$, but there was a positive trend; Figure 13 indicates on average, 33.3% of mice provided with no additional enrichment (control group) survived until the end of the study ($M = 74.333, SD = 8.731$), 25% of mice provided with a nestlet survived to the end of the study ($M = 57.750, SD = 4.761$), and lastly, 0% of mice provided with a cardboard tube remained group-housed by day 68 ($M = 45.500, SD = 11.296$). As indicated by the ✖ symbols in Figure 13, if a

population decrease was due to an event unrelated to aggression, such as an unrelated health condition, or there was a lack of involvement in an aggression incident, that animal's date of isolation was marked as "censored". In cohort two, reaching approximately 100 days marked the end of the study, and any remaining animals were marked as censored per lack of aggression, as seen in the Control and Nestlet groups. It is important to reiterate that by around day 68, all animals assigned to the Cardboard tube group had been isolated. Figure 14 visualizes the decay of each intervention group at a weekly count.

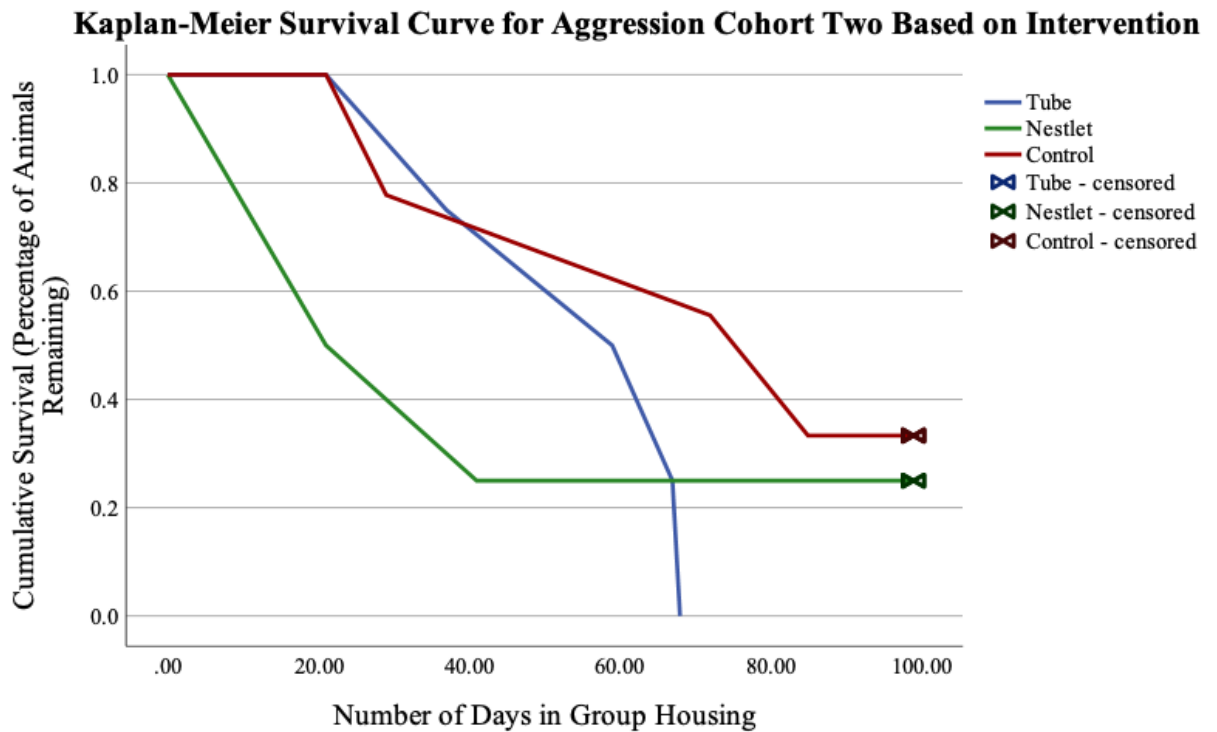


Figure 13 Cohort Two Survival Curve Based on Intervention

Note. Censored (X) indicates an instance where an animal was removed from the study for a reason unrelated to aggression, or because the study had completed and the remaining animals never experienced aggression.

