How is social dominance of Wildtype Groningen rats in a semi-natural social colony reflected in brain and behavior?

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Abstract

Animals are an essential model for research of chronic social stress. Groups of social animals for dominance hierarchies, which can cause physical and psychosocial stress. Subordinate animals experience the most stress which is reflected in their behavior, and the physiological and plasticity changes. Previous studies included females for an increase in aggression, while the current study includes the agonistic interactions by females.
36 male and 36 female Wild Type Groningen (WTG) rats were divided into 12 colonies of 4 male and 4 female rats and placed into a visible burrow system, which is a semi-natural environment. Behavioral and physiological markers for stress were examined and the spine density of the dominant and subordinate animals were counted, and the level of BDNF and proBDNF were determined through Western Blot.
The results show weight loss for both dominant and subordinate males, with subordinate animals having the highest weight loss. No difference was found between physiological stress markers, same as the spine density, except an increase in density further from the apical branch in the most subordinate animal of the BLA region. Due to malfunctions in the Western Blot process no conclusion can be drawn about the level of BNDF and proBDNF.
However, the conclusion can be drawn that, a dominance hierarchy is formed, but there is no indication of social stress. Rather there is an indication that animals are able to adapt to their stressful situation. Furthermore, female animals do interact with other females and males agonistically, but there is no indication of them forming a dominance hierarchy.
Finally, there does not seem to be a superior method of scoring the behavior to determine the dominance hierarchy, as some animals still share the same rank.

TABLE OF CONTENTS

1. Introduction

Understanding the socioeconomic gradient is one of the most difficult challenges in public health. In many Westernized societies, a lower socioeconomic status (SES) predicts increased risks of cardiovascular, respiratory, rheumatoid, and psychiatric diseases; low birth weight, infant mortality and all-cause mortality [1-4]. This relationship is primarily due to the influence of socioeconomic status on health. Disease incidences can be several times higher at the lower end of the SES spectrum. These physiological concerns are difficult to investigate in humans, hence a significant body of research has concentrated on animals. Significant inconsistency in resource access in these animal studies can result in groups of social animals forming dominance hierarchies. A dominance hierarchy is defined as a relationship between two individuals, in which one holds off the other in a contest [5]. Dominance relationships among males within adult, mixed-sex, rat groups typically develop within the first several days of grouping, and are often stable over the life-span of the group [6]. The dominance hierarchy is established by agonistic interactions, and it is influenced by different factors, such as the prior attributes and the winner-loser effect. The winning animals will have a changed neuroendocrine effect or it's perception of its own fighting ability is improved, and will now have more of a chance to win a fight [7]. an individual is more likely to lose again and vice versa after winning [8]. Fighting ability can also be altered by prior attributes. Prior qualities and the winner-loser effect are two examples of elements that can influence hierarchy formation. Some animals possessed prior attributes before the creation of the dominance hierarchy, which can influence fighting results and rank positions. It can include size, distinct physical traits, fighting ability, and sex [9]. Male animals are generally the animals who possess the highest rank. Although females are involved in dominance hierarchies, the low prior attributes cause the male to win an interaction more often, and outrank the female. Furthermore, females in general perform less aggressive acts than males [10]. Hence, female behavior is mostly ignored, as they are used to cohabitate male animals with to increase aggression [11].

However, female animals can become dominant over males. Moreover, the female dominance over males seems to increase with the percentage of males in the group. The explanation for this could be that a higher percentage of males in the group augments the number of interaction with high intensity, thus reducing the dominance of males relative to females, resulting in females being victorious over them. Furthermore, because of this altered sex ratio, interaction of females with males are of higher proportion which leads to incidental victories for females over males, and the higher intensity of these interactions also lead to stronger hierarchical differentiation among females, which can also be attributable to the prior attribute hypothesis, where its perception of its own fighting ability is improved [7]. A subordinate rank can result in unattainable resources, and in such cases, an animal's dominance rank can have a significant impact on the quality of its life [12], as animals of different ranks may experience different stress intensities [7]. There is no agreement yet about whether dominant or subordinate animals are more physiological stressed. Ranks that experience most physical and physiological stressors tend to display the most severe stress-related pathologies. In species such as dwarf mongooses, African wild dogs, and ring-tailed lemurs, their rank is physically demanding as the high-ranking individuals have to maintain their dominance over the subordinate cohort [13,14]. In other species it is less stressful as the rank is inherited, matrilineal dominance system is exhibited in which a female inherits the mothers' rank [15].

However, a different study states that the animals of a lower dominance rank experience more stress and carry the most significant risk of stress-related diseases. Instead of confrontation, high-ranking individuals maintain authority by psychological intimidation (where, for example, mere eye contact with the alpha individual might elicit subordination gestures).

Subordination is associated with the highest physiological indices in such cases (e.g., savanna baboons, rhesus and squirrel monkeys, mice, rats, and white-throated sparrows), possibly reflecting the frequent psychological stressors for subordinates and the scarcity of physical stressors for dominant individuals [12-14,16,17]. The study of rank-health relations in animals has frequently been framed in the context of stress and the idea that animals of different ranks experience different patterns of stress, such as maintaining the dominant rank or feeling extreme anxiety because of a dominant animal [12].

Stress is a cognitive perception of uncontrollability and unpredictability expressed in physiological and behavioral responses [18]. Different types of stressors engage various brain networks, including the Sympathetic-Adreno-Medullar (SAM) axis and the Hypothalamus-Pituitary-Adrenal (HPA) axis [19].
The first phase of the stress response goes through the SAM axis. The SAM axis provides a rapid physiological adaption, resulting in short-lasting responses, such as alertness, vigilance, and appraisal of the situation. The SAM axis enables an individual to make a strategic decision, enabling them to face the challenge in the initial phase of a stressful event, which is the description of 'fight or flight'. Secondly, the HPA axis results in an amplified and protracted secretory response [20]. The HPA axis is a dynamic system consisting of the central nervous system (CNS) and the endocrine system. The leading player in the system is the corticotropin-releasing factor (CRF). The hypothalamus releases CRF. After CRF binds to CRF receptors on the anterior pituitary gland, adrenocorticotropic hormone (ACTH) is released by the pituitary gland. ACTH then binds to the receptors on the adrenal cortex, stimulating the production and release of cortisol from the adrenal cortex [21]. Fifteen minutes after the beginning of stress, cortisol levels rise systemically and remain for several hours [22,23]. Increased cortisol levels result in the mobilization of glucose and tissue substrates used for fuel. The high cortisol level decreases inflammation to manage stressors effectively [24,25]. A stressor is a stimulus that threatens homeostasis, whereas stress is the response where homeostasis is the goal to be reached. Stress occurs when an organism senses a disruption or even a threat to homeostasis and will lead to a compensatory reaction. Thus "stress" is a condition where prior learning expectations do not meet the anticipated perception of the internal or external environment, resulting in compensatory responses. The levels of physiological activity that will reestablish or keep the homeostasis are different, including running, standing, or laying down. The physiological activity levels depend on the situation imposed on the organism. The amount of activity required for the individual to maintain stability through change or to adapt to the problem is called "allostasis" [26,27]. The "allostatic load" is the prolonged continuous or intermittent activation of effecters involved in allostastis[28], and is the result of chronic stress which can result in psychopathologies like depression or anxiety [29].

Stress is an important topic to research, and, as mentioned before, because it is hard to research in humans, animals are used as a model. Social defeat (the resident-intruder test) is a model that is used to understand the mechanisms of stress better. The model generates emotional and psychological stress by exploiting social conflict between members of the same species [30]. Animal models of social defeat can include acute and chronic stress models. In the resident intruder model, an episodic model, aggression is inflicted when residents attack the intruders. There is only one confrontation or a series of conflicts separated by more extended periods[31]. The confrontation will result in social defeat for the loser in the conflict. The stressed "loser" animal returns to its home cage or is left in a protected situation. The confrontations or protected exposures may be repeated on consecutive tests. A variant of this stress model includes intermittent defeat. In the periodic defeat model, animals are housed in

bordering areas within visual, auditory, and olfactory contact. The barriers are removed between the two animals in intervals so that the animals can interact directly. The direct interaction results in a victor and a defeated animal. Subsequently, the barrier is replaced, leaving the defeated animal in chronic sensory (except tactile) contact with the victor. The regular contact with the victor will result in chronic psychosocial stress exposure for the defeated animal, while initially, the first agonistic interaction was intermittent. The duration and severity of behavioral and physiological effects of single social defeat depends on whether animals are housed individually or communally. The unfavorable long-term impact of social defeat was significantly reduced in the group-housed rats compared to the defeated individually housed rats [32]. Animals can also be grouped and maintained in colonies. An example is the Visible Burrow system (VBS), a semi-natural habitat with tunnels and burrows, which will be explained in more depth further on. On the other hand it can include standard animal cages, housing multiple animals of one sex. Stress is then inflicted through a factor for animals to fight over. In a group where water and food are constantly available, there is no specific provocation for the ensuing agonistic interactions except the presence of females, where sexual interaction can increase the aggression in male rats. [11]. In general, in larger and natural habitats the level of fighting is higher, due to bigger competition over food and water or female animals, and due to the natural behavior of animals to form a dominance hierarchy [33].

As explained earlier, semi-natural habitats exist, in which the Visible Burrow System is a very prominent model. Such natural habitats will result in a wider and more natural range of behaviors, which may resemble the real world more closely. Furthermore, the VBS has burrow systems, that provide the possibility of escape from an attack and thereby reduces physical injury during a social conflict [34,35]. The visible burrows are a typical feature in the natural environment of many rodents [6,36], in which animals are held for approximately 10 days. The visible burrows mimic the burrow systems that laboratory rats create for themselves when given a dirt substrate. Rats quickly get used to the tunnel-chamber system. Once settled, they will sleep in the chambers and remain in the burrows during the light/dark cycle [37]. This setup allows for analysis of social group behavior that would normally occur in mixed sex-colonies [38]. Normal social group behavior of rats includes offense or aggressive attacks, with the attacking behavior characterizing the dominant and defensive behavior characterizing the subordinate animal. Some species have recognizable patterns in their communication towards their fighting partner to terminate aggression. Aggressive or offensive components include lateral attack, chase, and standing on top of other animals. Defensive components include flight/avoidance, defensive upright, and lying on the back, indicating defeat [39,40]. An aggressive contest is over when one animal flees (the loser), and one animal stays put (the winner). The major mechanism by which social experience produces stress is agonistic behavior. For most social grouping studies, agonistic behavior is a self-explanatory component in laboratory rats. Agonistic behavior can be measured directly, when observing an agonistic interaction between two male animals or indirectly, by counting the animals' wounds after the fight [41-43].

Scoring methods

In the current scoring method, performed by Miguel Puentes, the result showed many male animals sharing the same rank, as seen in appendix D4. This study aims to compare two different scoring methods, one based on the research of Miguel Puentes and one on the research of James Curley, and determine if there are any differences in the results and argue which one is preferable.

The scoring methods of Miguel Puentes consist of scoring the agonistic interaction in time slots of 10 minutes, 8 times a day, while also observing the specific behavior accompanied by this agonistic interaction, such as Patrolling, Tunnel guarding, Approach, Retreat, Status quo, Drinking. The scoring also consisted of the amount of time the animals would spend in the tunnels or the open area.

James Curley's method consist of merely scoring the behavior for a winning or losing outcome, and is done for multiple consecutive hours. No additional behavior is scored beside winning or losing a fight. Observations are made for 1-3 hours per day during the dark cycle, with the majority of them taking place in the first 4 hours after the dark cycle began. Fighting, chasing, mounting, subordinate posture, and induced-flee were recorded in order of priority [111].

Difference

The biggest difference between Miguel Puentes and James Curley is that in Puentes' method the behavior is scored more precisely, with the characteristics of the agonistic behavior included. Furthermore, it is done in time slots of 10 minutes. For James Curley's method, multiple hours of behavior are scored to attempt to score as many interactions as possible. No additional characteristics of behavior is scored.

In the study of Tamashiro et al. (2007), the aggressive and defensive behavior that dominant and subordinate animals exhibited were assessed by the number of bite wounds [44]. A study by Tamashiro in 2003 described that the least number of wounds characterizes the dominant animal, suggesting the animals do not experience stress as much as subordinate animals [45]. The number of wounds was also assessed in the study by Blanchard et al. (1993). The subordinate animals have a higher number of wounds compared to the controls. In contrast, dominant animals have slightly more wounds than subordinate animals, suggesting that both ranks experience aggression [6]. Females of both ranks have the absolute least wounds of the colonies. Females are mostly brought into the VBS to increase the aggression between males and do not engage in as many agonistic interactions as male animals [46].

Both dominant and subordinate animals are expected to lose weight, as they engage in the most agonistic interactions requiring the most energy, compared with females [10]. Studies have found that subordinate animals have a bigger decrease in body weight than dominant animals [6,47], which could be the result of decreased food intake due to insufficient food availability [46]. Part of this weight loss is attributable to a decrease in body fat percentage in both dominants and subordinates, where subordinates have an additional decrease in the percentage of lean mass [45]. In the same study conducted by Blanchard et al., the subordinates and dominant animals both had higher adrenal weights compared to controls. Higher adrenal gland can be expected when animals experience more stress or engage in more activity, as the adrenal gland produces Corticosteroid-binding Globulin (CBG) [48], which binds to cortisol to diffuse into cells and interact with intracellular corticosteroid receptors [49].In two studies, the thymus weight was lower in both dominant and subordinate males [6]. Tamashiro found that both dominants and subordinates show a thymus reduction and enlargement of the adrenal gland and a decreased testes weight for subordinates [44].

Dominant and subordinate animals show moderate elevations of corticosterone within the VBS [6,36]. It is expected that male subordinate animals, experiencing the most social defeat will have a bigger increase in corticosterone compared to male dominant animals. [45,50,51]. This is because social defeat creates a higher corticosterone response than social victory, as seen in figure 1. Dominant rats have elevated corticosterone levels relative to controls, indicating that they too experience some degree of stress, likely due to having to constantly defend their dominant social status [6,45,52]. A higher concentration of corticosterone in females at the end of the VBS is expected, as the corticosterone baseline is also already higher for females [53].

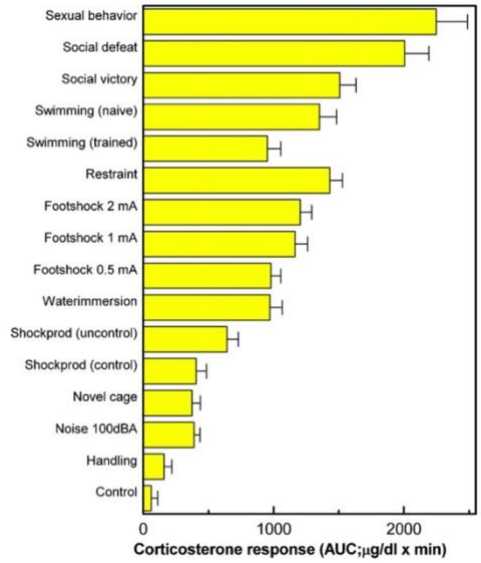


Figure 1 Plasma corticosterone responses of adult male Wistar rats to different test conditions {{533 Koolhaas,J.M. 1997;}}.

Early experiments conducted by Schuurman (1981) show that either winning or losing a social interaction leads to a different recovery speed of the corticosterone response, as seen in figure 4. The loser animal has a higher lingering level of corticosterone [54]. The lingering level could result in losing animals (subordinates) having a higher level of corticosterone at the end of the VBS than winning (dominant) animals.

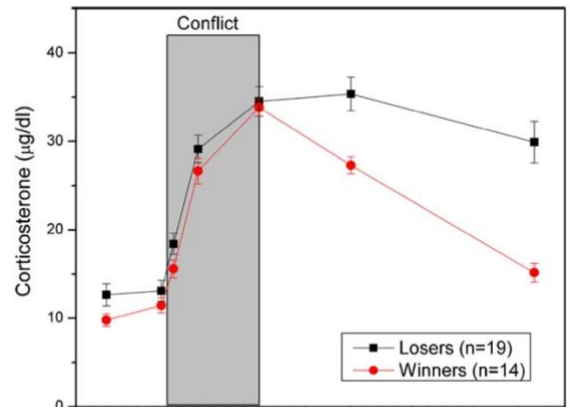


Figure 2 Time course of plasma corticosterone concentration in male rats either losing or winnen a social confrontation in a resident intruder paradigm {{328 Koolhaas,J.M. 2011}}.

In general, it seems that both ranks experience stress once in the VBS, but the subordinates show more stress associated changes than the dominant animals.

Chronic stress does not only influence behavior and physiology, but it may also influence neurons in distinct brain regions. The brain is the critical organ of the regulation of stress. Neural circuitry inside the brain decides if a situation is threatening and stressful. Brain systems involved in stressful and frightening situations include the hippocampus, amygdala, and prefrontal cortex areas [29]. These brain systems regulate the physiological and behavioral stress processes, which can be adaptative short term and maladaptive long term. Furthermore, the neurons in these brain systems are very susceptible to chronic stress, resulting in structural plasticity [55], which will be explained in the coming paragraphs.

The amygdala and hippocampus are part of the limbic system, which process experiences by interfacing with brain areas such as the hypothalamus and brainstem and higher cortical areas located within the prefrontal cortex. The amygdala and the hippocampus link to each other both anatomically and functionally [56,57]. For example, this amygdala and hippocampus link is seen in lesions of the basolateral amygdaloid nucleus that reduces long-term potation, a process that supports memory in the hippocampal dentate gyrus, and stimulation of this nucleus facilitates dentate gyrus long-term potation [58,59]. Moreover, the HPA axis is regulated by the hippocampus and amygdala, with the hippocampus being inhibitory and the amygdala being excitatory [60-62]. Other brain areas are also involved. As recent studies indicate, the medial prefrontal cortex (mPFC) also plays an important role in constricting the HPA axis under stress-related conditions.

The hippocampus

The hippocampus is a brain region that is very important for cognitive function and is a susceptible and shapeable region of the brain. Specific animal models have established that chronic stressful experiences, such as housing in dominance hierarchies, can remodel hippocampal neurons and result in changes in hippocampus morphology. Input from the entorhinal cortex to the dentate gyrus divides into connections between the dentate gyrus and the CA3 pyramidal neurons [63].

Amygdala

The amygdala is an almond-shaped structure formed by many nuclei, sorted into five major groups. The groups consist of the basolateral nuclei, cortical-like nuclei, central nuclei, other amygdaloid nuclei, and the extended amygdala. The amygdala is involved in many diseases, such as depression, anger, and neuropsychiatric diseases [64]. Animal studies have shown that stimulating the amygdala increases sexual and aggressive behavior. Moreover, studies using brain lesions have shown that harm to the amygdala may produce the opposite effect. Thus, it appears that this part of the brain plays a role in the display and modulation of aggression [65]. The amygdala and hippocampus work together to process information and store emotional memories [64]. The basolateral amygdala, or basolateral complex, consists of the amygdala's lateral, basal, and accessory-basal nuclei. The lateral nuclei receive most of the sensory information, which arrives directly from the temporal lobe structures, including the hippocampus and primary auditory cortex [66] Finally, the basolateral amygdala is involved in the regulation of the behavioral and physiological response to stress [67].

Structural plasticity

Stress can result in molecular changes in the brain. In the next paragraph, the changes in dendrites for the specific areas of interest, the hippocampus and the amygdala.

Hippocampus CA3&CA1

The dentate gyrus produces neurons throughout adult life, the “adaptive structural plasticity”. The dentate gyrus-CA3 pyramidal cells undergo a reversible remodeling of their dendrites in conditions such as hibernation and chronic stress. Acute and chronic stress results in the hippocampus undergoing several allostatic or adaptive changes, which may be to protect against permanent damage to the brain [52]. The most important form of neuroplasticity for the research of this thesis is the remodeling of dendrites in the hippocampus. Chronic restraint stress causes retraction and simplification of dendrites in the CA3 region of the hippocampus. Retraction of dendrites in the CA3 region is seen in both dominant and subordinate rats that undergo adaption of psychosocial stress in the VBS system. In terms of brain anatomy, dominant rats displayed a more widespread pattern of debranching of the apical dendrites of the CA3 pyramidal neurons in the hippocampus, compared to subordinate rats, who had less branching relative to cage controls [68]. This finding underscores that it is a complex combination of additional variables that influence neuronal structure rather than adrenal size or the expected amount of physiological stress that dictates dendritic remodeling [69]. Since dendritic remodeling is a reversible process, it can be said that the reorganization of the cytoskeleton is taking place rapidly and reversibly and that changes in dendrite length and branching are no "damage", but a form of structural plasticity, and it is one of the ways that stress hormones modulate function within the brain [68]. Neurotropic factors play an important role in the dendritic branching and length, and the enhancement of survival and differentiation of selective populations of neurons.

