Examining the relationship between social position, spatial learning, and memory in adult male and female rats

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Overview

Decades of neuroscience research has established the modulating role of stress in cognitive function, such as learning and memory, namely from severe stressors that invoke fear or physical stress. The effect on cognition from other types of stressors, such as stress stemming from social position, has yet to be understood and has received limited attention. Evidence stemming from the graded relationship of socioeconomic status on health outcomes, suggest one's relative social position may have adverse physiological consequences. Therefore, we used a simple rodent model to examine the relationship between social position on cognitive functions.

Objectives

This thesis aimed to 1) determine whether social hierarchy in rats could be reliably measured using the Tube Test, 2) examine whether social position mediates spatial learning and memory, and 3) assess the role of sex in the relationship between social position, spatial learning, and memory.

Methods

Eighty adult, male and female Sprague-Dawley rats were grouped, with either 4 males, or 4 females per group (N = 40 / sex). The Tube Test was conducted to determine the social position of groups over 3 days by examining pairwise dominance interactions. Afterwards, Morris Water Maze training was used to assess spatial learning over 4 days, memory through the probe test, and behavioural flexibility over 2 days of reversal training. Subsequently, a one-day Tube Test was conducted to examine the stability of the social positions. Following the completion of behavioral testing, body weight, and stress-sensitive organ weights (adrenal and thymus glands) were measured.

Results

The findings of our study provide no evidence that social position affects cognitive function as spatial learning, reversal learning and memory performance was not significantly different across social positions. We found no sex differences in relationship between social position and spatial performance, and our study found no differences in body weight and in stress-sensitive organs (adrenal and thymus glands) across social positions in male and female rats.

Conclusion

Our results suggest no evidence that social position within a group of same-sex housed rats altered learning and memory performance. Furthermore, we did not find sex differences in the relationship between social position and cognition. In our study, animals were not subjected to known socially stressful conditions such as social isolation or overcrowding, and were left alone to examine the social stress from natural occurring social stratification. Further research is needed to confirm our findings and explore when social position becomes a stressor. Furthermore, future directions can aim to explore the neurobiological mechanisms from social stress by comparing corticosterone levels and target proteins (e.g., AMPA receptors) across social positions.

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List of Abbreviations

AMPA(R)	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (receptor)
ANOVA	Analysis of variance
BW	Body weight
CAF	Central Animal Facility
CI	Confidence Interval
CPH	Cox Proportional Hazards
DS	David's Score
E	East
HPA-axis	Hypothalamic-Pituitary-Adrenal axis
HPC	Hippocampus
HR	Hazard Ratio
mPFC	Medial prefrontal cortex
MWM	Morris Water Maze
N	North
NE	Northeast
PFC	Prefrontal cortex
PH	Proportional hazards
S	South
SEM	Standard error of the mean
SES	Socioeconomic Status
SW	Southwest
VBS	Visible Burrow System
W	West

CHAPTER 1

INTRODUCTION

Social mammals commonly exhibit a phenomenon known as social hierarchy, wherein group members are organized based on their social rank. Long-standing social roles are thought to foster resource sharing, encourage cooperation, and reduce conflict among group members (Tibbetts et al., 2022; Vessey, 1984), and are often regarded as necessary factors of social systems. Dominance hierarchies are social structures in which more dominant individuals lie at the top of the hierarchy and more submissive individuals lie at the bottom. As stress has been shown to interfere with memory-related behaviour, particularly in male rats (Conrad, 2010; Luine et al., 2017), it remains unclear whether the constant stress of one's relative social position has comparable cognitive consequences.

1.1 The social environment as a stressor

In societies where living in groups is common, the social environment plays a crucial role in individual daily life. Negative social interactions, such as social isolation, or social rejection, could be a source of stress (Beery et al., 2020; Beery & Kaufer, 2015), while positive social interactions, such as social support, can buffer the effects of stress (Ozbay et al., 2007). Research from developmental studies has shown the importance of social interactions early in development as there are profound downstream effects on adult behaviour. For example, social deprivation was found to impede normal brain development, decrease pain sensitivity, and influence health risks such as developing psychiatric disorders (Begni et al., 2020; Fone & Porkess, 2008; Kawachi & Berkman, 2001; Kotch et al., 2008; Schneider et al., 2016; Snyder-Mackler et al., 2020; Varese et al., 2012).

Aside from its developmental effects, adverse social experiences may also affect social behaviour. Socially isolated male rats were found to be more dominant and aggressive than group-housed male rats. Socially isolated male rats won more matches against group-housed rats when tested on the Tube Test (a task that assesses social dominance by pairing rodents against each other in a tube) and displayed increased aggressive behaviour (Tada et al., 2016; Uyeno & White, 1967). Social rejection from peers during adolescence was modelled in female rats and was found to alter social behaviour in adulthood (Schneider et al., 2016). In particular, Wistar female rats that were reared with Fischer female rats during adolescence (Wistar is an outbred rat strain and Fisher is an inbred rat strain), faced peer rejection and were considered social outcasts relative to Wistar rats that were reared with same-strain members (control group). The effect of peer-rejection and improper rearing resulted in altered social behaviour in adulthood, such as a reduction in social exploration typically seen in rats (anogenital sniffing, and approach/follow behaviour) and social recognition (Schneider et al., 2016).

In humans, group-level interactions are complex as the social environment in humans pervade multiple facets of life and result in individuals' part of multiple networks (friends, family, work) simultaneously. The complexity of the social environment can therefore be challenging to study in humans, thus, we can turn to animal societies to understand how social position becomes a stressor, as nearly all mammals exhibit social stratification. At the group-level, the organization of individuals into a social order is known as a social hierarchy, and socially stressful experiences may occur depending on an individual's relative social position within a group. For example, lower-ranked rodents are typically chased by higher-ranked peers; chasing behaviour in adulthood is a common social behaviour in rodents that occurs before a fight (Calhoun, 1963; Schweinfurth,

2020; Williamson et al., 2019). As the social environment is indeed an important and broad area of research, our work focuses on one aspect of the social environment: social hierarchy.

1.2 Social hierarchy

The establishment of a social hierarchy naturally occurs within most social species and is thought to reduce conflict and promote social stability (Tibbetts et al., 2022). Conflict is minimized within a social hierarchy as individuals alter behaviour around more dominant individuals to avoid fights they are unlikely to win (Clutton-Brock & Huchard, 2013a; Kaufmann, 1983), and stability is promoted through sharing limited resources (e.g., food and territory) (Tibbetts et al., 2022; Vessey, 1984). A common feature of hierarchies is the asymmetry of resources granted to those at the top of the hierarchy, where higher ranked, or more dominant individuals enjoy extensive access and control of resources, and tend to have greater reproductive success compared to those lower in the hierarchy (Bonabeau et al., 1999; Koski et al., 2015; Sapolsky, 1995; Ziporyn & McClintock, 1991). For example, in male hierarchies of wild rats, the alpha male is typically the largest among their peers, has easier access to and control of food, can freely move around their environment, and are more likely to initiate attacks towards intruders (Calhoun, 1963; Schweinfurth, 2020). In contrast, wild male rats that occupy the lowest position in the male hierarchy have reduced access to food (which is reflected in their body weight), are less likely to reproduce, and are likely to be attacked by their higher-ranked peers (Calhoun, 1963; Schweinfurth, 2020). As a result, social interactions among individuals within a group may have profound long-term physiological and behavioural consequences. Further work needs to be done to understand the nuances of this effect as social interactions and physiology are context-dependent and sex-specific (Kondrakiewicz et al., 2019; Mendoza, 1993).

A characteristic of dominance hierarchies is linearity, which refers to transitive social orders where if A wins over B, and B over C, then A is presumed dominant over C (Strauss et al., 2022; Strauss & Shizuka, 2022; Tibbetts et al., 2022). Steepness, is another characteristic of social hierarchy, which refers to the social distance between individual members (de Vries et al., 2006; Karamihalev et al., 2020). When there are large differences, the social hierarchy is steep, whereas when the social distance is small, the social hierarchy is considered shallow (de Vries et al., 2006). Male hierarchies in rats and mice tend to form steep and despotic linear hierarchies, where one individual emerges as the most dominant over the male conspecifics of the group (Grant & Chance, 1958; Karamihalev et al., 2020). Individuals at the top of the social hierarchy are likely to stay in the same position, further creating a stable social hierarchy (Calhoun, 1963; Sapolsky, 1995; Tibbetts et al., 2022).

Generally, in animal societies, rank depends on pairwise interactions between members of the group that occur before the establishment of the stable hierarchy (Mendoza, 1993). The initial interactions between a pair reveal differences in social behaviour that set the relationship for subsequent interactions (Bonabeau et al., 1999; Mendoza, 1993). For example, in observational studies of pairs of non-human primates, the dominant animal is the most aggressive of the pair, while the other animal responds with submissive behaviours like avoidance with each subsequent interaction (Mason, 1993; Mendoza, 1993; Rowell, 1974). In laboratory settings, aggressive behaviour in male rats decreased as conspecifics became more familiar with each other (Adams & Boice, 1989). Dominance is generally defined as a consistent outcome from repeated agonistic interactions (social behaviours involved in displaying dominance, or submission) between two individuals within a group, resulting in favour of one individual and a yielding response from the other (Drews, 1993; Strauss et al., 2022; Strauss & Shizuka, 2022). Overall, the natural

phenomenon of social hierarchy formation may confer advantages such as access to essential resources and reducing conflict, which may outweigh the cost of being on the lower end of the hierarchy (Berdoy & Drickamer, 2007).

1.2.1 Social hierarchy of rats

Rats are popular animal models in various disciplines (e.g., neuroscience, pharmacology), and as a result, the social behaviour of rats has been extensively documented. There are numerous behavioural paradigms that examine social hierarchy in rodents such as the visible burrow system (VBS), which is a group-housing paradigm of an artificial burrow that consists of a large chamber interconnected with smaller chambers and corridors. Food and water would typically be located in the large arena, and dominance is determined based on the occupation of space of the rats and agonistic behaviours. The more dominant individuals typically take residence in the largest chamber, while the more subordinate animals spend the most time in the smaller chambers (Blanchard & Blanchard, 1989a). Another well-known paradigm is the resident-intruder paradigm, in which an unfamiliar rodent is introduced into the home-cage of an individual rodent or group (termed the resident). The paradigm takes advantage of the territorial nature of rodents, in which residents defend their territory from intruders. Dominance is determined based on the offensive and defensive behaviours from the interactions between the intruder and the resident(s) (Blanchard & Blanchard, 1989b; Koolhaas et al., 2013).

A study using the VBS paradigm where social hierarchy was yet to be established in a mixed colony of male and female rats found that the rats engaged in aggressive interactions to secure social positions, and found that the lowest ranking rats had the greatest proportion of wounds, while the dominant rats had the fewest wounds (Melhorn et al., 2017). Other studies have also reported differences in location of mealtimes between dominant and subordinate animals,

reflecting the differential access to food and space resources based on an individual's social position. Dominant rats tend to eat their food in larger and open spaces, while subordinate rats tend to consume their food in smaller enclosed spaces away from dominant individuals (Calhoun, 1963; Melhorn et al., 2017). Furthermore, rats engage in numerous types of social dominance behaviours such as chasing behaviour and piloerection. As previously mentioned, higher-ranked rats chase and initiate fights with intruders and lower-ranked rats (Calhoun, 1963; Schweinfurth, 2020; Williamson et al., 2019). Dominants observed from the resident-intruder paradigm are mostly residents, and were observed to display piloerection (where the fur along the back is upright) as they moved towards the intruder (Koolhaas et al., 1980). Subordinate animals are also more likely to retreat when dominant individuals display piloerection (Takahashi & Lore, 1983).

Social hierarchy among male rats typically depends on age, fighting ability, reproductive success, weight, and size (Clutton-Brock, 2007; Clutton-Brock & Huchard, 2013b). For example, in a semi-natural study of wild rats, age was a predictor of social rank (Berdoy et al., 1995; Calhoun, 1963). Older male rats were found to have the advantage of greater fighting experience and size over younger males and were able to maintain their dominance even after the young rats were fully grown (Berdoy et al., 1995; Calhoun, 1963). Dominant male rats were observed to have greater locomotor and exploratory activity compared to subordinate male rats (Calhoun, 1963; Olsson & Westlund, 2007). Mixed colony studies have found that although female rats also engaged in dominance interactions, they were not frequent enough for a dominance hierarchy to be observed (Berdoy et al., 1995; Ziporyn & McClintock, 1991). However, a dominance order was determined in female rats when tested on the Tube Test. Passing behaviour within the tube (right-of-way dilemma in which the more dominant of a pair would win the right-of-way) was found to strongly signal dominance in female rats and occurred more frequently than other social dominant

behaviours. For example, passing behaviour occurred 7 to 10 times more often than fighting and 3 to 5 times more often than crawl-over behaviours in female rats (Ziporyn & McClintock, 1991).

The establishment of female hierarchies in rats may have appeared more difficult to observe as researchers may have been looking for standard agonistic behaviours that are more reflective of male rats, such as fighting, or aggressive behaviour (Ziporyn & McClintock, 1991). Additionally, the complicated nature of measuring the oestrus cycle of female rats has historically led to female animals being excluded from animal research, which has led to limited research into female social behaviours, and a reduced understanding of the social nature of female rodents (Ziporyn & McClintock, 1991). Importantly, the oestrus cycle is thought to play a role in the dominant interactions between female animals and may be the reason why hierarchies among them may not be formed as strongly as male hierarchies (Schweinfurth, 2020). Furthermore, dominant females may suppress the reproductive function of subordinates temporarily, or completely, increasing the reproductive success of dominant females (Clutton-Brock & Huchard, 2013a; Holmes et al., 2009). For example, in a group of rats with a large difference in dominance, the dominant female was the first to become pregnant, while the most subordinate was the last female to become pregnant (Ziporyn & McClintock, 1991).

Since social hierarchy observations have primarily involved male animals, studies on other female animals may help further our understanding of the social dynamics of rodents and the potential sex differences in social interactions. In female animals, one's social position may be more influenced by social strategies such as securing and maintaining social support, which may partly explain why dominance hierarchies are not consistently observed among females (Benenson & Abadzi, 2020; Clutton-Brock & Huchard, 2013a). In one example, the most dominant female rat also had the highest frequency of social contact, such as anogenital sniffing (Adams & Boice,

1983). In mice, dominance was more evenly distributed in females compared to males, and displayed less linear and despotic hierarchies (Chance & Mead, 1988; Varholick et al., 2019; Williamson et al., 2019). Similarly, in humans, girls partake in relational aggression with each other by targeting interpersonal relationships (Koski et al., 2015). Nevertheless, as research into female social behaviours is underrepresented in the literature, more work needs to be done to further understand female-female interactions and the contextual factors that may affect female dominance relationships.

1.2.2 Consequences of social hierarchy

Despite the prevalence of social hierarchy in nearly all mammals the consequences of social position on the body are not fully understood. In humans, one notable metric of hierarchy is socioeconomic status (SES), which is determined by factors such as income, wealth, education, and occupation (Sapolsky, 2005; Shavers, 2007). Those on the lower end of the socioeconomic ladder tend to have reduced access to important resources such as health services, access to healthy food, education, and shelter, which contributes to increased health risks and mortality rates (Calixto & Anaya, 2014; Shavers, 2007). In the Whitehall studies of British civil servants, a gradient relationship was found between job status and health. For example, an increased prevalence of ischaemic heart disease was found as the job status decreased from Executive (highest status) to Office Support positions (lowest status) (Marmot et al., 1991). A 25-year follow-up from the first Whitehall study found men in the lowest employment grade had a mortality rate of 69% compared to 30% of men in the highest employment grade (Marmot & Shipley, 1996). Marmot's work has highlighted the importance of social factors such as occupation, income, and neighbourhood conditions, and showed how they are tied to relative social position, and health.

Unstable hierarchies, when the social hierarchy has not yet been established or when a new order emerges after the defeat of the previously dominant individual, are stressful as high levels of aggression and competition occur (Adams & Boice, 1989; Calhoun, 1963; Olsson & Westlund, 2007; Sapolsky, 1993). For example, unstable hierarchies in olive baboons and other captive primate species, were found to increase the basal cortisol levels within all members of the troop, with the highest level of cortisol typically belonging to the dominant male (Sapolsky, 1993; Sapolsky, 1995). In contrast, stable hierarchies in nonhuman primates reveal that the mean basal cortisol levels were higher in subordinate males and lower in dominant males (Sapolsky, 1995).

Similarly, unstable social conditions are stressful to rats, such as crowded housing conditions or constant changes to cagemates (Gerges et al., 2003; Gerges et al., 2004; Gerges et al., 2001; Haller et al., 1999). Stability, particularly in housing conditions, is important for rats, as chambers in underground burrows are important sites for rats to sleep and huddle together for warmth, and nesting sites for female rats (Schweinfurth, 2020). Male rats were reported to find crowded housing more stressful than individual housing, where higher corticosterone levels and thymus shrinkage were observed in crowded conditions (Brown & Grunberg, 1995; Haller et al., 1999). Similarly, in female rats, higher corticosterone levels, thymus shrinkage, and increased adrenal gland weights were found in female rats in unstable group-housing conditions (where new rats were constantly introduced) compared to female rats in stable housing pairs (Haller et al., 1999). However, individual housing was found to be more stressful than group-housing based upon higher cortisol levels when female rats were kept by themselves (Brown & Grunberg, 1995; Palanza et al., 2001). Indeed, there are consequences of social hierarchy that not only affects quality of life, but may have profound physiological outcomes depending on the social context of the environment. One's social position may be a stressor, however, as stress is known to influence

cognition, the relationship between social position stress and cognitive function is largely understudied.

1.3 Consequences of stress on cognition

Decades of stress research has provided evidence that stress can affect cognition. Stress has a modulatory effect on learning and memory function, enhancing or impairing function depending on the intensity and duration of the stressor. For instance, chronic stress has been reported to impair spatial learning and memory, particularly in male rats (Conrad, 2010). Studies have used a wide range of stressors (such as restraint and fear stress) and have generally found impairments in either learning a task, memory retention, or both. One study found chronic stress in male rats impaired spatial learning and memory performance in the radial arm water maze. Specifically, the non-stressed group rapidly learned the task, while the stressed group reached comparable levels of performance two days later than the non-stressed group (Park et al., 2001). In this case, the stressed group was chronically exposed to a cat, a natural predator, and placed in socially unstable and crowded housing conditions.

However, the effects of stress on cognition may not be the same in female subjects, as sex differences have been reported in both human and animal studies (Kudielka et al., 2004; Lupien et al., 2009; McCormick & Mathews, 2007). For example, chronic restraint stress does not seem to impair performance in female rats; rather, learning and memory abilities were enhanced in spatial tasks like the radial arm maze and the Morris Water Maze (MWM) (Luine et al., 2017). These findings agree with another study, where female rats subjected to 21 days of restraint stress, performed better on the memory task by spending approximately 28% more time in the target quadrant compared to the non-stressed group (Kitraki et al., 2004).

Stress triggers various responses within the body, one of which is the hypothalamic-pituitary-adrenal-axis (HPA-axis), a key system that modulates the neuroendocrine arm of the stress response. The HPA-axis is activated when a stressor stimulates neurons in the hypothalamus to release hormones (corticotropin-releasing hormone and arginine vasopressin) that go on to stimulate the pituitary gland (Stratakis & Chrousos, 1995). The pituitary gland, in turn, secretes adrenocorticotropic hormone into the blood, which stimulates the adrenal gland to release glucocorticoids (Sapolsky, 1995; Stephens & Wand, 2012; Stratakis & Chrousos, 1995). The hippocampus (HPC) and the prefrontal cortex (PFC) are involved in feedback regulation of the HPA-axis and act to turn-off the stress response by reducing levels of glucocorticoids (Herman et al., 2016; Sapolsky, 2005).

Early exposure to stress may affect the HPA-axis by altering the development of brain regions (the prefrontal cortex, hippocampus, and amygdala) involved in its regulation (Lupien et al., 2009). Altered development of the aforementioned brain regions results in altered function (e.g., altered glucocorticoid secretion) and regulation of the HPA-axis (Lupien et al., 2009; Sandi & Haller, 2015). For example, increased maternal investment such as licking and grooming of pups in the first 10 days of life after handling exposure was found to program the HPA-axis to have a decreased response to stress in adulthood (Liu et al., 1997). Aside from HPA-regulation, the PFC is primarily involved in executive function, decision-making, and social recognition. The HPC is involved in learning, memory, and information processing, while the amygdala has a role in emotional processing (Sandi & Haller, 2015). At the cellular level, chronic exposure to glucocorticoids results in dendritic atrophy in neurons in the PFC, HPC, and amygdala, and can eventually lead to neuronal death (McEwen, 2007; McEwen & Sapolsky, 1995; Woolley et al., 1990). Additionally, stress exposure also leads to a change in organ weights in stress-sensitive

organs such as the adrenal gland and the thymus, where stress causes adrenal hypertrophy and thymus involution (Gamallo et al., 1986; Zivkovic et al., 2005). Inevitably, stress has detrimental effects on brain regions involved in higher cognitive functions and key organs involved in neuroendocrine and immune function.

It is important to consider the nuances in response to stress to further our understanding of the consequences of stress. In particular, the work conducted in our study aimed to gain deeper insight into the chronic stress effect of the social environment and its impact on cognitive function, namely, learning and memory function. The social environment is a prime topic of interest in understanding the neurobiology of stress, as negative social interactions can be a potent stressor while having strong social networks can aid in an individual's resilience to the effects of stress (Beery & Kaufer, 2015). Stress, whether chronic or acute, influences behaviour and cognition in humans and animals alike. The effects of stress are dependent on its duration and the timing of exposure, as longer exposure during sensitive periods of development may alter normal brain development and typical age-related changes in the brain (Lupien et al., 2009).

1.4 Measuring social stress and cognition in laboratory conditions

1.4.1 Social dominance paradigms

Animal models have been proven to be useful tools in understanding the effect of social stress by allowing the control of experimental conditions, such as housing and social interactions. To understand the phenomenon of naturally occurring social hierarchies, research into the social interactions within groups should have some semblance of the natural environment of the species. The concept of ecological validity, which is an approach encouraged in behavioural research, has the potential advantage of reducing the likelihood of observing behaviours seen only in laboratory

environments (Kondrakiewicz et al., 2019; Schweinfurth, 2020). As such, the external validity of the study increases, and general conclusions drawn from animal data are more likely to be accurate and translated to other species like humans (Kondrakiewicz et al., 2019). For example, to research social hierarchy in rats under laboratory conditions, the experimental conditions should resemble the natural context of wild rats (burrow systems comprised of tunnels and chambers). Reports from seminatural studies have observed social interactions typically occurring within or near tunnels as rats navigate through the burrow (Schweinfurth, 2020). Therefore, behavioural paradigms that resemble the natural conditions of rats would be considered an ecologically valid measure.

Social dominance paradigms used to study social hierarchy in rodents include the VBS, resident-intruder paradigm, scoring of agonistic behaviours, food and water competition tests, and the warm-spot test (Fulenwider et al., 2021). The VBS and the resident-intruder paradigm have previously been described (1.2.1 Social hierarchy of rats*)*. Agonistic behaviours of rats such as chasing behaviour (initiate chases/being chased), fleeing behaviour, and freezing behaviour can be scored and used to determine social positions. More dominant animals typically initiate chases and are less likely to flee and freeze (Blanchard & Blanchard, 1990; Blanchard et al., 1984). Food and water competitions assess dominance based on which individuals take control and access of the resources. In the warm-spot test, there is one small area (typically a corner) of the test cage that can fit only one individual and is warmed to 34°C, while the remaining floor temperature is cooled to 0°C. Dominance is determined based on which individual takes control of the warm spot (Fulenwider et al., 2021). Notably, some of the paradigms may cause additional stress, such as fighting another conspecific as a means to score agonistic behaviour or fighting an intruder in the resident-intruder paradigm.