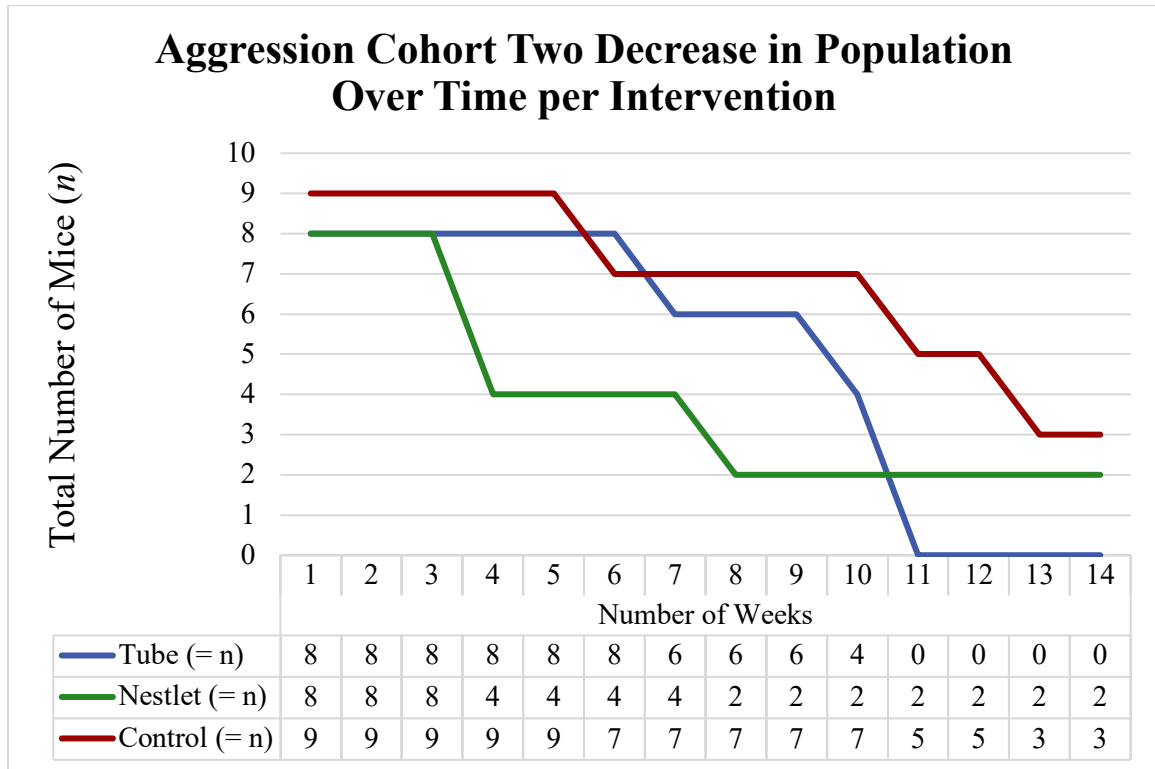


Figure 14 Cohort Two Decrease in Population Over Time

Note. Table represents number of animals left per intervention group at each week

Cohort two was also examined for any interactions with sex, to which there was no statistical significance in the survival distributions for male and female mice, $\chi^2(1) = 0.338, p = .561$ (see Figure 15). On average, 26.3% of female mice survived to the end of the study ($M = 61.842$ days, $SD = 6.838$), but by approximately 72 days, all male mice had been isolated (see Figure 15). As indicated by the \boxtimes symbols in Figure 15, any

remaining females were censored around day 100 to conclude the study. Figure 16 visualizes the decay of only the female population based on intervention at a weekly count, and Figure 17 visualizes only the male population decay. No males were present in the Nestlet condition for cohort two.

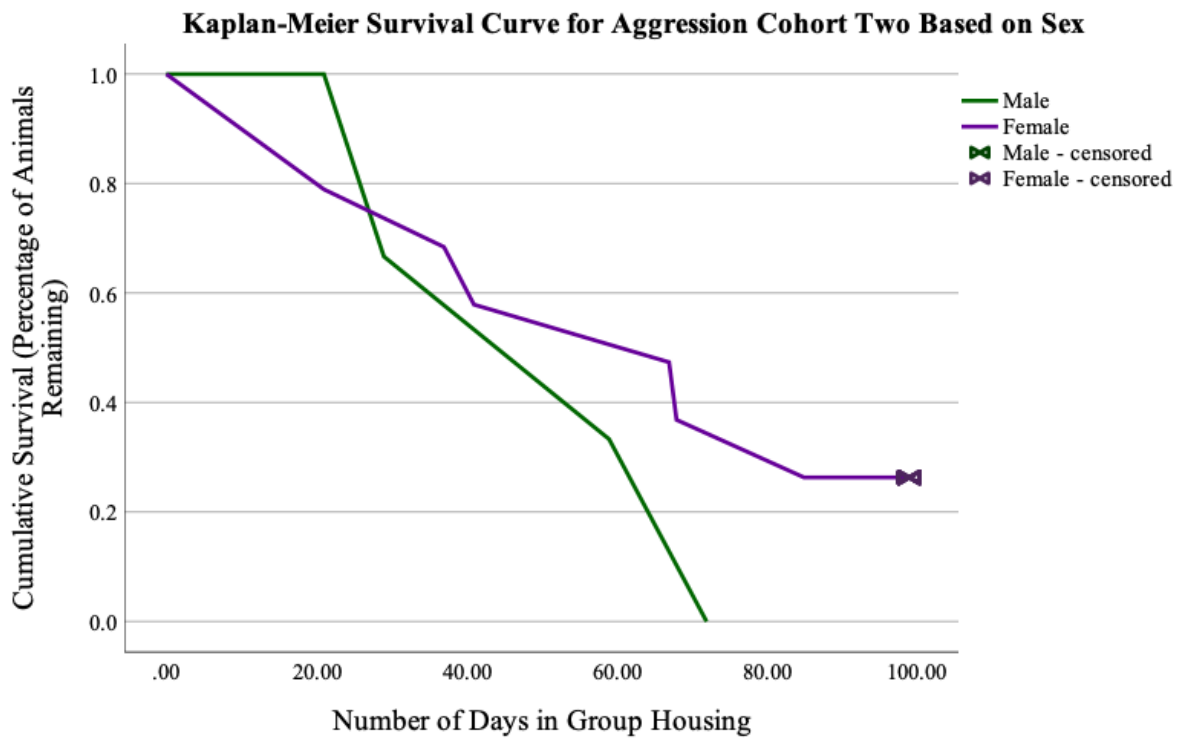


Figure 15 Cohort Two Survival Curve Based on Sex

Note. Censored (⊠) indicates an instance where an animal was removed from the study for a reason unrelated to aggression, or because the study had completed and the remaining animals never experienced aggression.