Furthermore, chronic stress tends to cause dendritic retraction, reduced branching of neurons in the pyramidal neurons in area CA3, and similar effects can be seen in the pyramidal neurons in the CA1 area and granule neurons in the dentate gyrus [70-75]. Furthermore, chronic stress results in the loss of spines [72,76]. Chronic stress not only promotes dendritic remodeling, but it also affects the form and density of the spine based on the length, severity, and kind of stressor. Evidence from diverse physical stresses has mostly revealed a reduction in spine density in CA3 and CA1 pyramidal neurons, which has been linked to depression-like behavior in animals [76-78]. However, there are just a few studies that reveal changes in dendritic spines caused by social stress. Chronic social defeat stress reduces dendritic spine density in neurons of the CA3 and dentate gyrus regions of the hippocampus in susceptible mice [79]. Repeated defeat of feral rats (wild-type Groningen rats) resulted in a significant reduction in spine density in the CA1 pyramidal apical dendrites [80].

Amygdala

Chronic immobilization stress that causes retraction of dendrites in the CA3 region of the hippocampus, produces dendritic growth in neurons of the basolateral amygdala.
Furthermore, besides chronic stress impairing hippocampal-dependent cognitive function [81], it also enhances amygdala-dependent unlearned fear and the fear conditioning processes [82]. These processes are consistent with the opposite effects of stress on hippocampal and amygdala structure. Hyperactivity of the amygdala might also cause chronic stress to increase aggression between animals living in the same cage [83,84].
Chronic and acute immobilization stress have both been shown to increase spinogenesis in the BLA across both primary and secondary branches of spiny neurons, whereas acute immobilization stress stimulates the progressive production of new spines over time but has no effect on dendritic arbors [85]. Adolescent rats showed a decrease in dendritic field and spine density in basal and lateral amygdala neurons after 5 weeks of social instability stress, but adult rats showed an increase in spine density. According to this study, social instability stress

impairs neuronal growth in the amygdala in the adolescent brain, but mature neurons in the amygdala can adapt to this sort of stress [86].

Molecular changes

There are multiple important proteins that are important in the structural remodeling. In this thesis BDNF will be highlighted. Brain-derived neurotropic factor (BDNF) is a neutrophin vital for the survival and growth of neurons in brain regions involved in emotional and cognitive functions [36] and it regulates neuronal plasticity and survival[87,88]. BDNF is reduced when atrophy of dendrites in the hippocampus has occurred as a consequence of chronic stress [89]. Mice bred to show reduced levels of Brain Derived Neurotropic Factor (BDNF) show a less branched dendritic tree and do not show a further reduction of CA3 dendrite length with chronic stress, contrary to Wild Type mice showing reduced dendritic [71]. Chronic restraint stress (CRS), however, reduces BDNF mRNA levels with some studies reporting a decrease in the hippocampus [90] and others have shown no change [91-94]. Mature BDNF is formed when it is cleaved by tissue plasminogen activator (tPA) from proBDNF. It is a plasticity-related serine protease, which enzymes that cleave peptide bonds in proteins [95]). tPA is able to cleave proBDNF when it converts plasminogen to plasmin. When stress induced raised levels of glucocorticosteriods are present, tPA and proBDNF are stimulated [96].

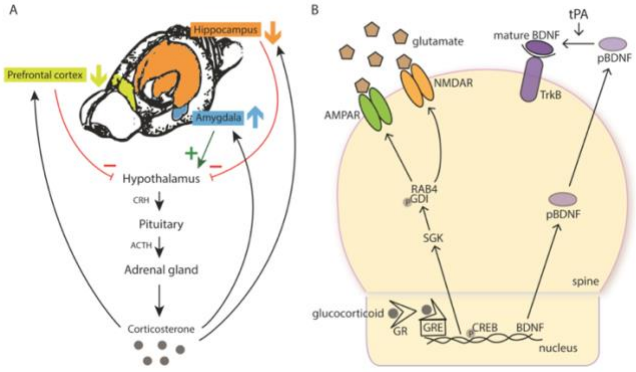


Figure 3 How the different brain circuitry work together. B. How BDNF, pro BDNF and tPA function together [97].

Research questions

Under the influence of stress, body weight, organ weight, corticosterone levels and structural plasticity take place. By observing the behavior, thus determining the dominance hierarchy, it will be possible to distinguish dominant animals from subordinate animals and examine the endocrine and molecular difference between the ranks.

The research question is: How is social dominance of Wildtype Groningen rats in a semi-natural social colony reflected in brain and behavior?

The sub-research question is: Is there any difference between the scoring method of Miguel Puentes and James Curley? With a sub-question: is there a difference in organ weight and corticosterone change results between the scoring of Miguel Puentes and James Curley?

To answer the research questions, sub-questions will be answered:
Is there a difference between males and females, dominant and subordinate animals on the amount of wounds inflicted?
Is there a correlation between the amount of wounds inflicted and the body weight loss?
Is there a correlation between the amount of wounds and the change in corticosterone?
Is there a difference in body weight loss between dominant and subordinate animals?
Is there a difference in organ weight (adrenal gland, thymus, retroperitoneal fat, seminal vesicle, testes) between dominant and subordinate animals?
Is there a difference in corticosterone changes between dominant and subordinate animals?
Is there a difference in corticosterone changes between male and female animals?
Is there a difference in the spine count between dominant and subordinate animals?
Is there a difference in BDNF and proBDNF levels between dominant and subordinate animals?

The expectation is that higher the intensity of aggression, the more wounds are inflicted, especially on subordinate animals and male animals.
The lower the dominance rank of the animal, the higher the weight loss is within the Visible Burrow System
The lower the rank of the animal, the heavier the weight of the adrenal glands, the lower the weight of retroperitoneal fat, thymus, seminal vesicles and testes.
The expectations were that due to higher level of stress subordinate animals would have a bigger change in corticosterone, specifically an increase in the percentage of corticosterone before the VBS compared to after the VBS. Furthermore, females would have almost no change in corticosterone level at the end of the VBS.
The lower the rank of the animals, the lower the spine count is in the hippocampal neurons (CA1 and CA3) at the end of the Visible Burrow system.
The lower the rank of the animals, the higher the spine count is in the amygdala neurons at the end of the Visible Burrow system.
The lower the rank of the animals, the higher the level of BDNF and proBDNF as a consequence of chronic stress in the dorsal hippocampus.

2. Methods

2.1 Visible Burrow System

2.1.1 Animals & animal procedures

In total, 12 colonies were observed in the VBS for ten days. Each colony contained four male and four female Wild Type Groningen (WTG) rats aged five months. The experiment was carried out in three batches of four colonies each (Batch 1 = colonies 1-4; Batch 2 = colonies 5-8; and Batch 3 = colonies 9-12). The females were sterilized by oviduct ligation before being placed in group housing so they would not have any offspring during the research. In addition, animals were marked by coloring different patterns on the back, allowing the animals in each colony to be distinguished from one another (figure 4). Before the VBS, the animals were housed in pairs, one male and one female, for one week to give them sexual experience before being placed in the VBS. Following that, fecal samples were collected, and the body weight was weighed in single housing for one day. In the VBS, the animals were placed in colonies after being single-housed. During their stay at the VBS, the animals' weights were measured on days 2, 5, 8, and 10. Finally, the animals were housed separately for one day after ten days in the VBS. The last weight and corticosterone measurements were done during the separate housing. The animals were sacrificed by decapitation after about 24 hours of being individually housed. The brains were collected and divided into two halves. Each brain was stored in Golgi-Cox fixative [appendix A1] for half an hour before being cut into 100m sections on the Vibratome (Campden) and stained for structural analysis [appendix A2]. The dorsal Hippocampus was collected for molecular analysis by snap-freezing the other half of the brains in isopentane, cooled by dry ice, and stored at -80 °C. The weights of the adrenal glands, thymus, and fat (retroperitoneal and epididymal) were measured. Additionally, the seminal vesicle and testes were measured of the male rats.

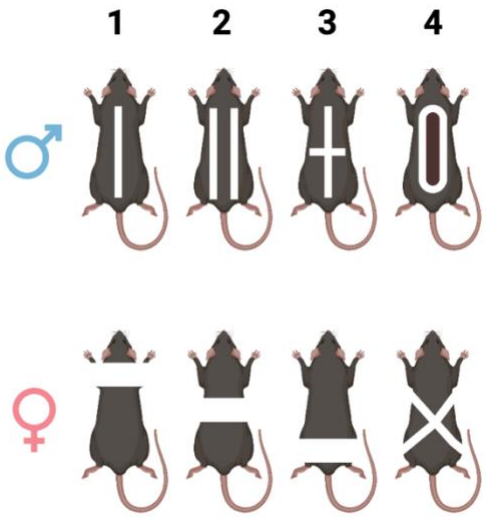


Figure 4 How the fur of the animals is painted to distinghuish them from one another in the experiment.

2.1.2 Experiment

The goal of the study is to find out how dominance in rats are reflected in brain and behavior. The Visible Burrow System (VBS) is used to create a dominance hierarchy within a colony of rats under semi-natural conditions to determine social dominance ranking. The VBS is made up of a continuous dark burrow with tunnels and chambers similar to those found in nature, and an open area with a circadian light pattern to simulate being outside in nature. The VBS resembles a natural environment, allowing the animals to behave as they would in the wild. The animals were observed using an infrared camera from above the VBS from the moment they were placed in it. Furthermore, two drinking points were initially located in the open arena in batch 1 colonies. However, some animals did not drink enough water so a third drinking point was added on day 5.

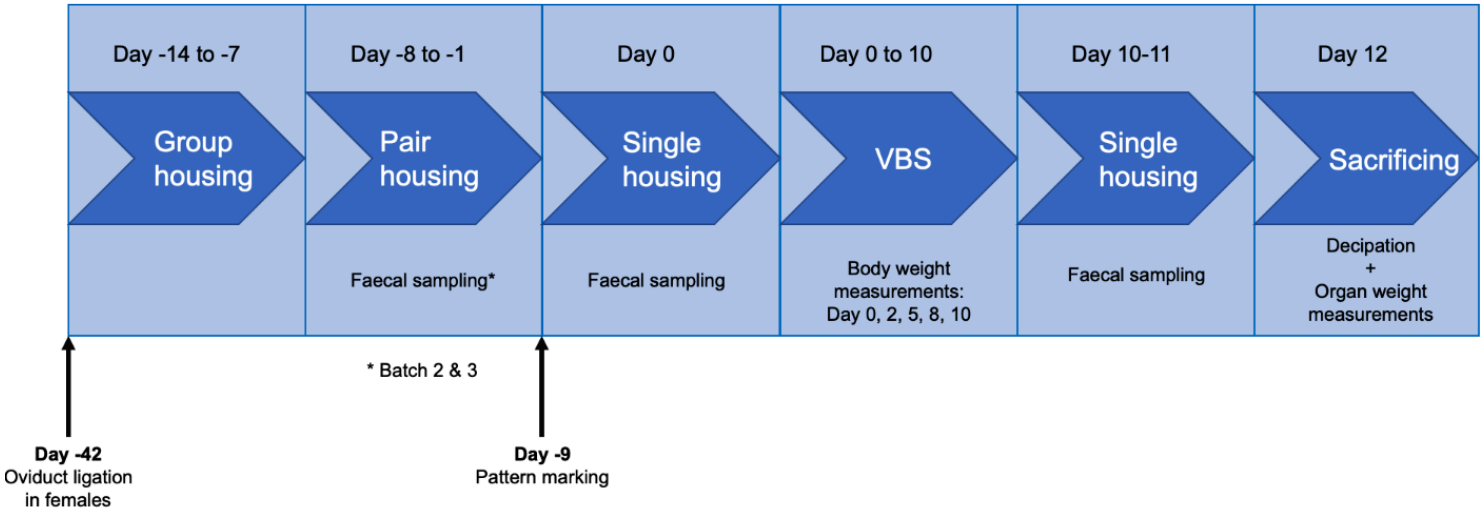


Figure 5 The entire experiment, from group housing to sacrificing

2.2. Behavioral Observations

To determine if there are any differences between the scoring methods of Miguel Puentes and James Curley, observations were made as James Curley has performed in his research. Observations were conducted in the 22 days the rats were housed in the Visible Burrow System. The observations were conducted for 2 hours per day (determined to 13h-15h), or more when no significant amount of interactions were found, during the dark cycle. Male-male, female-female and male-female interactions were all taken into account for the observations. Each contest between the animals lasted for 1-20 seconds. For each of these agonistic interactions the time, the initiator and the competitor were recorded. Fighting, chasing, mounting were counted as behavior in a contest. If an animal fled the contest, this would be seen as a losing experience. When an animal would guard the tunnel and subsequently the competitor would withdraw itself into the tunnel, this would also be seen as a losing experience. When each individual would separate, this would be considered the end of an aggressive interaction. This method was compared to the previous method of behavioral observations. In the previous method, all behaviors of the animals were observed during fixed time slots in the VBS to calculate the dominance rank in each colony. The light went on at 19.00h and off at 7.00h. At seven points of time lasting 10 minutes the animals were observed (12.00h, 12.30h, 14.00h, 16.00h, 18.00h, 05.00h, 07.00h and 08.00h). and The observations were done on day 1, 2, 5 and 10 in the VBS. Male-male, female-female and male-female interactions were all taken into account for the observations.

2.2.1 Dominance hierarchy

The average dominance index method is used to calculate the dominance hierarchy within a colony. This individual-based model, developed by Hemelrijk et al, generates a dominance hierarchy from a matrix representing the frequency of dominance interaction [98]. The higher the average dominance index value, the higher the colony's dominance position. The number of fights won over each other individual in the colony is used to calculate the ADI. The agonistic interactions of lost battles were captured and transformed into won battles. First, the individual dominance index is calculated, which establishes the win ratio per pair. This is accomplished by dividing the number of victories over the other members of the colony by the total number of fights in which the pair was involved. The average dominance index is calculated by taking the average of all individual dominance indices (equation). The greater the ADI number, the higher the rank in the hierarchy.

$$W_{ij} = \frac{X_{ij}}{X_{ij}X_{ji}} \qquad \boldsymbol{ADI} = \frac{1}{N}\sum_{j} Wij$$

Figure 6 Equation for the calculation of the Average Dominance Index. Wij = individual dominance index, i,j = individuals; ADI = average dominance index, N = number of agonistic partners.

2.3 Molecular analysis

First, the brain regions of interest (Hippocampus and Amygdala) were isolated. Second, brain tissue was lysed to allow the proteins to be detected. Finally, the concentrations of BDNF and proBDNF were determined using the western blot method.

2.3.1. Collection of brain regions

Using a Sliding Microtome, the frozen brain regions were first separated from one another (MICROM HM450; Thermo Fisher). One brain half from each animal was available for molecular analysis. The brain regions studied were the medial prelimbic cortex (mPRL), the agranular insular cortex dorsal (AID)/ventral (AIV), the accumbens nucleus core (AcBC)/shell (AcbSh), the basolateral amygdala (BLA), and the Hippocampus, which was divided into dorsal and ventral parts. However, only the levels of BDNF/proBDNF in the dorsal Hippocampus were measured. The punch location of these regions was determined using Bregma points and other brain characteristics [99]

2.4 Western blotting

The Western blot method is used to assess the BDNF and proBDNF levels in the dorsal hippocampus of the most dominant and subordinate animals of the 12 colonies. This includes lysing the samples, separating the proteins (electrophoresis), transferring the proteins to the membrane, and detecting them with antibody complexes.

2.4.1 Lysation

To create a uniform combination of the dorsal hippocampus material, the samples were first homogenized with metal beads in lysis buffer (pH 7,6) using the Tyssue Lyser (Qiagen) for 2 times 1 minute at 30 Hz [appendix C.3]. After that, the lysis buffer was agitated at room temperature for 30 minutes to lyse the cells and release primarily cytosol proteins by adjusting the osmolarity to rupture the cell walls. The lysis buffer (pH 7, 6) controls pH to prevent protein instability. To stop the proteins from being broken down by these enzymes, the lysis buffer also includes 1 tablet/10 ml of phosphatase inhibitor (PhosSTOP, Roche) and 1 tablet/10 ml of protease inhibitor (cOmplete ULTRA Tablets Mini, Roche). The samples were centrifuged for 30 minutes after being lysed, separating the released cytosol proteins in the

supernatant (liquid fluid) from the pellet made up of the leftover cellular debris at 13000g and 4 C. (cell organelles, membranes). By binding Commission Brilliant Blue (G250), a component of the Bradford reagents, to proteins, the Bradford method allows for the determination and incorporation of the protein concentrations in the samples. This method uses a spectrometer (SPECTROstar Nano Absorbance reader, BMG Labtech) to calculate the absorbance values. The samples' absorbance values were compared to a variety of known Bovine Serum Albumin (BSA) protein concentrations, including 0, 0.5, 1.0, 2.0, 3.0, and 4.0 g BSA. Each sample was mixed with three-fourths lysis buffer and one-fourth 4x LDS Sample Buffer to create a stock solution with a final protein concentration of 3.25 g/l protein (Novex NuPage). To allow the sample buffer to unfold the proteins and shield them from freezing artifacts, the prepared stock solution was then heated for 10 minutes at 70 C. In addition, to prevent repetitive thawing and freezing, which lowers the quality of the proteins, three 30 l aliquots containing stock solution were made for analysis. The original stock solution plus three additional aliquots of each sample were then frozen at -80°C in rack 15. For the Bradford analysis, only the first 24 lysed samples were frozen at -20 C [99].

2.4.2 Electrophorese

To analyze the BDNF and proBDNF levels in the samples, the proteins were separated using electrophoresis. The -80 °C frozen samples of interest were removed from the freezer and subsequently kept on ice throughout the procedure until they were implemented to the gel. Each sample (1 aliquot) was treated with 10x Sample reducing agent (Invitrogen NuPage) (0.11 l/l sample) and heated for 10 minutes at 70 C. The gel (1.5mm, 1.5% acrylamide gel) was made one day before the experiment to allow the gel to solidify as much as possible before the experiment. The gels were equipped into the electrophoresis setup (Bio-Rad Mini Trans-Blot® Cell) with 1x Running Buffer. 10 µl of each sample and 8 µl of Page Ruler Prestained protein ladder 10 to 250 kDa (Thermo Scientific) were transferred into the gel cells that had been washed with 1x Running Buffer. The ladder was always pipetted into the second or third cell and last cell to distinguish them from one another. The electrophoresis was performed at 60 V until a sharp front was formed, then at 120 V until the protein of the ladder of 10 kDa was still visible on the gel.

2.4.3 Protein transfer

The proteins were wet transferred from the gel to a nitrocellulose membrane in an electric field to detect the levels of BDNF and proBDNF in the samples. The PVDF membranes and the Whatman filters were cut to the same size, 8x10 cm. The membranes were then activated by immersing them in methanol for one minute and then in Towbin buffer for five minutes. After that, the sandwich was placed in the Mini Trans-Blot® Cell (Bio-Rad). To reduce the generation of heat, which can be problematic for blotting, a frozen cooling unit and stir bar were added, and the tank was filled with Towbin buffer and placed in the cold room. For 90 minutes, the membranes ran at 0.35 A.

2.4.4 Antibodies

To keep the membrane as clean as possible after blotting, it was washed three times with 1x TBS and then twice with 1x TBS-T for 10 minutes each step. Before using antibodies to detect proteins transferred onto the membrane, the remaining binding surface was blocked with 3% BSA to prevent non-specific antibody binding. Following that, the membranes were incubated overnight at 4 °C with the primary antibodies: purified rabbit anti-BDNF (Alomone Labs) 1:2000 in 3% BSA and purified rabbit anti-proBDNF (Alomone Labs) 1:1000 in 3% BSA. After incubation, the membranes were washed three times for ten minutes with TBS-T

before being incubated with the secondary antibody, anti-rabbit IgG - HRP (Cell signaling Technology, #7074) 1:7500 in I-blocking buffer (Cell signaling Technology, #7074). The same procedures were followed for actine, the housekeeping gene, with the exception of the following: The blots, after the incubation with BDNF or proBDNF antibody, were first stripped with stripping buffer (pH 2), which was heated in a 70 °C water bath until it reached at least 65 °C. After that, the membranes were incubated with the buffer twice for 15 minutes at room temperature. Following that, the blots were washed twice with 1x TBS-T. The blots were then incubated in I-Block for one hour before being incubated with the first antibody, actine anti-mouse 1:500.000 in I-Block. After another wash, the membranes were incubated with the second antibody mixture, anti-mouse 1:10.000 in I-Block.