In our study, ecological validity was promoted through the use of the Tube Test, which was first introduced in 1961 and used to show that different strains of mice (such as C3H, DBA/8, and A/alb) formed consistent and stable dominance hierarchies (Lindzey et al., 1961). The A/alb were found to more dominant over C3H animals, which in turn were more dominant than the DBA/8 animals. The test measures dominance using a long cylindrical tube, which resembles the burrowing tunnels where social encounters are likely to occur in wild rodents (Wang et al., 2014). These encounters result in the right-of-way dilemma where the animals must negotiate with each other in order to proceed through the tunnel (Ziporyn & McClintock, 1991). The Tube Test utilizes this dilemma and determines dominance based on the animal that asserts the right-of-way. While the majority of studies have predominantly used mice, the rankings determined from the Tube Test have been found to correlate strongly with rankings from other dominance measures such as the visible burrow system paradigm ($r = 0.98$), agonistic behaviours ($r = 0.95$), and ultrasonic vocalization tests ($r = 0.96$) (Fulenwider et al., 2021; Wang et al., 2014; Wang et al., 2011). The Tube Test is a less stressful paradigm compared to other standard tests, such as the resident-intruder test, as the goal of the Tube Test is for one animal to reach the opposite end of the tube, while the other animal retreats. Since determining social position is one of the main factors of interest in this thesis, the Tube Test is an ideal behavioural paradigm with ecologically relevant properties for measuring social hierarchy in rats.

1.4.2 Neural correlates of social position and cognition

Relative social status and stability of a hierarchy may be stressful as social interactions often influence means of survival. Two main stress coping strategies, proactive and reactive, have been observed across species (e.g., humans, rats, chickens, pigs). Proactive or active strategies of stress in rodents include aggressive behaviours and low-HPA activity, and are characteristics of

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dominant individuals (Koolhaas et al., 1999; LeClair & Russo, 2021). In contrast, subordinates have higher activation of the HPA-axis and display reactive or passive stress coping behaviours (e.g., avoidance) (Koolhaas et al., 1999; LeClair & Russo, 2021). For example, lower-ranked rats were observed to take indirect routes to their destination as they avoided, or retreated whenever a higher ranked rat blocked the tunnel path (Calhoun, 1963; Schweinfurth, 2020). The findings align with earlier work using the visible-burrow system, which found high-HPA activity in subordinate rats by comparing the adrenal glands of subordinate rats to single-housed control rats. The study found the adrenal glands were larger in subordinate rats compared to control rats (1.18 and 1.45 times larger) (Blanchard et al., 1995).

Aside from its role in the HPA-axis, the hippocampus has been a thoroughly studied region of the brain in understanding learning and memory, which are impaired in humans with hippocampal damage and in rodents with hippocampal lesions (Knierim, 2015). With the discovery of place cells, decades of electrophysiological research suggest a role for the hippocampus in forming a spatial cognitive map of the environment (Epstein et al., 2017; O'Keefe & Dostrovsky, 1971). Notably, the current consensus suggests that the hippocampus supports the function of memory through a spatiotemporal framework (Knierim, 2015). Researchers suggest this framework includes social representations and the formation of a social cognitive map (Montagrin et al., 2018). Within this map, social dynamics, such as hierarchies, are formed through a network of neural representations of social interactions and observations of conspecifics (Leblanc & Ramirez, 2020). For example, social place cells in the hippocampus of rats and bats were selectively active in observing the location of another conspecific relative to their own position (Danjo et al., 2018; Omer et al., 2018; Wirth et al., 2021). In mice, impaired social memory and recognition of conspecifics was observed when hippocampal neurons in the CA2

subregion were silenced (Meira et al., 2018). The evidence outlined above suggests that the function of the hippocampus in representing the social environment, and in learning and memory, may provide an adaptive response that advances survival chances, which justifies further investigation into the hippocampus.

As social hierarchies are dynamic and can change over time, they demand behavioural flexibility, which is a function mediated by the prefrontal cortex (Bicks et al., 2015). The prefrontal cortex governs executive functions such as decision-making and social behaviour; however, the medial prefrontal cortex (mPFC) may be particularly important in social position (Amodio & Frith, 2006). Although there is controversy about whether functional homology of the mPFC exists between rodents and humans, the current consensus suggests the rodent mPFC is homologous to Brodmann's area 32, which is found within the anterior cingulate cortex (Bicks et al., 2015). The mPFC is thought to play a role in perceiving the status of the self relative to a group and the status of others (Bicks et al., 2015; Watanabe & Yamamoto, 2015). In one study, high mPFC activation was found when hierarchical ranks were updated throughout a game simulation based on the participant's performance relative to other participants (Zink et al., 2008). Studies that have manipulated the mPFC region have observed changes in social position. For example, manipulating AMPA receptor-mediated synaptic transmission within mPFC neurons of male mice resulted in changes in social position in the Tube Test. In particular, increasing the amplitudes of AMPA receptor-mediated postsynaptic currents resulted in an upward shift in rank, whereas decreasing AMPA receptor currents resulted in a downward shift in rank (Wang et al., 2011). Similarly, social ranks were lowered from their original positions when mPFC neurons were inhibited in male mice, and social rank increased when mPFC neurons were activated (Zhou et al., 2017).

To examine hippocampal and prefrontal cortex function in relation to social position, a behavioural paradigm that can assess both functions is needed. One such test is the Morris Water Maze. Through this task, hippocampal function is examined through spatial learning of rats as they learn the position of the hidden platform in a pool of water (Morris, 1984). This test also examines memory of the platform through the probe test, where the platform is removed and the swim location of the rats are assessed. Furthermore, PFC function is examined through the reversal phase of the Morris Water Maze. In this phase, the learned hidden platform is moved to an opposing location, and rats learn the new platform location while suppressing the memory of the old platform location (Vorhees & Williams, 2006). The reversal phase examines the relative behavioural flexibility in rats, which is a function of the mPFC. Therefore, the Morris Water Maze is a behavioural paradigm suited to assess neural correlates of social position and was used in this thesis. Thus, as stress has modulatory effects on learning and memory, our work aimed to explore the stress from an individual's social position on cognition.

CHAPTER 2
STUDY RATIONALE

Recent developments in neuroscience have established the influence that stress can have on the brain and, in turn, how stress may influence behavioural and physiological outcomes in life. Particularly, stress modulates learning and memory function, enhancing, or impairing cognition depending on the intensity and duration of the stressor. Despite decades of stress research in understanding the effect of stress on our biology, further research is needed to understand the nuances of stress and the underlying neurobiological mechanisms, especially in relation to learning and memory. One type of stressor is social stress, where social interactions with other individuals can induce the stress response. As the social environment is a key feature of daily life, social stress can occur at various points across the lifespan and become a chronic stressor. For our study, we are interested in understanding the effect of social position, as a chronic psychosocial stressor, on learning and memory function.

Notably, our study aimed to investigate social position in rats by using an ecologically relevant paradigm, the Tube Test, as a means to determine social hierarchy in rodents. Furthermore, cognition was measured by utilizing the Morris Water Maze, which is a standardized behavioural model in neuroscience that examines spatial learning and memory function. Moreover, as male subjects have preferentially been used in behavioural studies, there is limited knowledge on social hierarchies of female rats, which motivated the inclusion of female rats in our study. Thus, our study aimed to address these knowledge gaps by answering the following question:

Does social position affect spatial learning and memory in group-housed adult male and female rats?

2.1 Objectives

There were three specific aims of this thesis. The first objective was to confirm that the Tube Test can clearly and reliably measure social position in small groups of same-sex rats. If, as expected, the Tube Test acted as a reliable measure of social hierarchy, the second objective was to assess whether an animal's social position mediated its spatial learning and memory ability in the Morris Water Maze task. The third objective was to further explore whether the relationship between spatial memory and social position was moderated by the sex of the animal. Furthermore, there were two secondary objectives of this thesis. The first objective was to determine the stability of social positions over time, particularly after one week. The second sought to uncover anatomic markers of social position, specifically, whether body weight and organ weights (adrenal and thymus glands) differed by social position.

2.2 Hypotheses

As the first main objective pertained to successful determination of hierarchy using the Tube Test, no hypothesis was needed. For the remaining objectives, our hypotheses were:

1. We hypothesized that the most dominant animal would learn the location of the platform faster and outperform the lowest-ranked animal in the memory test of the Morris Water Maze task.

2. We hypothesized that the performance in the water maze between the most dominant and the least dominant rats would be different between male and female animals, such that less differences in performance was expected between social positions in the female animals.

3. We predicted that social positions would remain stable after 1 week.

4. We hypothesized that the least dominant animals would be more stressed, and this would be reflected in decreased body weight, thymus shrinkage, and enlarged adrenal glands.

CHAPTER 3
METHODS

3.1 Study design

As limited work has been conducted on rodent models of social hierarchy, this thesis presents an exploratory study on adult rats with the aim of measuring social position using the Tube Test. The first objective was accomplished by conducting the Tube Test on cage mates to determine their social position within a small group. Once the hierarchy was established, the second objective was achieved by using the Morris Water Maze to assess and compare spatial learning and memory performance of high-ranking and low-ranking rats. Furthermore, the third objective was to examine whether sex differences occurred by conducting statistical analysis of the behavioural data. See Appendix I for an overview of the experimental design.

3.2 Animals

Each week for ten consecutive weeks, a batch of 8 adult Sprague-Dawley rats (4 male and 4 female) arrived at the Central Animal Facility (CAF). The rats, purchased from Envigo, were approximately 12 weeks of age upon arrival and were same-sex housed in cages measuring 65 cm in length, 50 cm in width, and 21.5 cm in height. Bedding and nesting were consistent with standard protocols at the University of Waterloo CAF and the rats had ad libitum access to standard rodent chow and tap water. Each housing cage included enrichment items, specifically two small tubes, that may help habituate the rats to the apparatus of the Tube Test. The light/dark cycle of the housing room was kept at a 12-hour reverse cycle, with lights off from 7 am to 7 pm. Housing rooms were kept at a constant temperature of ~21°C and humidity at 45-56%.

3.3 Behavioural experiments

All behavioural testing occurred during the dark cycle with dim light (Tube Test room: 6-10 lux, Water Maze room: 10-35 lux). For context, when the room lights are on, the light in the Tube Test room ranges from 680-1100 lux, and 410-1000 lux in the Water Maze room. Behavioural testing took place after the animals were housed for two weeks, which allowed sufficient time for a social hierarchy to form (Beery et al., 2020; Blanchard & Blanchard, 1990; Williamson et al., 2019). Only one group of animals underwent behavioural testing at a time, with the 4 male rats tested first. The animals were identified using tail stripes marked with a permanent marker. All procedures and methods were approved by the Animal Ethics Committee of the University of Waterloo, Waterloo, Canada (ORE 44254).

3.3.1 Tube Test apparatus

Social position and social hierarchy structures were measured using the Tube Test. The protocol for the Tube Test followed the structure outlined in an earlier report that used mice (Fan et al., 2019), but was modified to be more suitable for rats. A clear plexiglass tube (length 100 cm, diameter 7 cm) with two holding chambers attached at either end (length 20 cm, width 20 cm, height 15 cm) was placed on a table in the testing room. The diameter of the tube was large enough to fit one adult male rat (~400-500g at 14 weeks of age), limiting movement and direction. Given the smaller size of the female rats (~230-350g at 14 weeks of age), two separate plexiglass tubes (length 44.5 cm, diameter 5.5 cm) were joined together and inserted inside the main tube, which reduced the diameter of the tube and imposed the same movement restrictions that faced male rats. Three sliding doors (length 15.2 cm, width 6.5 cm) were positioned along the tube, one door in the middle, and one at either end of the tube – separating the holding chambers from the tube. See Figure 1 for an illustration of the Tube Test apparatus.

3.3.2 Tube Test procedure

Two days before the Tube Test, each group of rats underwent habituation to the tube, followed by three days of the Tube Test. During the habituation phase, animals were transported to the testing room at approximately 9:00 am and left to acclimatize to the room for 15 minutes. Afterward, each rat was handled for 5 minutes and the tail was marked using a permanent marker (e.g., I, II, III, IIII). After the handling period, a rat was placed inside the holding chamber on the right side of the tube and was allowed to explore the apparatus in one direction, as all three doors of the apparatus were lifted. Crossing the tube to the opposite end was counted as 1 trial. After a total of 10 trials (5 trials from each holding chamber) were completed, each rat was placed in a separate cage (length 43 cm, width 29 cm, height 20.1 cm). Habituation was conducted for a total of two days and followed the same procedure on the second day with two notable changes. On the second day, the animal order was reversed, beginning with Rat 4 and ending with Rat 1, and the animals were placed in the left holding chamber of the tube as a counterbalance measure. The apparatus was cleaned using 1% (v/v) Micro-99 in T1 water between male and female groups.

After habituation, the Tube Test was conducted for three consecutive days. The test was organized in a round-robin fashion, in which all 4 rats from the same housing group were paired and tested against each other. Six different match-ups took place (1 vs 2, 3 vs 4, 1 vs 3, 2 vs 4, 1 vs 4, 2 vs 3), with each pair tested twice in alternating start positions, for a total of 12 trials. The order of matches for the testing days (Days 1-3) was randomized, resulting in three distinct match orders. The order of matches was consistently followed for all group of rats, ensuring standardization in the sequence of matches (Table 1). The animals were transported to the testing room at approximately 9:00 am and left to acclimatize to the room for 15 minutes. After the acclimatization period, each rat was weighed and placed into separate cages. For the first match-

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up, the corresponding pair of rats were placed inside the holding chamber at either end of the apparatus. All doors along the tube were initially closed, with each rat in its corresponding chamber. To start the trial, the two outermost doors at the end of the tube were lifted simultaneously, and the middle door was lifted once the animals reached the center of the tube. The rats pushed against each other until one rat successfully managed to force the other rat out of the tube and back inside the holding chamber. For each trial, 1 point was awarded to the winner that reached the opposite end of the tube and pushed the other rat out, while no points were awarded to the losing rat. The rats were returned to their cages at the end of each trial. The next pair of rats were tested according to the order of the match-ups of the day, and this process was repeated until all 12 trials were completed for each day. The apparatus was cleaned using 1% (v/v) Micro-99 in T1 water and dried before the next group of animals was tested. After one week, the rats underwent a one-day Tube Test to test the stability of the social hierarchy (see Figure 1 for a schematic of the Tube Test procedure).

3.3.3 Morris Water Maze

After three days of the Tube Test, the Morris Water Maze (MWM) was conducted. The task assessed spatial learning function as the subjects learned the location of a hidden platform in a pool of water over repeated trials (Morris, 1984; Vorhees & Williams, 2006). Memory function was examined by observing the preference for the quadrant of the pool where platform was located. Additionally, behavioural flexibility was assessed through reversal learning, testing animals' ability to suppress prior memory of the platform and learn its new location. A black circular pool (diameter 175 cm) with a platform (diameter 17.5 cm) placed in the SW quadrant of the pool was filled with water at a temperature of 22°C ± 1. The water was filled 2 cm above the surface of the platform. An overhead camera and a computer with Noldus Ethovision XT software v.17 was used

to record the time (escape latency) and distance for each trial. The overhead lights were turned off, and two lamps provided the minimum required lighting for camera tracking of the animals in the pool.

All Morris Water Maze testing followed a similar acclimatization protocol to the Tube Test protocol, where animals were transported to the testing room at approximately 1:00 pm and left to acclimatize to the testing room for 15 minutes. Afterwards, each rat was placed into individual cages, which was equipped with a towel, and an additional towel was used to cover each cage. The order of animal testing was shifted daily as a means of pseudo-randomization. For instance, on Day 1, the order started with Rat 1, 2, 3, and ended with Rat 4, while on Day 2, the order began with Rat 2, 3, 4 and ended with Rat 1. To ensure standardization, the order of release positions (N, S, E, W) was randomized for Day 1 to 4 of the spatial learning phase and Day 6 to 7 of the reversal learning phase (see Table 2). The resulting order of release positions was consistently followed for each group.

Spatial learning spanned the first four days of testing, where each rat underwent 4 trials per day at different release positions of the pool, for a total of 16 trials across four days. The rats were gently released facing the pool wall at a specific release position, and given 60 seconds to find the platform. Failure to find the platform within the allotted trial time resulted in the subject being guided to the platform, where they stayed for 30 seconds. If the animal found the platform before 60 seconds elapsed, the animal was removed from the platform after 30 seconds and placed back into its cage. All four rats were tested at the same release position before moving on to the next position, until all rats underwent a total of 4 trials for the day.

The probe test occurred on Day 5, where the platform was removed from the pool, and memory of the location of the platform was tested. Each rat performed one 30-second trial and was

released at the furthest position (N) from the platform. Day 6 and 7 of the Morris Water Maze focused on reversal learning. The procedure closely resembled the spatial learning phase on Days 1 to 4, with one significant change – the platform was relocated to the opposite quadrant of the pool (NE) (see Figure 2 for a schematic of the Morris Water Maze procedure).

3.4 Tissue and blood collection

Twenty-four hours after the last behavioral experiment, each group of rats was euthanized through CO_2-mediated anesthesia (3.4 flow rate for ~2 minutes), followed by decapitation. At sacrifice, body weight, trunk blood, and organ weights (adrenal and thymus glands) were collected from the highest and lowest-ranked animals. The collected blood was allowed to clot for 20 minutes at room temperature and centrifuged at 1800 x g for 20 minutes. The supernatant (i.e., serum) was collected and flash-frozen in liquid nitrogen. The mPFC was isolated from the brain using a stainless-steel brain mold. A coronal slice of the prefrontal cortex was removed, and the mPFC was carefully dissected and collected. The right and left hippocampus was carefully dissected from the intact brain and collected separately. The mPFC and the right and left HPC tissue were flash-frozen in liquid nitrogen. The adrenal and thymus glands were dissected from the body and the weight of each organ was recorded. In middle-ranked animals, the whole brain was extracted and flash-frozen in liquid nitrogen, and no further dissections took place. All collected brain tissue and blood were stored at -80°C for future experimental work.

3.5 Sample size estimate

The primary research questions of this project focused on examining the relationship between social position, spatial learning and memory, as well as the potential modulation by sex in this relationship. As such, the two key experimental factors were social position and sex. For

social position, four different positions were possible (alpha, beta, gamma, and delta), while for sex, there were two groups to consider: male and female. To our knowledge, there are no studies that have investigated learning and memory performance by social position using the Morris Water Maze, which limits guidance in calculating sample size. As such, we assumed the existence of a larger minimum difference in performance between the most dominant and least dominant subjects. Considering these factors, a two-tailed t-test was appropriate to estimate the sample size needed to detect relevant differences across social position and sex. In line with exploratory research, the significance level and power were set to $\alpha = .20$, $\beta = .80$ in the estimation of the sample size. The G*Power method (Faul et al., 2009; Faul et al., 2007) estimated that a sample size of 9 groups would be needed for spatial memory analysis using ANOVA. An additional group was added given the exploratory nature of this project, resulting in 10 groups. Overall, with 4 animals per group, 40 males and 40 female rats were used, with a total sample size of 80 animals. Although additional power calculations would have been needed to estimate the sample size for spatial learning and reversal learning using survival analysis, the calculated sample size was used as a proxy due to financial constraints and time limitations.

3.6 Statistical analysis

3.6.1 David's Score

The overall social position from the Tube Test matches was confirmed using David's Score (DS). Compared to other methods, David's Score is better suited to calculate dominance rank because it can consistently determine hierarchal rank in both an unbalanced and balanced number of interactions. In balanced tournaments like the match-ups for the Tube Test, where the number of encounters is the same for all players, David's Score reduces to row-sum scoring, which allows social position to be determined by the number of wins from each subject (David & Andrews,

1993). Row-sum scoring occurs because each paired interaction is assumed to be independent of other paired interactions, reducing the likelihood of artificially inflated, or deflated rankings (David, 1971; Gammell et al., 2003). The David's Score calculation of individual subjects also considers the proportion of wins and losses of each opponent the individual subject faced. Lastly, David's Score can determine hierarchical rank where reversals in wins and losses occur between paired interactions (Bang et al., 2010), which is beneficial for this exploratory project, as knowledge of the reliability of the Tube Test, female hierarchy, and stability of rank is limited. Thus, the David's Score for each rat was calculated for each testing day of the Tube Test, where a win from each match was worth 1 point, and a loss was worth 0 points. The average score over Days 1 to 3 was used as the final social position metric for subsequent analysis. In the case where a winner was not able to be determined (e.g., a tie result, where both animals manage to squeeze past each other), these matches were removed from the analysis (11 matches). See Appendix II for an example of calculation of David's Score.

3.6.2 Stability analysis of Tube Test scores

The stability of social positions was examined by comparing the average 3-day David's Score and the retest scores. A correlation analysis was conducted to investigate the relationship between the two groups of scores, and line-plots were utilized to visually compare the trends and average differences of both groups of scores. A bootstrapping test was conducted to confirm the differences between the two groups of scores. If the scores were stable, then the pair of scores (a subject's average 3-day DS and retest DS) would be as likely as the reverse order, and the distribution of the two groups of scores would be the same. The test involved randomly swapping values from the average 3-day DS and the retest scores and was reiterated 5000 times. The

distributions were compared based on the average difference of the scores and the standard deviation.

3.6.3 Survival analysis to examine Morris Water Maze data

The spatial learning and reversal learning phases of the MWM were analyzed using survival analysis, which examines time-to-event. In the context of the MWM, the event is whether the subject has reached the platform within the allotted time of 60 seconds (also known as the escape latency). Survival analysis was used instead of standard analysis of variance (ANOVA) because of the presence of censored data from the MWM trials. When subjects fail to reach the platform within 60 seconds, these trials are considered 'censored' (Andersen et al., 2021; Davari et al., 2023), as we do not know the true time the subject would have taken to find the platform. Notably, subjects fail to find the platform more frequently during earlier training days, as the animals are still learning the location of the platform. Subjects that found the platform at 60 seconds are not considered different from subjects censored at 60 seconds, which results in an underestimation of the sample mean time to find the platform (Davari et al., 2023; Jahn-Eimermacher et al., 2011). Although ANOVA modelling is the standard method of analyzing MWM data in neuroscience, by comparing means of different groups, the underestimation of sample means from censored data can introduce systematic biases and lead to misleading conclusions (Andersen et al., 2021; Davari et al., 2023; Jahn-Eimermacher et al., 2011). Furthermore, as the animals learn the platform location, censored trials decrease over time, and the animals accomplish the task at a faster time with reduced swim path. The trajectory of learning leads to a skewed distribution in the data with heterogeneous variance, which violates the assumption of ANOVA modelling that the data should follow a normal distribution with homogeneous variance (Andersen et al., 2021; Jahn-Eimermacher et al., 2011). Thus, the standard

statistical method of analyzing MWM data using ANOVA improperly handles censored data, which biases the analysis of the spatial learning phase.

A more suitable statistical method to analyze MWM data is survival analysis, which is typically used in clinical health research in investigating the probability an event occurs as a function of time (e.g., time to death). Survival analysis addresses the limitations of ANOVA modelling (e.g., event did not occur within the time period of the study) by handling censored data, which is common in time-to-event data. The typical survival analysis models (e.g., Cox Proportional Hazard) do not nearly have as many assumptions as ANOVA and do not require normality of the residuals (Andersen et al., 2021; Clark et al., 2003; Jahn-Eimermacher et al., 2011). As there are different types of survival models, the Cox Proportional Hazards (CPH) model in particular, has been proposed to analyze MWM data (Davari et al., 2023; Jahn-Eimermacher et al., 2011). The CPH can investigate the effect of multiple variables on spatial learning performance and can compare performance distributions through hazard ratios. In CPH, the proportionality assumption, which assumes that the hazard (i.e., event – in this case, finding the platform) between groups is equal over time. If the assumption of proportionality is violated, there are multiple solutions that can be used, such as stratifying for variables that do not satisfy the proportionality assumption, or including time-dependent variables. Upon analyzing spatial learning, the proportionality assumption was not satisfied for social position and training day, and a modified Cox model was fitted with social position and training day as time-dependent covariates. The model also included sex as a variable and was stratified for batch to account for the effect of the animal group. A CPH model was also used to analyze reversal learning data, however as there was no evidence to support the effect social position in the model, it was not included as a covariate.

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Thus, the model included training day, with sex as a time-dependent variable, and was stratified for batch.

3.6.4 Probe test analysis of the Morris Water Maze

The probe test of the MWM was analyzed using two-way ANOVA to examine the effect of sex and social position, on quadrant preference of the animals during their swim trials. If an interaction effect was found to be non-significant, the main effects of sex and social position were examined using Fisher's Least Significant Difference Test. Furthermore, the effect of batch was used as a blocking variable in the ANOVA test and the interaction of batch with factors such as sex and social position, was not tested.