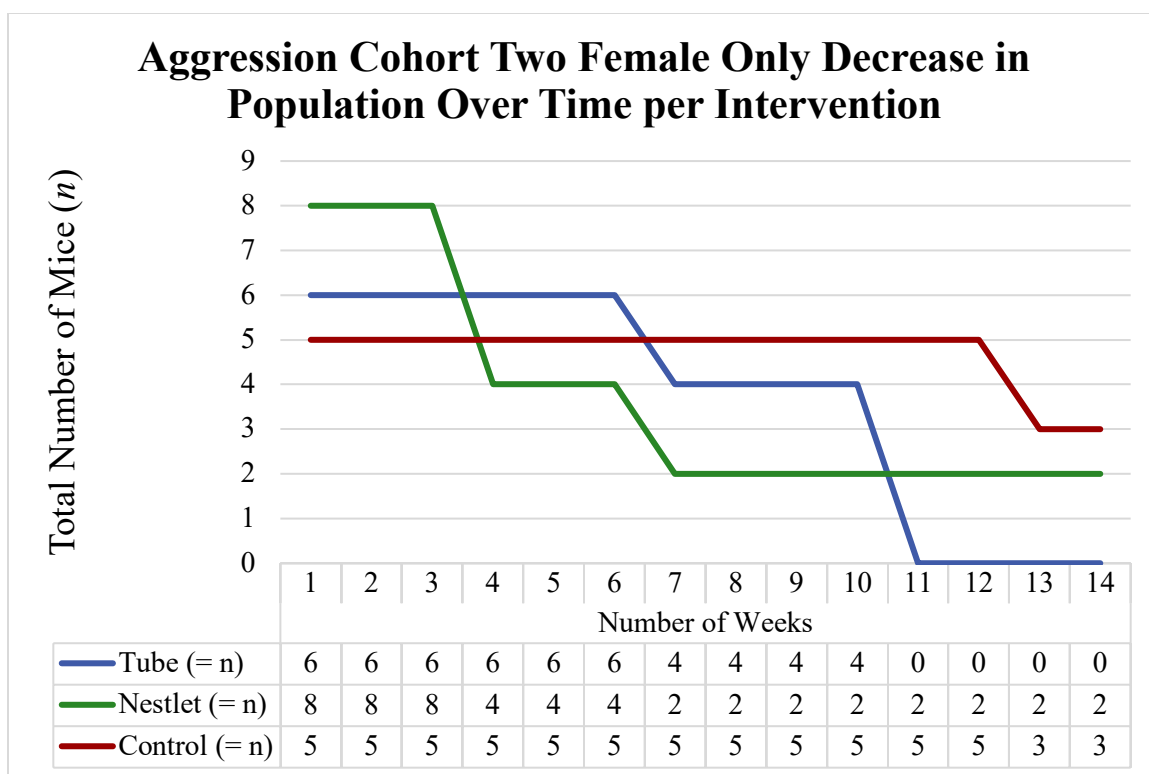


Figure 16 Cohort Two Female Only Decrease in Population Over Time

Note. Table represents number of animals left per intervention group at each week

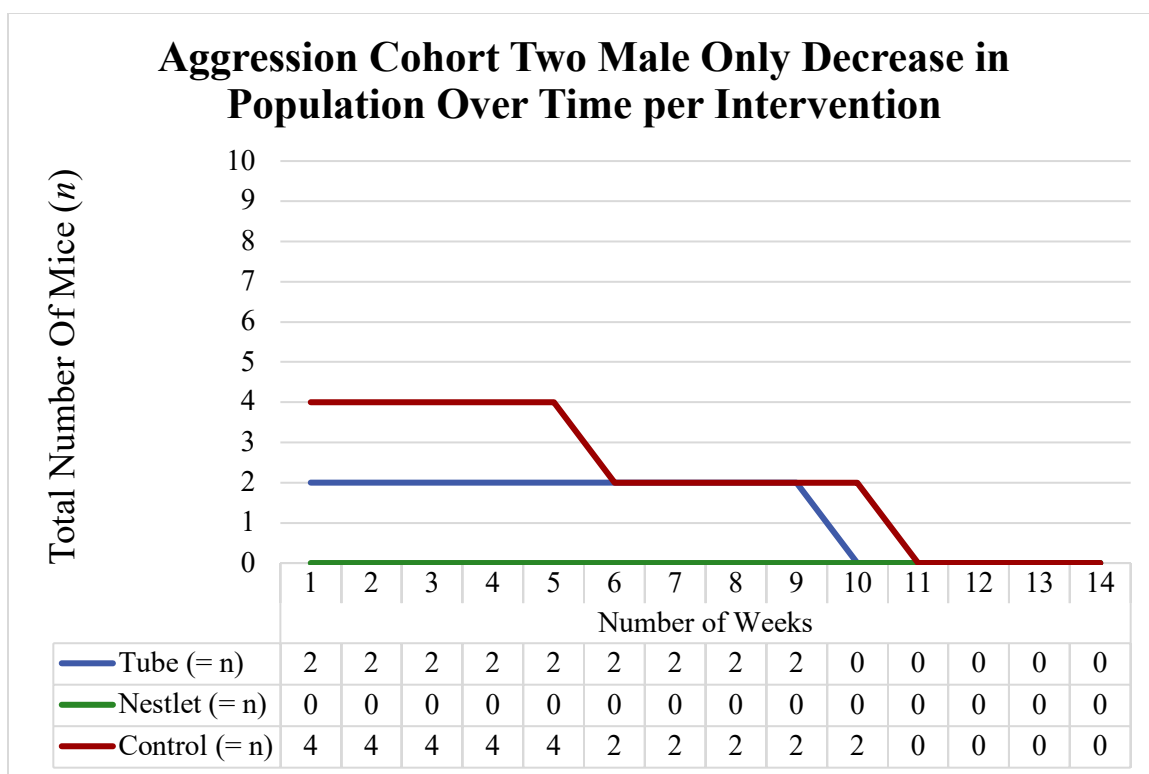


Figure 17 Cohort Two Male Only Decrease in Population Over Time

Note. Table represents number of animals left per intervention group at each week