2.4.5 Detection & analysis

ECL was used to detect the antibody complex, as it produces light when it comes into contact with the HRP-conjugated secondary antibodies bound to the membrane. ECL was applied to the membranes for 4 minutes. Dura-ECL (Super Signal West Dura Extended - Duration Substrate; ThermoScientific) was used to detect BDNF and proBDNF, and Normal ECL (Pierce ECL Western - Blotting Substrate; ThermoScientific) was used to detect actine proteins. The chemiluminescence was detected using the ChemiDoc (Bio-Rad), and membrane recordings were made until the resulting bands were fully saturated, indicated with red on the membranes. Image Lab (Bio-Rad, version 6.1) was used to analyze the levels of BDNF, proBDNF, and actine from the detected bands, beginning with the first record before saturation of one of the samples. To begin, the size of the resulting protein bands was determined by comparing their position to the known protein molecular weight of the ladder proteins (BDNF=17kDa, proBDNF=32kDa [100], actin = 42 kDa).

2.5. Dendritic spine density

A Golgi-Cox staining was performed to determine dendritic spine density, followed by morphological analysis and dendritic spine analysis.

2.5.1 Modified Golgi-Cox staining

All experimental animals were quickly decapitated and sacrificed. The brains were then removed and placed in the Golgi-Cox fixative, adapted from [101] [80]. Using a fixed tissue vibratome, 120 m thick coronal sections were obtained (Leica VT 1200S). The color was developed by sodium carbonate after the sections were serially collected, and the brain sections were dehydrated using absolute alcohol, cleared in xylene, and cover-slipped, as adapted from Suvrathan et al, 2013412 Suvrathan,A. 2013;. The slides were coded, but the experimenter was not aware of the code. After the morphological analysis was completed, the codes were broken [80].

2.5.2 Morphological analysis

Pyramidal neurons were chosen from the amygdala's BLA region, the Hippocampus's CA1 region, and the hippocampal CA3 region. 5 pyramidal neurons from each animal (6 animals per group) were analyzed for morphological quantification. The analysis of BLA, CA1, and CA3 neurons is limited to those within the bregma -1.92 to -2.64mm, -2.40 to -3.96mm, and 3.7 to 2.7mm, respectively [80].

2.5.3 Analysis of dendritic spine density

The same NeuroLucida software was used to analyze dendritic spine density, which was attached to an Olympus BX61 microscope (100X, 1.3 numerical aperture, Olympus BX61;

Olympus, Shinjuku-Ku, Tokyo, Japan). Dendrites that originate directly from the main shaft are classified as primary apical dendrites, and they were used to calculate the primary apical dendrite spine. The spine density analysis, on the other hand, was performed on secondary basal dendrites that emerged from the primary basal dendrite. Spines were manually counted along an 80-meter stretch of the selected dendrite, beginning at the branch's origin and moving away from the cell soma. Furthermore, a detailed segmental analysis was used for this spine density analysis. The segmental analysis involved counting the number of spines in 10m steps for a total of 8 steps (i.e. a total length of 80 m). The values for each segment at a given distance from the branch's origin were then averaged across all neurons in the experimental group [80,102].

2.6 Statistics

Statistical analyses and graphs were made using both IBM SPSS Statistics version 28 (one way ANOVA, independent T-tests, Pearson's correlation) and Graphpad Prism 8 (unpaired t-test) [all statistical tests can be found in appendix E]. All data are present as mean + standard error of the mean (SEM). The difference between individual rank positions with each other was determined with one way-ANOVA and unpaired t-tests. For testing hypothesis, a probability level of $p \leq 0.05$ was considered significant.

3. Results

3.1 Weight change

To determine any difference in body weight, the differences between female and male rats were analyzed by using the statistical unpaired T-test. Means and SEM were calculated with Excel. There is no significant difference in body weight, between female dominant and subordinate animals on day 2 (p=0.450), day 5 (p=0.252), day 8 (p=0.311) and day 10 (p=0.682). No significant difference was found in body weight between male dominant and subordinate animals on day 2 (p=0.436), day 8 (p=0.108) and day 10 (p=0.153), except on day 5 where the male most dominant animals are statistically different (p=0.043) from the subordinate animals.

Table 1 Average percentage of body weight change during the VBS period. Values represent the mean ± SEM.

	Day in VBS	Weight change (%)
SUB-M	2nd	-4,2133333 ± 0,73589497
	5th	-8,8183333 ± 1,45949235
	8th	-9,2891667 ± 1,72376854
	10th	-8,735 ± 1,74420708
SUB-F	2nd	2,3763636 ± 0,83090014
	5th	2,7254545 ± 0,64324937
	8th	5,3336364 ± 1,00329482
	10th	7,2018182 ± 0,99590144
DOM-M	2nd	-3,9608333 ± 0,72123049
	5th	-6,5708333 ± 0,85326419
	8th	-6,5508333 ± 0,92907698
	10th	-6,5508333 ± 1,21509038
DOM-F	2nd	0,7766667 ± 0,92717258
	5th	2,1166667 ± 0,98659526
	8th	4,5733333 ± 0,76317452
	10th	5,9958333 ± 1,09896677

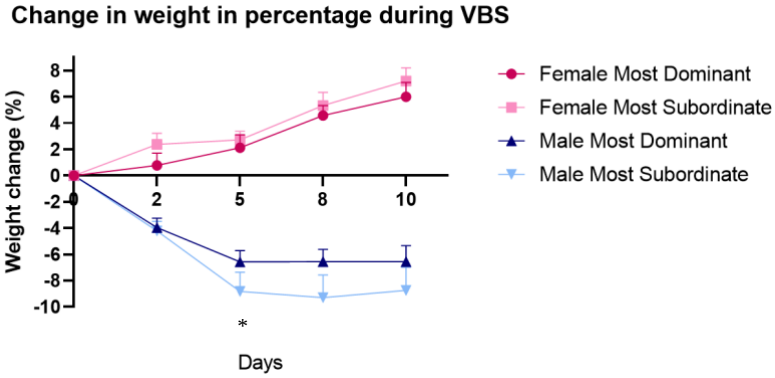


Figure 7 Average percentage of body weight change for females and males per dominance status (n=12). Data are expressed as means ± SEM.

The weights of Male Most Dominants and Subordinates seems to remain stable during pair housing, while Female Most Dominant and Subordinate animals seem to gain weight increasingly. Subsequently, during their time in the VBS, the male animals lose the most weight, especially the subordinate males. Meanwhile, the females continue to gain weight throughout the VBS experiment.

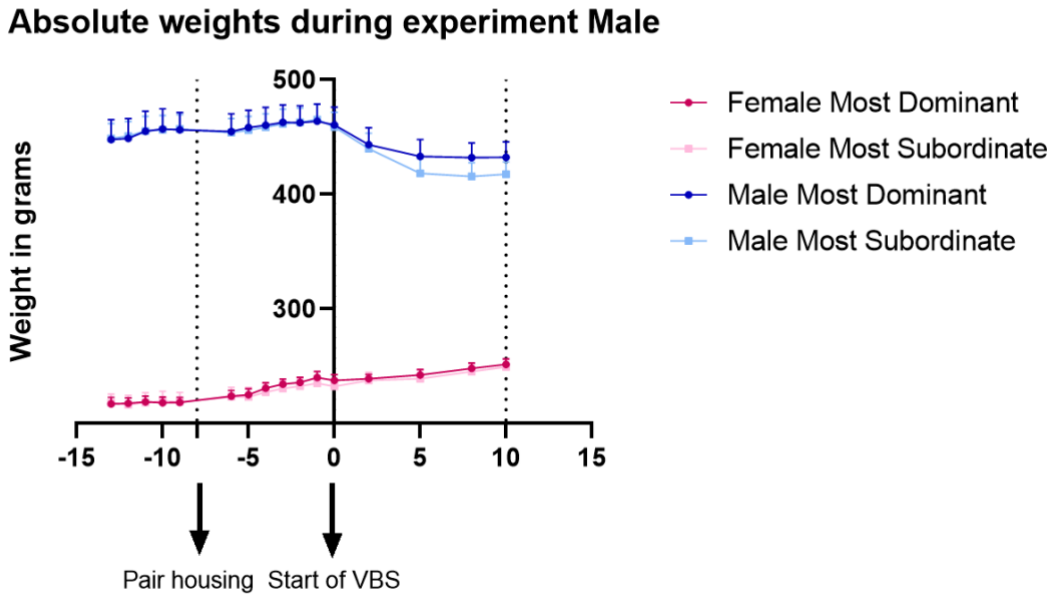


Figure 8 Absolute weights during the whole experiment, including pair housing and the time inside of the VBS. Day10 Female Most Dominant (mean: 251,5±4,76651833), Day10 Male Most Dominant (mean: 432,25±13,5138117), Day10 Female Most Subordinate (mean: 249,4167±6,797346), Day10 Male Most Subordinate (mean: 417,5833±10,79173). N=12

No significant difference in body weight between Female Most Dominant and Female Most Subordinate (p=0.6109) on the last day of the VBS (day 10).

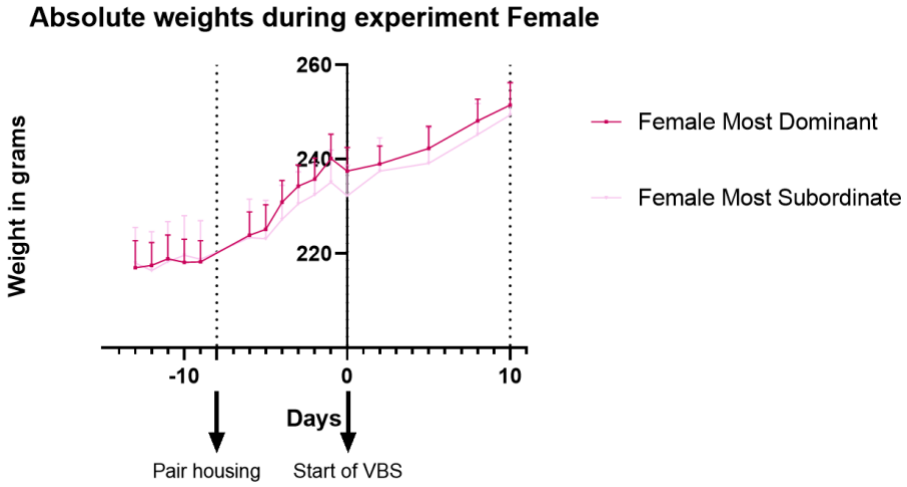


Figure 9 Absolute weights during the whole experiment, including pair housing and the time inside of the VBS. Day 10 Female Most Dominant (mean: 251,5±4,76651833), Day 10 Female Most Subordinate (mean: 249,4167±6,797346). Data are expressed as means ± SEM. N=12

No significant difference in body weight between male most dominant and male most subordinate (p=0.5168) on the last day of the VBS (day 10).

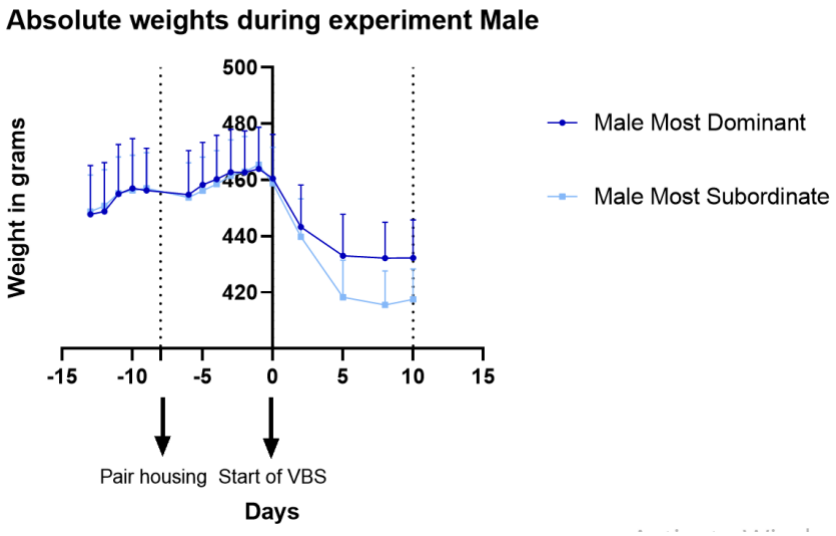


Figure 10 Absolute weights during the whole experiment, including pair housing and the time inside of the VBS. Day 10 Male Most Dominant (mean: 432,25±13,5138117), Day 10 Male Most Subordinate (mean: 417,5833±10,79173). Data are expressed as means ± SEM. N=12.

To show how much weight animals loose or gain during the whole experiment, the delta weight was calculated [the calculation can be found in Appendix E4a]. The delta weight was calculated by setting the base point on day -13. All the consecutive days were then subtracted by the base weight.

Table 2 Delta weight change throughout the whole experiment. Values represent the mean ± SEM.

Day	Male Most Subordinate	Female Most Subordinate	Male Most Dominant	Female Most Dominant
-13	0 ± 0	0 ± 0	0 ± 0	0 ± 0
-12	2,08333333 ± 0,92489421	-1,5833333 ± 1,15114129	1 ± 1,40345893	0,5 ± 1,63993718
-11	6,5 ± 0,88334763	0,33333333 ± 1,37252992	7,25 ± 1,40413351	1,91666667 ± 1,17717132
-10	7,58333333 ± 1,47431717	1,66666667 ± 1,55861892	9,25 ± 1,46744986	1,16666667 ± 1,55131095
-9	8,33333333 ± 2,45669565	0,83333333 ± 1,75737982	8,41666667 ± 3,2670493	1,25 ± 1,86322057
-6	5 ± 2,18118678	5,41666667 ± 1,91666667	7 ± 2,34197534	6,91666667 ± 1,58811081
-5	7,41666667 ± 1,88879605	5,16666667 ± 1,69148193	10,4166667 ± 2,94509951	8,16666667 ± 1,92603114
-4	9,75 ± 2,60281025	9,25 ± 2,1395447	12,5 ± 2,92196488	13,9166667 ± 1,99034919
-3	12,75 ± 2,76647955	12,5833333 ± 2,68965001	15 ± 3,19564097	17,3333333 ± 2,11893818
-2	14,1666667 ± 2,17016035	14,5 ± 2,26468273	14,8333333 ± 3,29791792	18,8333333 ± 2,13141486
-1	16,6666667 ± 2,03876572	17,1666667 ± 2,50706074	16,0833333 ± 3,39218874	23,1666667 ± 1,89029953
0	10,0833333 ± 2,3563529	14,25 ± 2,49886338	12,6666667 ± 2,6294755	20,5 ± 2,15849274
2	-9 ± 4,79425161	19,5 ± 1,99050778	-4,5833333 ± 4,10922527	22 ± 2,4832774
5	-30,5 ± 8,58248745	21,1666667 ± 1,57072331	-14,833333 ± 4,08959014	25,3333333 ± 2,17539036
8	-33,25 ± 9,68607639	27,25 ± 2,43747572	-15,666667 ± 6,14513029	31,1666667 ± 2,07376591
10	-31,166667 ± 9,76452557	31,4166667 ± 2,25112205	-15,583333 ± 6,72338562	34,5 ± 2,43241992

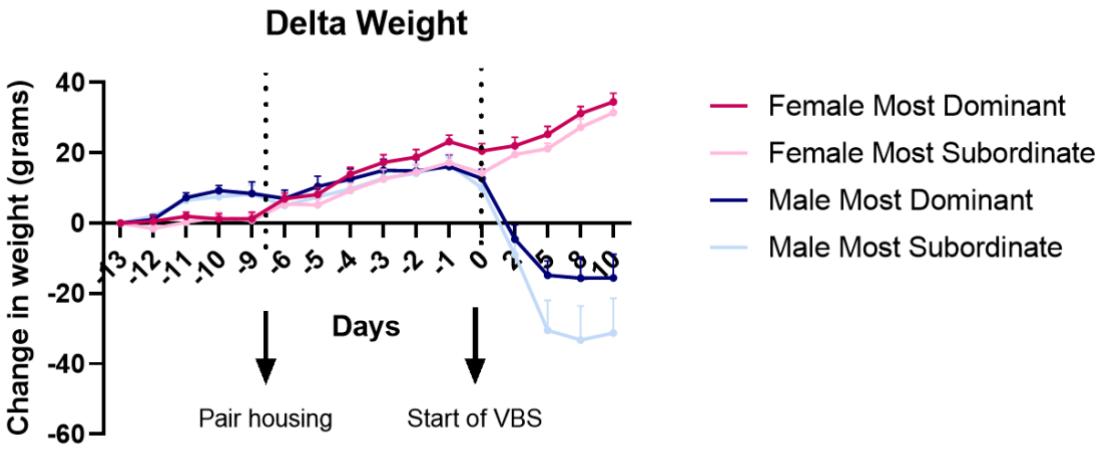


Figure 11 Delta weight change in grams for the whole experiment. Data are expressed as means ± SEM. N=12

3.2 Aggression

Because the intensity of aggression may have effect on the animals in the colonies, the amount of wounds were documented [appendix E3] and analyzed based on sex and rank.

Table 3 Average amount of wounds inflicted on animals in all colonies. Values represent the mean ± SEM.

	mean of wounds
VBS1	1,625 ± 0,56497471
VBS2	0,875 ± 0,61054718
VBS3	4,375 ± 1,8892317
VBS4	0,375 ± 0,18298126
VBS5	1,375 ± 0,41992771
VBS6	0,125 ± 0,125
VBS7	3 ± 1,40152978
VBS8	1,25 ± 0,6196197
VBS9	2,25 ± 1,0479572
VBS10	2,125 ± 1,05960741
VBS11	1,75 ± 0,70076489
VBS12	1,25 ± 0,6196197

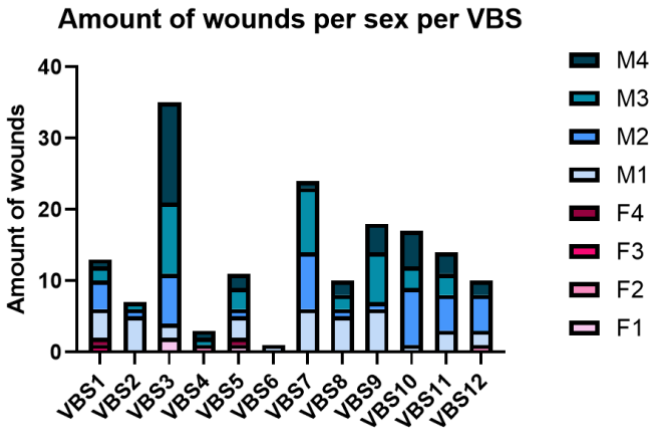


Figure 12 Amount of wounds for both male and female for every colony. Data are expressed as means ± SEM. N=12

Male animals had a significant higher mean of wounds compared to female animals (p=0.041).

Table 4 Average amount of wounds inflicted sorted on sex. Values represent the mean ± SEM.

	mean of wounds
F1	0,25 ± 0,17943514
F2	0,16666667 ± 0,11236664
F3	0,08333333 ± 0,08333333
F4	0,16666667 ± 0,11236664
M1	3,16666667 ± 0,5881833
M2	3,41666667 ± 0,89998597
M3	3,41666667 ± 0,98055591
M4	2,91666667 ± 1,09723021

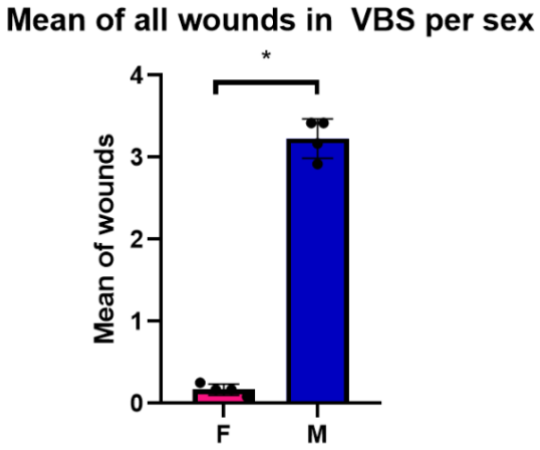


Figure 13 Difference on the average wound inflected on male and female animals. N=12 Data are expressed as means ± SEM.