CHAPTER 4
RESULTS

4.1 Behavioural observations of the Tube Test

The first day of the Tube Test trials was markedly longer than the following two days, as the pair of animals did not always meet in the middle of the tube at the same time. There were cases where one rat would scurry to the middle and scurry back to the holding chamber before the other rat could make it to the middle. Another scenario was where one rat would wait in the middle, while the other rat would not enter the tube for long periods of time (~ 5min). A third scenario we encountered was when the pair of animals would alternate entering the tube from their respective holding chambers. By the second day, however, the animals became familiar with the procedure and would instantly head to the middle of the tube as soon as the doors from the holding chamber were lifted. The trials were relatively fast at approximately 2-3 seconds. The third and retest days followed a similar pattern.

At the end of each trial, both animals were typically in the holding chamber of the losing animal. Out of all the matches of the Tube Test and retest trials across male and female groups, we observed 881 clear matches where the losing animal fled from their own holding box as soon as the winning animal occupied the same holding box (92.8%). Although we were typically fast enough to close the door to the holding chamber as soon as an animal fled to the opposing chamber, there were some cases where the winning animal chased the losing animal through the tube before we could close the door.

Aside from observations of fleeing by the losing animal, we also recorded whether there were any "power struggles" during the match. We defined power struggle as a match where the

winner was not obvious, as there seemed to be a prolonged fight where, depending upon the moment, each one rat seemed to be winning over the other. Power struggles did not occur as frequently as the fleeing behaviour, occurring at 18.4% of the trials.

We observed various strategies employed by winning rats. For example, some female rats would back out near the tube on their own and then proceed to charge forward, using their momentum to drive their opponent out of the tube. In another case, one male rat pushed his opponent by executing a flurry of left and right nudges, resulting in the opponent being pushed backwards. In another case, a male rat placed their head overtop of their opponent's head and then pushed all the way to the end of the tube. Another female rat took a similar strategy, however, instead of only pushing from the top, this rat nudged their opponent from both the top and bottom angles.

Lastly, we observed a few cases in different female groups, where one or two rats were able to learn how to lift the doors of the Tube Test apparatus to continue to interact with their fellow opponent. These rats used a combination of their nose and their paws to lift the sliding door that separated their holding chamber from the tube. In one case, one rat was able to open two doors very quickly, making their way to the door in front of their opponents holding chamber. As well, we observed both male and female rats had an apparent increase in energy during the Tube Test trials and retest trials compared to their energy during the Morris Water Maze.

4.2 David's Score rankings

The animal's performance on each Tube Test Day was calculated using David's Score and the average 3-day DS obtained. The DS ranged from ±6, with higher scoring animals winning a greater proportion of matches, while lower scoring animals lost a greater proportion of matches.

4.3 Social hierarchy phenotypes

The average David's Score was used to determine whether the hierarchy followed a linear, or non-linear order across the male and female groups. Linear groups were apparent when each animal in a group had their own distinct score, and the highest score was labelled as Alpha, second highest as Beta, third as Gamma, and the lowest score as Delta. Non-linear groups were present when there were two, or more animals in a group that had the same David's Score. In the male groups, 8 out of the 10 groups formed a linear hierarchy, while in the female groups, 7 out of the 10 groups were linear. In addition, 2 out of the 10 male groups were non-linear, and one of these took the form where there was a clear Alpha and Delta, however, the middle-ranked animals had the same David's Score (this formation also occurred in one of the female groups). Furthermore, two of the female groups (and one male group) displayed another non-linear hierarchy phenotype, where there was a clear Alpha and Beta in the group, however, the Gamma and Delta ranks had an equivalent average David's Score (Figure 3).

4.3.1 Changes to the social hierarchy phenotypes after 1 week

After a week, the Tube Test was conducted again to assess the stability of positions and hierarchy phenotypes. The retest scores were used to determine the hierarchy phenotypes of the groups after one week from the initial Tube Test trials. In the male groups, half of the groups displayed a linear hierarchy, while the remaining half displayed a non-linear hierarchy. In female groups, only 3 of the 10 groups displayed a linear hierarchy, while the remaining 7 groups were non-linear. Notably, the male and female groups had a similar number and direction of changes in their hierarchy phenotypes. Among the male animals, 4 groups changed from a linear hierarchy to a non-linear hierarchy, while 1 group displayed the reverse transition, from a non-linear to a linear hierarchy. Similarly, among the female animals, 4 groups also transitioned into a non-linear

hierarchy phenotype, while no changes were observed in the reverse direction. Thus, the changes resulted in a greater proportion of non-linear phenotypes in female groups. In terms of consistent hierarchy phenotypes, 5 of the male groups displayed the same phenotype on retest compared to their previously established structure, while 6 of the female groups displayed consistent hierarchy phenotypes. Generally, the phenotypic dynamics in the hierarchies of male and female groups were similar (Figure 4).

The Tube Test and the retest revealed different hierarchical phenotypes, with six different non-linear structures emerging from the groups. Triangular formations were most common, with four different variations observed. The first variation occurred in two male groups and three female groups, and was where the Alpha and Beta positions were linear, and the Gamma and Delta positions were tied. The second variation occurred in one male and one female group, where there was only clear Alpha, and the remaining positions were tied. The third triangular variation was an inverted formation from the one previously described, where Alpha, Beta, and Gamma positions all tied, and a clear Delta was observed (this occurred in only one male group). The last triangular variation occurred in one female group, where the Alpha and Beta tied, while the remaining positions followed the linear hierarchy structure. Aside from the triangular formations, two square-like groups were also observed. The first square-like group occurred in two female groups and had only two distinct positions, such that the Alpha and Beta positions tied, and the Gamma and Delta positions tied. Lastly, in one male group, the retest scores were the same in all four members of the group, resulting in a formation where all subjects tied.

4.4 Stability test of average 3-day DS vs retest

Stable social positions would indicate that if a subject's pair of scores (average 3-day DS and retest DS) was swapped, the distribution of the two scores would not be different. First, we

examined the stability of social positions by comparing the relationship and trends between the average 3-day DS and the retest scores. A bootstrapping test was conducted to confirm the differences between the two groups of scores by comparing the average difference distribution and standard deviation distribution. Specifically, the test involved randomly swapping values 5000 times from both groups of scores and comparing the distribution.

In male rats, a strong positive linear correlation ($r = \sim 0.70$) between the 3-day average DS and the retest score was found (Figure 5a). That is, a higher 3-day average DS is highly associated with a higher retest DS, and a lower 3-day average DS is highly associated with a lower retest DS. The line plot for the average 3-day DS and the retest score followed a similar trend (Figure 5b), and the difference between the scores resulted in a standard deviation of 2.7 (Figure 5c). The line plots of the scores indicated there may be differences between the average 3-day DS and the retest score. As a result, the differences were analyzed using the bootstrapping method of randomly swapping the values, which revealed large p-values in the average difference distribution ($p = 0.99$) and in the standard deviation ($p = 0.20$) distribution of the average 3-day DS and the retest score (Figure 6a-b). In other words, there is no persuasive evidence to support a difference in the distribution between the average 3-day DS and the retest scores for male rats when comparing the average difference and the standard deviation. Based on the analysis, the average DS from initial testing appears stable after one week in male rats, and any difference between the two groups of scores is likely due to chance.

In female rats, a moderate positive linear correlation ($r = 0.48$) between the average 3-day DS and the retest score (Figure 7a) was found. The relationship of 3-day average DS is linearly associated with retest DS, such that as the average 3-day DS increases, the retest score also increases, however, the relationship is not as strong compared to male groups. The line plot of the

average 3-day David's Score and the retest score displays slightly different trends (Figure 7b) and the difference between the two groups of scores results in a standard deviation of 3.32 (Figure 7c). The line plots suggested that there were differences between the 3-day average scores and the retest scores. As a result, the differences between the two groups of scores were analyzed using the bootstrapping test of randomly swapping values, which found the distributions based on the mean difference of the average 3-day David's Score and the retest were not different (p = 1.0) (Figure 8a). However, a weak difference was found when comparing the distribution based on the standard deviation (p = 0.057) (Figure 8b). Overall, the analyses suggest that the distribution of the David's score was more or less stable after one week.

4.5 Biometric effects on social position

The data were judged to have met the assumptions for ANOVA modelling and as biological differences are expected between male and female rats (and were thus not of interest in the current study), a two-way ANOVA with sex in the model, was used to examine the effects of social position on body weight, and two-sample t-tests was used to examine adrenal gland and thymus weights. Body weight was collected for all animals; however, adrenal gland and thymus weights were only collected for the highest and lowest scoring animals of each group.

The two-way ANOVA found social position was not a significant predictor of body weight (p = 0.65). Although, social position was not significantly different across social positions, Tukey's post hoc comparison test was used to compare the body weight of the most dominant and least dominant animals (male groups: p = 0.99, female groups: p = 0.99). In male rats, adrenal gland and thymus weights were not different between the highest scoring average and lowest scoring average male animals (adrenals: p = 0.68, thymus: p = 0.23). The effect size for body weight and adrenal gland weight was negligible (BW: g = ~ 0.070, adrenals: g = 0.18). However, the effect

size for thymus weight between Alpha and Delta males resulted in a medium effect size of 0.53. In female rats, body weight was also not found to be significantly different across all social positions (p = 0.38). Similarly, differences in adrenal gland weight and thymus weight were not significantly different between Alpha and Delta positions (adrenal: p = 0.14, thymus: p = 0.21). The effect size for body weight was small (g = 0.35) and medium for adrenal glands (g = 0.66) and thymus glands (g = 0.56). Overall, the biometric results of both male and female rats were not found to be significantly different across social position in stress-sensitive physiological parameters. The biometric effects are summarized in Table 3 for male rats, and Table 4 for female rats.

4.6 Spatial learning performance

Survival analysis (CPH model) was used to assess the impact of sex, social position, and training day on spatial learning performance. As the PH assumption was violated for training day and social position, an extended Cox model with time-dependent variables was used. The PH assumption held true for sex, social position and training day in the extended model when the data was split from 0-25 and 25-60 seconds. The effect of animal batch was accounted for by including it as a stratified variable in the survival analysis model. The standard approach of using ANOVA modelling was also conducted to analyze spatial learning performance.

4.6.1 Effect of social position on spatial learning

The extended Cox Proportional Hazards model was used to analyze the effect of social position on spatial learning. By the last training day (Day 4), a 1-unit increase in the average 3-day DS would not result in a statistically significant change in the spatial memory and learning (HR = 1.030, CI: 0.996, 1.066) in both male and female groups. In other words, by Day 4, rats

with a 1-unit increase in the average DS, on average, would not have a higher chance in finding the platform within the first 25 seconds. However, in the last 35 seconds by Day 4, a significant difference in the hazard ratios was found, on average, when comparing a 1-unit increase in the 3-day average DS in male and female rats, as the hazard ratio was 1.085 with a 95% confidence interval of (1.04, 1.14). The finding indicates that while adjusting for other variables, on the last 35 seconds of the trial on Day 4, a 1-unit increase in the average DS score for male and female rats increases the instantaneous rate of finding the platform by a factor of 1.085.

As the average difference between the highest average DS and the lowest average DS was approximately 7-units for male groups and 6-units for female groups, the hazard ratios were calculated to examine the rate of finding the platform of more dominant animals compared to less dominant animals. By Day 4, in the first 25 seconds of training, the hazard ratio for an average DS of up to a 7-unit increase was not significantly different from 1 (HR = 1.23, CI: 0.972, 1.560). That is, on average, there is no difference in the rate of finding the platform in the first 25 seconds with an average DS of up to 7-units. However, in the last 35 seconds, rats with up to a 7-unit increase in their average DS, were faster at finding the platform by a factor of 1.77 (HR = 1.77, CI: 1.28, 2.43). Overall, male and female animals in our study had similar average differences between the highest and lowest average DS, and by the last training day, higher scoring rats had a greater rate of finding the platform in the last 35 seconds of training. The difference in rates materializes in the survival function in Figures 13a-b.

A two-way ANOVA model with social position and training day was stratified for sex. In male groups and female groups, spatial performance between the most dominant and least dominant animals were not significantly different (male groups: p = 0.21, female groups: p = 0.94) (Figures 14a-b).

4.6.2 Effect of sex on spatial learning

The extended Cox Proportional Hazards model was used to calculate the hazard ratios between male and female rats in the learning phase of the Morris Water Maze. The interaction term between training day and sex was not statistically significant and was excluded in the model. As such the hazard ratio for male and female rats was 1.28 across all learning days, with 95% confidence intervals of (1.13, 1.45), indicating that male rats were 1.28 times faster at finding the escape platform than female rats. Figure 9 represents the spatial learning performance of male rats compared to female rats, where the estimate of the survival function for male rats is consistently lower over time compared to the female's curve. The survival function at any time point t0 is the likelihood that it takes longer than t0 for the rat to find the platform. As a result, the lower this probability, the better the performance.

A two-way ANOVA model found training day and sex as significant predictors of spatial learning based on escape latency performance (training day: $p < 0.0001$, sex: $p = 0.0040$) (Figure 10). Tukey's post-hoc test confirmed significant differences between Day 1 and Day 4 ($p < 0.0001$). Similarly, a two-way ANOVA model found training day and sex as significant predictors of spatial learning performance based on the distance travelled (training day: $p < 0.0001$, sex: $p = 0.0007$) (Figure 11). Tukey's post-hoc test confirmed significant differences between Day 1 and Day 4 ($p < 0.0001$). A correlation analysis comparing the escape latency and distance travelled found both measures have a strong linear relationship ($r = 0.96$) and the large correlation coefficient indicates that escape latency and distance travelled are dependent measures that would lead to similar scientific conclusions in assessing spatial learning performance (Figure 12). Overall, the results from the ANOVA model suggest the animals learned the location of the

platform as performance (based on time and distance) differed from Day 1 and Day 4, and spatial learning performance was significantly different between male and female groups.

4.6.3 Censoring as a measure of spatial learning performance

As the learning trials were limited to 60 seconds, censoring occurs when animals do not find the platform within the given time and the true time to find the platform is unknown. However, censored trials decrease as animals learn the location of the platform. As a result, we examined the frequency of censored trials based on the time to find the platform (escape latency, measured in seconds) for all trials (1280 trials) over the four learning days (320 trials per day) (Figure 15).

On Day 1, the frequency of censored trials was 50%, indicating that in 160 trials, rats were unable to find the platform before 60 seconds or found the platform at 60 seconds. The frequency of censored trials decreased over the learning days, with 24% of the trials on Day 2 were censored, 9% on Day 3, and 6% on Day 4. The reduction of the censored trials over the four learning days provide evidence that the animals learn the location of the platform over time, with some censoring still present on the final training day.

4.7 Spatial memory performance

4.7.1 Effects of sex and social position on spatial memory

A two-way ANOVA was used to assess the memory phase of the Morris Water Maze with sex and social position as covariates. Although a weak interaction between sex and social position ($p = 0.054$) was found, the main effects of sex and the main effects of social position were examined and were not observed to be statistically significant ($p = 0.23$ and $p = 0.87$, respectively). The results from the ANOVA model indicate that male and female rats spent a similar average

amount of time in the target quadrant (SW) (Figure 16). As well, the results indicate a similar average time was spent in the target quadrant across Alpha, Beta, Gamma, and Delta (Figure 17).

4.7.2 Relationship between average distance to platform and time spent in platform quadrant

Spatial memory is typically examined by the average time spent in the target quadrant, however, the average distance to the platform during the Probe test has been considered a more accurate measure of memory. We compared the two measures using a correlation analysis to examine the relationship between average distance to platform and time spent in the target quadrant. We found a strong negative relationship between average distance from platform and time spent in the SW quadrant ($r = -0.84$) (Figure 18). In other words, as average distance to the platform decreased, there was an increase in time spent in the target quadrant. The large correlation coefficient is a strong indication that inference based on distance or time would most likely result in a similar scientific conclusion.

4.8 Spatial reversal learning performance

As spatial reversal was conducted similarly to spatial learning (with the exception being the change in the platform location) the effect of sex and social position on the average time to find the new platform was examined using a Cox Proportional Hazards model. As the PH assumption was violated for sex, an extended Cox model was used with sex as a time-dependent variable, with training day as a covariate in the model. The PH assumption held true when the data was split from 0-15 and 15-60 seconds. Social position was not a significant variable and was not included in the model. Two-way ANOVA modelling was also conducted to analyze reversal learning.

4.8.1 Effect of social position on reversal performance

From the Cox Proportional Hazards model, social position was not a significant predictor of reversal learning and was removed from the model. Figures 21a-b visually represents the survival function during reversal learning, where the probability of finding the new platform location was not different between the most dominant and least dominant animals.

A two-way ANOVA with social position and training day in the model was stratified for sex. In male and female groups, reversal learning between the most dominant animal and least dominant animal was not significantly different (male groups: p = 0.85, female groups: p = 0.57) (Figures 22a-b).

4.8.2 Effect of sex on reversal learning

Hazard ratios were calculated from the extended Cox model with sex introduced as a time-dependent variable. In the first 15 seconds, the hazard ratio for male and female rats was 1.58, with a 95% confidence interval of (1.23, 2.04), which indicates that, on average, male rats were 1.58 times more likely to find the platform in the first 15 seconds of reversal training compared to female rats (HR: 1.58, CI: 1.228, 2.037). In the last 45 seconds of training, the hazard ratio for male and female rats was not significantly different from 1 (HR = 0.984, CI = 0.790, 1.23), indicating that, on average, male and female rats had equal rates of finding the platform (HR: 0.984, CI: 0.790, 1.225) (Figure 19).

A two-way ANOVA model found spatial reversal learning performance was significantly different between sex and across training days (sex: p = 0.026; training day: p < 0.0001) (Figure 20).

4.8.3 Censoring as a measure of spatial reversal performance

Similar to the learning trials, the reversal learning trials were limited to 60 seconds. We examined the frequency of censored trials for all reversal learning trials (640 trials) over the two reversal days (320 trials per day) (Figure 23).

On Day 6 of the Morris Water Maze, the frequency of censored trials was 21%, indicating that rats were unable to find the platform at the new location before 60 seconds or found the platform at 60 seconds, in 21% of the trials. The frequency of censored trials decreased over the reversal days, with censored trials decreasing to 6% on Day 7. The reduction of the censored trials over the two reversal days provide evidence that the animals learned the new location of the platform rapidly, with some rats still censored at 60 seconds.

CHAPTER 5
DISCUSSION

5.1 Overview

In group-living species, the social environment plays a large role in every day life, as social interactions with other members influence development and shape behaviour at the individual level. For instance, studies have found juvenile rats reared in isolation displayed cognitive deficits in adulthood, however, the cognitive deficits were reversed when juvenile rats were exposed to daily interactions with peers (Einon, 1980; Einon et al., 1981; Einon & Morgan, 1977; Pellis et al., 2023). Social hierarchy is one facet of the social environment that is present and naturally occurs in group-living species, where interactions between individuals establish an order, granting those at the top of the hierarchy priority access to resources such as territory, food, and mating opportunities (Bonabeau et al., 1999; Koski et al., 2015; Sapolsky, 1995; Ziporyn & McClintock, 1991). Prior to the formation of a socially organized group, aggression levels are high as individuals engage in interactions, such as fights, to establish their positions within the hierarchy. Then, social behaviours, such as agonistic behaviours displayed by dominant individuals, reinforce and maintain the social structure. Observations of rats have found dominant male rats display more aggressive behaviours by initiating chases and fighting intruders, while subordinates are typically chased by higher ranking conspecifics and display submissive postures during fighting (Adams & Boice, 1989; Berdoy et al., 1995; Calhoun, 1963; Koolhaas et al., 1980).

In humans, one metric of social hierarchy is socioeconomic status, which has been found to be predictive of health outcomes (Farah, 2017). In particular, there is a graded relationship between SES and health, such that mental, physical, and life expectancy outcomes improve with

increased SES (Adler & Stewart, 2010; Marmot & Shipley, 1996; Marmot et al., 1991). Similarly, in rats, the effects of social stress are reflected in immune function, where subordinate rats have been found to have adrenal hypertrophy, thymus involution (or shrinkage), and increased disease risk (Barnett, 1958; Bartolomucci et al., 2005; Tamashiro et al., 2004; Tamashiro et al., 2005). One's relative social position not only influences social interactions with other members of the group, but also impacts health and overall quality of life.

Although animal social behaviour has been extensively researched, understanding the neurobiological mechanisms of social hierarchy has only recently become a topic of interest in neuroscience. Indeed, the interest has grown out of advancements in neuroscience research that have identified the consequences of stress on the brain, particularly on cognitive function. Stress has been consistently reported to reduce dendritic branching in the mPFC, HPC, and amygdala, impairing functions such as learning, memory, decision-making, and emotional responses (McEwen, 2007). Numerous studies have also manipulated glucocorticoid levels and tested learning and memory function, such that blocking glucocorticoid receptors impaired spatial learning and memory in the Morris Water Maze (Oitzl & de Kloet, 1992; Roozendaal & McGaugh, 1997; Sandi & Pinelo-Nava, 2007). Thus, stress has a key role in modulating cognition, however the effects may differ between sexes.

Sex differences have also been reported on learning and memory function, where male rats perform better in learning the platform location than female rats (Jonasson, 2005). While chronically stressed (e.g., restraint) female rats tend to perform better on the Morris Water Maze than stressed males (Luine et al., 2017). However, the consequences of stress depend on the type of stressor and the duration, and since social hierarchy is a pervasive aspect of everyday life, the chronic effects of social stress on cognition remain to be fully understood. Thus, our objectives

were to examine whether social position influenced spatial learning and memory, and to explore the role of sex in this relationship. Additionally, we were interested in the stability of social positions over time, and potential biomarkers of social position.

5.2 Effect of social position on spatial learning and memory

To our knowledge, no previous studies have directly assessed the effect of social position on spatial learning and memory in rats, leaving us with limited guidance on what to expect. We predicted that the most dominant animal would perform better than the least dominant animal in the MWM task. We used survival analysis (Cox Proportional Hazards) to examine the effect of social position on the time taken to find the platform for spatial learning and reversal learning phases of the MWM task. Surprisingly, our results did not provide evidence that social position could influence a rat's ability to learn the platform location in either phase of behavioural testing. However, our results found an advantage of dominant individuals during the learning phase, where if the platform was still not found after 35 seconds, animals with at least a 1-unit increase in their DS would find the platform faster.

Additionally, we investigated the effect of social position on spatial memory. We predicted that more dominant animals would display better spatial memory compared to less dominant animals. Contrary to our expectations, we did not find evidence that social position impacts spatial memory, as all social positions demonstrated similar time spent in the target platform. These unexpected findings highlight the complexity of the relationship between social position, stress, and spatial cognition in rats, warranting further exploration and investigation into the underlying mechanisms that govern these interactions.

One possible explanation for the unexpected results is rooted in the social nature of rats. Group housing of rats together with the same cagemates for an extended period of time may have provided a stable and secure environment, resulting in minimal stress and may explain no impairment to spatial learning and memory function. In a recent report, stable social housing of male rats was found to enhance learning and reversal learning performance in the Barnes maze (a standard spatial learning and memory paradigm) compared to individually housed male rats (Heimer-McGinn et al., 2020). In our study, the animals were group-housed with the same conspecifics and left undisturbed for two weeks before behavioural testing, and no changes were made to the housing conditions during behavioural testing. This timeframe has been shown to be sufficient for a stable hierarchy to form in rats, leading to reduced aggression and competition (Beery et al., 2020; Blanchard & Blanchard, 1990; Gerges et al., 2003; Gerges et al., 2004; Gerges et al., 2001). As a result, the stability of the housing environment may in fact contribute to a low stress environment and may explain the lack of impairment in spatial learning and memory performance (Heimer-McGinn et al., 2020). In addition to the stability of the housing environment, resources such as food and water were constantly available, inhibiting any survival motivation to compete or engage with one another over a food or water source (Calhoun, 1963; Costa et al., 2021; Fulenwider et al., 2021). Nonetheless, there is a chance that the social environment of our sample may not have been stressful enough to induce impairments in spatial learning and memory.