Combined exploratory data. The data from aggression cohorts one and two were also combined for an exploratory analysis to determine overall treatment effect on levels of aggression within a larger population. A Kaplan-Meier Survival Curve under a Breslow test was run to determine if there were differences in the survival distribution for the different forms of enrichment used to attenuate aggression for both cohorts. When examining the effects of treatment on the overall population, there was no statistical significance in the survival distributions for the three enrichment groups, $\chi^2(2) = 0.562, p = .755$; Figure 18 indicates on average, 31.5% of mice provided with a nestlet survived to

the end of the study ($M = 51.353$, $SD = 9.015$), 30% of mice provided with no additional enrichment (control group) survived until the end of the study ($M = 61.800$, $SD = 8.486$), and lastly, 16.7% of mice provided with a cardboard tube remained group-housed by day 68 ($M = 56.333$, $SD = 6.266$). As indicated by the ✖ symbols in Figure 18, if a population decrease was due to an event unrelated to aggression, such as an unrelated health condition, or there was a lack of involvement in an aggression incident, that animal's date of isolation was marked as "censored". In the exploratory combined cohorts, reaching approximately 100 days marked the end of the study, and any remaining animals were marked as censored per lack of aggression, as seen in all three groups. Figure 19 visualizes the decay of each intervention group at a weekly count.

Chi-square tests of independence were conducted between aggression intervention and removal from group housing at Month 1, Month 2, and Month 3. At Month 1, there was a statistically significant association between intervention and removal from group housing, $\chi^2(2) = 6.447$, $p = .040$. This significant effect was largely driven by the female mice, with post-hoc tests showing a difference between intervention groups. Female mice assigned to the nestlet condition experienced higher levels of aggression than any other group, resulting in higher rates of separation. At Month 2, there was a marginal association between intervention and removal from group housing, $\chi^2(2) = 4.666$, $p = .097$, with female mice assigned to the nestlet condition again experiencing higher levels of aggression than any other group, resulting in continually higher instances of separation. At Month 3, there was no significant association between intervention and removal from group housing, $\chi^2(2) = 2.502$, $p = .286$.

Exploratory Kaplan-Meier Survival Curve for Combined Cohorts Based on Intervention

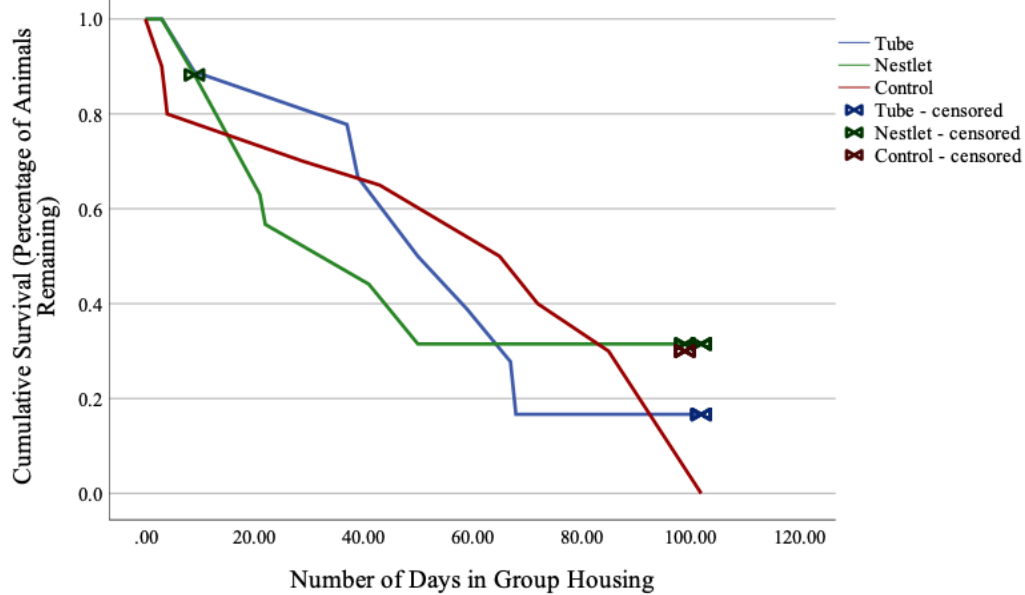


Figure 18 Combined Cohorts Survival Curve Based on Intervention

Note. Censored (⊠) indicates an instance where an animal was removed from the study for a reason unrelated to aggression, or because the study had completed and the remaining animals never experienced aggression.

Table 5 No. of Animals Remaining in Each Intervention Group in the Combined Cohorts at Every 20 Days

	0 days	20 days	40 days	60 days	80 days	100 days
Tube (= n)	18	16	12	7	3	3
Nestlet (= n)	17	14	9	5	5	3
Control (= n)	20	16	14	13	8	3