Significant difference was found between male most subordinate animals with the female most subordinate animals (p=<0.001), and male most dominant animals (p=0.015) of all colonies.

Table 5 The average amount of wounds inflicted on the most dominant female and male, and most subordinate female and male.

	mean of wounds
DOM-F	0,16666667 ± 0,11236664
DOM-M	2,08333333 ± 0,73297167
SUB-F	0,16666667 ± 0,11236664
SUB-M	5 ± 1,03718734

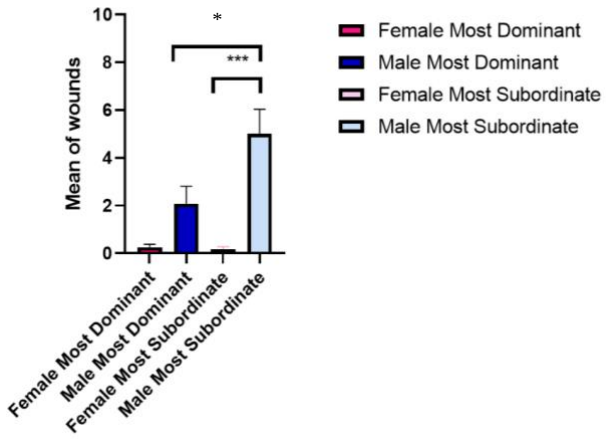


*Figure 14 The average wounds inflected on male and female and dominant and subordinate. Data are expressed as means ± SEM. *p<0.05 *p<0.05, **p<0.01, ***p<0.001 N=12*

To find out if there is any correlation between the amount of wounds inflicted on the animals and the corticosterone change [appendix E3f], and the change in body weight [appendix E3g], the Pearson Correlation was performed.
No correlation was found between wounds and change in body weight (r=-0.261, p=0.087).

There is no significant correlation between wounds and corticosterone of the male most subordinate animals (r=-0.492, p =0.104), the male most dominant animals (r=0.221, p=0.491), the female most subordinate animals (r=-0.118, p=0.714), and the female most dominant animals (r=-0.122, p=0.706). Graphs not pictured.

3.3 Organ weight

To determine if there are any differences in organ weight between the female and male animals and their ranks, the organ weights are calculated (in Excel) to 100 gram of body weight for each animal on the day of sacrifice. To determine any significancy between the animals, the statistical unpaired T-test was performed. There is no statistical difference in adrenal gland weight between the female most dominant and female most Subordinate (p= 0.124), and between male most dominant and male most subordinate (p = 0.373), in thymus weight between female most dominant and female most subordinate (p = 0.929), male most dominant and male most subordinate (p = 0.387), in retroperitoneal fat weight between female most dominant and female most subordinate (p = 0.250) and male most dominant and male most subordinate (p = 0.180). There is also no significant difference in seminal vesicle weight and male most dominant and male most subordinate (p = 0.976) testes weight male most dominant and male most subordinate (p = 0.973).

Table 6 Average weight of organs (%body weight)

weight of organs per 100 gram body weight				
	Female Most Dominant	Female Most Subordinate	Male Most Dominant	Male Most Subordinate
adrenal gland	0,02833257 ± 0,00185907	0,03074831 ± 0,00129997	0,01579438 ± 0,00103931	0,01672066 ± 0,00145096
thymus	0,11174903 ± 0,00801101	0,1020814 ± 0,00746217	0,04826568 ± 0,00584984	0,06346004 ± 0,00627153
fat	2,54755457 ± 0,35585837	2,53520303 ± 0,24822388	2,27314702 ± 0,18949006	3,3320399 ± 0,26129781
vesicle			0,2671113 ± 0,02631477	0,2462688 ± 0,02396997
testes			0,71850536 ± 0,05021534	0,69976007 ± 0,0475904

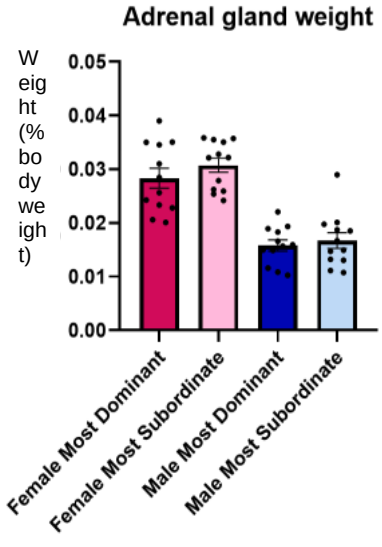


Figure 15 The percentage adrenal gland weight of body weight of dominant and subordinate males and females (n=12). Data are expressed as means ± SEM.

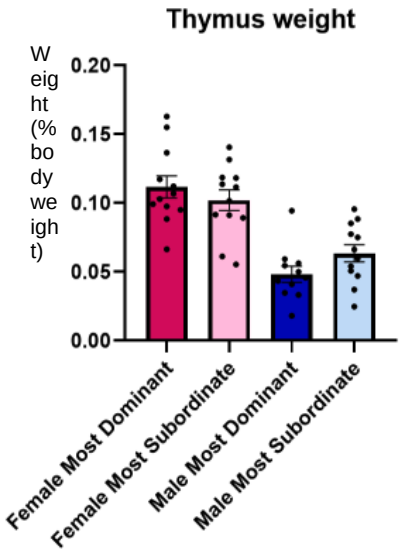


Figure 16 The percentage thymus weight of body weight of dominant and subordinate males and females (n=12). Data are expressed as means ± SEM.

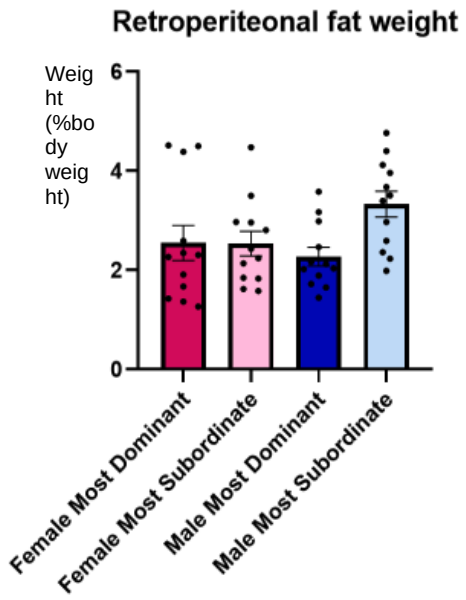


Figure 17 The percentage retroperitoneal fat weight of body weight of dominant and subordinate males and females (n=12). Data are expressed as means ± SEM.

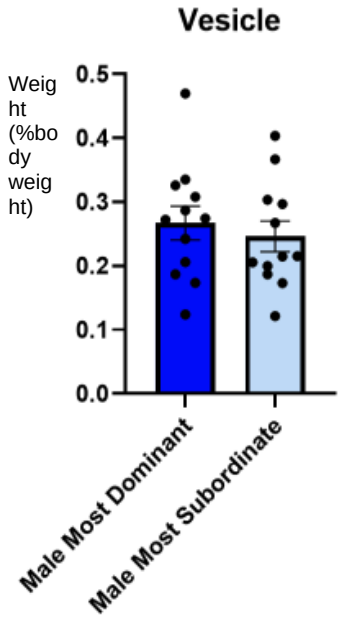


Figure 18 The percentage adrenal gland weight of body weight of dominant and subordinate males (n=12). Data are expressed as means ± SEM.

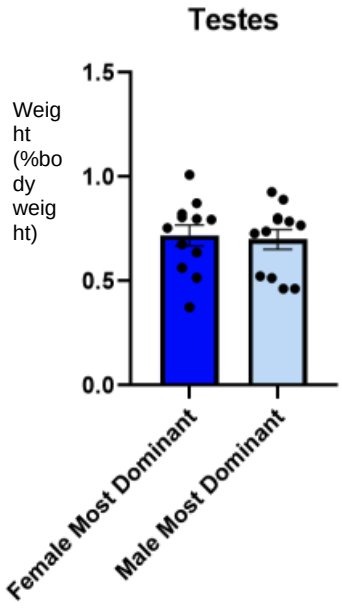


Figure 19 The percentage testes weight of body weight of dominant and subordinate males (n=12). Data are expressed as means ± SEM.

3.4 Endocrine changes between social dominance ranks

To determine if there are any significant differences between the sexes and ranks, the statistical test One-way ANOVA was performed. No significant difference in the percentage of change of corticosterone from pre VBS to post VBS (p=0.297) for all sexes and ranks.

Table 7 Average corticosterone % from pre VBS to Post VBS

	Corticosterone change
Female Most Dominant	191,965924 ± 63,12011
Male Most Dominant	87,628074 ± 56,8982
Female Most Subordinate	208,137988 ± 61,93784
Male Most Subordinate	76,5386591 ± 57,48888

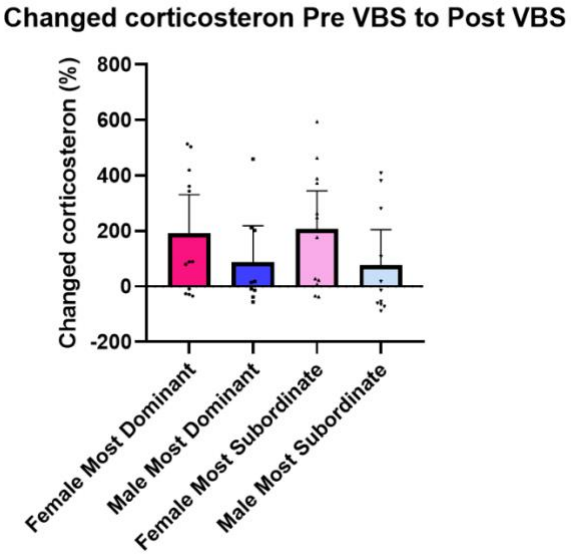


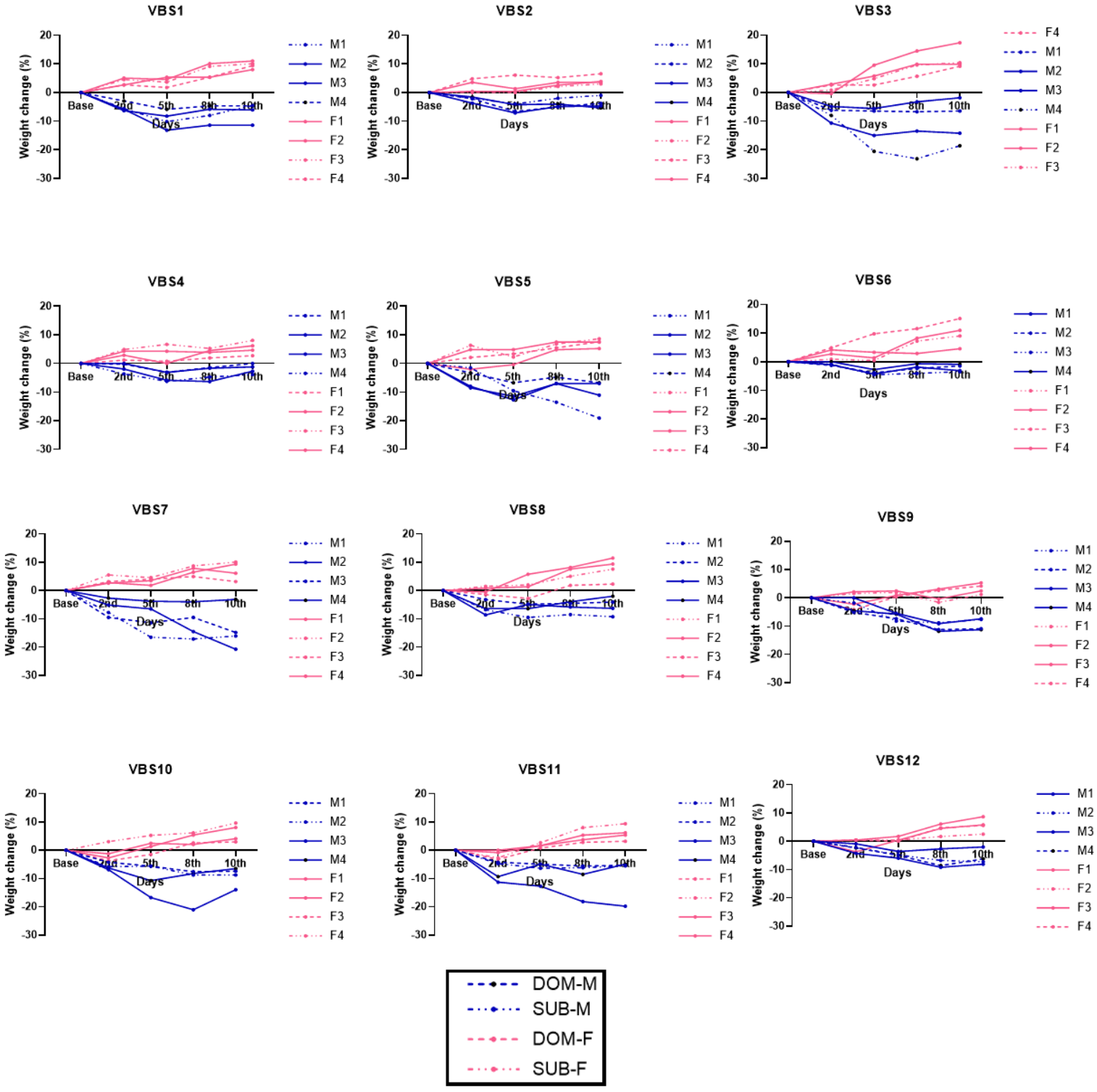
Figure 20 Average concentration of corticosterone change for females and males during the time in the VBS per dominance status as most dominant and most subordinate. For Male Most Dominant two outliers were not included; 7218,681772 and 2223,843284. For Male Most Subordinate one outlier was not included; 7281,681772. Data are expressed as means ± SEM. N=12

3.5 Weight, organ changes between social dominance ranks and sex based on previous scoring with a comparison for aggression

For every rank and sex, the body weight changes are vastly different. Females gain weight, while males lose weight.

When examining the weight changes in the colonies, five come forward where the weight change seems significant when comparing them with the other colonies. VBS 3, 5, 7, 10, and 11 all seem to have at least one male animal who lases weight pas the percentage of 20%, while in other colonies this remains around 10-15%.

When looking at the most wounds inflected on an animal, shown in figure 9, VBS 3 and VBS 7 are the ones far above the other colonies, and this does overlap with VBS3&7 showing the male animals losing the most weight. This is why VBS 3 and 7 are selected to examine further. To compare VBS 3 and 7 to colonies with less aggression and weight loss, VBS 2 and 6 are selected. In these colonies the weight loss is the most minimal for the male animals, and the least wounds are inflicted compared to the other colonies. In the following section these colonies will be analyzed more closely, by looking at the weight differences in the organs and fat. It is expected that adrenal weights are elevated in both dominant and subordinate rats, thymus weights are reduced, an additional decrease in fat percentage for subordinate animals, and testes weights are elevated in dominant animals, and decreased in subordinate animals. Furthermore, it is expected that the corticosterone level will increase for all animals, and a slightly higher change for subordinate animals.


VBS1
VBS2
VBS3
VBS4
VBS5
VBS6
VBS7
VBS8
VBS9
VBS10
VBS11
VBS12
Weight change (%)
Days
Base
2nd
5th
8th
10th
M1
M2
M3
M4
F1
F2
F3
F4
DOM-M
SUB-M
DOM-F
SUB-F

When looking at the adrenal weights of the animals of VBS 3, it can clearly be seen that the weights of all animals are higher than the other colonies. Especially M4, the subordinate animal has an adrenal gland weight of above 0.10 grams, while other animals remain below this level. Female adrenal glands also seem enlarged in the most aggressive colony, rising way above the adrenal gland weights of the other animals. However in the other aggressive colony (VBS7) there is almost no difference with the other colonies, for both males and females, dominants and subordinates. The subordinate and dominant female of VBS3 both seem to have higher thymus weights than the other colonies, with the dominant animal having a slightly higher weight than the subordinate animal. The retroperitoneal fat weight seems slightly higher in male rats, which would make sense as they do weigh significantly more. The subordinate animal (M1) in VBS7 has a higher amount of fat, compared to the dominant animal (M3). There does not seem to be a clear line of higher seminal vesicle weights in a subordinate or dominant animal, same as in the other colonies. In all colonies, except for VBS7, the testes weights seem to be higher for dominant animals compared to subordinate animals.

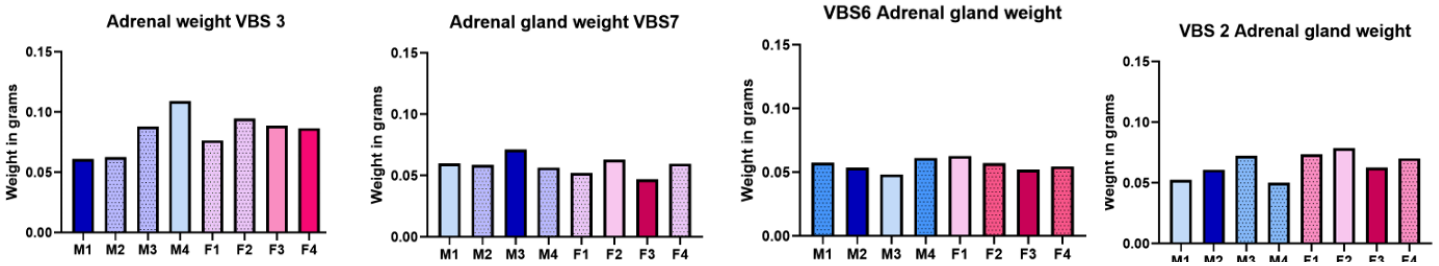


Figure 21 Adrenal gland weight voor VBS3, VBS7, VBS2 and VBS6.

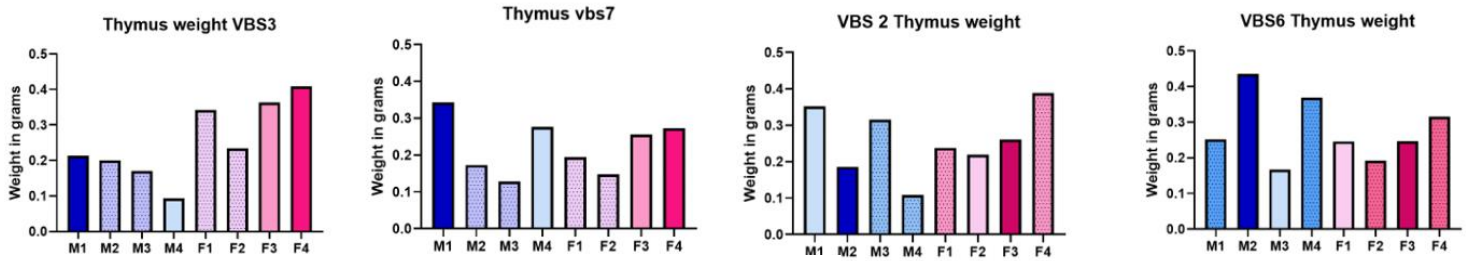


Figure 22 Thymus weight for VBS3, VBS7, VBS2 and VBS6.

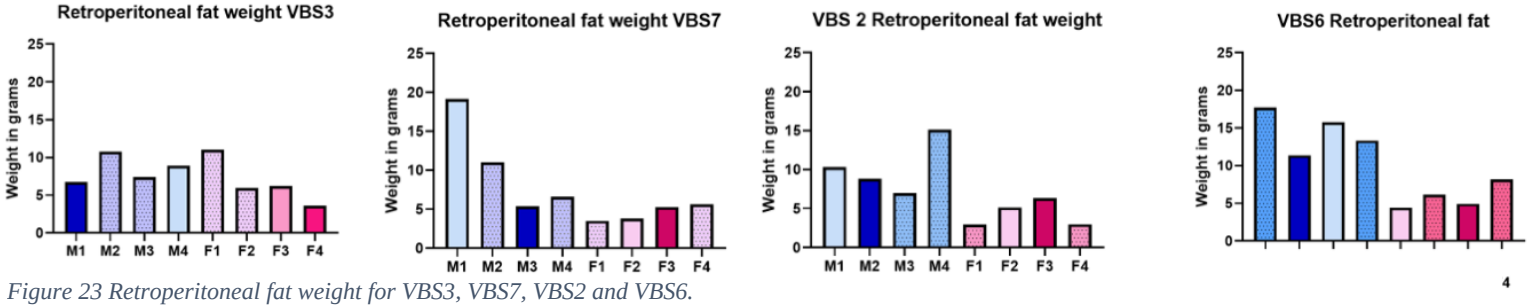


Figure 23 Retroperitoneal fat weight for VBS3, VBS7, VBS2 and VBS6.