5.3 Sex effects on spatial learning and memory

In spatial navigation literature involving rodents, male rats have been found to learn the platform location faster than female rats in the Morris Water Maze (Jonasson, 2005; Saucier et al., 2008). However, studies have found that stress reverses this advantage, where female rats that were chronically stressed through restraint, were found to make fewer errors than male rats

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subjected to the same stress condition when tested in the radial arm maze (Luine et al., 2017). To examine the effect of sex in spatial learning and reversal learning, we used survival analysis (CPH) to analyze the Morris Water Maze data. In our study, we found male rats indeed performed better than female rats in learning the platform location during the learning and reversal phases of the Morris Water Maze. In particular, male rats were 1.28 times more likely in their probability to find the platform compared to female rats during the learning phase. In the reversal learning phase, male rats were 1.58 times more likely to find the platform during the first 15 seconds of the trial compared to female rats (after 15 seconds, male and female rats displayed an equal rate of finding the platform).

A two-way ANOVA model was used to explore whether there were any sex differences in the average time spent in the target quadrant (SW), a measure for memory of the platform location. We found no sex differences during the memory test, as male and female rats spent a similar amount of time in the target quadrant. Overall, our results are consistent with learning and memory literature of non-stressed rats, where male rats have an advantage in spatial learning and navigation compared to female rats. Furthermore, as chronically stressed female rats typically outperform male rats, the absence of this observation in our results may indicate that the rats were not socially stressed during our study.

5.4 Sex effects on social position and spatial learning and memory

In our study, we hypothesized that the effect of sex on the relationship between social position and spatial cognition would result in less differences in performance across ranks in female groups compared to male groups. In other words, we expected the differences in performance between the most and least dominant animal would be bigger in male groups, and smaller in female groups. To test this hypothesis, we used survival analysis to explore the effect

of sex and social position on spatial learning performance. We found no evidence for an interaction of sex and social position in all phases of the Morris Water Maze, suggesting that the effect of social position on spatial learning is not influenced by sex. Thus, our results indicate that social position in both male and female rats result in similar patterns of spatial learning and memory performance.

5.5 Other measures of examining spatial learning of the platform

We assessed spatial learning and reversal learning in the Morris Water Maze other than the time it took to find the platform. We found that distance to platform and frequency of censored trials are both measures that indicate spatial learning took place over the training days. Distance to platform was strongly correlated with the time to find the platform over time, and suggest similar conclusions can be drawn from using either distance or time. Censored trials indicate that the rats did not find the platform before 60 seconds, and observing a reduction in the frequency of censored trials over the training days indicate whether spatial learning took place. In our study, half of the trials on Day 1 were censored, however by Day 4, censoring decreased to 6%, indicating that a small proportion of rats remained unable to find the platform even after four days of training. Similarly, censored trials reduced from 21% Day 6 to 6% on Day 7 during reversal learning; which indicate that although the animals were familiar with the task and rapidly learned the platform location, censoring was still present in a small proportion of the trials.

5.6 Tube Test strategies, scores, and stability

The Tube Test is a tool to assess social hierarchy in rodents and is highly correlated with other standard social dominance tests (Fulenwider et al., 2021; Wang et al., 2011). We used the Tube Test to determine social positions based on a 3-day round-robin tournament, where pairs of

animals from the same cage group were tested in a right-of-way challenge. We observed different strategies used by winners, and observed interesting behaviours from losing animals. Rats that won their matches employed various strategies, notably pushing and nudging their opponent repetitively until they were out of the tube. Interestingly, as soon as the winning rat pushed the losing rat out of the tube and into the holding chamber, the winning rat would often enter the same holding chamber. With both rats occupying the same space, the losing rat would immediately leave the chamber, this fleeing behaviour occurred in 92.8% of the matches. Typically, as soon as the losing rat fled from the holding chamber, the doors were immediately closed. However, in cases where the doors were not closed promptly, the winning rat would chase after the losing rat. We also observed scenarios in which both rats struggled to win the match, where one rat would take control of the match and subsequently the opponent would take over. We defined these matches as 'power struggles', which were less common than fleeing behaviour, occurring only in 18.4% of all matches.

Based on the outcomes of the Tube Test matches, the David's Score was calculated for each testing day and the average 3-day David's Score was determined. Hierarchical phenotypes of all groups were visualized as either linear, or non-linear, with a greater proportion of linear hierarchy phenotypes during initial testing in both male and female groups. However, using the retest scores, there was a shift of non-linear hierarchy phenotypes. In female groups, we observed a higher proportion of non-linear hierarchy phenotypes compared to the hierarchy phenotypes from initial testing. In contrast, an equal number of linear and non-linear phenotypes occurred in male groups. The change in hierarchical phenotypes suggest that rodent social hierarchies are dynamic with slight changes in social position. In terms of social positions, we compared the average 3-day David's Score and retest scores, and we found a strong linear correlation in male groups and a

moderate correlation in female groups, which suggest that retest scores of female rats may be more variable. Our results provide evidence to suggest that the scores obtained from initial testing days are stable after one week, indicating stability of the social hierarchy of both male and female groups. Overall, our results suggest evidence of sex differences in social hierarchy phenotypes and social position scores after one week, where female groups had greater variability in their retest scores and displayed a greater shift towards non-linear hierarchy phenotypes.

5.7 Biometric effects of social position

Another goal of our study was to explore anatomical biomarkers of social position in rats. Previous social hierarchy studies have documented larger body weights in more dominant male rats while smaller weights were observed in more subordinate male rats (Berdoy et al., 1995; Calhoun, 1963). Body weight is considered an indicator of dominance, as dominant individuals were found to take priority access of food, and successfully displace others during feeding (Karamihalev et al., 2020). However, our results found no difference in body weight across social positions in male rats and female rats. The lack of body weight difference may indicate no individual rat took over priority access of food and water resources in their group. Additionally, the discrepancy from our findings and earlier reports from Berdoy et al. (1995) and Calhoun (1963) may stem from the different experimental conditions and rodent strain. Namely, Berdoy et al. and Calhoun observed the social hierarchy of wild trapped rats in seminatural conditions, while our study observed social hierarchy of Sprague-Dawley rats, a docile strain commonly used in laboratory experiments, in laboratory conditions.

Furthermore, we examined adrenal and thymus gland weights because of their functional role in the stress system (Smith & Vale, 2006; Yaribeygi et al., 2017). The adrenal gland is a key organ involved in the HPA-axis, releasing glucocorticoids in response to stress (Smith & Vale,

2006), and examining adrenal gland weights is a useful measure to reflect organ activity. For example, hypertrophy in the adrenal glands was observed in male rats subjected to crowded housing conditions (10 rats per cage) compared to the control group (5 rats per cage) (Gamallo et al., 1986). The role of immune function in response to stress has been well-documented, where chronic stress has been found to suppress the immune response (Dhabhar, 2008). As the thymus gland has a role in producing immune cells (T-cells), suppression of the immune response leads to shrinkage of the thymus gland (Dhabhar, 2008). For example, decrease in thymus gland weight was observed in male rats that underwent forced swim test for 21 days compared to control rats (Zivkovic et al., 2005). In our study, we found no difference in adrenal and thymus gland weights across the highest and lowest social positions in male and female rats. Since our group size (4 animals per cage) was the maximum number of animals for the home-cage size, it did not subject the animals to socially stressful crowding situations, and may have been optimal for group-housing conditions. As such, the biometric results provide further evidence that the animals in our study were not severely stressed to alter biological parameters. The other biological materials collected in this study (blood and brain tissue) were not analyzed and will be assessed in future work.

CHAPTER 6
CONCLUSIONS

6.1 Limitations

Despite the contributions and insights of this study, it is important to acknowledge and discuss the limitations of this work. In this section, three main limitations will be discussed. The first limitation concerns the disadvantages of purchasing animal subjects with unknown information regarding their early life, and the effects of transportation stress as a result of shipping the animals to the Central Animal Facility at the University of Waterloo. The second limitation concerns the relatively small sample size of the study, and the third limitation concerns the Tube Test as a heuristic for determining social rank and the lack of a secondary test to confirm the ranks from the Tube Test.

As the animals were transported to the University of Waterloo's animal facility as adults at 12 weeks old, there was little information on the lives of the animals before we received them. Notably, there is increasing evidence that early life stress such as a lack of maternal interaction, may alter normal development and behaviours seen in adulthood (Sandi & Haller, 2015). For example, high maternal investment such as increased frequency in grooming or licking pups during early life (first 10 days) was found to program the HPA-axis to have a reduced response to stress in adulthood (Liu et al., 1997). Thus, the unknown early life conditions of our subjects are concerning as the animals may have experienced conditions that could have altered programming of HPA-axis and behaviours in adulthood (Fan et al., 2021; Sandi & Haller, 2015; Welberg et al., 2006; Workman et al., 2011).

As the animals were transported to the University of Waterloo Central Animal Facility, transportation may have stressed the animals. For example, stress may have been caused by changes in temperature and lighting conditions, traveling conditions, exposure to new animal handlers, and new conspecifics (Arts et al., 2012). In adult male rats, increased plasma corticosterone was still present 3 weeks after transport, suggesting that transportation stress may have enduring effects (Arts et al., 2012). Physical and behavioural changes have also been reported from transportation stress in adult male rats, including matted fur and rough hair, reduced social interactions, and anxiety-like and depressive-like behaviours (J. Wang et al., 2019; L. Wang et al., 2019). The ideal solution to address this limitation would be an in-house animal colony, however, such a solution requires resources that involve financial and institutional investment, time, and constant personnel to set up, breed and maintain the animal colony. Although ideal, creating and maintaining a rat colony for my thesis would not have been feasible. To mitigate the effect of transportation stress, all animal groups underwent a week-long acclimatization process at the University's animal facility. Subsequently, an additional week of undisturbed housing was allocated to allow sufficient time for the establishment of a social hierarchy.

The sample size of the study, which was composed of 10 male and 10 female groups may also be a limitation. Although our sample size was statistically sufficient for the goals of this thesis, a larger sample size would increase statistical power and would be able to detect smaller differences in effects, particularly if differences occur as a gradient effect (e.g., gradient effect of social position and health as seen in Marmot's Whitehall studies). Furthermore, a larger sample size would result in a better representation of the rodent social scene and provide more accurate data regarding social behaviours of dominance, sex differences in social hierarchy formation, and the effect on spatial learning and memory. However, as one of main responsibilities of animal

research is to minimize the number of subjects as possible, a larger sample size would not have been ethical in our study given the exploratory nature of this thesis, and would not have been feasible within the duration of my graduate studies.

The heuristic nature of the Tube Test is also a limitation of our study. As the testing conditions occur inside a laboratory environment, the Tube Test serves to mimic the burrowing environment of rodents and utilizes the right-of-way problem as one measure of dominance. However, since we are merely observing the interactions between pairs of rats in the tube, we cannot truly know the hierarchy within the groups. Instead, we can only approximate the ranks of the animals based on the outcomes from the interactions of the Tube Test. In addition, we did not conduct a second dominance test that utilizes space to measure dominance, which could have confirmed the rank-order obtained from the Tube Test. For example, the visible burrow system or the warm-spot test are two standard social dominance paradigms that use space as a measure for social dominance (Fulenwider et al., 2021). However, other studies have reported a high correlation to the Tube Test with other known social paradigms including the visible burrow system, the warm-spot test, and territory urine marking assay (Fulenwider et al., 2021; Sabanovic et al., 2020; Wang et al., 2011). Despite the noted limitations, we believe the Tube Test remains a useful tool to study social dominance.

Although this thesis provides insight into the relationship between social rank and cognitive function in rats, there are practical and heuristic limitations that confine the scope of this study. Firstly, the acquisition and transportation of adult animals to our facility combined with the limited information regarding their early life hinders our understanding of the stressors they may have experienced and how these stressors may have affected them in adulthood. Secondly, the sample size of this study may have failed to detect finer nuances in social behaviour and the

relationship between social rank and cognition. Lastly, the Tube Test is a heuristic tool to measure social dominance in rodents. As such, we can only determine an approximation of social position within each group. Therefore, future work assessing social dominance should employ methodologies that can mitigate these limitations.

6.2 Future directions

Given the exploratory nature of this study, addressing the limitations identified previously for future studies would contribute to a more comprehensive understanding of the insights gained from the experiments that were conducted. As previously mentioned, mitigating unknown factors, such as transportation and husbandry-related stressors that have the potential to alter adult behaviour, can be achieved by sourcing animal subjects for studies from an in-house rodent colony or from a vendor that has a documented life history of the animals. Additionally, increasing the sample size and conducting a second dominance test to validate the rank-order results obtained from the Tube Test would help to address the limitations previously discussed.

Future work could also introduce new methodologies such as recording the home-cage environment and the Tube Test, or automating the test. By observing animal activity within the home-cage, we can gather further insight into the social dynamics of the groups, such as social exclusion, and observe behaviour that correlates with social position, such as feeding behaviour, or barbering behaviour (where the most dominant animal would trim, or pull whiskers, or fur of subordinates). Another area where recording behaviour would be advantageous, would be to record the exploration of the tube and the Tube Test matches. By doing so, we could gain a better understanding of other dimensions of behaviour, such as assessing anxiety through exploration of a novel environment, pushing strategies of winners, and conversely, the retreat behaviours of

losers. Speed could potentially be another factor that could be recorded to determine whether speed of exploration of the tube could be related to social position. Additionally, recording the speed of matches could correlate with distance between social positions. Furthermore, automating the task, in which sensors would open and close each door along the tube instead of the researcher, would limit confounding effects introduced by human interference and would create a more controlled and standardized environment.

Aside from practical improvements, there are two key areas that warrant further investigation. Future work could focus on assessing physiological characteristics such as corticosterone and hormones, and analyzing proteins (e.g., AMPA receptors) of key brain regions involved in stress and in social navigation (particularly the PFC and the HPC). Examining levels of corticosterone across rank and between the sexes, may provide insight into the underlying biological mechanisms of social position and how social stress affects male and female rats. Measuring hormones, such as testosterone, and monitoring the estrous cycle in female rats could provide valuable insights into the potential mechanisms underlying social position in rats and its impact on social dynamics between rats. In other rodent species, such as naked mole rats, dominant females were reported to have elevated testosterone levels and to suppress reproductive function of subordinate females (Clutton-Brock, 2007; Clutton-Brock et al., 2006; Holmes et al., 2009). In Syrian Hamsters, increased submissive behaviour was also found with elevated estradiol concentrations (Solomon et al., 2007).

Furthermore, exploring the role of proteins in brain regions such as the PFC and HPC that are not only involved in regulating stress, but also functionally involved in navigating social interactions, could also contribute to a deeper understanding of the biological mechanisms of social stress and social position. In particular, investigating AMPA receptor expression would be a

suitable candidate for further research as AMPA receptors in the mPFC have been found to modulate social position when expression of GluR4 was manipulated (Wang et al., 2011). Social dominance was also found to correlate with phosphorylation of AMPA receptors in the mPFC, notably in the GluA1 subunit (Park et al., 2018).

6.3 Implications and conclusions

Based on the findings of our results, we do not have evidence to suggest that social position influenced spatial learning and memory performance. Sex was also found to be an insignificant factor in the relationship between social position and cognition. The evidence from our results suggests that the male and female groups in our study may not have been socially stressed to induce an impairment in learning and memory function. Furthermore, the results from the biometric analysis did not confirm stress-related changes as no significant differences in body weight, adrenal gland, and thymus gland weights were found across social positions. Thus, the results from our study suggest that differences in social position in a group of same-sex rats, did not impair learning and memory function. In terms of stability of social positions, we found the average score from the Tube Test matches were linearly associated, suggesting that male and female groups in our study were in similar social positions over time. However, social positions in female groups were found to be slightly less linearly associated and displayed a greater proportion of non-linear hierarchy phenotypes after 1 week compared to male groups. The findings from the stability analysis suggest that female hierarchies may be more dynamic than male hierarchies.

Understanding social position is a complex yet important topic to understand, as the effects of social stratification influence psychological and physiological outcomes. For example, agonistic behaviours of dominant and subordinate rats are similar to bullying and hazing behaviour seen in

humans. Bullying can be a severe physical and psychosocial stressor, which can erode mental health in those who are bullied, and result in long-lasting effects (Arseneault et al., 2010; Buwalda et al., 2011). Adverse social experiences or lack of social engagement has also been linked to developing post-traumatic stress disorders, psychiatric mood disorders such as depression and anxiety (Sandi & Haller, 2015). Social relationships can also buffer the effects of stress as a strong social support has been found to lessen negative reactions to stressful events, and is considered a protective factor in the development of neurological diseases such as Alzheimer's Disease (Fabrigoule et al., 1995; Kawachi & Berkman, 2001). Understanding social stress also has important implications in preclinical research practice, as animal models of psychiatric illnesses can further the understanding of the disorder of interest and further the development of potential therapeutics.

While the findings of our study do not indicate consequences of social stress in cognition, the absence of stress-related behavioural and physiological changes may suggest that the group-living conditions in rats are socially organized in a stable manner, where stress levels are low. Our exploratory work makes valuable contribution to the limited literature on social hierarchy using the Tube Test in rats, as this test has primarily been used on mice. Moreover, by including both male and female rats in our study, we were able to explore potential sex differences and gain insight on the social dynamics of female rats. However, our work has barely scratched the surface in understanding the effects of social stress on cognition. Therefore, future work is needed explore the relationship between social position and cognition further.