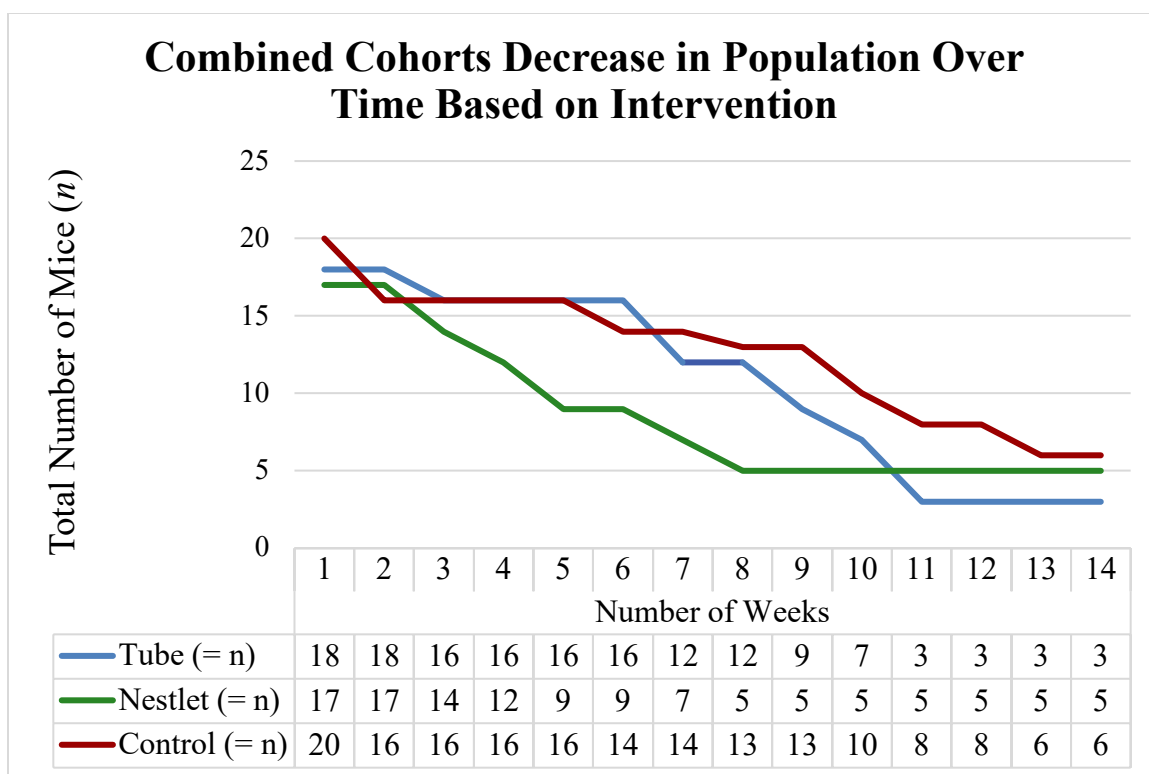


Figure 19 Combined Cohorts Decrease in Population Over Time

Note. Table represents number of animals left per intervention group at each week

The exploratory combined cohorts were also examined for any interactions with sex, to which there was a statistically significant difference in the survival distributions for male and female mice, $\chi^2(1) = 16.259, p < .001$. On average, 32.2% of female mice survived to the end of the study ($M = 74.172$ days, $SD = 5.990$), but by approximately 72 days, all male mice had been isolated ($M = 37.149$, $SD = 4.846$) (see Figure 20). As indicated by the ☒ symbols in Figure 20, if a population decrease was due to an event unrelated to aggression, such as an unrelated health condition, or there was a lack of

involvement in an aggression incident, that animal’s date of isolation was marked as “censored”. This is seen at approximately day 9 as the male uninvolved with an aggressive incident was isolated as a result of removal of the aggressor and aggressed mouse, and around day 100 when all remaining female mice concluded the study. Figure 21 visualizes the decay of only the female population based on intervention at a weekly count, and Figure 22 visualizes only the male population decay.