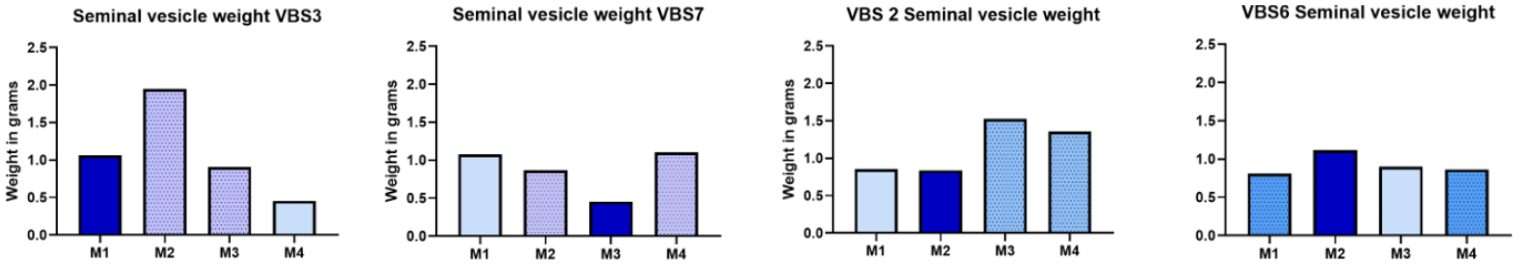


Figure 24 Seminal vesicle weight for VBS3, VBS7, VBS2 and VBS6.

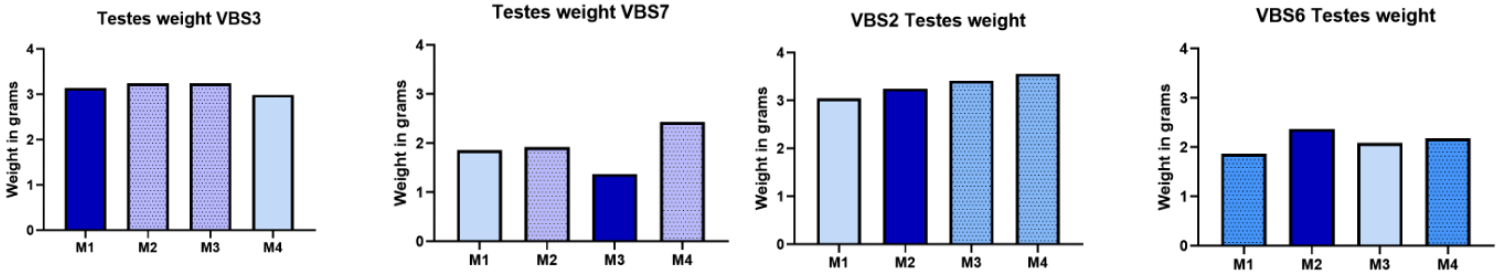


Figure 25 Testes weight for VBS3, VBS7, VBS2 and VBS6.

VBS3 and 2 seem to have the lowest change in corticosterone, while VBS7 and 6 have some of the highest percentage. There were some outliers which were excluded from the calculation and the graph, including M2 in VBS7 and M4 in VBS2.

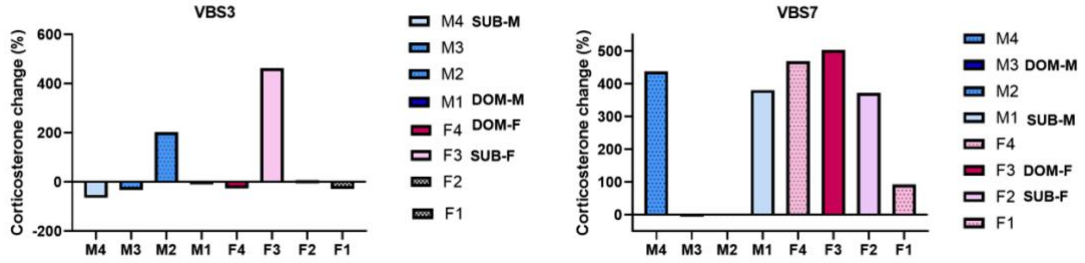


Figure 26 Percentage of corticosterone change in VBS3 and VBS7.

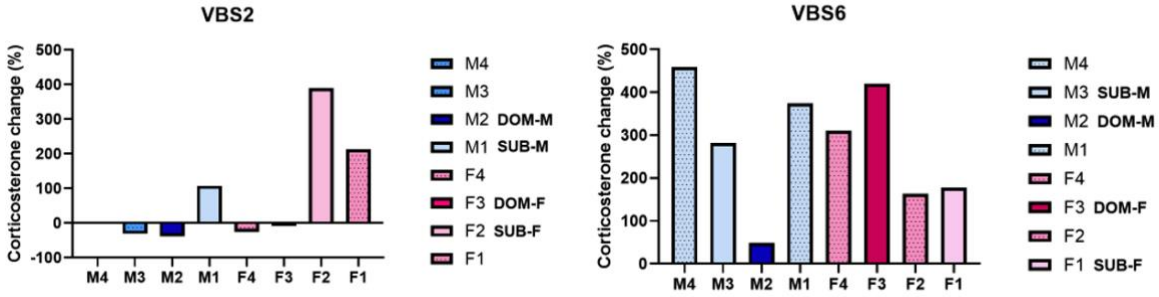


Figure 27 Percentage of corticosterone change in VBS3 and VBS7.

3.6 Difference in scoring methods

To determine if there is a difference in the scoring methods explained previously, the weights of the organs are compared to see if there would be a significant difference in the determined most dominant and most subordinate animals. To determine any significant difference the independent T-test was performed. There is no significant difference in the adrenal weight for the female dominant animal from the scoring of Puentes compared to Curley (p = 0.195), or male dominant animals (p = 0.505), female subordinate (p = 0.862), male subordinate (p =0.612). For the thymus weight, there is also no significant difference between the methods for the female dominant animals (p = 0.683), or male dominant animals (p = 0.994), female subordinate (p = 0.945), male subordinate (p =0.592). For the fat weight, there is no significant difference between the methods, for the female dominant animals (p = 0.717), or male dominant animals (p = 0.462), female subordinate (p = 0.316), male subordinate (p =0.936). No significant difference in the vesicle weight between the methods, for dominant male (p = 0.475) and subordinate male (p = 0.528), same as the testes weight for dominant male (p = 0.552) and subordinate male (p = 0.640).

Table 8&9 Average adrenal gland, thymus, retroperitoneal, seminal vesicle and testes weights for both the most dominant and subordinate animals determined by the methods of Miguel Puentes and James Curley. Values represent the mean ± SEM.

Miguel Puentes' method			
adrenal		DOM-F	0,07155 ± 0,0052
		DOM-M	0,068225 ± 0,0050
		SUB-F	0,07725833 ± 0,0048
		SUB-M	0,06931667 ± 0,0056
thymus		DOM-F	0,28075 ± 0,0206
		DOM-M	0,20851818 ± 0,0265
		SUB-F	0,25554167 ± 0,0215
		SUB-M	0,2644 ± 0,0260
fat		DOM-F	6,52096667 ± 1,0045
		DOM-M	10,055275 ± 1,1367
		SUB-F	6,37185 ± 0,6659
		SUB-M	14,0767417 ± 1,2827
seminal vesicle		DOM-M	1,14251667 ± 0,1003
		SUB-M	1,01761667 ± 0,0886
testes		DOM-M	3,11001667 ± 0,2304
		SUB-M	2,90203333 ± 0,1891

James Curley's method			
adrenal		DOM-F	0,070525 ± 0,00426451
		DOM-M	0,06894167 ± 0,00565469
		SUB-F	0,07374167 ± 0,00417961
		SUB-M	0,06628333 ± 0,00452208
thymus		DOM-F	0,26833333 ± 0,0182771
		DOM-M	0,22332727 ± 0,02467454
		SUB-F	0,2987 ± 0,02136613
		SUB-M	0,28411667 ± 0,02171842
fat		DOM-F	6,89676667 ± 1,00413748
		DOM-M	9,70384167 ± 0,9930268
		SUB-F	7,41880833 ± 0,91831689
		SUB-M	13,0497167 ± 1,34563326
seminal vesicle		DOM-M	1,15338333 ± 0,07939885
		SUB-M	1,05911667 ± 0,06934295
testes		DOM-M	3,19626667 ± 0,1815301
		SUB-M	2,84115833 ± 0,19580094

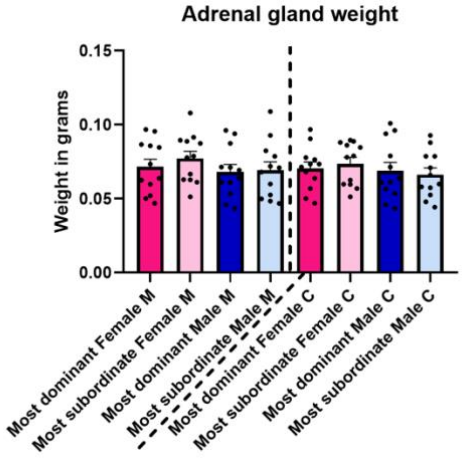


Figure 28 The average adrenal gland weight of dominant and subordinate males and females (n=12) determined by the two scoring methods. Data expressed as mean ± SEM. (M= Method by Miguel Puentes. C= Method by James Curley)

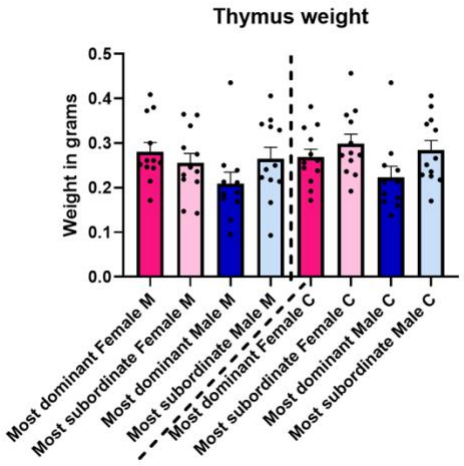


Figure 29 The average thymus weight of dominant and subordinate males and females (n=12) determined by the two scoring methods. Data expressed as mean ± SEM. (M= Method by Miguel Puentes. C= Method by James Curley)

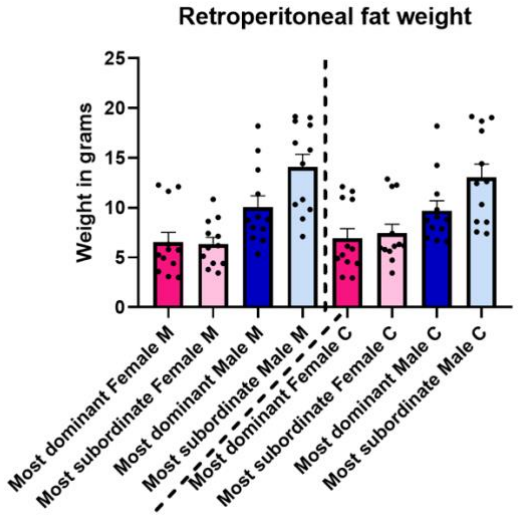


Figure 30 The average retroperitoneal fat weight of dominant and subordinate males and females (n=12) determined by the two scoring methods. Data expressed as mean ± SEM. (M= Method by Miguel Puentes. C= Method by James Curley)

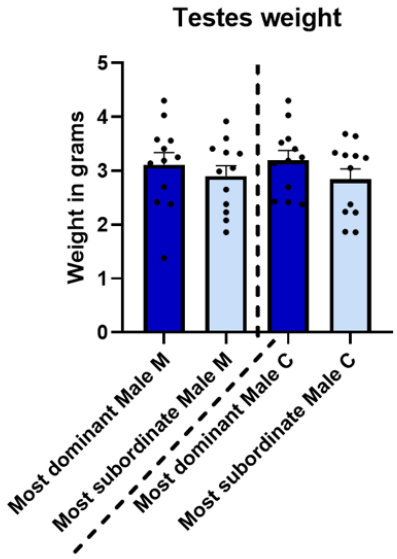


Figure 31 The average testes weight of dominant and subordinate males (n=12) determined by the two scoring methods. Data expressed as mean ± SEM. (M= Method by Miguel Puentes. C= Method by James Curley)

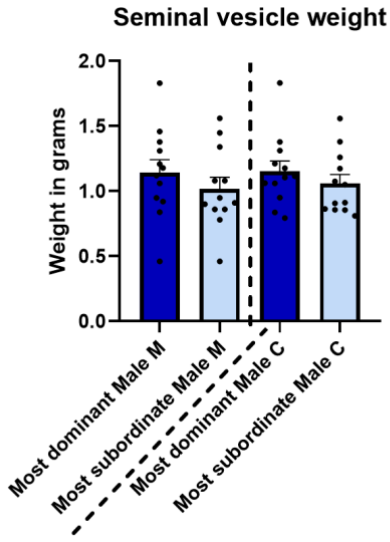


Figure 32 The average seminal vesicle weight of dominant and subordinate males (n=12) determined by the two scoring methods. Data expressed as mean ± SEM. (M= Method by Miguel Puentes. C= Method by James Curley)

For the following section of the results, the dominance scoring based on Miguel Puentes will be used, as the plasticity research was already being conducted after the other method by James Curley was carried out.

3.7 Plasticity changes in pyramidal neurons in different brain areas

To determine any changes and differences in the plasticity changes of the CA1, CA3 and BLA region between females and males and their ranks, the spine density was determined.

Table 10 Average spine count for regions CA1, CA3 and BLA for females and males and dominant and subordinate.
Table 11. Average spine count for the distances from the main shaft in μm. Values represent the mean ± SEM.

CA1	SUB-M	81,3 ± 3,20281127
	DOM-M	78,7333333 ± 3,9180494
	SUB-F	77,7333333 ± 3,88146593
	DOM-F	77 ± 2,64020201
BLA	SUB-M	71,5333333 ± 1,44006173
	DOM-M	67,4777778 ± 1,95546086
	SUB-F	73,6666667 ± 3,30238971
	DOM-F	74,4666667 ± 3,59765356
CA3	SUB-M	52,5 ± 2,33794782
	DOM-M	59,4 ± 2,06106768
	SUB-F	51,9666667 ± 4,77225779
	DOM-F	57,4 ± 3,53647659

	Distance (μm)			Distance (μm)	
SUB-M CA1	10	9,56666667 ± 0,70694963	F-DOM CA1	10	9,7 ± 0,76724616
	20	9,9 ± 0,68847658		20	9,86666667 ± 0,66063942
	30	10,7 ± 0,80787788		30	10,1333333 ± 0,50771821
	40	10,1666667 ± 0,66616648		40	10 ± 0,17888544
	50	10,6666667 ± 0,22310934		50	9,46666667 ± 0,34896673
	60	10,0666667 ± 0,27162065		60	9,76666667 ± 0,38787169
	70	9,83333333 ± 0,37028518		70	9,2 ± 0,27808871
	80	10,4 ± 0,58878406		80	9 ± 0,56803756
DOM-M CA1	10	9,43333333 ± 0,9762741	F-SUB CA1	10	8,46666667 ± 0,92616293
	20	9,93333333 ± 0,67263991		20	9,38333333 ± 0,5192409
	30	10,7333333 ± 0,58802872		30	8,8 ± 0,68166463
	40	9,8 ± 0,57735027		40	10,7166667 ± 0,81503238
	50	10,4333333 ± 0,46595183		50	9,96666667 ± 0,46380072
	60	9,73333333 ± 0,48074017		60	10,35 ± 0,70793126
	70	9,73333333 ± 0,35276684		70	9,40833333 ± 0,69238918
	80	8,93333333 ± 0,54812813		80	9,49166667 ± 0,53329427

To determine the difference in spine density between the females and males and their ranks, the one-way ANOVA was performed. No significant difference in the mean of the spine counts of the neurons in the CA1 region between any of the sexes or ranks (p=0.828). No significant difference between the distances for all ranks and sexes (dominant female (p=0.737), dominant male (p=0.562), subordinate female (p=0.320), subordinate male (p=0.838).

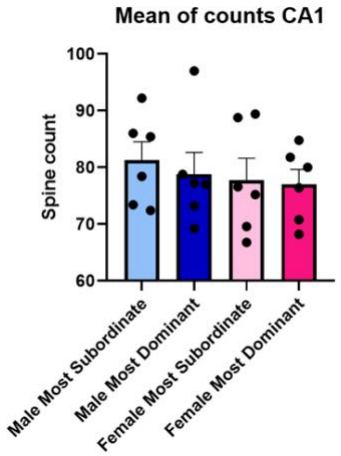


Figure 33 The average amount of spines in the CA1 region for males and females both dominant and subordinate (n=6). Data expressed as mean ± SEM.

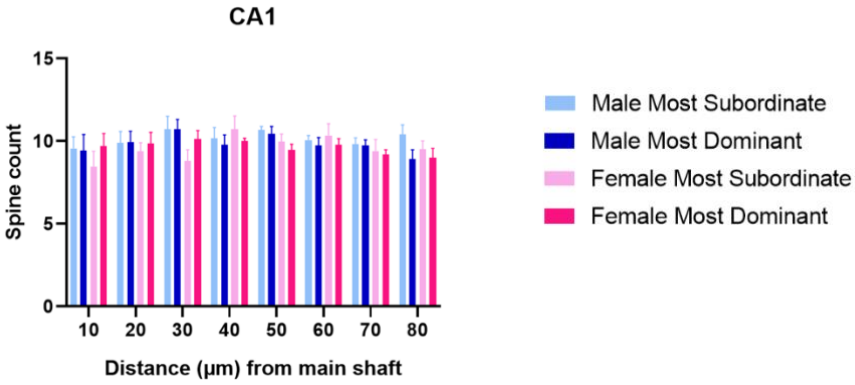


Figure 34 The average amount of spines in the CA1 region for every 10 μm in dinstance for males and females both dominant and subordinate (n=6). Data expressed as mean ± SEM.

No significant difference in the mean of the spine count from the neurons of the BLA region (p=0.297). dominant female (p=0.856), dominant male (p=0.195), subordinate Female (p=0.717), except in the subordinate male (p=<0.001). Significant differences were found in the Subordinate Male in the BLA region between 10 cm and 60 cm (p<0.001), 10 cm and 70

cm (p<0.001), 10 cm and 80 cm (p<0.001), implying that the spine count does increase with more distance in subordinate males.

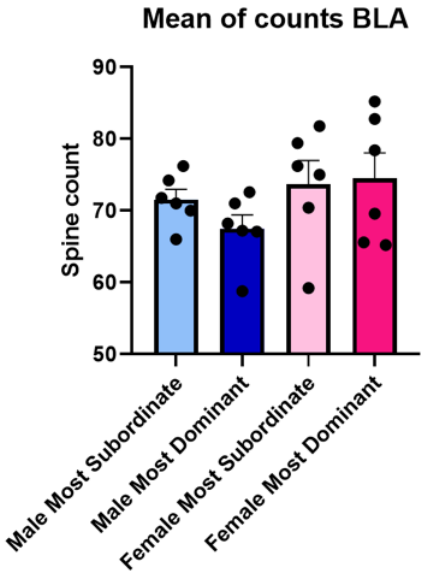


Figure 35 The average amount of spines in the BLA region for males and females both dominant and subordinate (n=6). Data expressed as mean ± SEM.