FIGURES AND TABLES
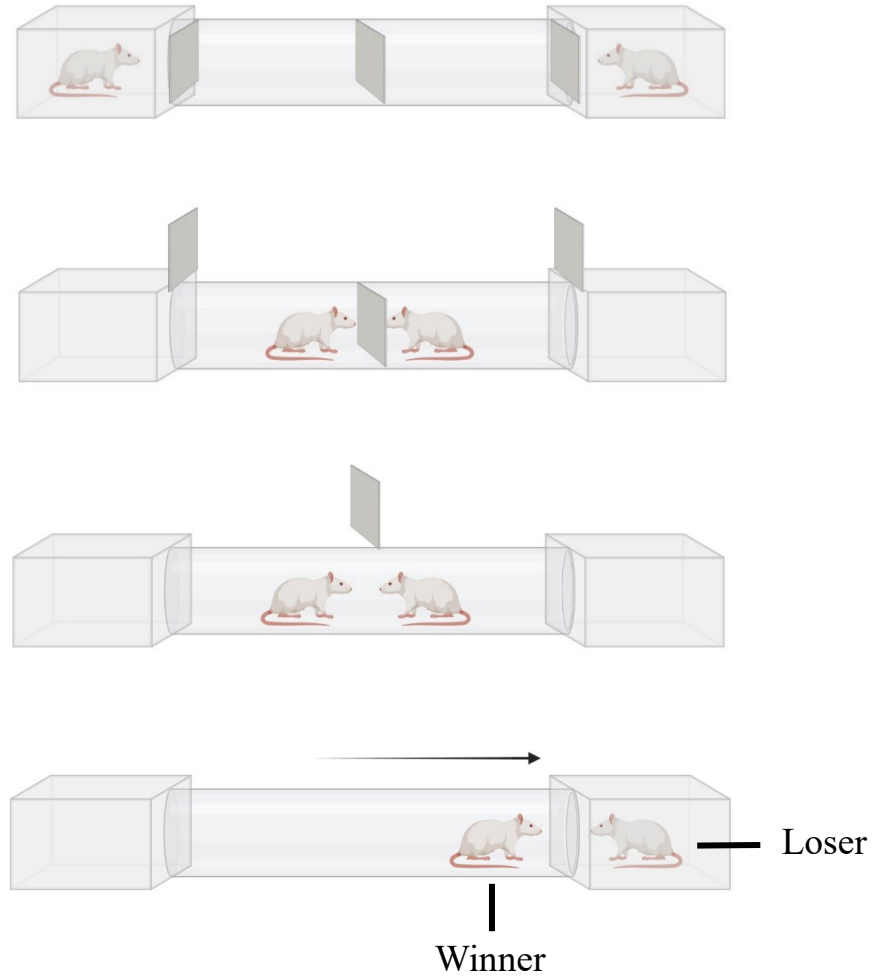
Figure 1. Schematic of the Tube Test procedure courtesy of Eden Solomon.
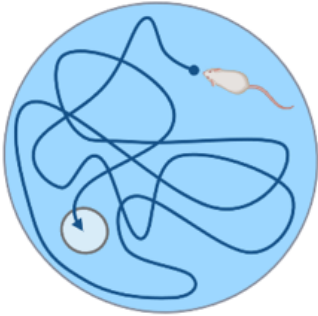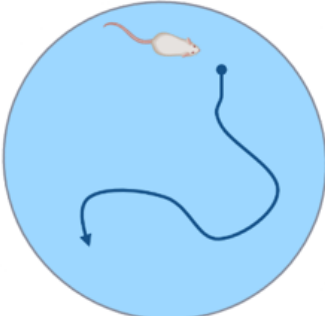
Figure 2. Experimental schematic of the Morris Water Maze.
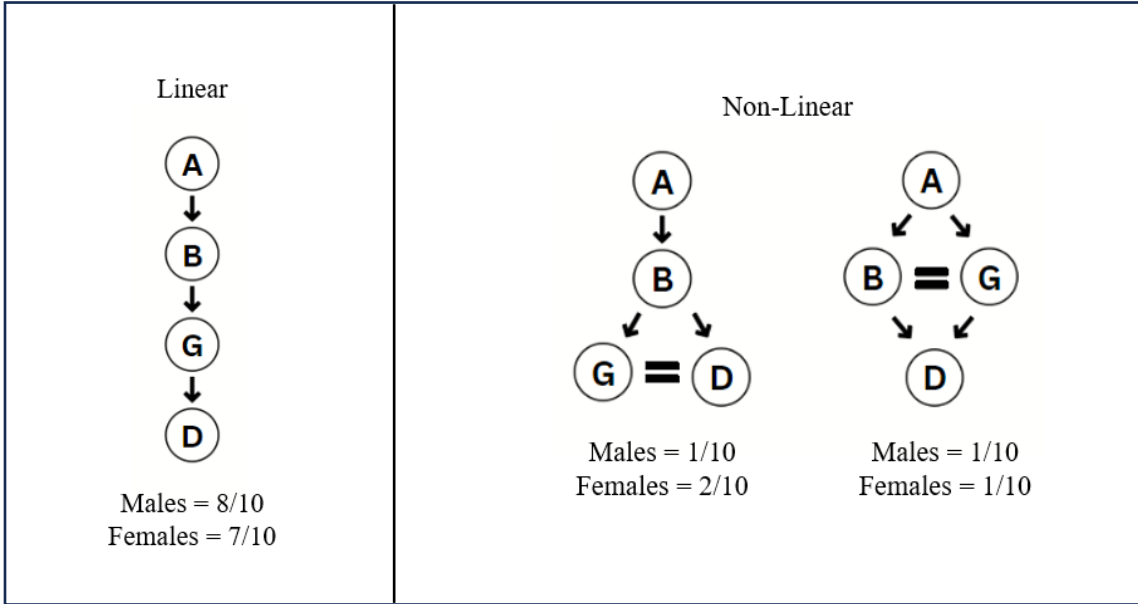
Figure 3. Social hierarchy phenotypes based on average 3-day David's Score.
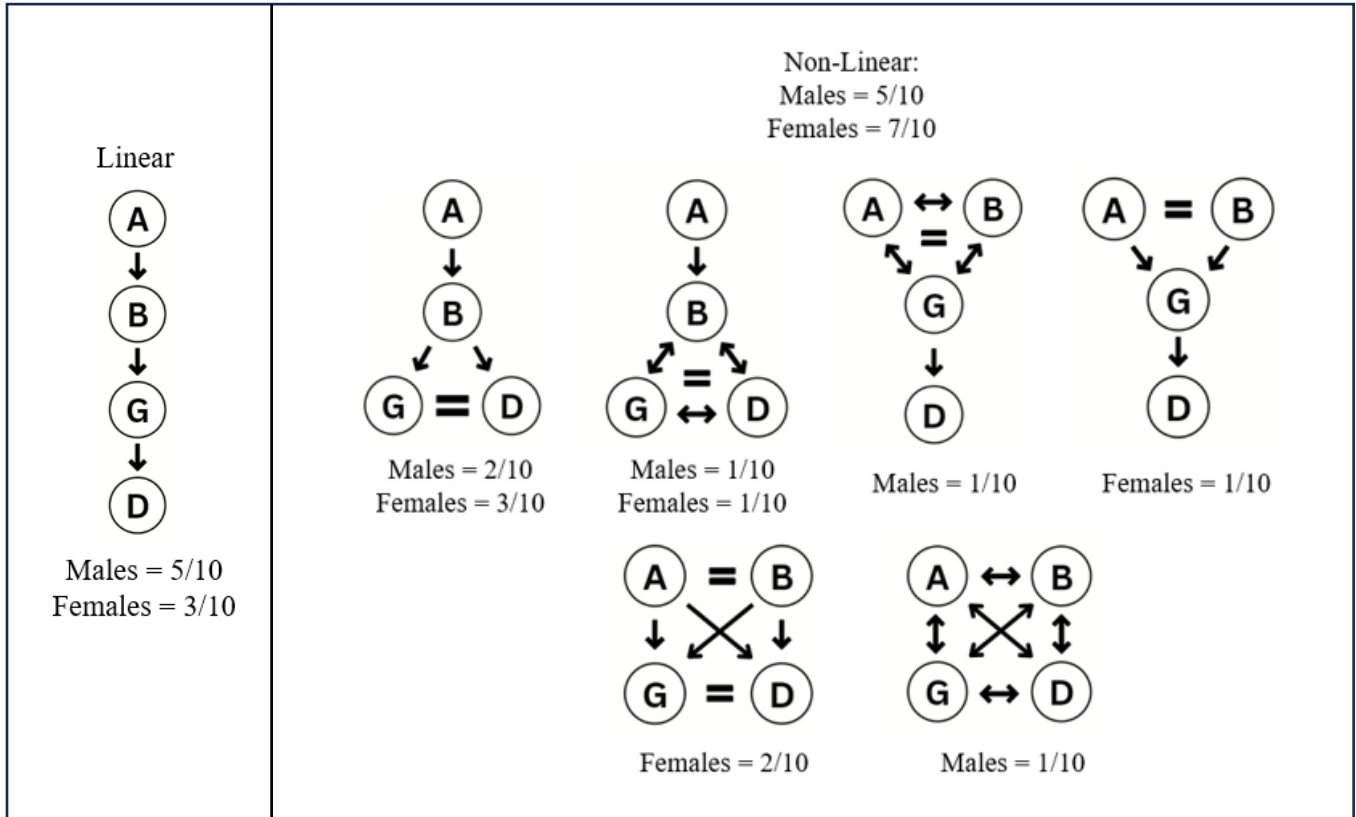
Figure 4. Social hierarchy phenotypes of based on retest David's Score.
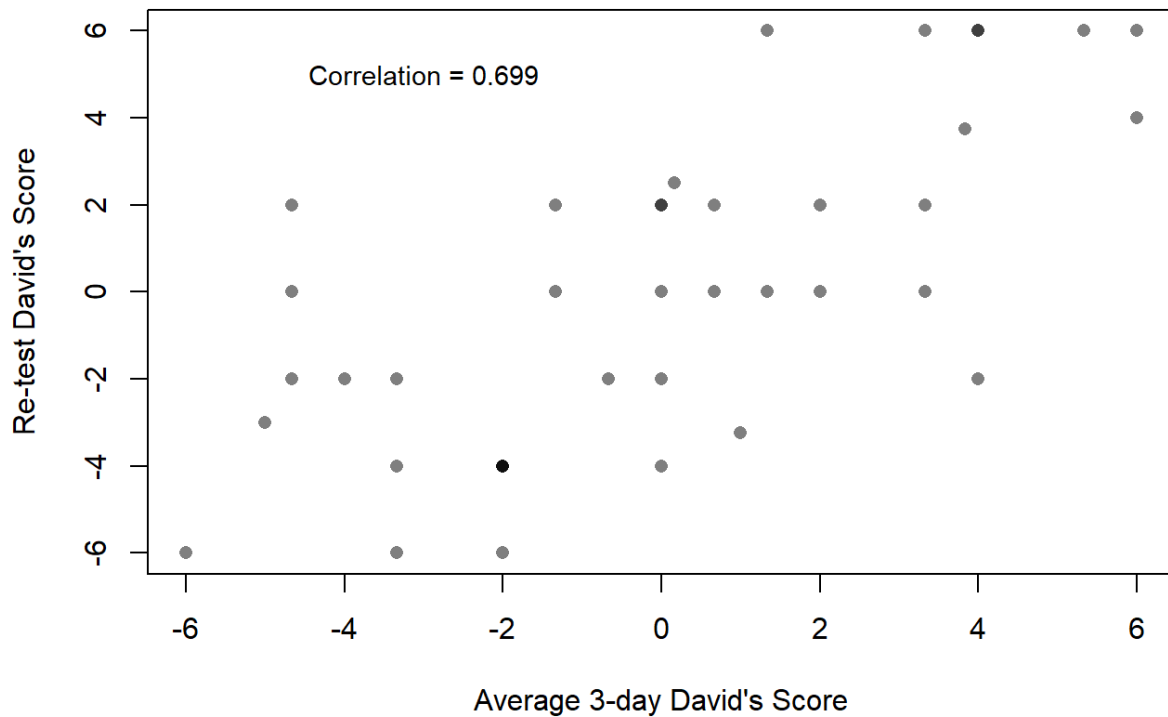
Figure 5a. A correlational analysis was conducted to investigate the relationship between the average 3-day David's Score and retest scores of male rats (n = 40). The two groups of scores follow a strong, positive linear relationship (r = ~0.7). The intensity of the dots corresponds to density of values, with darker dots indicating higher density and lighter dots indicating lower density of values.
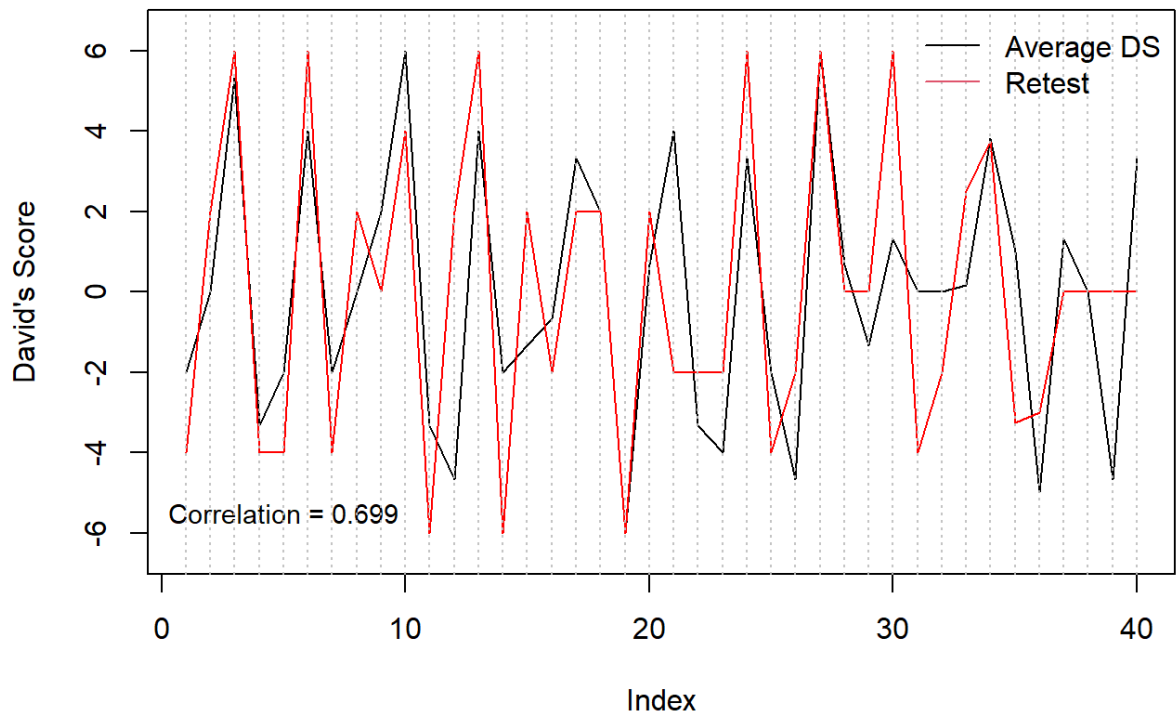
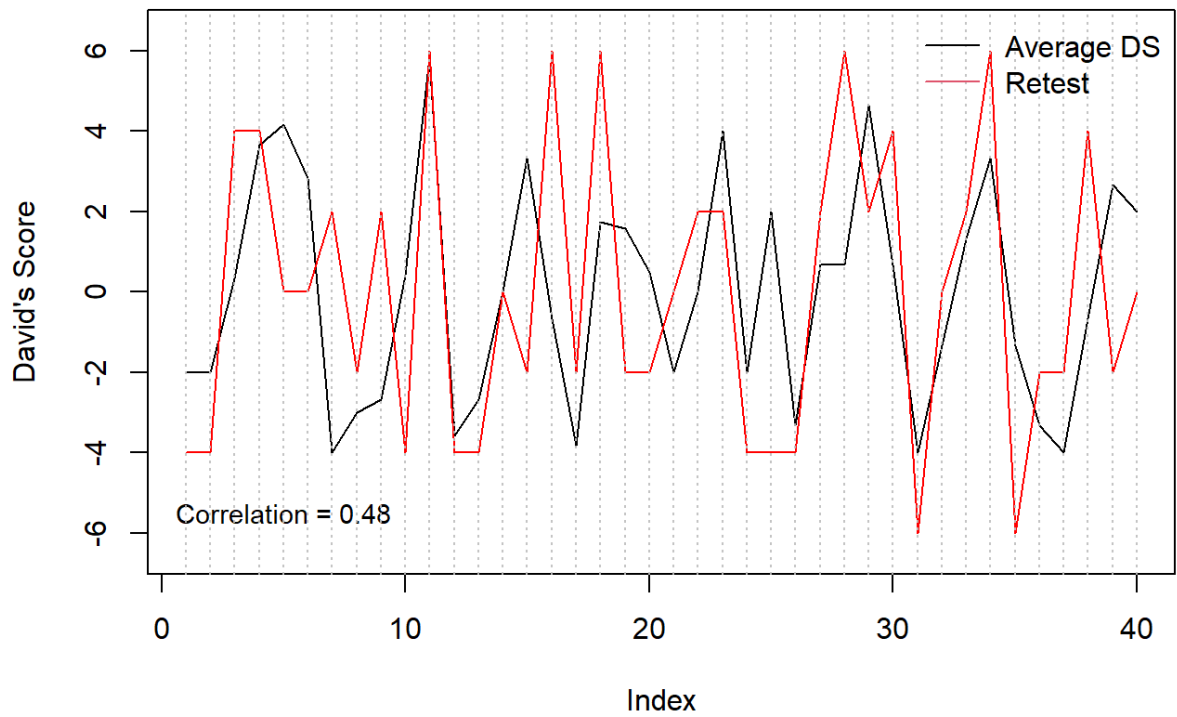**Figure 5b.** A line plot comparing the trends of the average 3-day David's Score (black) and retest scores (red) of male rats, illustrate that the two groups of scores generally follow a similar trend, with slight differences in the scores.
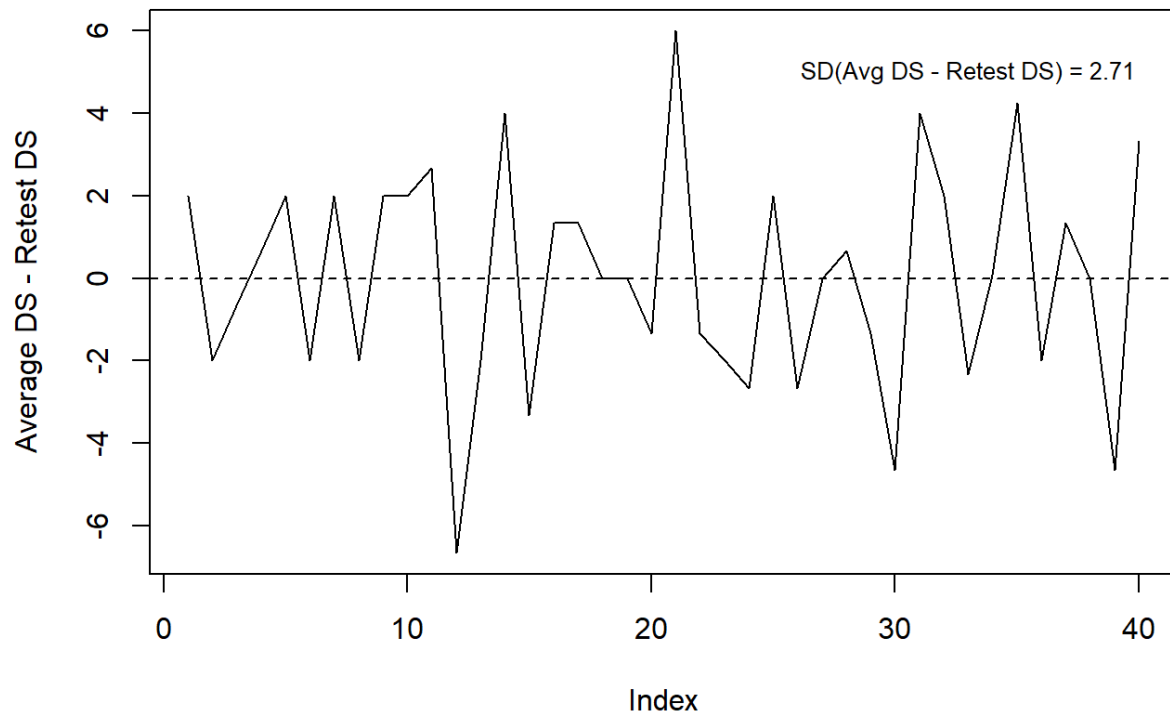
Figure 5c. A line plot comparing the average difference between the average 3-day David's Score and retest scores, indicate there are slight differences between the scores (SD = 2.71) of male rats.

Randomly Swapped Populations

p-value=0.992
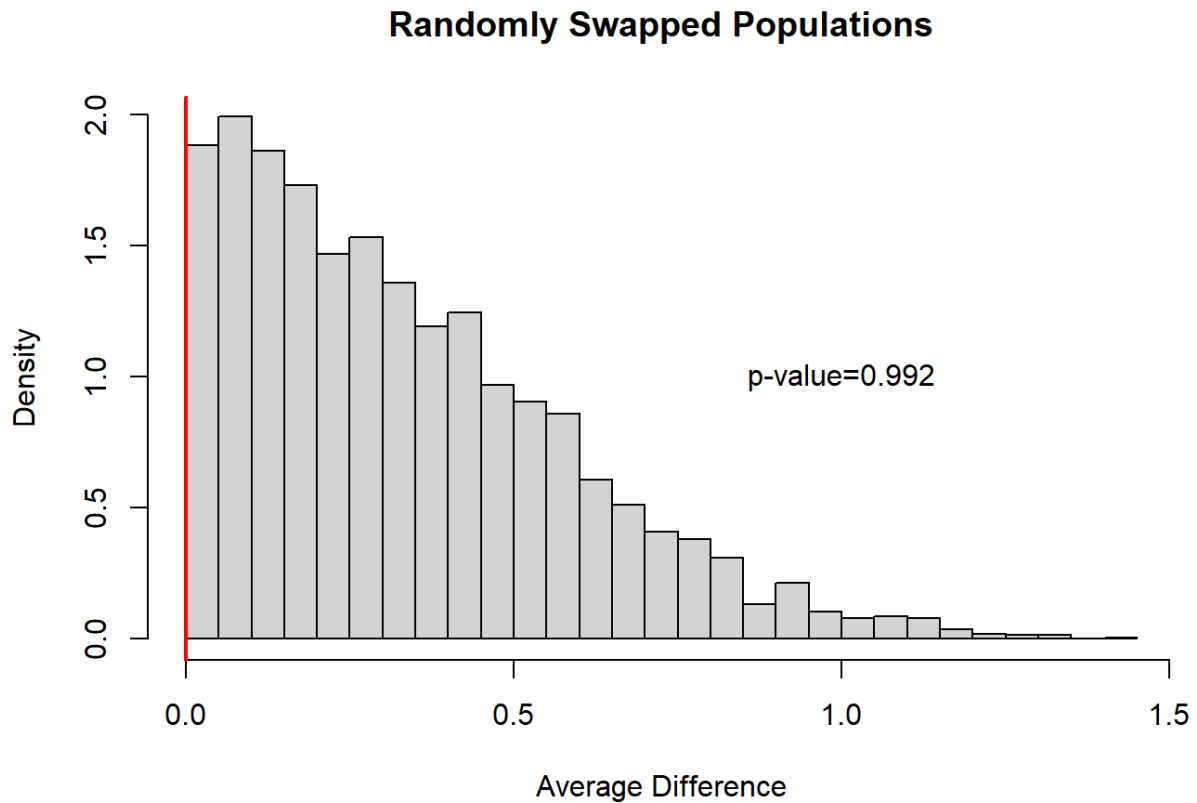
Figure 6a. Bootstrapping test of randomly swapping values 5000 times between average 3-day DS and retest scores of male animals. The histogram shows the distribution of the average difference in randomly swapped populations. The red line refers to the average difference from the original population.
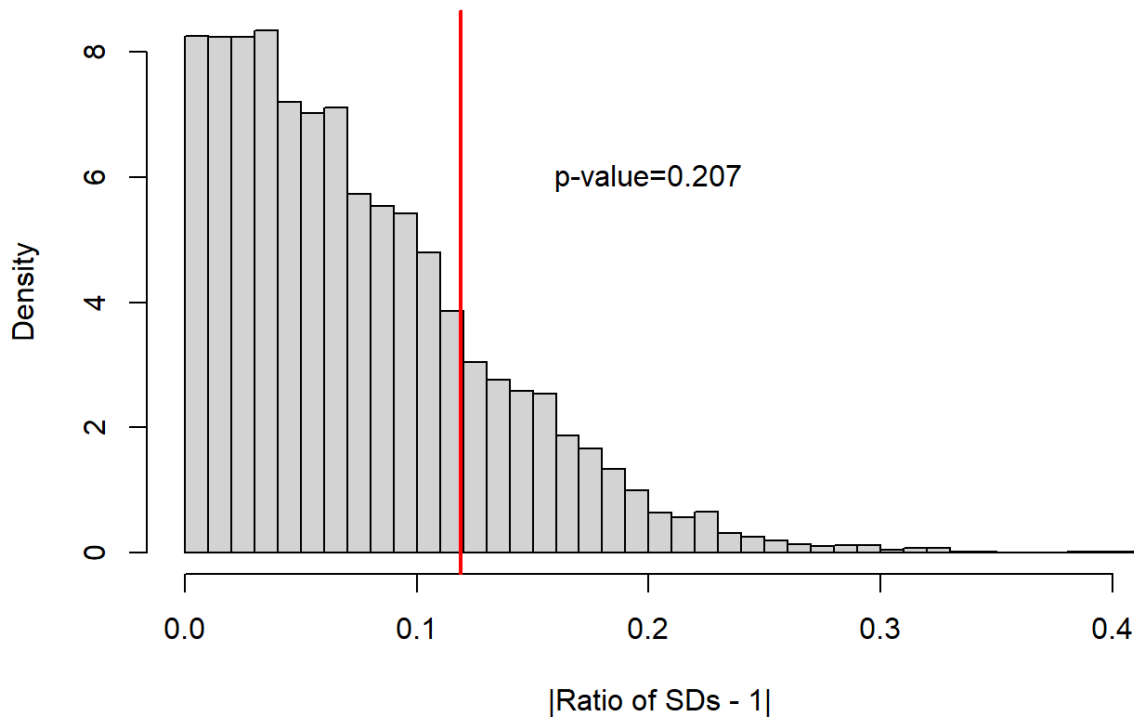
Figure 6b. Bootstrapping test of randomly swapping values 5000 times between average 3-day DS and retest scores of male animals. The histogram shows the distribution based on the standard deviations in the randomly swapped populations. The red line refers to the ratio of the standard deviation minus 1 from the original population.
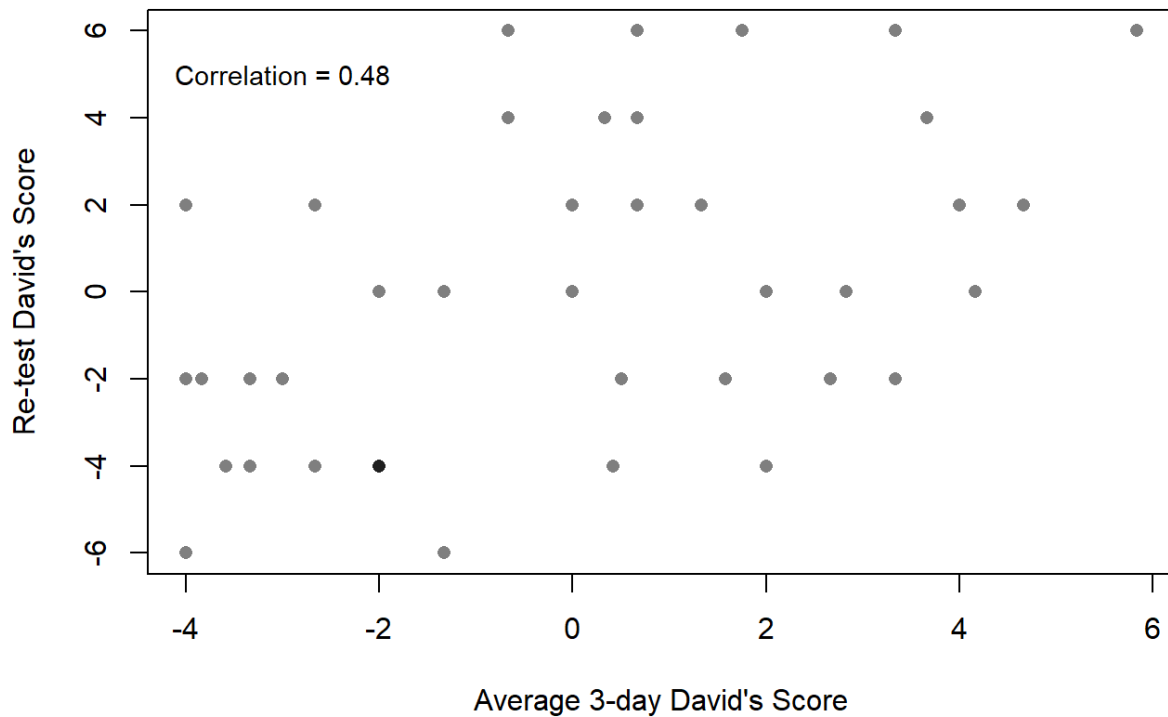
Figure 7a. A correlation analysis between the average 3-day David's Score and retest scores of female rats (*n* = 40), depict a moderate, positive linear relationship between the two groups of scores. The intensity of the dots corresponds to the density of values, with darker dots indicating higher density and lighter dots indicating lower density of values.