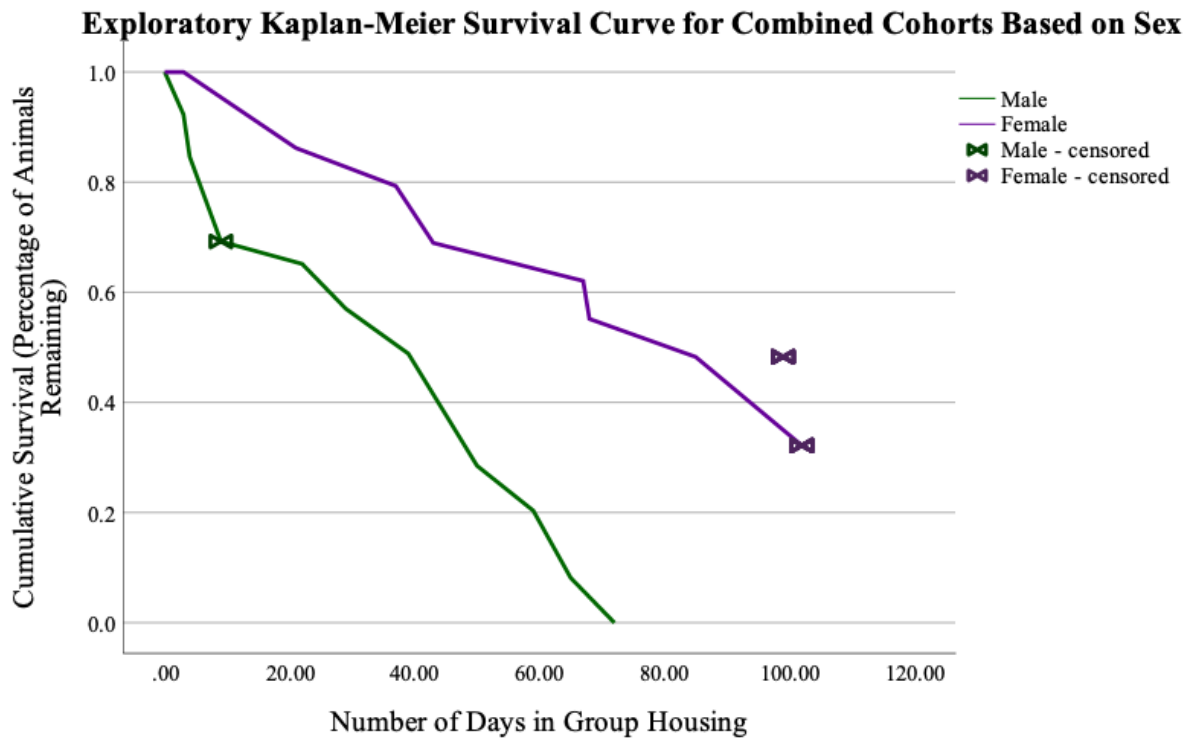


Figure 20 Combined Cohorts Survival Curve Based on Sex

Note. Censored (☒) indicates an instance where an animal was removed from the study for a reason unrelated to aggression, or because the study had completed and the remaining animals never experienced aggression.

Table 6 No. of Female and Male Mice Remaining in the Combined Cohorts at Every 20 Days

	0 days	20 days	40 days	60 days	80 days	100 days
Female (= n)	29	29	23	20	16	9
Male (= n)	26	17	12	5	0	0

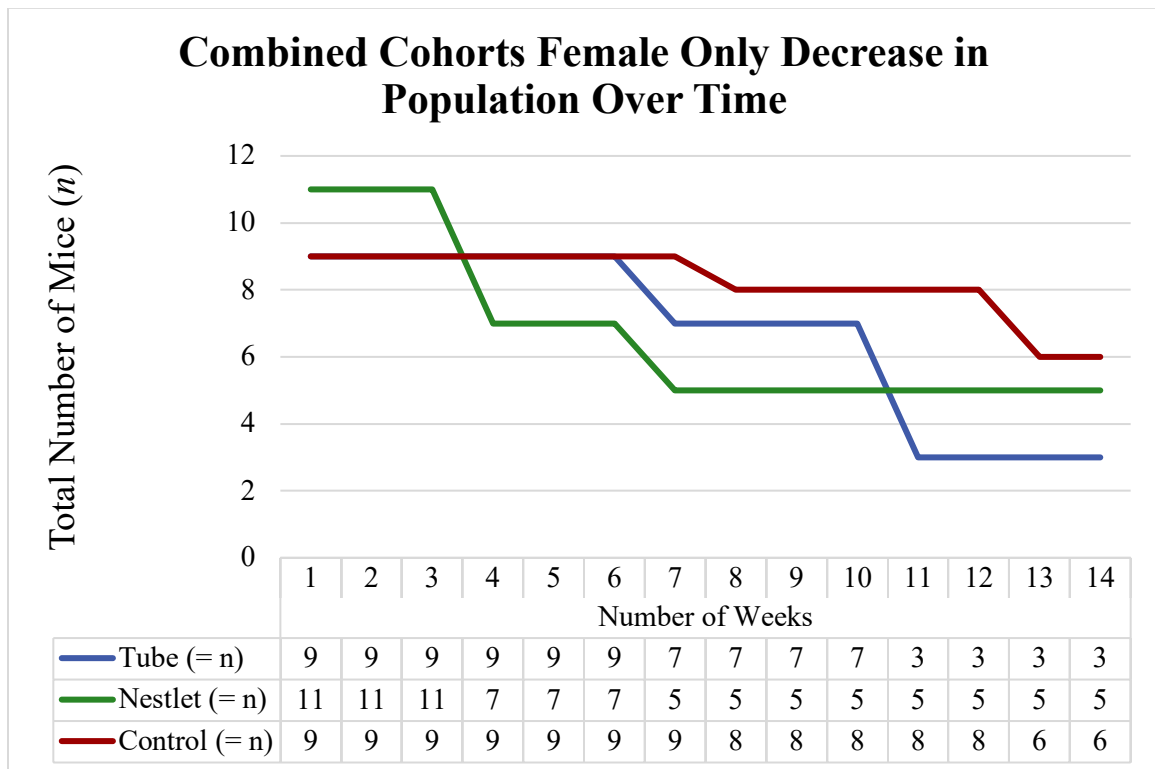


Figure 21 Combined Cohorts Female Only Decrease in Population Over Time

Note. Table represents number of animals left per intervention group at each week.