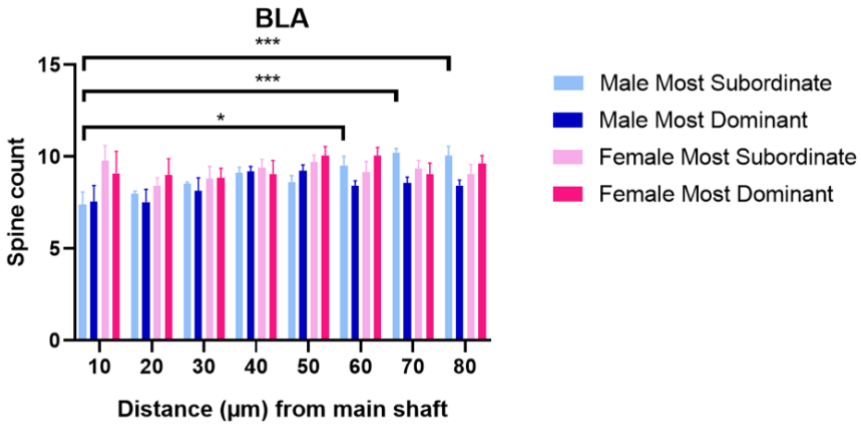


Figure 36 The average amount of spines in the BLA region for every 10 µm in distance for males and females both dominant and subordinate (n=6). Data expressed as mean ± SEM.

No significant difference between the mean of the spine counts of the neurons in the CA3 region between the ranks and sexes (p=0.339). Between the different distances there was also no significant difference found for female dominant (p=0.631), male dominant (p=0.242), female subordinate (p=0.880), male subordinate (p=0.691).

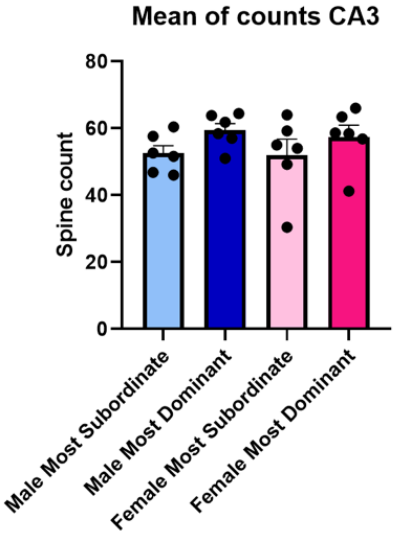


Figure 37 The average amount of spines in the CA3 region for males and females both dominant and subordinate (n=6). Data expressed as mean ± SEM.

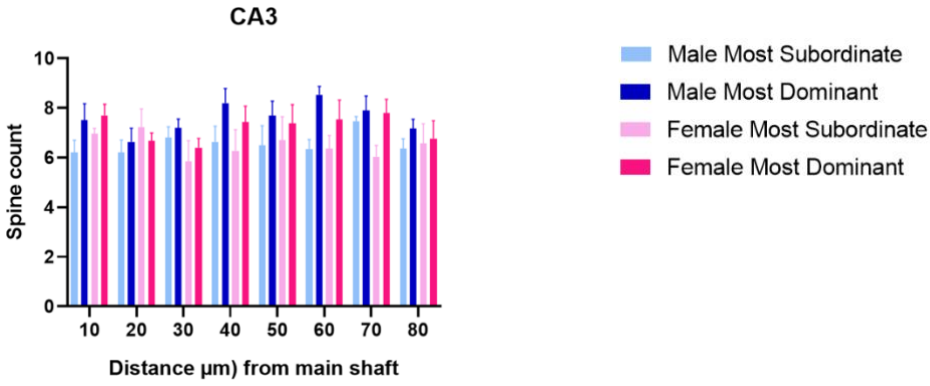


Figure 38 The average amount of spines in the CA13region for every 10 µm in distance for males and females both dominant and subordinate (n=6). Data expressed as mean ± SEM.

3.8 Western Blot

To determine the BDNF and proBDNF levels in the dorsal hippocampus tissue, Western Blot was performed.

Dorsal hippocampus		
Colony	Sex-rank	Sample
VBS2	M-DOM	1
VBS3	M-SUB	4
VBS2	M-SUB	5
VBS4	M-DOM	14
VBS2	F-DOM	24
VBS3	F-DOM	29
VBS1	F-SUB	31
VBS6	M-SUB	38

Figure 39 The example samples of the dorsal hippocampus used in the Western Blot

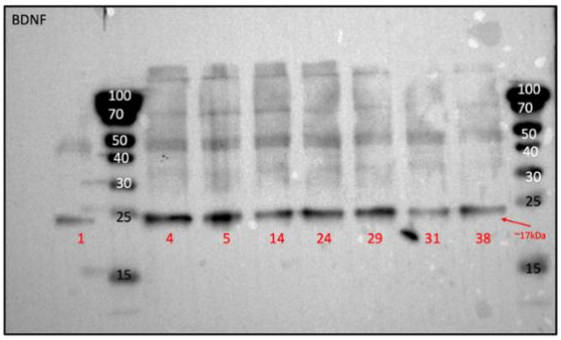


Figure 18 Example Blot of detection with BDNF antibody

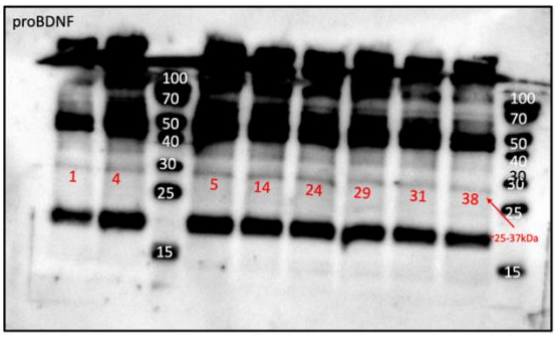


Figure 41 Example Blot of detection with proBDNF antibody

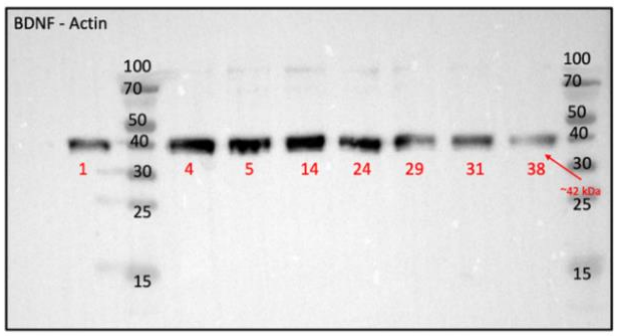


Figure 19 Example Blot of detection with actin antibody

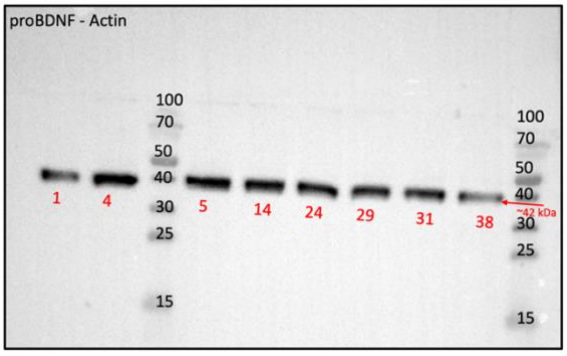


Figure 43 Example Blot of detection with actin antibody

4. Discussion

This research aims to determine how the social dominance of Wildtype Groningen rats in a semi-natural social colony is reflected in brain and behavior. Furthermore, this research aims to determine if there is any difference between two dominance scoring methods, by Miguel Puentes and by James Curley. Research is done into the physiological and endocrine changes such as body and organ weight change, fecal corticosterone measurements, spine density and levels of BDNF and proBDNF. To research these characteristics, the VBS is utilized, which is a social stress model that mimics natural settings. Using the VBS is the closest possible way to mimic chronic social stress in humans, specifically socioeconomic stress. Once in the VBS, animals will form a dominance hierarchy when there is competition for food, water, and female rats [33]. However, research has shown that it is possible for females to form a dominance hierarchy, so for this research, female interactions are also considered to see if females participate in the dominance hierarchy [7].

4.1 Aggression

The intensity of aggression in the different colonies is based on the average proportion of fierce fights. When potential or actual physique damage is inflicted, in this case, wounds, it is seen as a fierce fight. In colonies, most injuries are inflicted on the subordinate animal [6,36], and the dominant animals have the lowest number of wounds [45].
Furthermore, female animals are expected to have far fewer wounds than male animals [103].
The findings of this research do go in line with the expectations. A significant difference was found between the male most subordinate animal and all other sexes and ranks. The wounds of female dominant and subordinate rats, or female animals in general, are negligible, and as expected, far less than male animals. These findings establish that female and male rats do not experience the same aggression in the VBS, and that females may not participate in the dominance hierarchy, as they do not partake in as much agonistic interactions [45]. Furthermore, these findings prove that dominant animals are the most aggressive animals, inflicting wounds to subordinate animals, as a way to maintain their dominance in a physical form [12].

4.2 Stress markers

Stressful periods can be recognized with prototypical stress markers, such as weight loss, organ weight loss or gain, and the activity of the HPA axis, measured as the level of corticosterone in feces [103].

4.2.1 Body weight

Subordinate males are characterized by severe weight loss, associated with a reduced eating [36], due to reduced access to recourses [12]. Dominant animals are characterized by the least body weight loss over the course of the experiment [45]. Females are expected to remain stable in their weight or even gain it, as they engage in little agonistic behavior [45] which would lead to a less amount stress and no avoidance of other animals, increasing their accessibility to food. Finally, subordinate animals with a higher amount of wounds would be expected to lose the most weight, as they are intimidated the most through fighting and subsequently experience the most stress.
In the current study it is very noticeable that the moment the male animals are put into the VBS, they start losing weight. This could be an indication that males start experiencing stress once put into the VBS. Most of the body weight loss happens in the first few days of the VBS, from day 0 to 5. After that the weight loss stabilized. This stability after the fifth day was seen in a similar study with WTG rat colonies, however with less females in the colonies[104]. The

stabilization indicates that subordinate animals experience the most stress in the first few days and afterwards adapt to their situation, and even find comfort in their situation by affiliating with other subordinates, as seen in apes [12].
The subordinate animals lose far more weight than dominant animals throughout the whole time they are in the VBS. Because it is assumed that subordinate animals are the most stressed, and weight loss is related with chronic stress, these findings are consistent with expectations [46,47]. Dominant animals also lose weight, but far less than subordinates, which does go in line with the hypothesis [45]. It can clearly be stated that both male animals lose weight during their time in the VBS, with the subordinate animal losing the most weight. It can said for certain that this weight loss is due to the amount of stress the animals experience. Energy expenditure may also play a significant role in weight loss, which could explain the dominant animal also losing weight.
Finally, females gain weight during their time in the VBS. Before pair housing, the weight of the females seems to remain very stable. Once the females are put into pair housing, pairing them with a male animal, their weight gain increases majorly, and keeps increasing throughout the VBS.
One possible explanation for this sudden weight gain is that for females the key stressor is being alone. In a crowding experiment conducted by Brown et al., where four females were housed together and four males were housed together [105]. Female rats who were in crowded housing did not show a stress reaction, but instead had lower levels of corticosterone than female rats that were separately housed [105,106]. Moreover, females do not go in hiding like subordinate male animals, and remain moving freely throughout the VBS, giving them more access to resources like food.

Finally, there was no correlation between wounds and body weight. Which suggests that the amount of wounds inflicted on an animal does not necessarily make it loose the most weight as a consequence of stress.

4.2.2. Organ weight

It is expected that both subordinate and dominant animals have higher adrenal weights and lower thymus weights than controls. Furthermore, it is expected that testes weights are lower in dominant animals than subordinate animals [6,44]. For the adrenal gland and thymus, there was no difference in weight between the sexes and the ranks. Furthermore, there was no difference in weight for the testes and seminal vesicle weight between the most dominant and the most subordinate males. This goes somewhat in line with the hypothesis, except the results obtained by both Blanchard et al. and Tamashiro et al. included control animals. In this study no control animals were included in the experiment, which can be considered as a limitation to the results.
When looking closely at the animals individually in the colonies, the adrenal weights of the animals of VBS 3, it can clearly be seen that the weights are higher than the other colonies. Especially M4, the subordinate animal has an adrenal gland weight of above 0.10 grams, while other animals remain below this level. This does go in line with the hypothesis that subordinate animals in aggressive colonies experience more stress, and might go in line with more production of CBG which is synthesized by the adrenal gland, enlarging the organ, and could be the result of hypersecretion of adrenocorticotropic hormone (ACTH), as stress stimulates the release of ACTH and subsequently stimulates the adrenal cortex to release more corticosterone [107]. Female adrenal glands also seem enlarged in the most aggressive colony, rising way above the adrenal gland weights of the other animals. However in the other aggressive colony (VBS7) there is almost no difference with the other colonies, for both

males and females, dominants and subordinates. This could also be attributed to the fact that VBS7 is an aggressive colony but a more stable one than VBS3.
The subordinate and dominant female of VBS3 both seem to have higher thymus weights than the other colonies, with the dominant animal having a slightly higher weight than the subordinate animal. In the other colonies There is no particular animal, both male and female, that has a thymus increasement, which does go in line with previous findings, stating that both subordinates and dominants have a higher thymus weight than controls. This study however does not include controls. The decreased thymus weights and increased adrenal weights of subordinates suggest hypersecretion of ACTH as well as CORT and confirm at a neuroendocrine level that subordinate animals were chronically stressed relative to control (and sometimes dominant) rats [36].
The retroperitoneal fat weight seems slightly higher in male rats, which would make sense as they do weigh significantly more physically. The subordinate animal (M1) in VBS7 has a higher amount of fat, compared to the dominant animal, same as in the other VBS'es. Which goes in line with the expectation. the percentage of body fat in both dominants and subordinates decreases, where subordinates have an additional decrease in the percentage lean mass. However, they retain a higher percentage of visceral fat (which includes retroperitoneal, perirenal, mesenteric and epididymal fat pads) than control and dominant animals at the end of the VBS housing [45]. This weight loss is attributable to a decrease in the percentage of body fat in both dominants and subordinates, where subordinates have an additional decrease in the percentage lean mass [45].
There does not seem to be a clear line of higher seminal vesicle weights in a subordinate or dominant animal. A study conducted by Iamsaard et al. determined that the atrophy of the Seminal Vesicle was not obviously observed in chronic stress but it demonstrated the significant decreases of the weight [108]. However this study was conducted in Sprague Dawley rats which should be noted, as it is a different strain than the WTG rats.
In all colonies except for VBS7, the testes weights seem to be higher for dominant animals compared to subordinate animals. This does go in line with a study conducted by Ribeiro et al. [109]. The study states that in animals who were subjected to chronic stress, testicular atrophy occurred. However, this experiment was conducted in Wistar rats, limiting the substantiation for the current research. In VBS7 the dominant rat has a lower testes weight than the subordinate rat. This could also indicate that the dominant animal in this colony actually experienced more stress than the subordinate animal, as it might had to have to defend its title, causing big amounts of stress, seen in for example dwarf mongooses [12].
Previous research findings are often compared to a control group, to be able to compare the effects of dominance hierarchy on animals who are not affected by rank. However, controls were not included in this research.

4.2.3. Corticosterone levels

The expectations were that due to higher level of stress both dominant and subordinate animals would have an increase in the percentage of corticosterone, with the subordinate animals having a higher increase, before the VBS compared to after the VBS. This is expected because all animals experience stress, but because social defeat elicits a higher corticosterone reaction than social victory, higher levels of corticosterone are expected for subordinates [50].It is also expected that more wounds inflicted on an animal will result in more stress and thus a higher corticosterone change. Furthermore, less effect in corticosterone levels is expected between females, as they might not participate in the dominance hierarchy [45]. However, in the current study there is no significant difference in the percentage of change of corticosterone from before the VBS to the end of the VBS, in both females and males, which was also found by McKittrick et al. Important to note though is that if the animals in McKittrick's study were

let to rest one hour before sampling the level of corticosterone for dominant animals fell to that of the control animals, while the levels of subordinate animals remained elevated. This remaining elevated level of corticosterone can also be seen in an resident intruder experiment conducted by Koolhaas et al [50], where the "loser animal" has a remaining higher level of corticosterone compared to the "winner" animal. Finally, there is no significant correlation between wounds and corticosterone, which can be contributed to the fact that all animals experience stress in the VBS, and it does not matter if there is more aggression imposed on an animal. VBS3 and 2 seem to have the lowest change in corticosterone, while VBS7 and 6 have some of the highest percentage. There were some outliers which were excluded from the calculation and the graph, including M2 in VBS7 and M4 in VBS2. Part of the corticosterone experiments had some unreliably high amounts of corticosterone, making it a limitation to include in the study.
All in all it can be said that the change in corticosterone from before the VBS to after the VBS is the same for all animals and ranks, suggesting that they all experience similar amount of stress. Building on this conclusion is the fact that there is no correlation between the aggression (measured in wounds) and the change in corticosterone. It is then also important to note that corticosterone is not only comprised of stress, but also the amount of energy that is needed for a response, such as sexual behavior, eliciting even a higher corticosterone response than social defeat or social factory [50]. In addition, the corticosterone baseline is also higher for females [53].

Taking all the results into account, it can be concluded that a higher or lower rank within the dominance hierarchy in this study does not influence the social stress level.

4.3 Difference in behavioral scoring methods

In the scoring of Miguel Puentes some dominant males shared their first or last place in the dominance rank. For this reason a second scoring method was investigated to see if it would clarify the dominance hierarchy. In Curley's method, the behavior of the rats is scored in real time for 1-3 hours per day during the dark cycle, with the majority of them taking place in the first 4 hours after the dark cycle began [111]. Compared to Puentes' method, where scoring the agonistic interaction took place in time slots of 10 minutes, 8 times a day, Curley's method could result in a higher amount of interactions as there would not be any missed. The hypothesis is that, to score for a longer amount of time, as in Curley's method, a clearer dominance hierarchy will arise with a clear first and last place in the hierarchy. Furthermore, it is then expected that there will be a difference in parameters of the animals, with a focus on adrenal gland, thymus, retroperitoneal fat, testes, seminal vesicle weight and the level of corticosterone.
The current study shows that there is a more clear first place in the dominance rank, however not in the last place of the dominance hierarchy. This could be due to a lower amount of agonistic interactions in the last days of the VBS, resulting in some animals not engaging in any interactions at all, making it impossible to place them in the dominance hierarchy. However, the differences between the dominance hierarchy outcomes were very little, which can be seen when comparing the animals who are most dominant and subordinate.
Furthermore, to test the differences in the hierarchies, the organ weights and corticosterone change were compared between the methods. It was shown that there is no significant difference in the adrenal weight in both the dominance scoring methods, same for the thymus weight, the fat weight, the vesicle weight, the testes weight.
This concludes that there is a slight differences in the outcomes of the dominance hierarchies. This can be contributed to the fact that more agonistic interactions are taken into account with the Curley method than with Puentes' method. However, it should be noted that the

observations made with the Curley method were conducted by two observers. There are established characteristics that make up an agonistic interaction. However, with some interactions it is difficult to distinguish between for example an agonistic interaction or a sexual one. Furthermore, winning a contest was characterized by the loser animal fleeing the site of the fight. This fleeing was not always clear, as sometimes the animal would walk away. Making it possible for one observer to score this as a win while the other observer would not document it at all. All these factors could limit the results of the second scoring method.

4.4 Female dominance

Normally, females are included in the VBS to elicit aggression in male animals [11]. However, in the current study females were observed equally to male animals. Male-female and female-female interactions were all taken into account when scoring the behavior. It is expected that females may not participate in the dominance hierarchy, as they do not partake in agonistic interactions [45]. Agonistic behavior was detected in females, but this did not appear to result in avoidance behavior, as they did not avoid the open arena and spent the same amount of time there independent of dominance rank. This lack of avoidance behavior also suggests that females do not feel intimidated by the most dominant males. Furthermore, the agonistic interactions they do participate in are either fighting of a male animal who mounts the female animal for sexual interaction or it fights with a female animal. However in these female-female interactions, the "losing" females rarely flee which makes it questionable if it was an agonistic interaction or a playful interaction. Finally, females gain weight when put into the VBS, contrary to males who lose weight. Suggesting that females are in fact comfortable in the VBS. Females did suffer some amount stress, but it had no effect on the dominance hierarchy or behavior. Which goes in line with the hypothesis stating that females do not engage in the dominance hierarchy [45].