Figure 7b. A line plot comparing the trends of the average 3-day David's Score (black) and retest

scores (red), reveal slightly different trends in the scores of female rats.
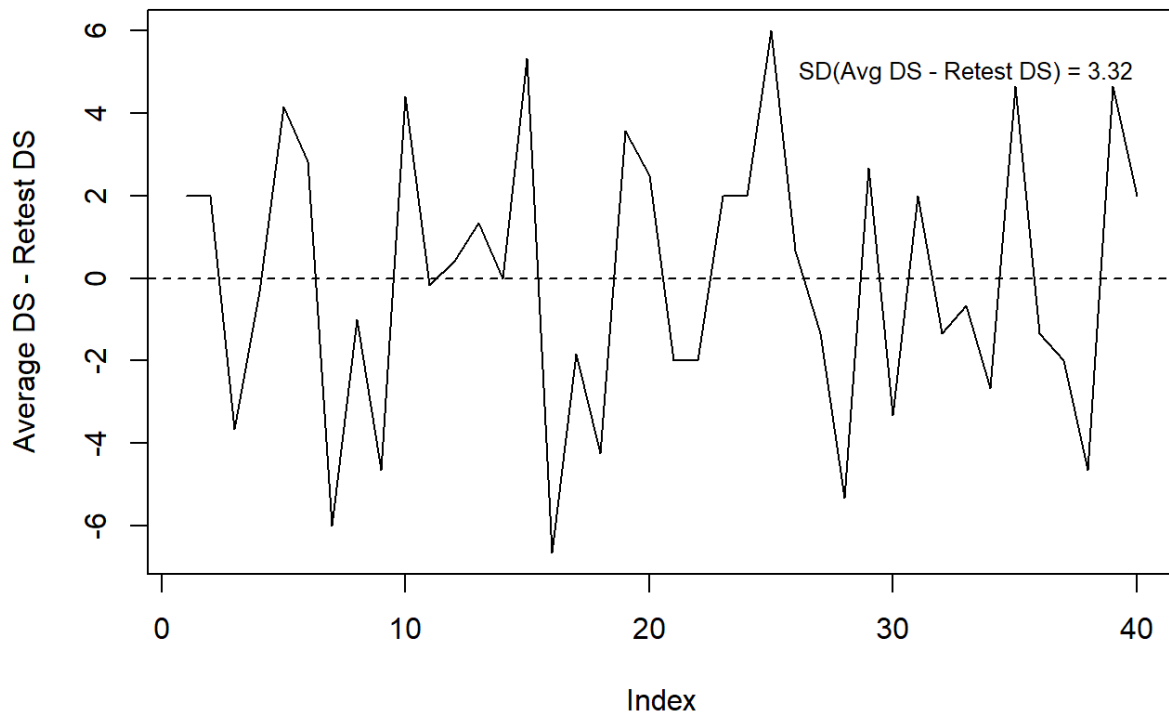
Figure 7c. A line plot of the average difference between average 3-day DS and retest scores of

female animals, indicate there are differences between the scores (SD = 3.32).
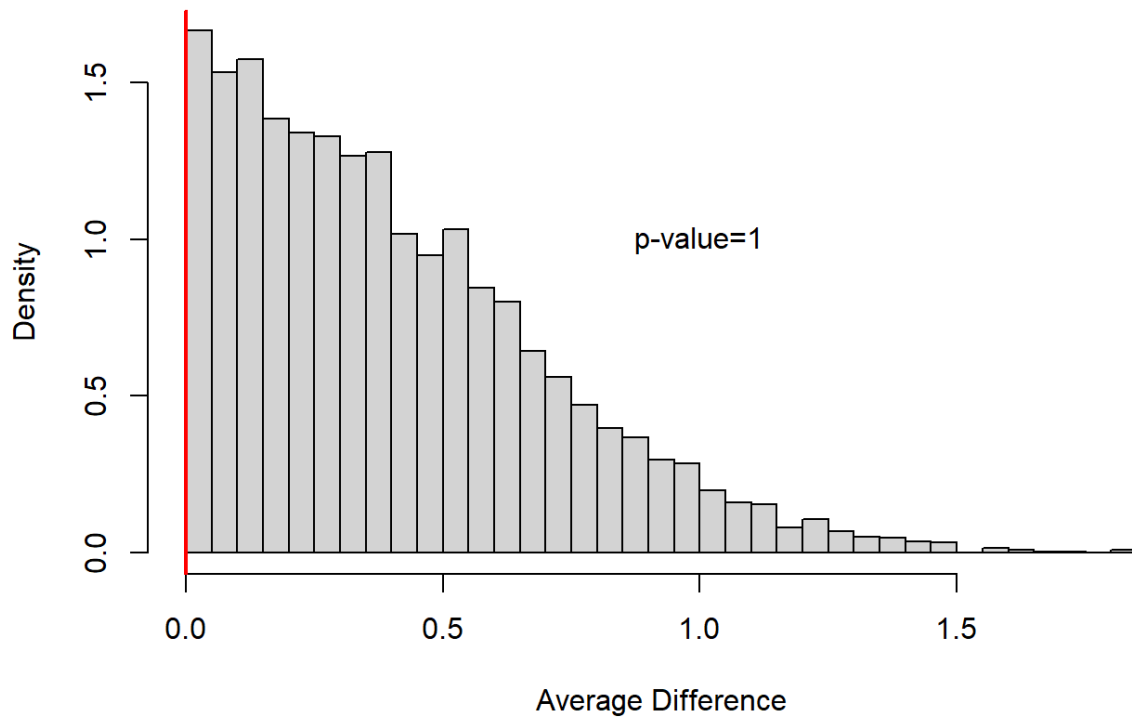
Figure 8a. A histogram based on the average difference distribution from the bootstrapping test of randomly swapping values 5000 times from the average 3-day DS and retest scores of female rats. The red line depicts the average difference from the original population of scores.

Randomly Swapped Populations

p-value=0.058

|Ratio of SDs - 1|
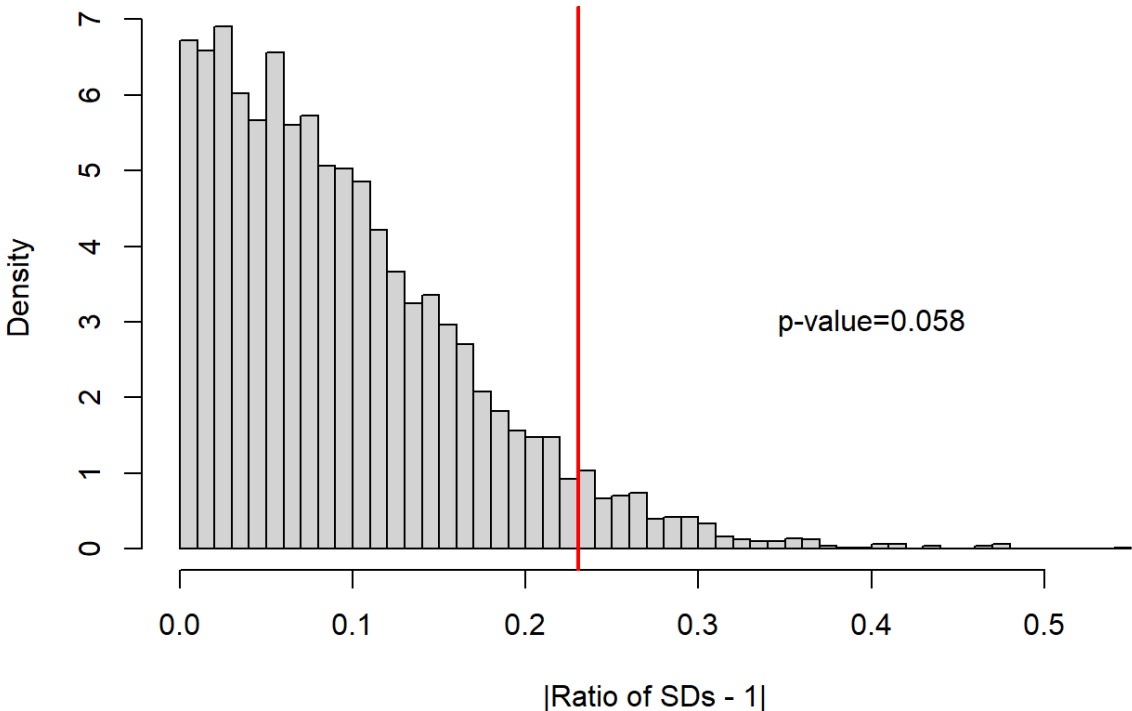
Figure 8b. A histogram based on the standard deviation distribution from randomly swapping the average 3-day DS and retest values of female rats 5000 times. The red line depicts the standard deviation from the original set of scores.
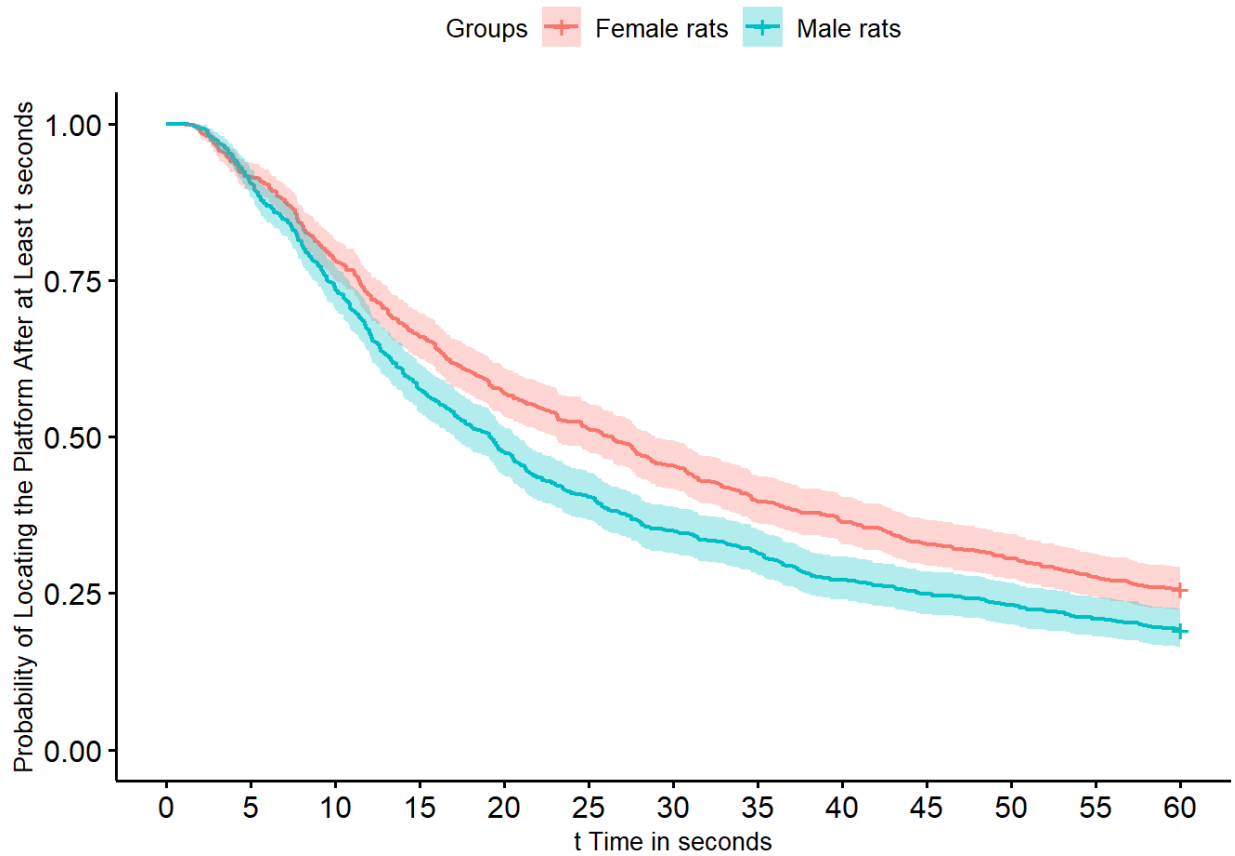
Figure 9. Probability of locating the platform displayed by male (blue) and female (red) rats for all trials of the Learning phase. The estimated survival function for the male rats is consistently lower over time compared to female rats. The shaded regions correspond to the 95% confidence bands.
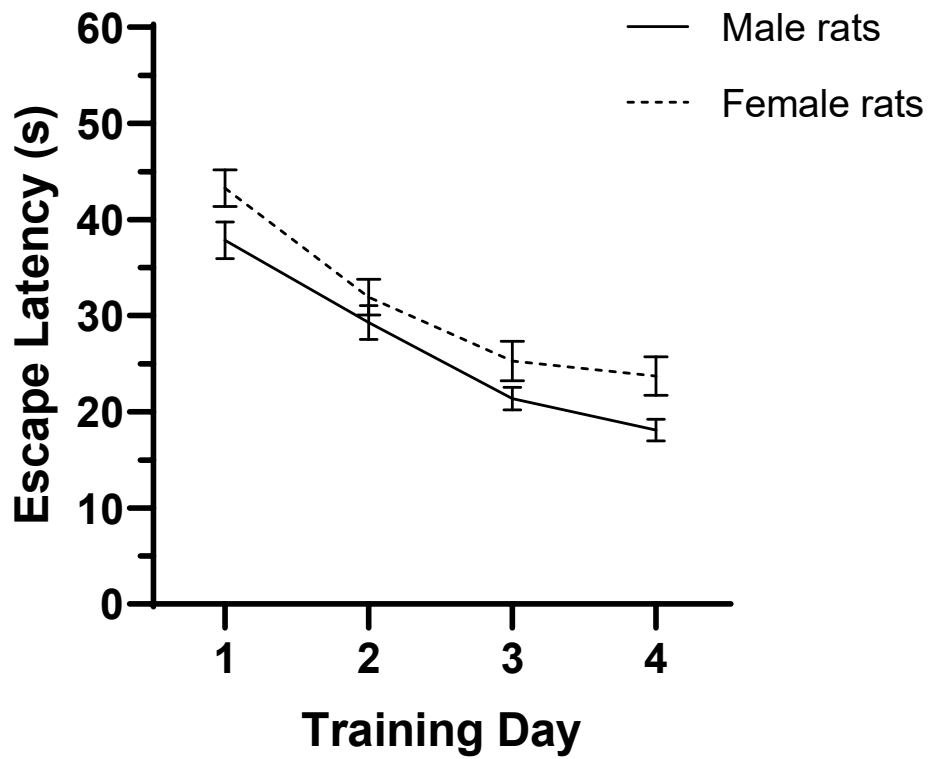
Figure 10. Standard representation of average escape latency of male and female rats across training days of the Learning phase. Performance was significantly different across training days (p < 0.0001) and across sex (p = 0.0040) using two-way ANOVA. Data points represent mean ± SEM of each training day. n = 40 animals per group.
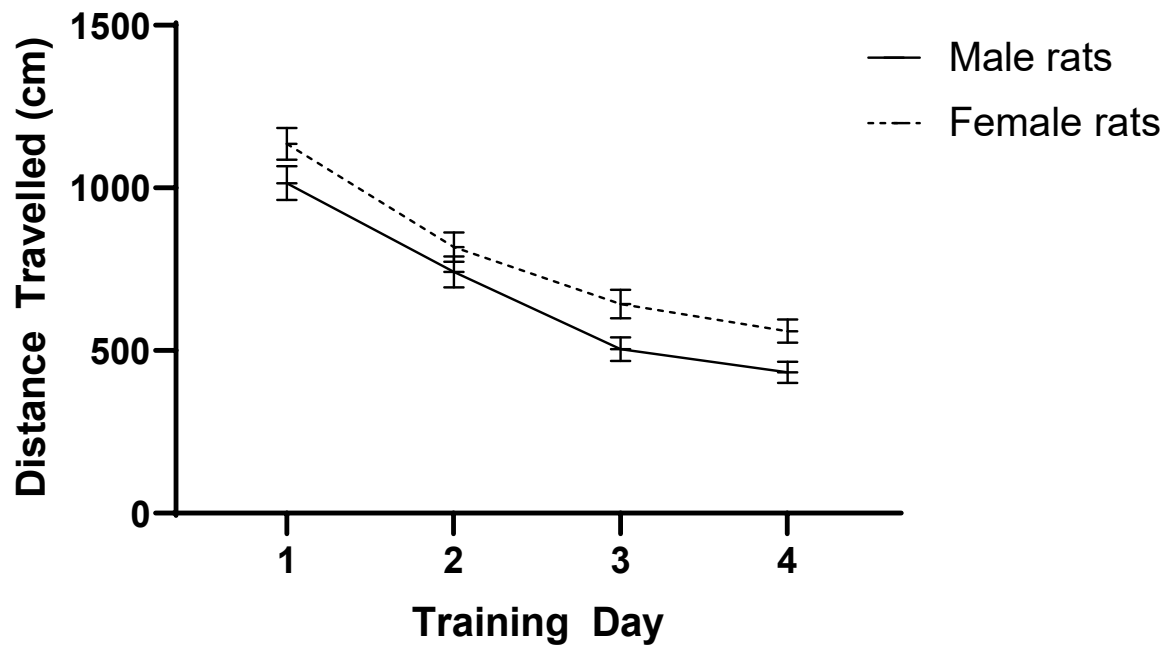
Figure 11. The distance travelled across training days of the Learning phase. Distance travelled was significantly different across training days ($p < 0.0001$) and across sex ($p = 0.0007$) using two-way ANOVA. Data points represent mean ± SEM of each training day. *n* = 40 animals per group.
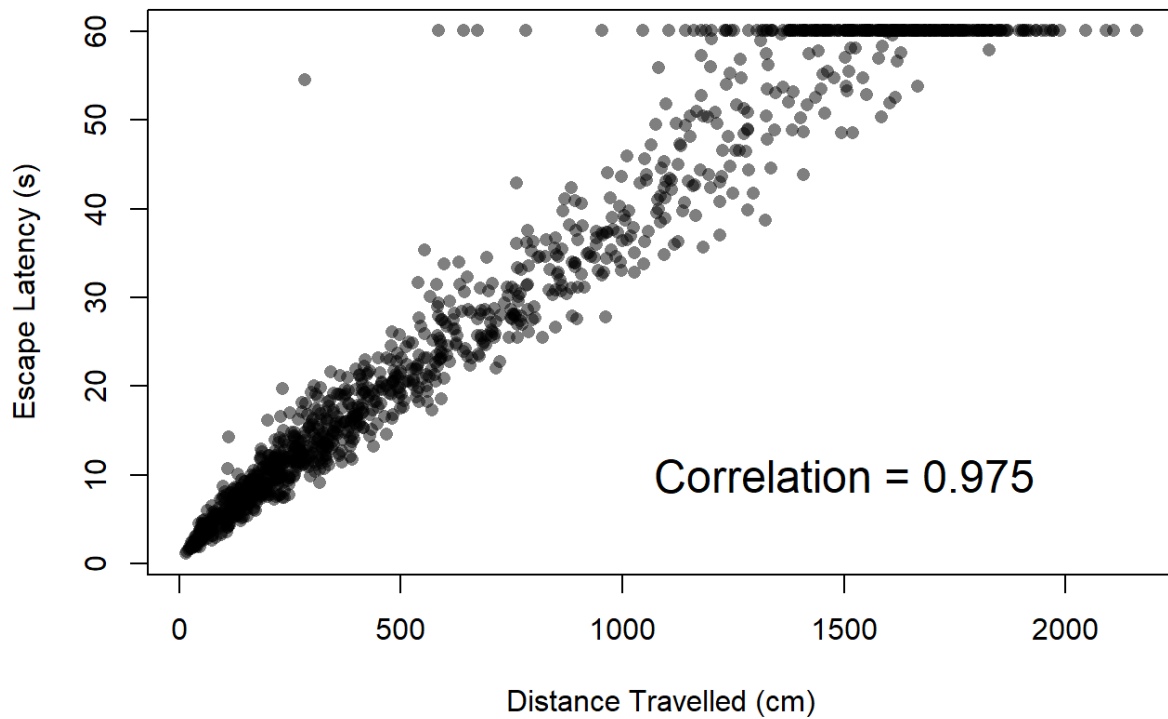
Figure 12. The correlation between the escape latency and distance travelled for all trials of the Learning phase is strong and linearly associated (r = 0.98). Each dot represents performance on one trial. The darker dots indicate overlapping values, while lighter dots indicate less or no overlap of values. *n* = 1280 trials.
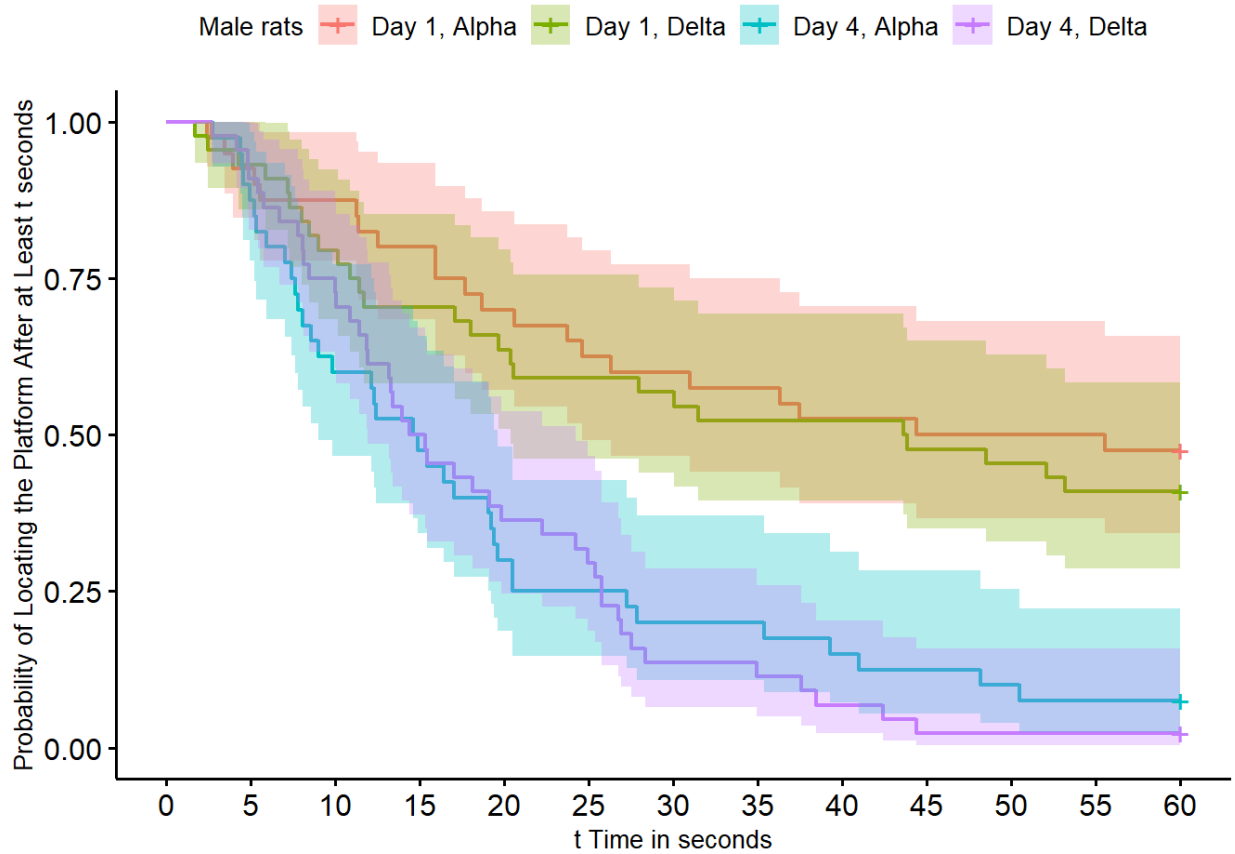
Figure 13a. Probability of locating the platform in male Alpha and Delta rats for trials on Days 1 and 4 of the Learning phase. The upper survival curves represent the survival function on Day 1 (Alpha = red, Delta = green), while the lower survival curves represent the survival function on Day 4 (Alpha = blue, Delta = purple). The survival functions of the most dominant and least dominant animals were similar over time, and indicate similar likelihood in finding the platform. The shaded regions correspond to the 95% confidence bands.
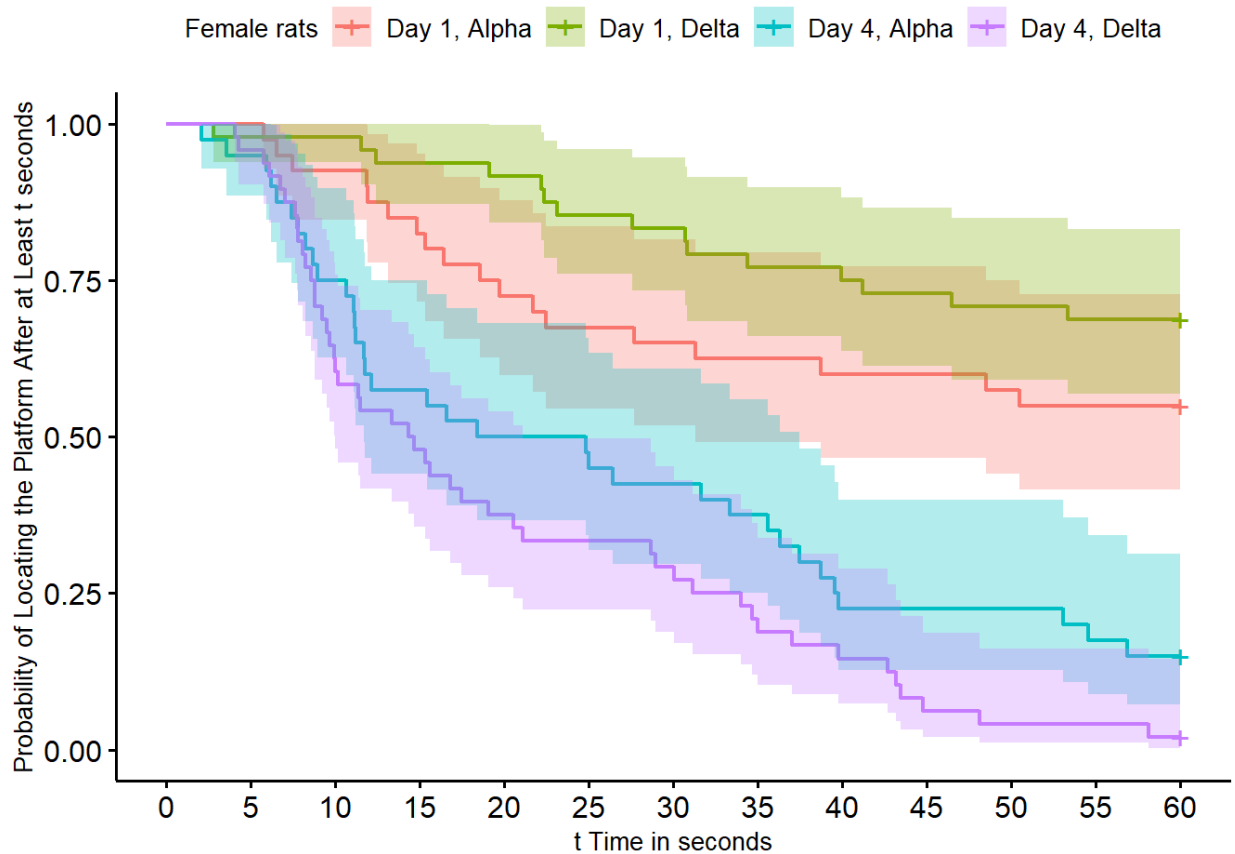
Figure 13b. Probability of locating the platform in female Alpha and Delta rats for trials on Days 1 and 4 of the Learning phase. The red (Alpha) and green (Delta) curves at the top represent the probability of locating the platform on Day 1, and the two blue (Alpha) and purple (Delta) curves below represent the probability of locating the platform on Day 4. The curves indicate that the most dominant and least dominant female rats had similar probabilities in finding the platform. The shaded regions correspond to the 95% confidence bands.
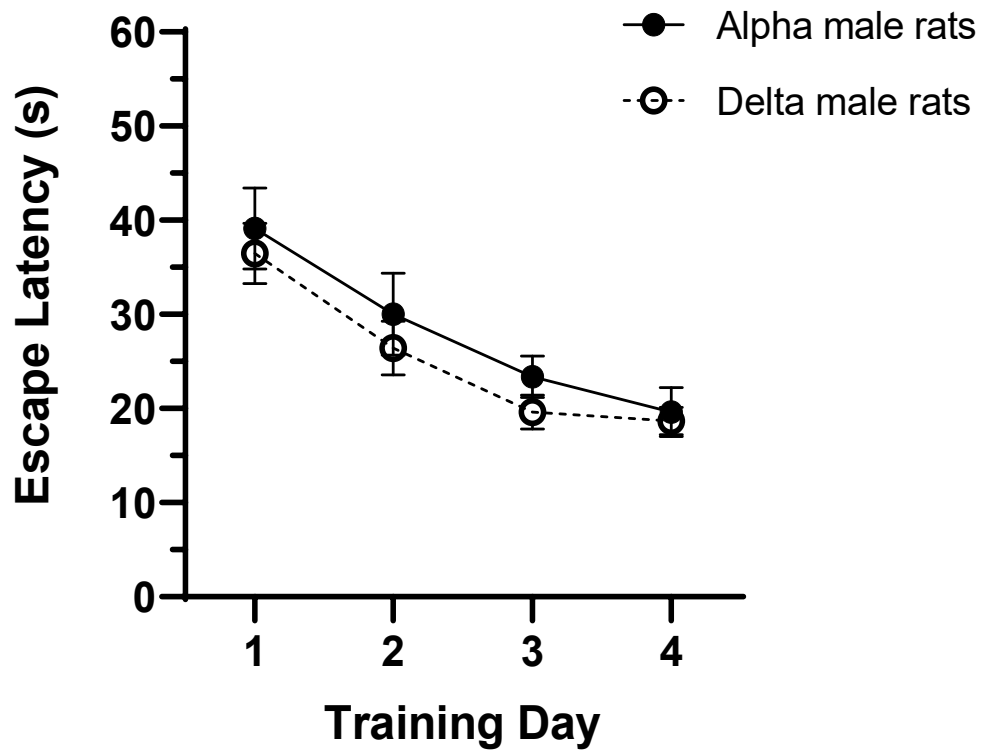
Figure 14a. Standard graph for displaying average escape latency of Alpha and Delta male rats across all training days of the Learning phase. Spatial learning performance was not significantly different between Alpha and Delta male animals. Data points represent mean ± SEM. Alpha: $n =$ 10, Delta: $n = 11$.
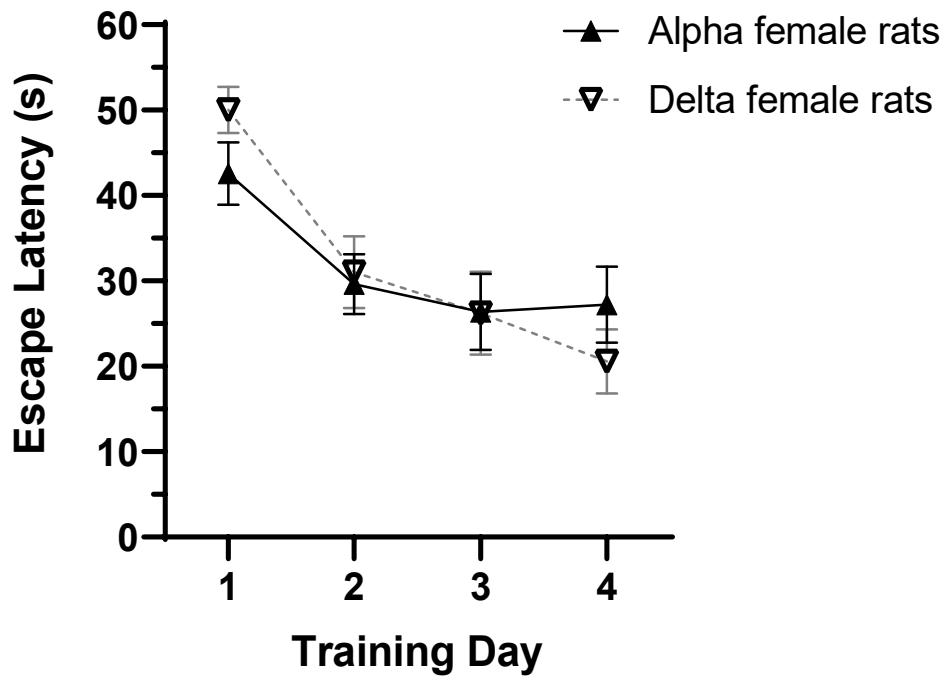
Figure 14b. Standard graph for displaying average escape latency of Alpha and Delta male rats across all training days of the Learning phase. Spatial learning performance was not significantly different between Alpha and Delta female animals. Data points represent mean ± SEM. Alpha: $n = 10$, Delta: $n = 12$.
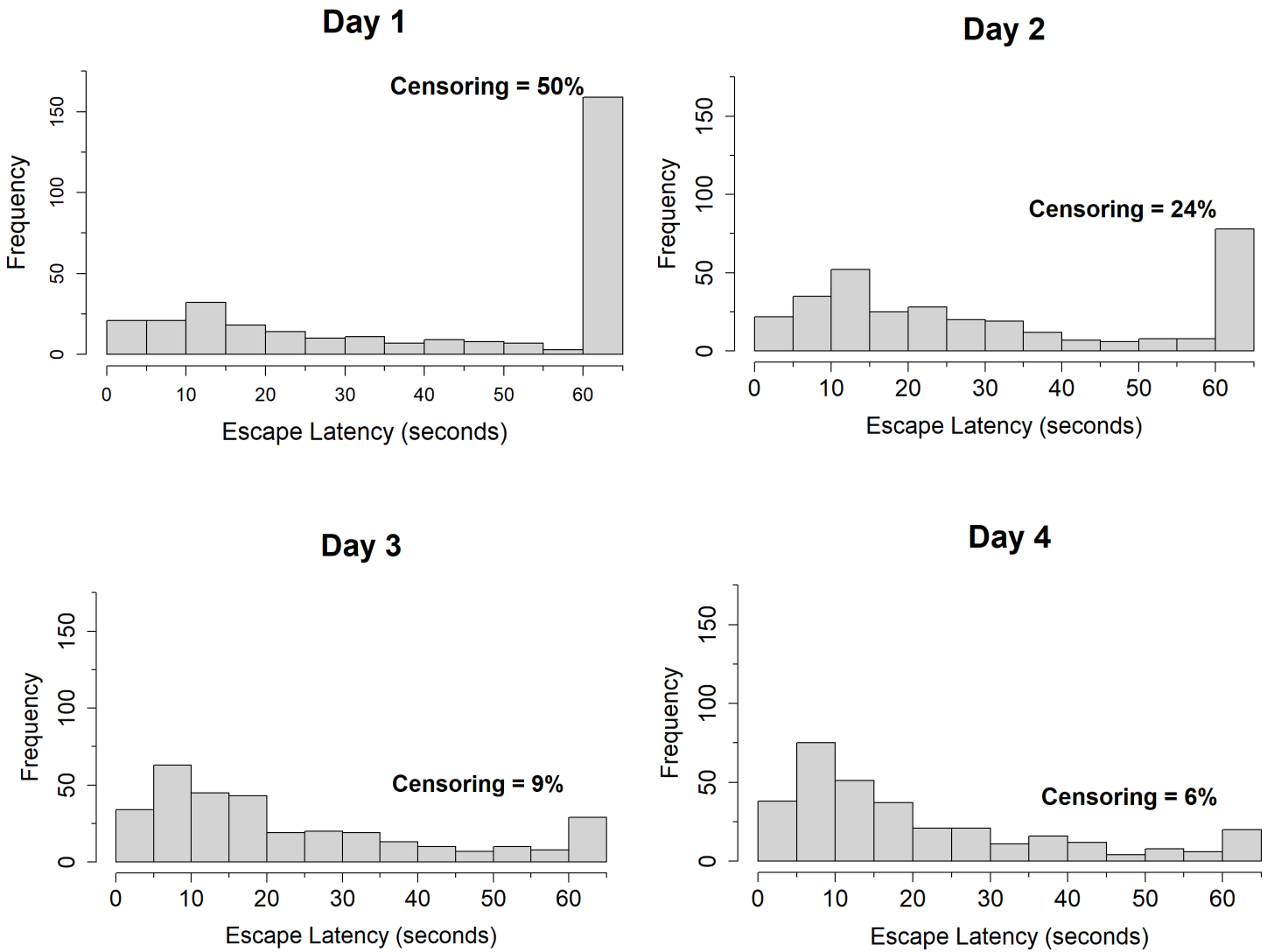
Figure 15. The frequency of censored trials from all trials of each training day of the Learning