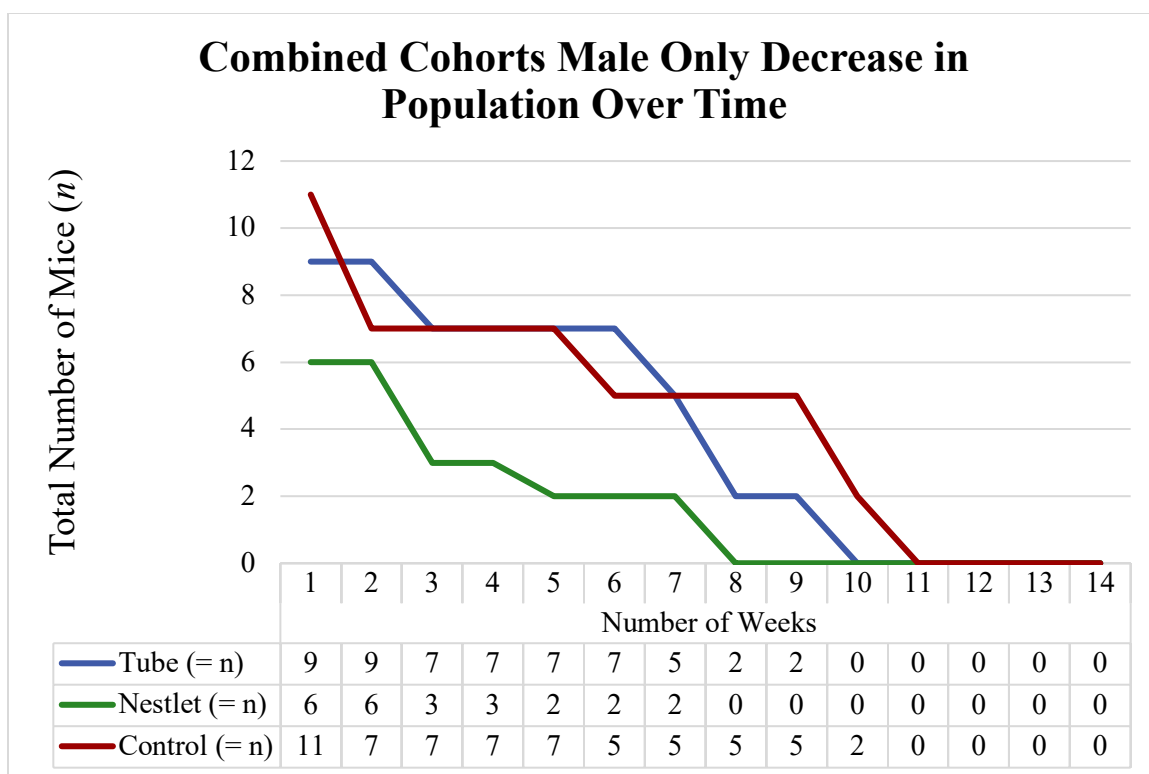


Figure 22 Combined Cohorts Male Only Decrease in Population Over Time

Note. Table represents number of animals left per intervention group at each week.

CHAPTER FOUR

This chapter will discuss the findings from the nesting and aggression studies, exploring the significance of the interventions on modifying behavior in AD-modeling mice. The outcomes will be critically analyzed with consideration to broader implications regarding animal welfare and research practices. Contextualizing these results with the previously discussed literature and identifying potential limitations will ultimately help to contribute to the ongoing discussion on optimizing and improving the use of animals in research to collect sound and reliable data.

DISCUSSION

Nesting Study

The aim of the nesting study was to determine if repeated exposure to a nesting task would prevent the degradation of the nesting task over time in hTau-expressing mice. While it was hypothesized that the mice that received a nestlet and had repeated exposure to the nesting task would perform better than the mice who were never exposed to a nestlet; the two-way repeated-measures ANOVAs determined the repeated exposure to a nestlet resulted in nest building skills that showed a positive trend, indicating a positive influence of priming. At the two-hour measurement, the mice that received a nestlet were perceived to have built worse nests than the control group and used less material to construct their nests. After 18 hours, there were still no statistically significant

differences. However, mice that received a nestlet were perceived to have improved over time with their average subjective score raising, and with overall performance better than the control group. The mice that received a nestlet also increased the quantity of materials used to construct their nests compared to the two-hour quantity, and overall used more nesting material in the final nest than the control group.

The significant effect of time was to be expected; progress at a task as time went on is typical across any animal, but it is interesting that, while not significantly, the repeated exposure mice did continue to improve over time and would likely have continued to improve their nests if time had been extended. While the effects of the nestlet intervention were not statistically significant, the experimental group of mice that had repeated exposure to the nesting task did marginally improve during the assay. It cannot be affirmed that repeated exposure to the task prevented the loss of nesting abilities, but it did provide insight on the effects of enrichment in Alzheimer's-modeling mice. Though the goal of this study was to find a method to preserve a behavioral ability that is known to deteriorate in AD models (Neely et al., 2019), this study instead brought up the question of the consequences of repeated exposure to a task on study validity. Instead of the repeated-exposure mice outperforming the control group, their similar objective and subjective scores for both time measurements demonstrated that reinforcing behaviors that are necessary for animal wellbeing does not appear to significantly affect how the animal will perform during scored behavioral assays.

It is well documented how nesting plays important and innate roles in rodent wellbeing; from thermoregulation to reproductive purposes, failure to complete such an

ingrained task can often indicate the progression of brain damage or neurodegeneration (Jirkof, 2014). Nesting assays can also be vital parts of animal-based Alzheimer's research studies, so it is important not to artificially alter that behavior using regular enrichment, just as it is important to provide laboratory rodents with materials that promote a healthy and necessary activity. Ultimately, in the case of mice from strain rTg4510 with the P301L-CAMKII-carrying genotype, it is appropriate to provide these animals with access to nest-building materials through nestlet enrichments to promote their overall health and wellbeing without concern of compromising any future behavioral assays.

However, limitations extend to only P301L-CAMKII-carrying mice from JAX strain rTg4510. As this specific genotype from this specific strain was the sole participant in the study, these results can only be applied to them. It is possible that these results are unique to this genotype due to the accumulation of tau tangles and damage to regions of the brain needed to produce the ability. Future studies could implement the same design across more diverse populations, such as including more genotypes or various strains of Alzheimer's-modeling mice, to determine what affect repeated exposure has on ability acquisition and retention. As there was a positively trending effect of treatment, future studies should also utilize larger samples and a longer time to complete the nesting behavioral assay to identify any more robust effects of repeated exposure.

Aggression Study

The goal of the aggression study was to utilize two different forms of enrichment in attempts to decrease levels of aggression in noncarrier/noncarrier hTau mice. Two

cohorts of mice were used to complete the study and then combined for an exploratory analysis. At the beginning of the first cohort, there was a delay in receiving the cardboard tubes that were meant to be administered the day the mice were weaned. The mice in the first cohort subsequently received their first enrichment intervention (either a tube or a nestlet) five days after weaning, necessitating a second cohort to test an immediate-delivery approach. The second cohort of mice received their first enrichment intervention the day they were weaned. In both cohorts, mice were kept in group housing until aggression incidents were recorded, after which the mice needed to be isolated.