4.5 Spines

Evidence from diverse physical stresses has mostly revealed a reduction in spine density in CA3 and CA1 pyramidal neurons, which has been linked to depression-like behavior in animals [71,76,78]. Chronic and acute immobilization stress have both been shown to increase spinogenesis in the BLA across both primary and secondary branches of spiny neurons [71]. However, there was no significant difference between the rank or the sex in spine count for both CA1 and CA3 neurons, or in the BLA region. This is contradictory to the hypothesis stated above. The reason behind this could be that structural remodeling in the hippocampus is not entirely linked to stress. Many things can influence plasticity besides stress, such as for example exercise. For future studies it would be interesting to also take into account the activity levels in animals. Finally, an explanation could be that the neurons are capable of adapting to social instability stress. A study found that that social instability stress inhibits neuronal growth in the amygdala in the adolescent brain, but mature neurons in the amygdala can adapt to this sort of stress [86].

4.6 Molecular changes

BDNF regulates neuronal plasticity and survival [87,88].
In presence of raised levels of glucocorticoids (induced by stress), the expression of pro-BDNF and tPA is stimulated [90], implicating that BDNF levels are also raised. The following hypothesis is expected: The lower the rank of the animals, the higher the level of BDNF and proBDNF as a consequence of chronic stress in the dorsal hippocampus. To examine this hypothesis, multiple Western Blots were performed on the most dominant males and females, and the most subordinate males and females. Western Blots are used to detect and quantify

specific samples within tissues. Gel electrophoresis is used to first separate the denatured proteins based on their molecular weight. Subsequently the proteins are transferred unto a membrane, and probed using antibodies that are specific for the protein of interest [112].
Due to the large lack of samples, the database is not suitable to be analyzed. The lack of samples consists of BDNF and proBDNF membranes that developed accurately, but with the data of housekeeping gene Actin missing. Additionally, in some cases the original BDNF and proBDNF membranes did not develop validly, with for example one blot containing bubbles (appendix B4.2.2), making it unfeasible to analyze. The membranes that did not develop accurately could be a result of multiple complications that occurred during the experimental time of the Western Blot. These complications which will be explained in the next paragraph. Due to lack of time there was opportunity to process all the samples with the Western Blot, resulting in a database which in incomplete. Other complications include the antibody for proBDNF detecting the wrong kDa. In literature and on the manufacturers website (Alomone) it is stated multiple times that proBDNF is supposed to be detected at approximately 32 kDa [100], or in between 25 and 32-37 kDa [113]. However, on the western blot membranes (appendix C4.2.1) it can be seen that proBDNF is not detected at this amount of kDa. There are slight indications that the protein is located at this distance, however the analyzing tools did not show saturation at these locations, meaning they are not adequate to analyze. Unusual or unexpected bands can be due to protease degradation, which produces bands at unexpected positions. However, this does not seem likely, as the samples were kept at -80°C while they weren't used. Similarly, blurry bands are mostly caused by high voltage while running the electrophorese or air bubbles during the transfer from gel to membrane. For future experiments it is important to ensure that the gel is running at a lower voltage. Refreshing the running buffer could perhaps also help the problem.
No bands can arise due to many reasons related to antibody, antigen, or buffer used. If the wrong antibody is used, either primary or secondary, the band will not show. In addition, the concentration of the antibody should be appropriate as well; if the concentration is too low, the signal may not be visible. Buffers can also contribute to the problem, so it should be ensured that buffers are all noncontaminated. If the buffers are contaminated with sodium azide, it can inactivate HRP [114]. The antibody for BDNF however did work properly, as the protein has a weight of 17 kDa and this is confirmed on the western blot membranes. Moreover, the antibody for the housekeeping gene Actin also had the propriate weight of 42 kDa which can be seen on the membranes. However, presumably due to different circumstances, some membranes where actin would be detected, did not develop well, with some samples faded away. This could be due to the buffers not having the right composition. Another circumstance could be the sandwich in the western blot not being wet enough to not allow bubbles inside during the transfer from the gel to the membrane. Wet conditions are usually more reliable as it is less likely to dry out the gel, and is preferred for larger proteins [114]. The bubbles on the membranes were eliminated in a next experiment, when the filter paper was made wet with buffer before constructing the sandwich, not allowing any air inside. In a few instances the actin antibody did not work, however it eventually became clear that the mistake was not in the antibody but presumably in the buffers. When the actin body was ready to use, still some membranes came out with a weak signal. This is most likely due to the membranes left in buffer for 1-2 weeks in wait of the actin antibody experiment. Washing is very important as it minimized background and removes unbound antibody [114]. However, the membrane should not be left to wash for a really long time, as it can also reduce the signal.

4.7 Limitations and recommendations

It is important to consider is that there were no control groups in this study, resulting in that the dominant and subordinate animal characteristics cannot be compared to a control animal

that does not experience any stress. Next, for this research not all spine date for all animals were analyzed. Reducing the strength of this part of the research slightly. However, once all animals are analyzed, strong conclusions can be made about the spine density in future research.
For future experiments, it would be interesting to look further into the other brain regions. As indicated by recent studies the medial prefrontal cortex (mPFC) also plays an important role in constricting the HPA axis under stress-related conditions. It would be of interest to examine the BDNF and proBDNF concentration in this brain region.
In addition to BDNF and proBDNF, it would be interesting to take a closer look into tPa, as it also has an important role in structural remodeling.
Finally, it is important that for the samples of the dorsal hippocampus, the BDNF and proBDNF protein Western Blots will continue, so that there will be a complete database of the samples which will result in a clear overview if BDNF and proBDNF are increased in either dominant or subordinate animals.

4.8 Conclusion

The current study tried to investigate how social dominance in rats is reflected in brain and behavior. The expectation was that a dominance hierarchy would form, and in the process chronic stress would occur for the subordinate animals. Dominant animals do seem more stress when considering their body weight loss and the amount of wounds inflicted, especially when compared to dominant animals. However, contrary to this statement, there was no difference found in organ weight, corticosterone change before to after the VBS and spine density, except an increased spine density further away from the apical branch in the most subordinate males in the BLA region. The explanation for this could be that the animals all experience the same amount of stress or adapt to their situation. To conclude, a dominance hierarchy is formed, but there is no indication of social stress. Furthermore, female animals do interact with each other and males agonistically, but there is no indication of them forming a dominance hierarchy.

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Appendices

Appendix A1: Golgi-Cox fixative

50 ml Golgi-Cox fixative / brain half → 5% potassium dichromate solution, 5% Mercuric chloride solution, 4% Potassium chromate solution (5:5:4).

Appendix A2: Golgi-Cox staining

ddH_2O – 5 minutes
ddH_2O – 5 minutes
5% Na_2CO_3 – 20 minutes
ddH_2O – 5 minutes
ddH_2O – 5 minutes
70% C_2H_5OH (ethanol) – 20 minutes
100% C_2H_5OH – 5 minutes 100%
C_2H_5OH – 5 minutes
100% C_8H_{10} (xylol) – 2,5 minutes
100% C_8H_{10} – 2,5 minutes

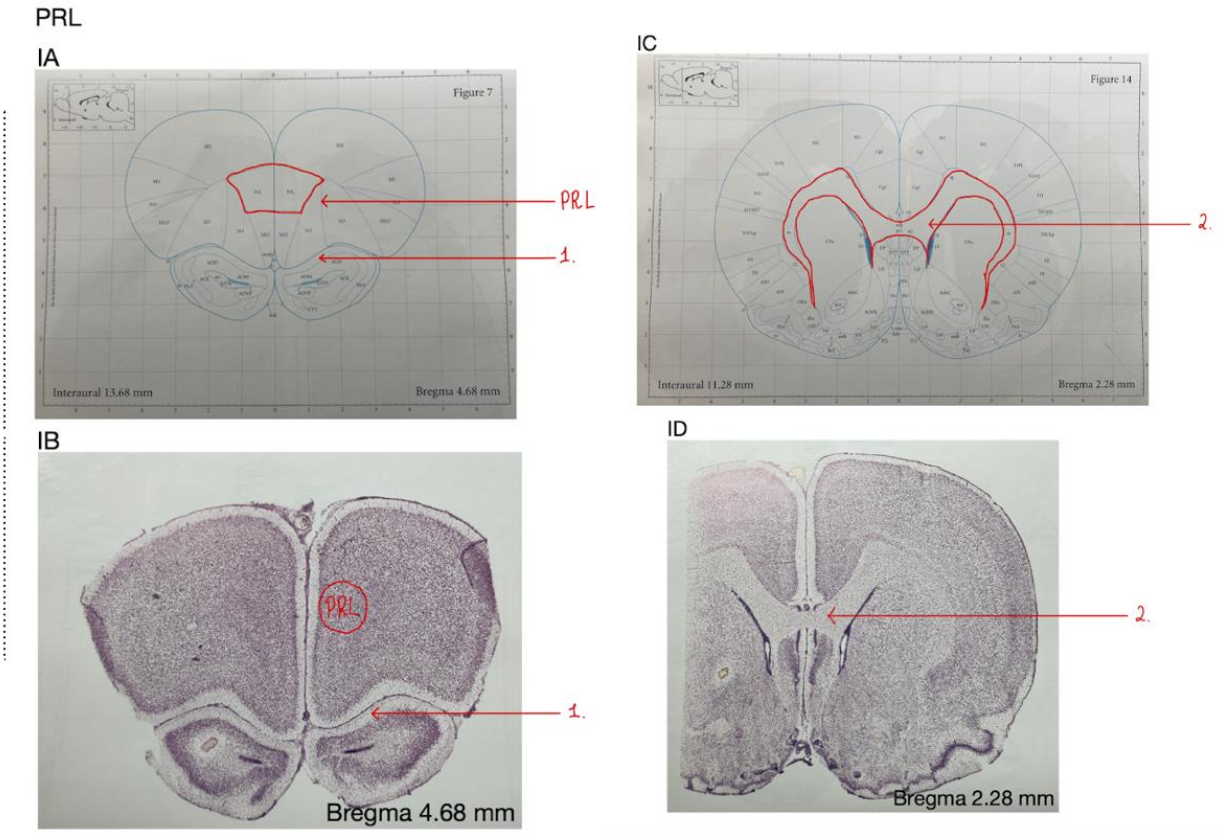
B. Punched location data

Procedure: The samples were kept frozen on dry ice all the time during the collection of the regions. Initially, the cerebellum of each sample was removed and the remaining part was separated vertically in two sections just in front of the hippocampus. From dry ice, one of the sections was transferred on the -20°C cooled cutting block of the microtome and fixed using Tissue-Tek (Labtech). Next, the samples were shaved with a knife in steps of 100

Table B1. Location brain regions based on Bregma points

Region	Start point Bregma (mm)	Endpoint Bregma (mm)	Atlas Figure (Start point)
Prelimbic cortex (PRL)	4,2	2,52	8
Agranular insular cortex dorsal (AID); Agranular insular cortex ventral (AIV)	4,2	2,76	8
Accumbens nu, core (AcBc); Accumbens nu, shell (AcbSh	2,76	0,6	12
Basolateral amygdala (BLA)	1,72/-1,80	-3,36	47
Hippocampus Dorsal	-2,04	-5,52	50
Hippocampus Ventral	-4,68	-6,84	

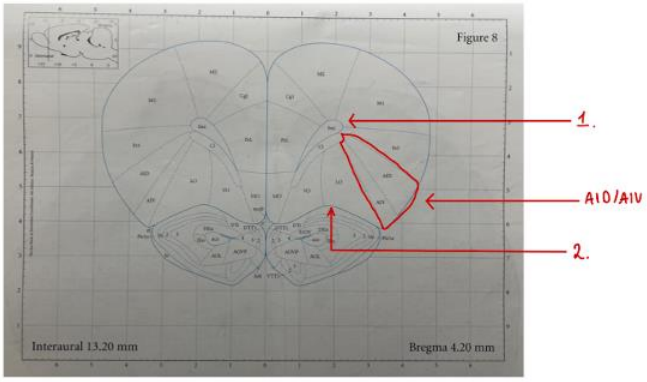
B I) PRL



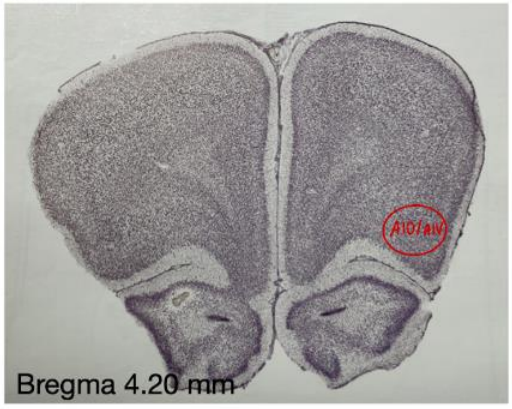
B II) AID/ AIV

AID/AIV

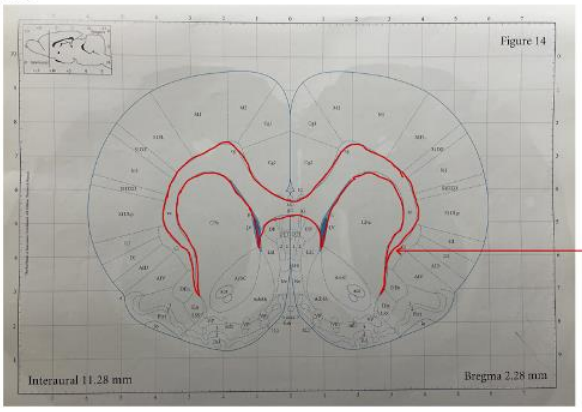
IIA



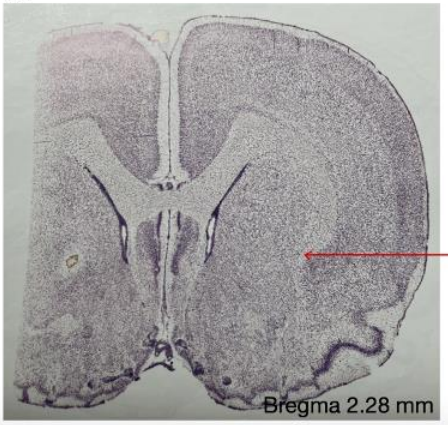
IIB



IIC



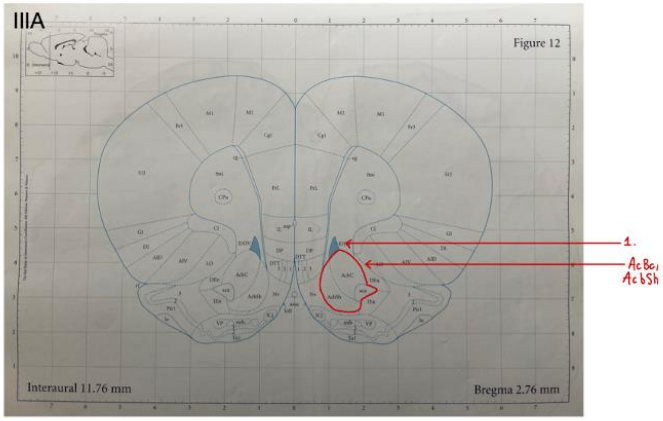
IID



B III) AcBc/AchSh

III. AcBc/AchSh

IIIA



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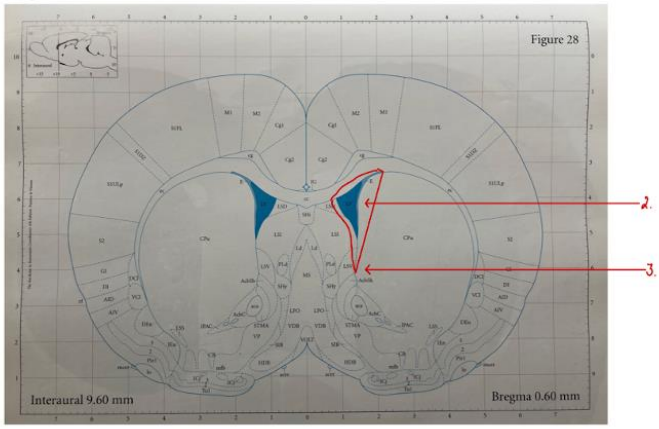
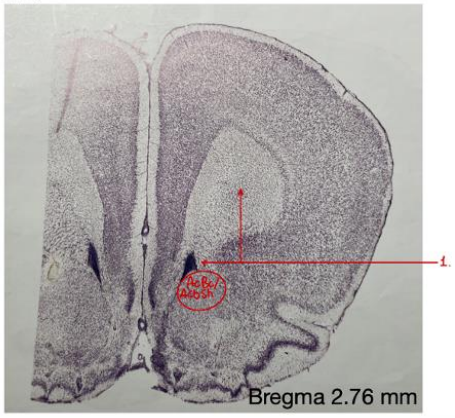


IIIB



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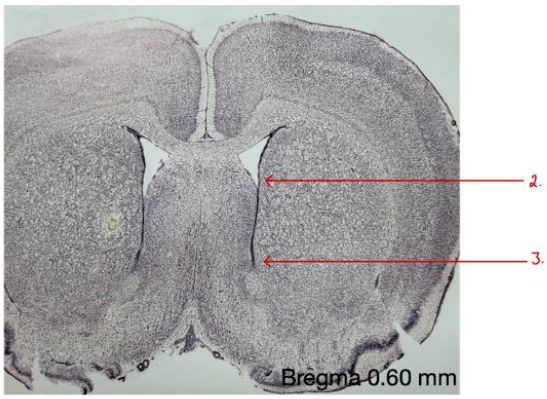
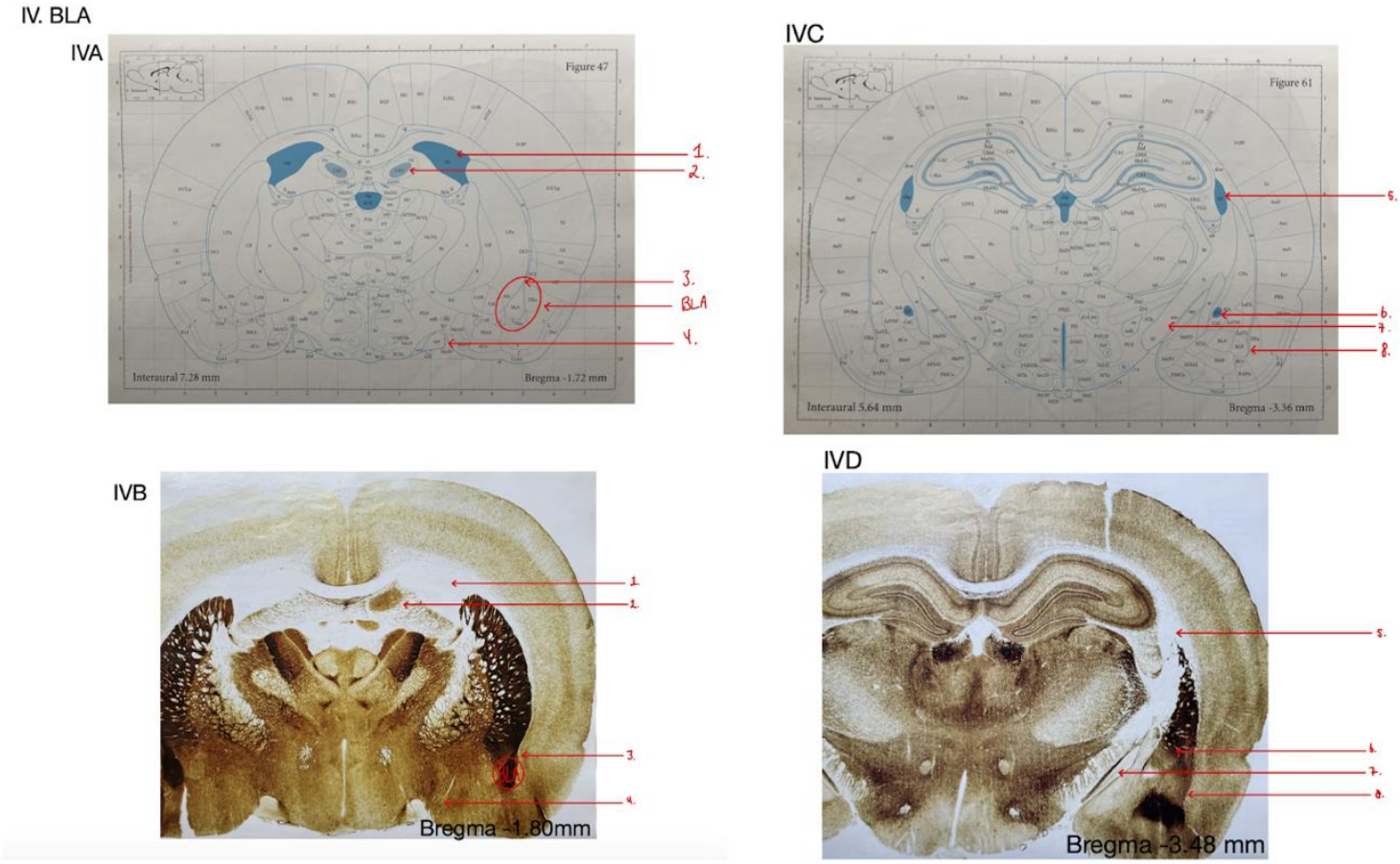
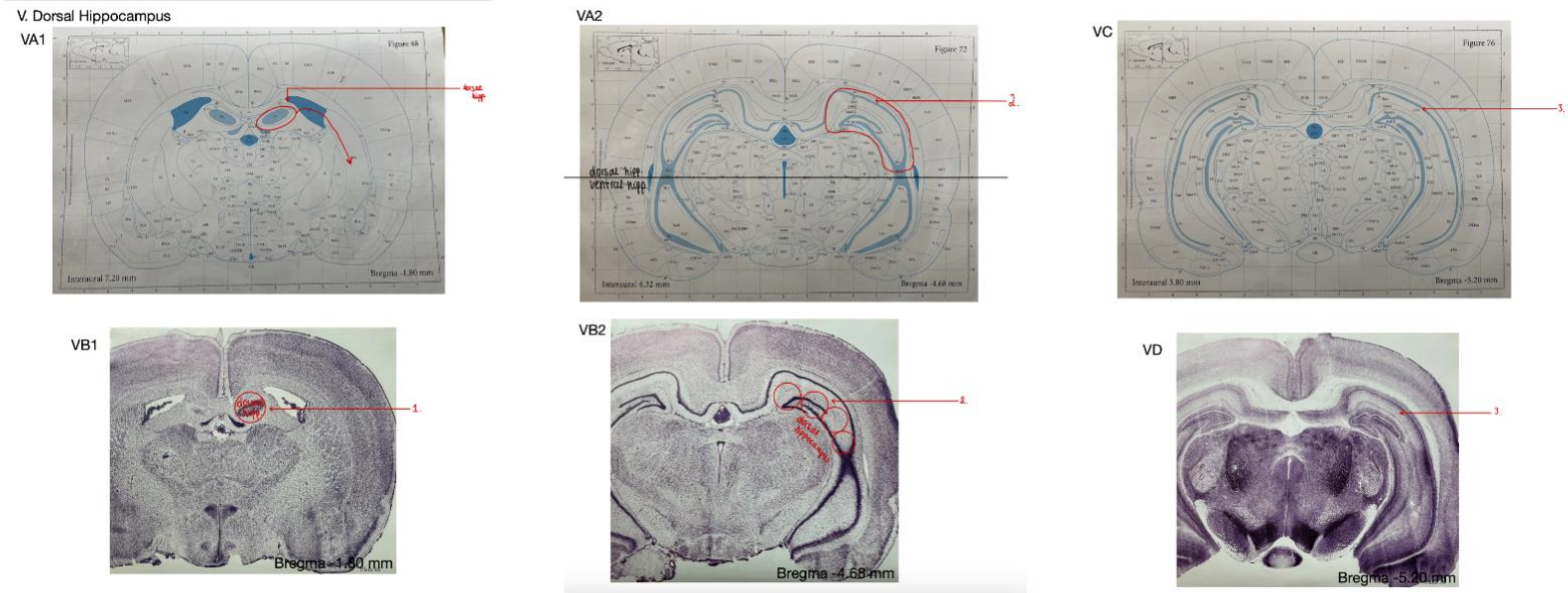