phase decreased from 50% on Day 1 to 6% on Day 4. Trials = 320 trials per day, *n* = 80 animals.
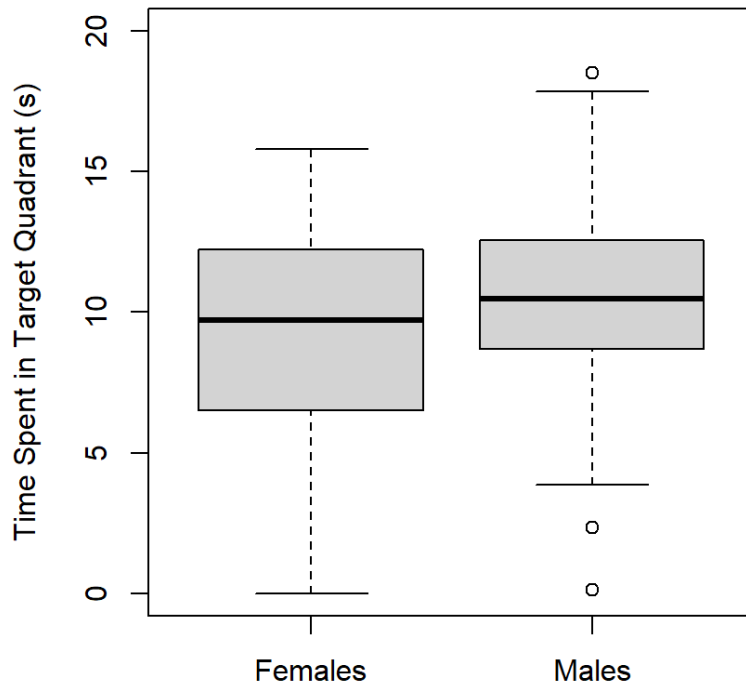
Figure 16. The time spent in the target quadrant during the Probe test in male and female rats. The black horizontal line represents the median values, and the whiskers represent minimum and maximum values of the performance times in male and female rats. The dots that lie outside the boxplot for male rats, represent outliers in the data. $n = 40$ animals per group.
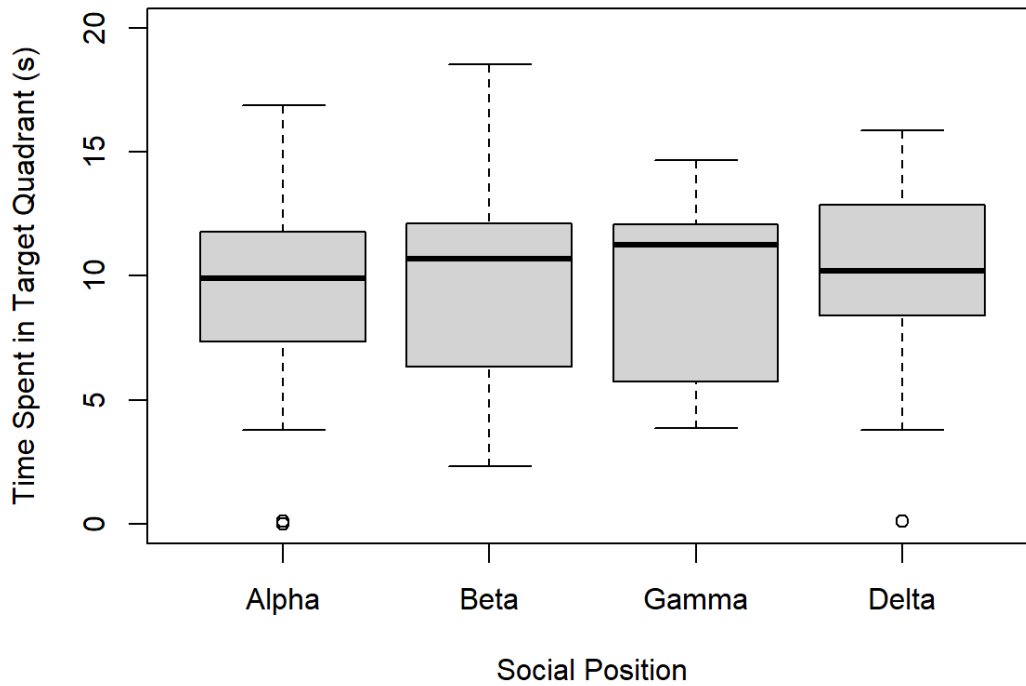
Figure 17. The time spent in the target quadrant during the Probe test across social positions for all animals. The black horizontal line represents the median values, and the whiskers represent minimum and maximum times spent in target quadrant. Alpha: $n = 20$, Beta: $n = 22$, Gamma: $n = 15$, Delta: $n = 23$.
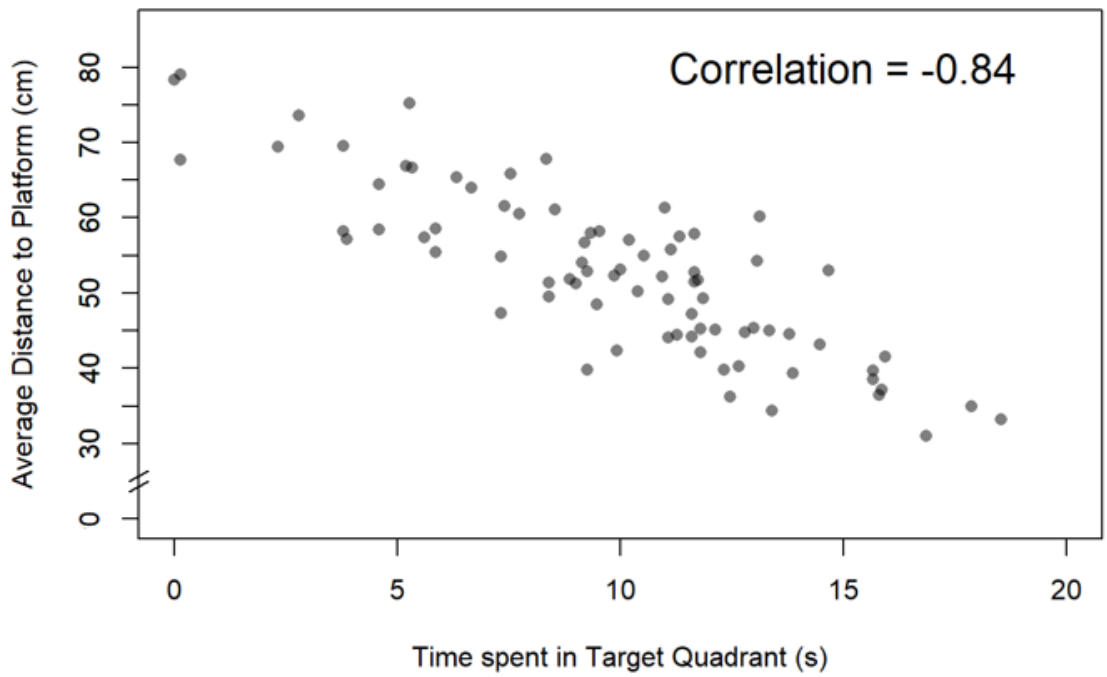
Figure 18. The relationship between average distance to the platform and time in the target quadrant (SW) is strongly correlated. As average distance to platform decreases, time spent in the platform quadrant increases. Each dot represents the performance of a rat during the Probe test of the Morris Water Maze, *n* = 80 trials.
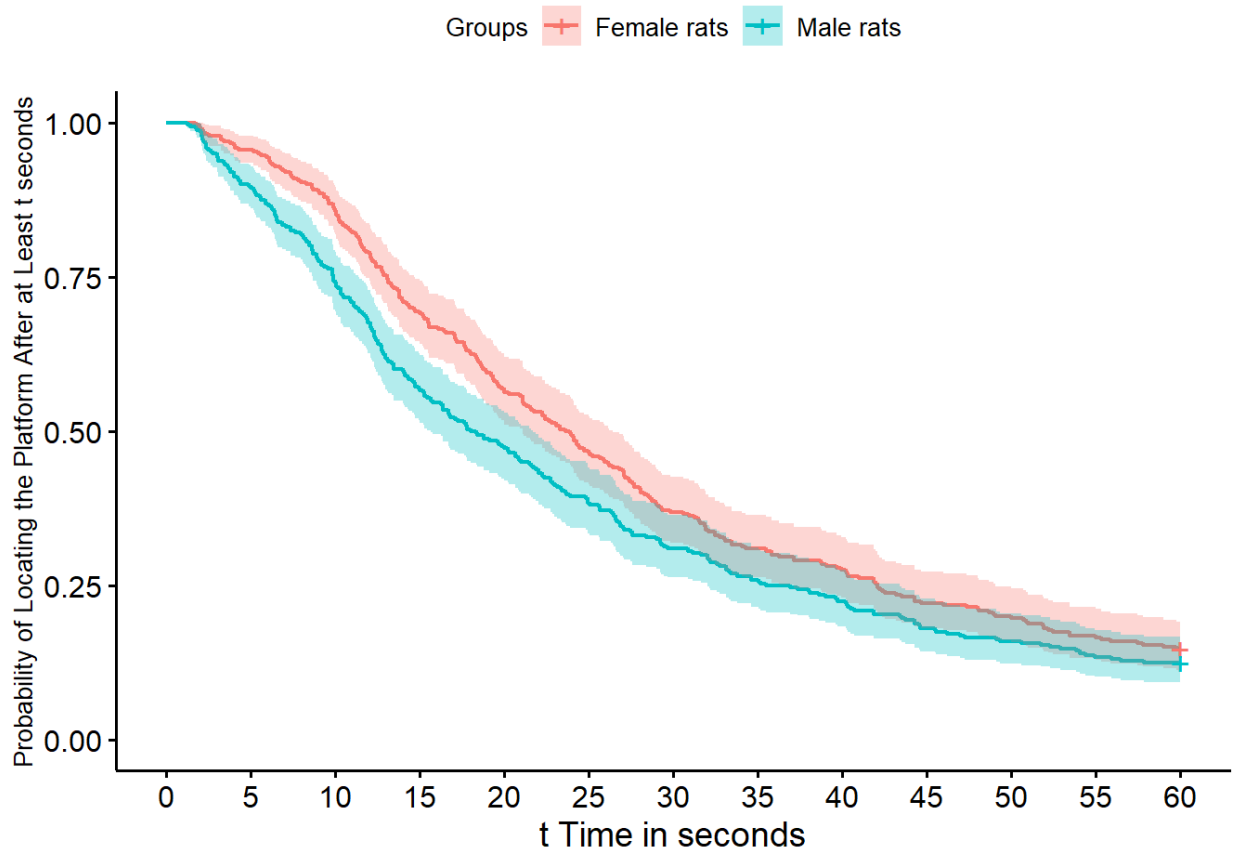
Figure 19. Probability of locating the new platform located in the NE quadrant in male (blue) and female (red) rats for all trials of the Reversal phase. The survival curve for male rats was consistently lower than the female curve over time. The shaded regions correspond to the 95% confidence bands.
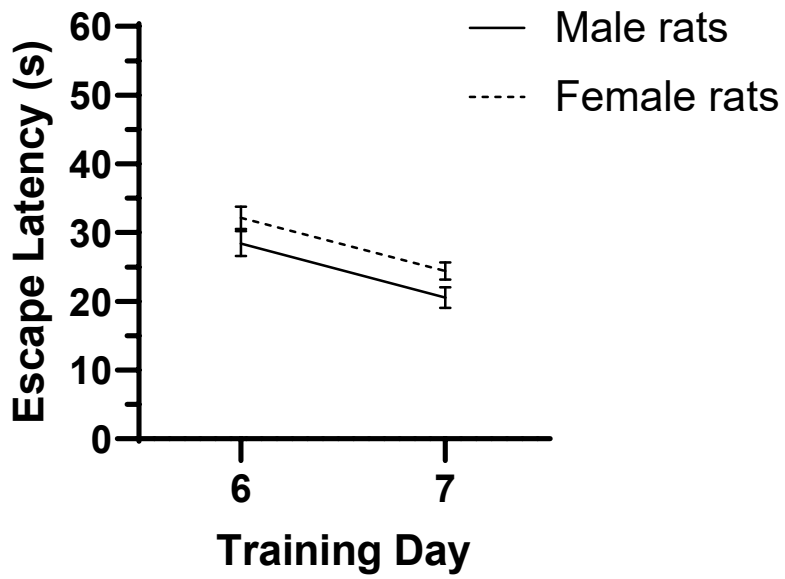
Figure 20. Standard representation of the average escape latency for male and female rats during training days of the Reversal phase. Data points represent mean ± SEM of each training day. *n* = 40 animals per group.
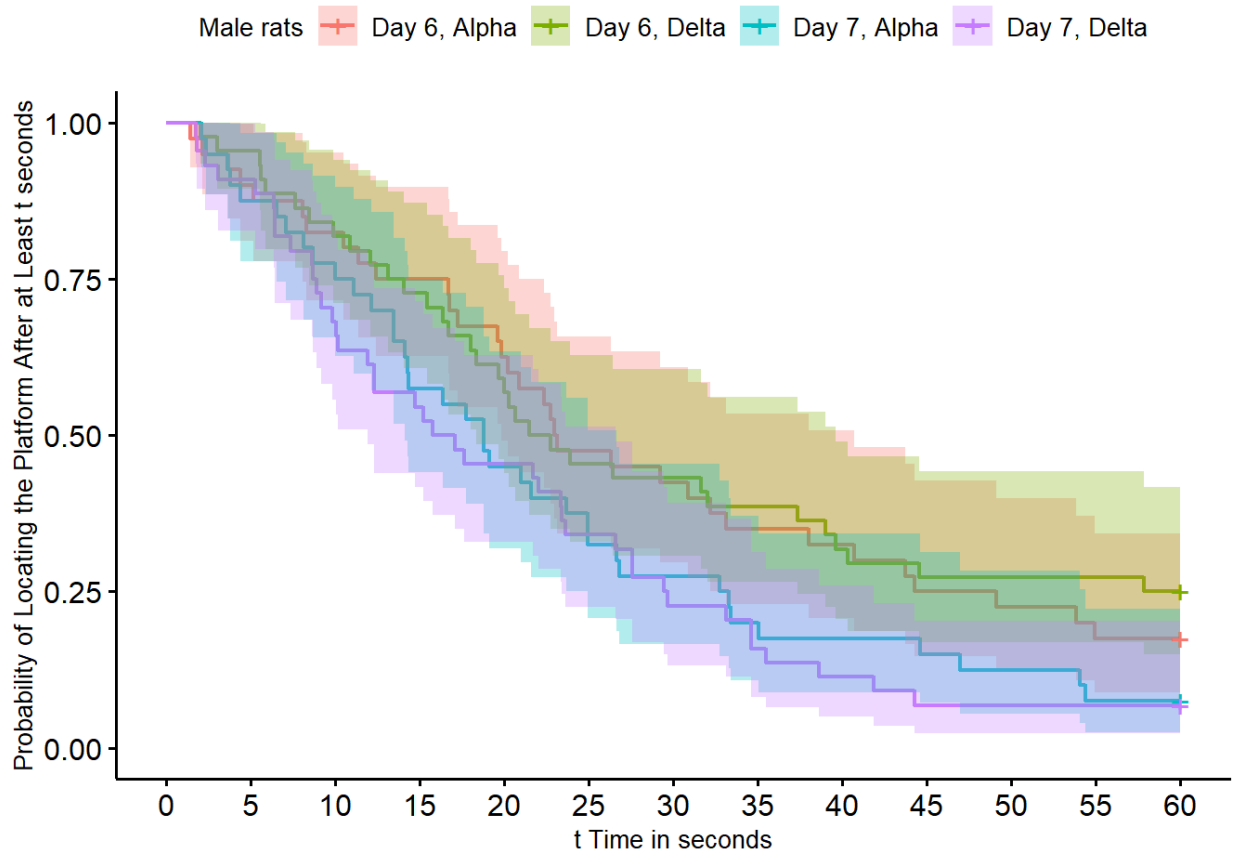
Figure 21a. The two uppermost survival curves represent the probability of locating the platform on Day 6 displayed by Alpha (red) and Delta (green) male rats during the Reversal phase. The two bottom survival curves represent the probability of locating the platform on Day 7, displayed by Alpha (blue) and Delta (purple) male rats. The Alpha and Delta male rats had similar probabilities in locating the platform over time. The shaded regions correspond to the 95% confidence bands.
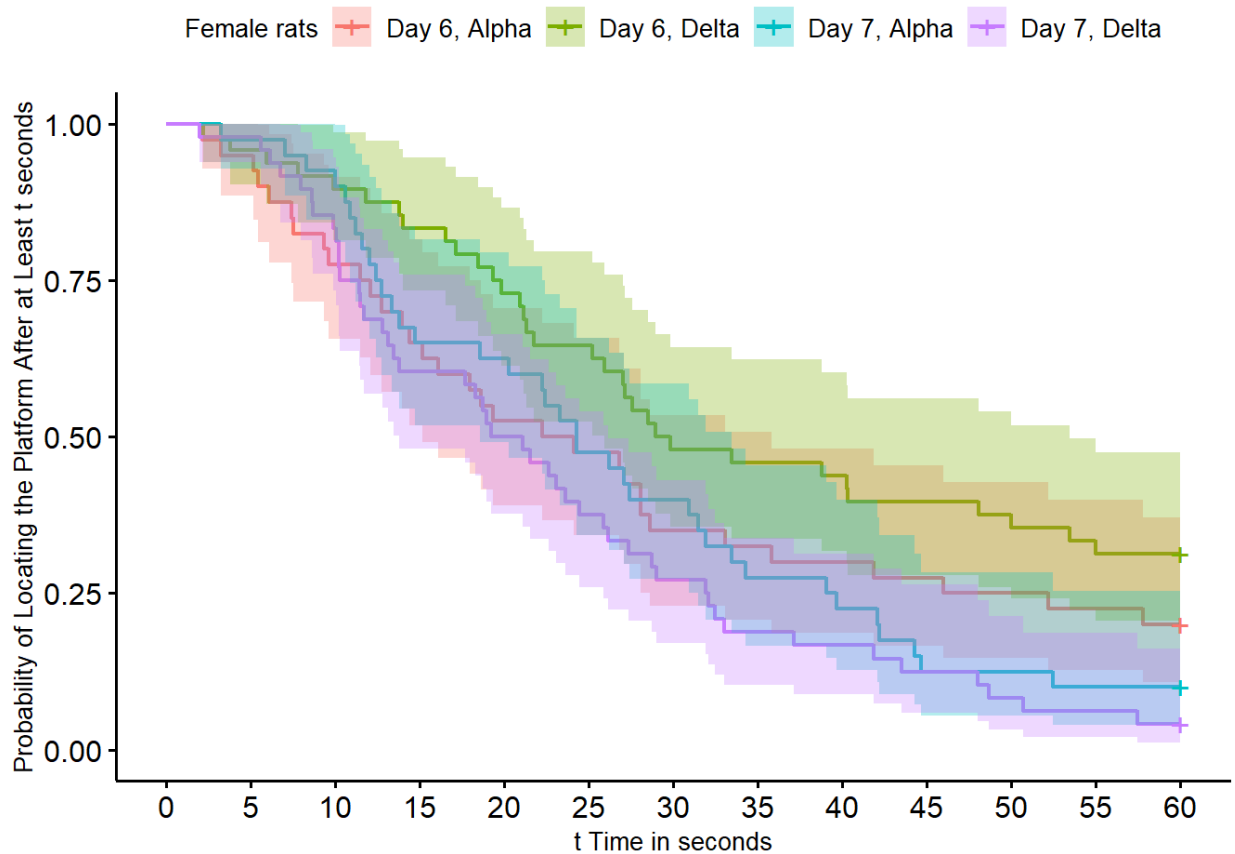
Figure 21b. The probability of locating the escape platform in the new location displayed by Alpha and Delta female rats, for training days of the Reversal phase. The red (Alpha) and green (Delta) survival curves at the top represent the probability of locating the platform on Day 6, and the blue (Alpha) and purple (Delta) survival curves represent the probability of locating the platform on Day 7. The survival curves across Alpha and Delta positions are similar, indicating similar probabilities in finding the platform during the Reversal phase. The shaded regions correspond to the 95% confidence bands.
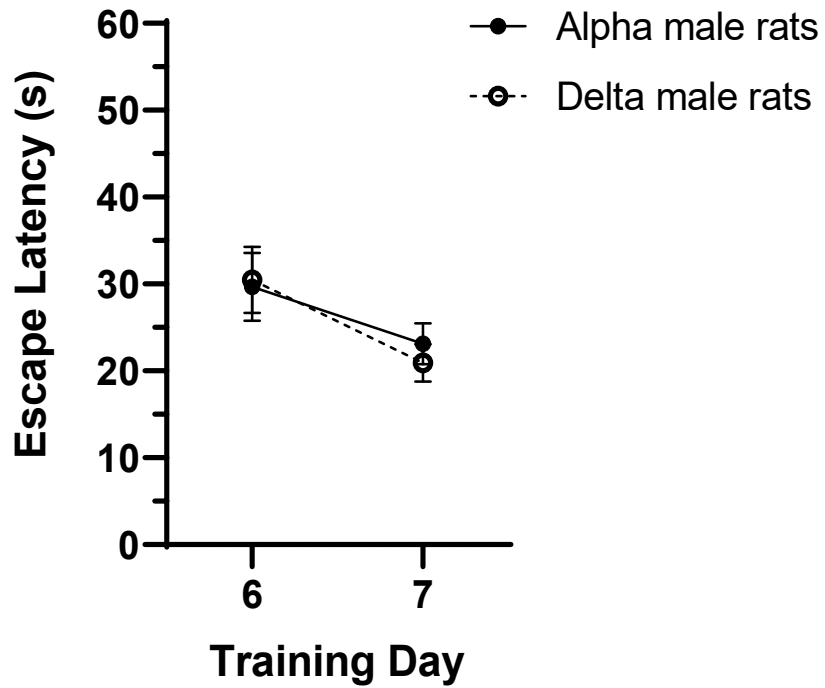
Figure 22a. Standard graph for displaying average escape latency across all Reversal phase days for Alpha and Delta male rats. No significant differences between Alpha and Delta male rats were found in learning the new platform location (NE) across training days. Data points represent mean ± SEM. Alpha: *n* = 10, Delta: *n* = 11.
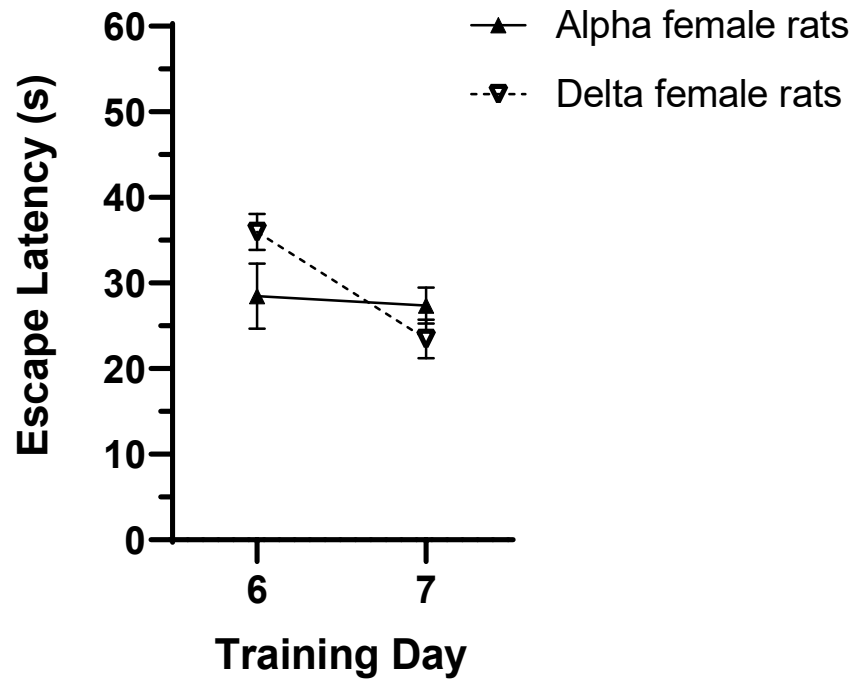
Figure 22b. Standard graph for average escape latency for Alpha and Delta female rats for all trainings days of the Reversal phase. The differences in performance were not statistically significant between Alpha and Delta female rats in learning the new platform location (NE). Data points represent mean ± SEM. Alpha: $n = 10$, Delta: $n = 12$.
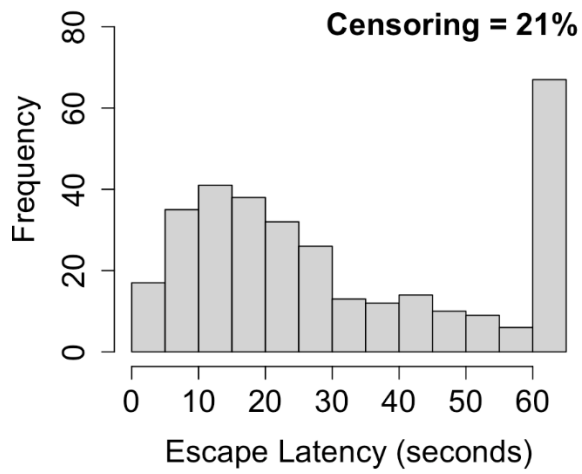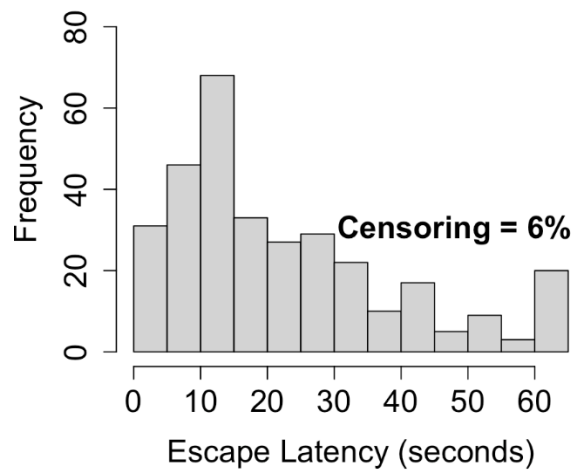
Figure 23. Frequency of censored trials from all trials of each training day of the Reversal phase. Censored trials decreased from 21% on Day 6 to 6% on Day 7. Trials = 320 trials per day, *n* = 80 animals.