Both aggression cohorts were also combined to examine an exploratory analysis with a greater sample size. When the cohorts were combined, there was still no significant effect of intervention throughout the duration of the study. All three treatment groups declined over time, but a Chi-square analysis determined there were statistically significant differences within the groups. Female mice assigned to the nestlet condition had significantly fewer mice left in group housing at Month 1, and by Month 2, this effect was still trending with female mice displaying increased aggression over the nestlets. There were no other significant differences between the three groups while accounting for sex.

By the time the experimental mice were weaned and introduced to the study, many had likely already entered sexual maturity. When exposed to each other's chemical scents or vocalization, these passive interactions between males and females can trigger estrus to begin in sexually mature females (Asaba et al., 2014). As the increased aggression is greatest during the first months of the study, any female mice in estrus may

have competed over a resource they deemed valuable for reproductive needs. Nests are important not only for shelter, but to provide a safe place to birth and nurse pups, thermoregulate, and keep pups closely together. Though there was no possibility for an experimental female to become pregnant or be directly exposed to a male, nesting is still an innate behavior that is driven on instinct and need, which may explain why these females fought over the limited resource.

While it was hypothesized originally that the additional enrichment interventions would decrease aggression levels, in actuality, aggression incidents were so violent that animals would need to be isolated after the first instance. In a typical cage of three mice, there would be one aggressor mouse, one aggressed mouse, and one mouse that was uninvolved. However, as the aggression cases were so violent that the aggressor mouse needed to be isolated for the others' safety, the aggressed mouse needed to be isolated to allow it to heal and not be preyed on, and as a result, the uninvolved mouse would become isolated. Once animals were isolated, they were unable to be reintroduced to group housing, so data collection for each animal consisted of the number of days it lasted in group housing until the first instance of aggression.

There was a significant effect of sex in cohort one regarding survival in group housing, with females exceptionally outperforming males and exhibiting minor levels of aggression. While female mice tend to demonstrate fewer aggressive behaviors than males, they are still susceptible to displays of dominance and disputes over territory (Takahashi, 2021). This effect may also largely be due to unequal sample sizes of sex across the cohorts, with males outnumbering females 2:1 in cohort one, leading to higher

probabilities that aggressive behaviors will occur. Cohort two consisted of 19 females and only six males and did not experience a significant effect of sex. Sex is also a difficult factor to control for in animal studies because of its unpredictable nature, especially in breeding paradigms that produce limited pups of certain desirable genotypes.

In cohort one, there was no statistically significant difference in the number of days each mouse lasted based on what type of enrichment they were provided. The late introduction of the enrichment for cohort one may have influenced the levels of aggression, but aggression levels did not appear to differ greatly from the mice in the second cohort that immediately began receiving their enrichment. It is most interesting that out of each group, the control group in cohort two did marginally better than the different enrichment groups, indicating the additional enrichment instigated some aggression.

While sex was a difficult factor to control for, the number of animals housed together was a critical decision. As the mice were primarily housed in groups of three, each cage received one or two nestlets or tubes. A cage of two mice received one intervention, a cage of three received two interventions, and a cage of four received three interventions. It is likely that the mismatch in the number of items provided to the number of mice in the cage resulted in territorial or dominance disputes. In a similar study conducted previously at George Mason University, noncarrier/noncarrier hTau mice that were provided with foraging crumbles displayed significantly decreased amounts of aggression. Because foraging crumbles are small granules of food spread

throughout the cage bedding that mice can search for and eat, mice did not display the same levels of aggression as when enrichment in limited quantities were provided. However, foraging crumbles may not always be an appropriate enrichment, especially if the focus of a study is based on what or how much food an animal consumes. While this present study was unsuccessful in minimizing aggression, it is possible that if each mouse received its own enrichment intervention or if there was a greater abundance of enrichment interventions, there would be fewer territorial disputes and less overall aggression. However, limited funding may constrain the use of enrichments like foraging crumbles, as they often need to be ordered from specialty manufacturers, must be replenished frequently, and may require specific storage conditions.

Future studies aiming to attenuate aggression in laboratory rodents may find more success when ensuring there is equal access to enrichment within group-housed mice. However, there can be limitations to this approach. Such as what was experienced in this study, cage sizing constraints may limit the number of resources that can be provided; while it would have been ideal to provide a cage of three mice with three cardboard tubes, there was not enough room in the animals' home cages to fit that amount alongside their regular cage inserts. Nestlets were also limited to remain consistent but can normally be used plentifully. It may also be beneficial to house fewer animals in a cage at a time, but constraints on facility availability and funding may limit the number of cages allowed per study.

Overall, there is a limited selection of proactive interventions designed to improve laboratory rodent aggression that are fully successful and readily accessible. To prevent

territorial disputes and resource guarding, and limit displays of dominance, an ideal behavioral modifier is an enrichment that can be widespread throughout a cage, occupies the animals' attention, is rewarding, and encourages natural behaviors that overall improve the animals' wellbeing. It is also important that this ideal modifier be easy to access and administer, while adhering to lab-specific financial and space constraints.

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BIOGRAPHY

Alison Perlberg received her Bachelor of Science in Psychology from George Mason University in 2022. After receiving her Master of Arts in Psychology from George Mason University in 2024, she will be enjoying some well-deserved time off before seeking opportunities to expand her education in pursuit of earning her Doctor of Philosophy in Neuroscience.