Tube Test Match-ups			
Day 1	Day 2	Day 3	Day 11 (Retest)
4 vs 1	1 vs 3	4 vs 2	1 vs 4
1 vs 3	2 vs 3	3 vs 1	2 vs 3
4 vs 2	4 vs 1	2 vs 4	4 vs 1
3 vs 2	1 vs 2	4 vs 1	2 vs 1
3 vs 1	2 vs 4	1 vs 4	3 vs 1
2 vs 3	2 vs 1	4 vs 3	4 vs 3
4 vs 3	3 vs 2	3 vs 2	3 vs 4
3 vs 4	3 vs 1	1 vs 3	1 vs 2
2 vs 1	4 vs 2	3 vs 4	3 vs 2
1 vs 4	1 vs 4	2 vs 3	2 vs 4
1 vs 2	4 vs 3	1 vs 2	4 vs 2
2 vs 4	3 vs 4	2 vs 1	1 vs 3

Table 1. The order of Tube Test match-ups on Days 1 to 3 and during retest one week later. The numbers 1-4 refer to the ID of each animal in each group.

Learning – Hidden Platform (SW)				
Trials	Day 1	Day 2	Day 3	Day 4
1	E	S	W	N
2	N	E	E	E
3	S	W	S	W
4	W	N	N	S

Memory	
Day	Trial 1
5	N

Reversal – Hidden Platform (NE)		
Trials	Day 6	Day 7
1	E	S
2	S	W
3	W	E
4	N	N

Table 2. Start positions for learning, memory, and reversal phases of the Morris Water Maze across each day.

	Alpha Males (mean ± SEM, N)	Delta Males (mean ± SEM, N)	p value	Effect size (g)
Body weight (g)	369 ± 3.8, 10	370 ± 5.5, 11	.99	.070
Adrenal Glands (%BW)	0.0117 ± 0.00034, 10	0.0120 ± 0.00035, 10	.68	.18
Thymus gland (%BW)	0.0743 ± 0.0055, 10	0.0838 ± 0.0053, 10	.23	.53

Table 3. Physiological characteristics of male with the highest and lowest average 3-day David's Score. BW, body weight; SEM, standard error of the mean; N, number of male rats; g, Hedge's g corrected effect size measure of Cohen's d.

	Alpha Females (mean ± SEM, N)	Delta Females (mean ± SEM, N)	p value	Effect size (g)
Body weight (g)	234 ± 3.2, 10	231 ± 2.8, 12	.99	.35
Adrenal Glands (%BW)	0.0220 ± 0.00080, 10	0.0239 ± 0.00096, 10	.14	.66
Thymus Gland (%BW)	0.104 ± 0.0049, 10	0.115 ± 0.0072, 10	.21	.56

Table 4. Physiological characteristics of female rats with the highest and lowest average 3-dat David's Score. BW, body weight; SEM, standard error of the mean; N, number of male rats; g, Hedge's g corrected effect size measure of Cohen's d.

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APPENDIX I

Experimental Schematic:
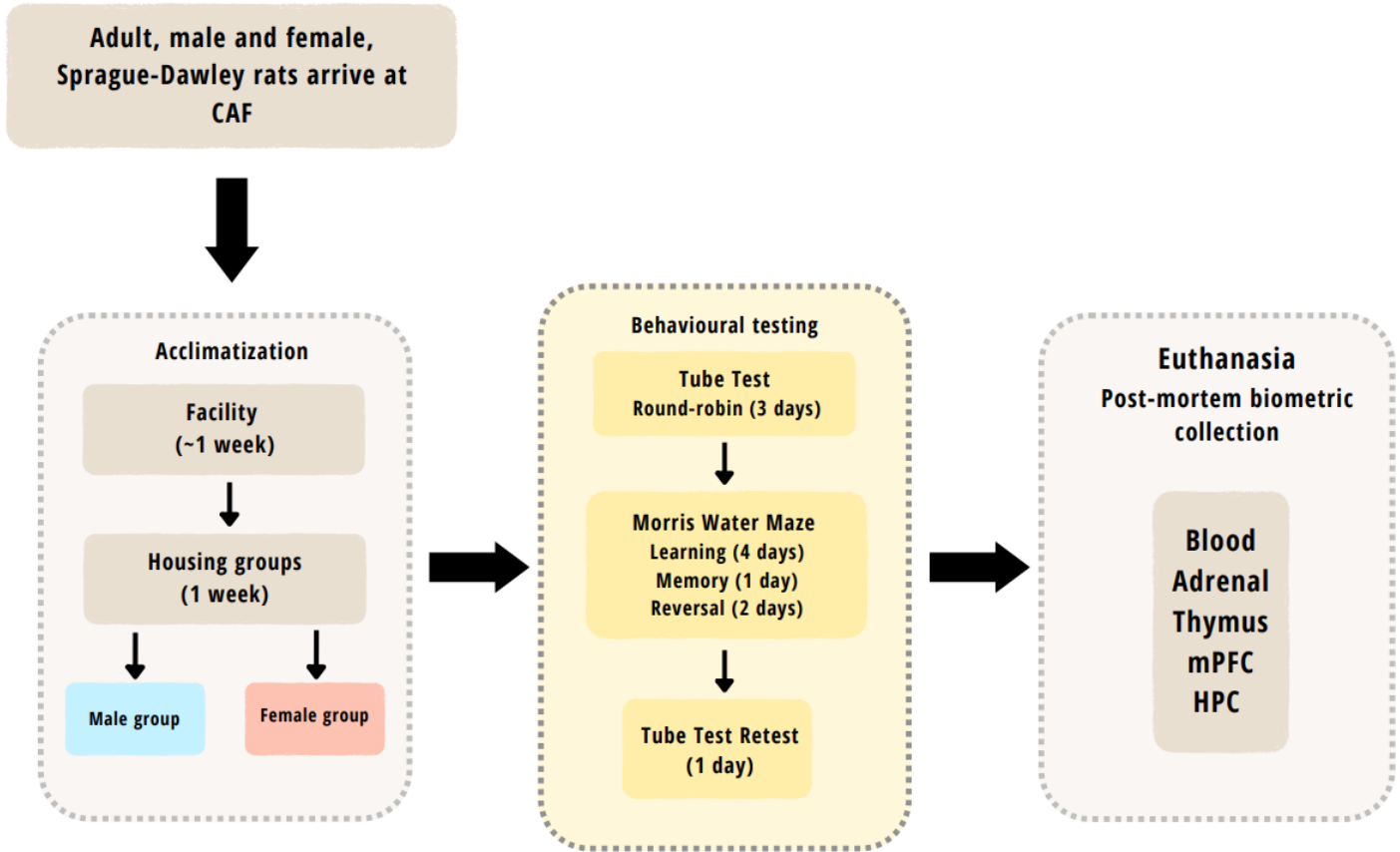
David's Score:

The general formula for David's Score is:

$$DS = w + w_2 - l - l_2$$

Calculation steps:

1. Each dyadic interaction will be calculated using the below formula:

$$P_{ij} = \frac{a_{ij}}{n_{ij}}$$
$$P_{ji} = 1 - P_{ij}$$

Where

P_{ij} is the proportion of wins by subject i for every interaction with j.

a_{ij} is the number of wins from subject i for every interaction with j.

n_{ij} is the total number of interactions between subjects i and j.

P_{ji} is the proportion of losses by subject i for every interaction with j.

2. Once the proportions have been calculated, David's Score can be determined:

$$DS = w + w_2 - l - l_2$$

Where

w is the sum of the ith subject's proportion of wins, P_{ij}.

w_2 denotes the sum of the weighted proportion of wins for every subject defeated by the ith subject.

l is the sum of the ith subject's proportion of losses, P_{ji}.

l_2 denotes the sum of the weighted proportion of losses for every subject that defeated the ith subject.

Example: The table below summarizes number of wins and losses between dyads, with the

proportion of wins/losses in the parentheses (Bang et al., 2010).

		Win					
		α	β	γ	δ	l	l_2
Loss	α	—	1 (0.50)	0 (0.00)	2 (0.40)	0.90	0.93
	β	1 (0.50)	—	4 (0.67)	0 (0.00)	1.17	1.84
	γ	2 (1.00)	2 (0.33)	—	3 (0.75)	2.08	1.92
	δ	3 (0.60)	0 (0.00)	1 (0.25)	—	0.85	1.06
	w	2.10	0.83	0.92	1.15		
	w_2	2.03	1.35	0.84	1.53		
	DS	2.30	−0.83	−2.24	0.77		

In the case of subject α, the first column indicates that α won against some interactions between β, γ, δ respectively. w is calculated by taking the sum of the proportion of wins from every interaction that involved subject α.

$$w = (0.50) + (1.00) + (0.60) = 2.10$$

w_2 is the sum weight of the proportion of wins from every individual that subject α interacted with. In other words, the proportion of wins from β, γ, δ is multiplied by each respective w value, and the total sum will output the w_2 value for subject α.

$$w_2 = (0.50 \text{ x } 0.83) + (1.00 \text{ x } 0.92) + (0.60 \text{ x } 1.15) = 2.025 \rightarrow 2.03$$

To calculate the proportion of losses, l, the rows of the table indicate the proportion of losses of subject α against β, γ, δ respectively. l is calculated by taking the sum of the proportion of losses from every interaction that involved subject α.

$$l = (0.50) + (0.40) = 0.90$$

l_2 is the sum weight of the proportion of losses from every individual that subject α interacted with. In other words, the proportion of losses from β, γ, δ is multiplied by each respective l value, and the total sum will output the l_2 value for subject α.

$$l_2 = (0.50 \text{ x } 1.17) + (0.40 \text{ x } 0.85) = 0.925 \rightarrow 0.93$$

Balanced Data: Row-Sum scoring

Since the Tube Test is a balanced tournament, each subject had the same number of interactions such that all subjects competed against the same individual a total of 6 times over 3 days. The overall results of the round-robin tournament can be expressed as a tournament matrix *T*, with each row vector corresponding to the number of wins for the ith subject with every interaction over the jth subject:

$$T = \begin{bmatrix} 0 & 6 & 5 & 3 \\ 0 & 0 & 3 & 2 \\ 1 & 3 & 0 & 2 \\ 3 & 4 & 4 & 0 \end{bmatrix}$$

Rank can be calculated by taking the sum of each row vector, known as Row-sum scoring:

$$a_i = \sum_{\substack{j=1 \\ j \neq i}}^{t} \alpha_{ij}$$

Where
a_i is the sum of the ith row of the tournament matrix *T*.
α_{ij} is the number of wins of T_i over T_j.

Example:

Using the above matrix as an example, with subjects A, B, C, D corresponding to each column from left to right and top to bottom, the rank order can be determined using the Row-sum score.

Row-sum	Total	Rank
A = (6+5+3)	14	1
B = (3+2)	5	4
C = (1+3+2)	6	3
D = (3+4+4)	11	2