B IV) BLA



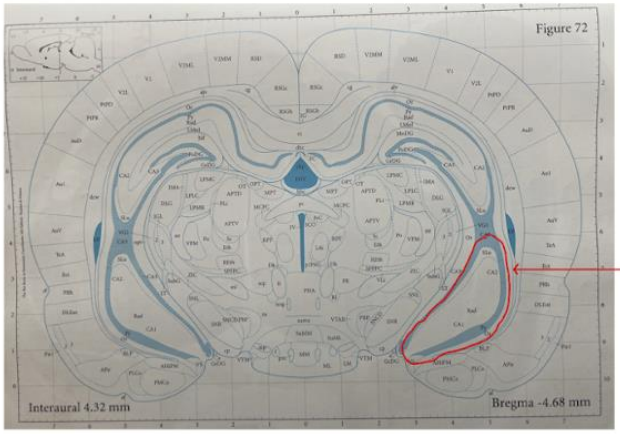
B V) Dorsal hippocampus



B VI) Ventral hippocampus

VI. Ventral Hippocampus

VIA



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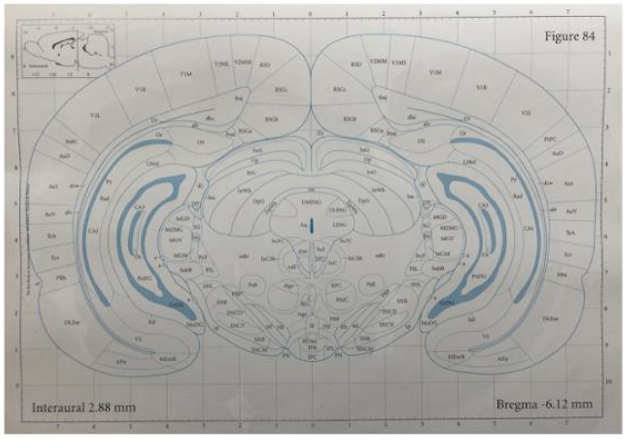

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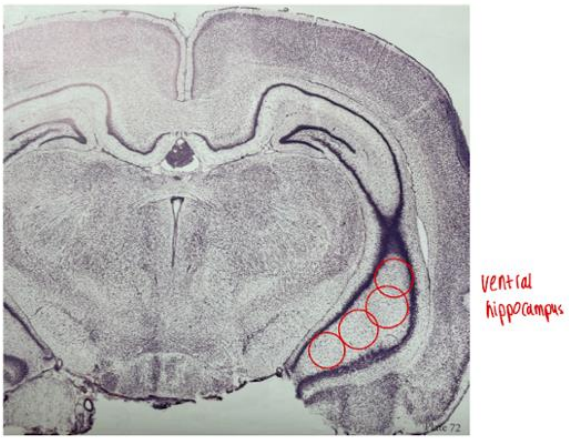

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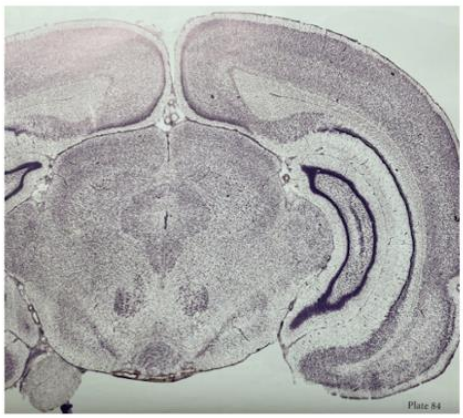


C. Buffers and reagentia

C.1 Bradford reagens:

- 2,5 mg Coommassie Brilliant Blue (G-250)
- 2,5 ml 96% ethanol (dissolve thoroughly)
- 5 ml 85% phosphoric acid (be careful, strong acid)
- Add ddH2O to a total volume of 50 ml.
- Filter 2x before use !!!!!!!

C.2 Electrophorese gels

Seperation gel

1.5 mm gel (more sample) , approximately 11,0 ml solution for each gel.

	5% gel	7.5% gel	10% gel	12% gel
UP water	5.8 ml	5.25 ml	4.3 ml	3.55 ml
40% acrylamide mix	1.5 ml	2.25 ml	3.0 ml	3.75 ml
1.5 M tris (pH 8.8)	2.5 ml	2.5 ml	2.5 ml	2.5 ml
10%SDS	100 µl	100 µl	100 µl	100 µl
TE ED	10 µl	10 µl	10 µl	10 µl
10% Ammonium persulfate (APS)	100 µl	100 µl	100 µl	100

Stacking gel

1.5 mm acrylamide stacking gel (more sample)

	1 gel	2 gels	4 gels	6 gels
UP water	4.36 ml	8.72 ml	*17,44* ml	26,16 ml
40% acrylamide mix	750 µl	1,50 ml	3,00 ml	4,50 ml
0.5 M tris (pH 6.8)	750 µl	1,50 ml	3,00 ml	4,50 ml
10% SDS	60 µl	120 µl	240 µl	360 µl
TEMED	6 µl	12 µl	24 µl	36 µl
10% APS	60 µl	120 µl	240 µl	360 µl

C.3 Buffers

<u>PBS 5x</u>
41,15g $Na2HPO_4x2H_20$
11,72g $NaH2PO_4xH20$
20gNaCl
Dissolve in 950 ml Ultra pie (UP) H20
pH 7,3-7,4
Fill up to 1 liter
Filtrate though 0,25 pm filter

Store at room temperature (RT)

Blocking buffer (I-block)

- 1 g 1-block (TROPIX)
- 100ml5xTBS/50m1 10xTBS
- Fill up to 500 ml with UP H20
- Heat to roughly 55°C (no particles should be visible anymore)
- Cool down to5°C
- Add 0,5 ml Tween20
- Store at -20°C

Blocking buffer (5% BSA)

- 5 g BSA (Bovine Serum Albumin - Sigma)
- 80 ml TBS-T (preheated 37°C)
- On the magnet stirrer solve the BSA
- Fill up to 100 ml with TBS-T
- Store at5°

Wash buffer (TBS-T)

- 200 ml5xTBS

1 ml Tween20 pure/10ml Tween 10%
Fill up to 1 liter with MilliQ
Store at 5 °C

Running buffer 5x

- 94 g glycin
- 15,lgTrisbase
- Fill up to 900 ml with UP H_20
- Add 50mll0%SDS
- Fill up to I liter with UP H_20 • Store at 5°C

Sample buffer 5x

- 50 g glycerin (50%)
- 3,72 g Tris/HCI pH 6,8 (312,5 mM)
- IOg SDS (10%)
- 25 g (313-mecaptoethanol (25%)
- 0,1 g brome phenol blue (0,1%)
- Fill up to 100 ml with UP H20
- Store at 5°C

Towbin buffer (Blotting buffer)

- 3gtris
- 14,4gglycin
- 200 ml methanol
- 2m110%SDS
- Fill up to I liter with UP H20
- pH8,6±0,2
- Store at 5°C

SDS 10%

Dissolve 10 g SDS in 100 ml

Store at RoomTemperature
•Note: Wear a mask, SDS dashes APS
• 100 mg APS
• Fill up to I ml with UP H_20
• Store at 5°C (can be used up to 1 week)

Tris 1.5M
• 18,17 g tris base (not tris/HCL)
• Fill up to 85 ml with UP H_20
• Set pH to 8,8 (let pH stabilize over 25 minutes roughly!)
• Fill up to 100 ml with UP H_20
• Store at 5°C
Tris 0.5M
• 6,06 g tris base (not tris/HCL) • Fill up to 85 ml with UP H_20
• Set pH to 6,8 (let pH stabilize over 25 minutes roughly!)
• Fill up to 100 ml with UP H_20
• Store at 5°C

10XTBS
• 24,2 g Tris base
• 80g NaCl
• Fill up to I L with UP HzO
• PH 7,6

RIPA Buffer
25 mM Tris/HCL pH 7.6 Merck 1.08219 mw 157.6
• 150mMNacl Merck 1.06404 mw 58.44
• 1% Nanidet P-40 Roche 79051393
• 0.5% sodium deoxycholate Sigma D6750 mw 414.55
• 0.1% SIDS BioRad 161-0301 mw 288.38
0.004% sodium azide stock 10% Merck 1.06688 mw 65.01
Add fresh on the day of use, immediately prior to lysing cells:
• Protease Inhibitors Roche 04693116001
• Phosphatase inhibitors Roche 04906845001
100 mL RI PA buffer stock
- Add 790 mg TrizBase to 75 mL MilliQ or UltraPure H20
- Add 900 mg NaCl
-Add 1 Ml NP-40
- Add 2,5 mL Na- deoxycholate
-Add 0.1 gSDS
-Adjust pH to: 7.6
Matrices 1=Most Dominant 8= Most Subordinate

C.4 Western Blot pipetting schemes with membranes

All samples used in Western Blot
Number = sample number used

Batch	Colony	Gender	Number	Rank	Rank Male/Female	Batch	Colony	Gender	Number	Rank	Rank Male/Female
1	2	M	1	1	1	2	8	F	51	1	1
1	4	M	2	8	4	2	5	F	52	1	1
1	1	M	3	1	1	2	7	F	55	1	1
1	3	M	4	8	4	2	8	F	57	7	4
1	2	M	5	8	4	2	5	F	58	6	4
1	3	M	13	1	1	2	6	F	59	7	4
1	4	M	14	4	1	2	7	F	60	8	4
1	1	M	16	8	4	2	6	F	62	2	1
1	4	F	17	6	4	3	9	M	65	1	1
1	2	F	24	3	1	3	11	M	67	2	1
1	2	F	25	7	4	3	10	M	68	8	4
1	4	F	26	1	1	3	10	M	69	2	1
1	3	F	27	2	1	3	11	M	70	8	4
1	1	F	28	3	1	3	12	M	71	2	1
1	3	F	29	7	4	3	9	M	72	8	4
1	1	F	31	7	4	3	12	M	80	8	4
2	7	M	35	2	1	3	11	F	81	1	1
2	5	M	37	3	1	3	9	F	82	7	4
2	6	M	38	8	4	3	12	F	83	1	1
2	6	M	41	1	1	3	10	F	84	7	4
2	5	M	46	8	4	3	10	F	93	1	1
2	7	M	47	7	4	3	11	F	94	7	4
2	7	F	50	3	2	3	12	F	95	6	4
						3	9	F	96	4	1

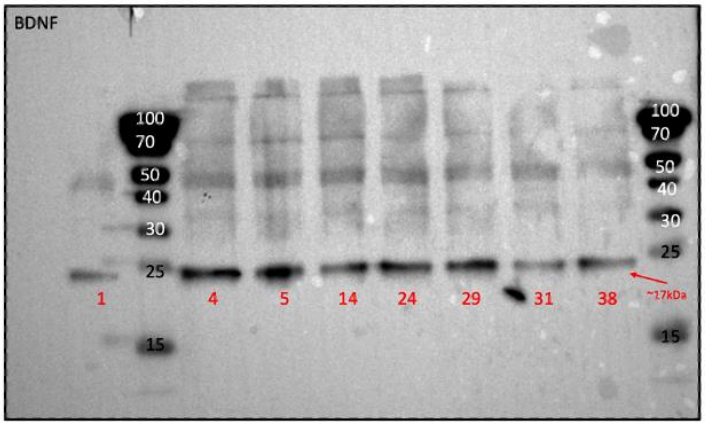
C.4.1.1 WB 1 Scheme

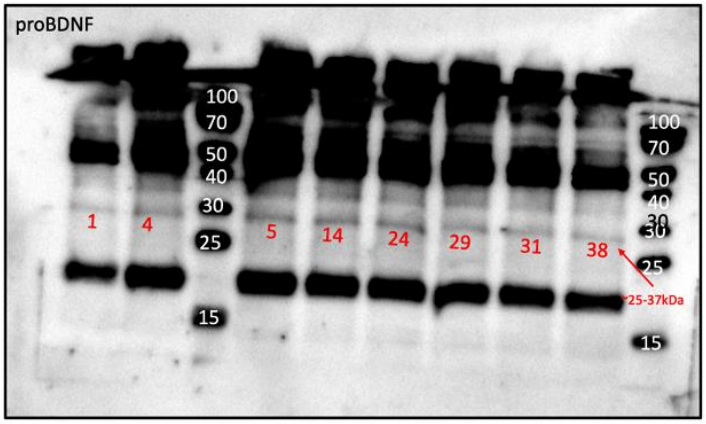
14-apr-22

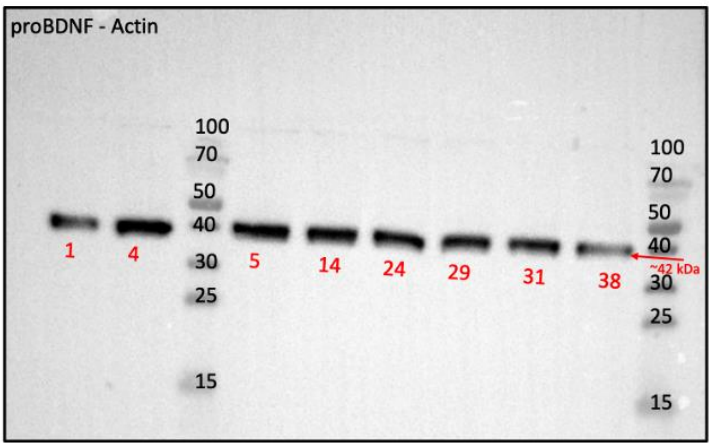
lane nr.	1	2	3	4	5	6	7	8	9	10
sample:	1		4	5	14	24	29	31	38	
ladder:		Ladder								Ladder
volume on gel (µl):	10	8	10	10	10	10	10	10	10	8
tissue	dorsal hipp.		dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	
protein	BDNF		BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	

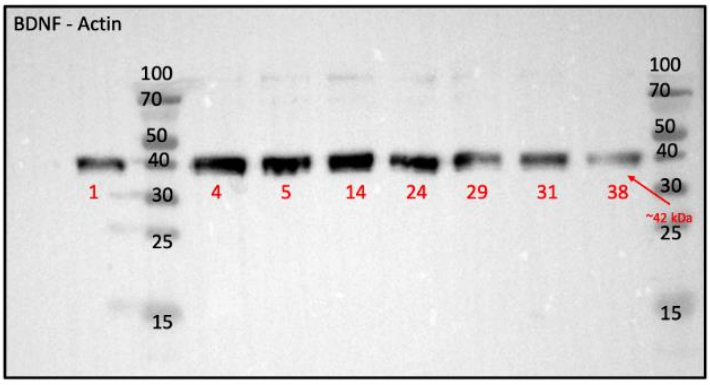
lane nr.	1	2	3	4	5	6	7	8	9	10
sample:	1	4		5	14	24	29	31	38	
ladder:			Ladder							Ladder
volume on gel (µl):	10	10	8	10	10	10	10	10	10	8
tissue	dorsal hipp.	dorsal hipp.		dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	
protein	proBDNF	proBDNF		proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	

C4.1.2 WB1 Membrane









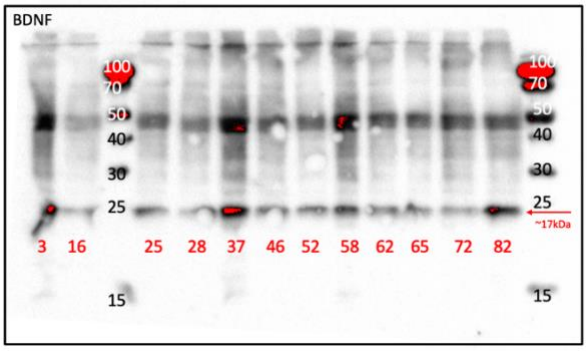
C.4.2.1 WB 2 Scheme

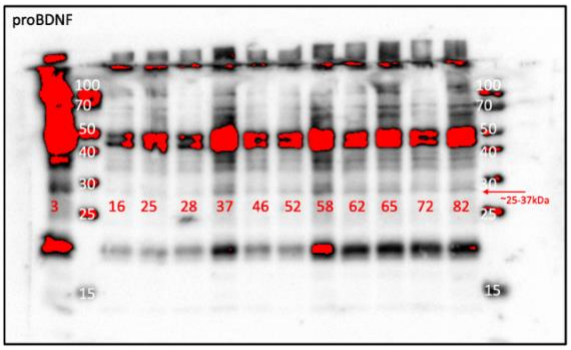
02-mei-22

lane nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
sample:	3		16	25	28	37	46	52	58	62	65	72	82		
ladder:		Ladder												Ladder	
volume on gel (µl):	10	8	10	10	10	10	10	10	10	10	10	10	10	8	
tissue	dorsal hipp.		dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.		
protein	BDNF		BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF		

lane nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
sample:	3	16		25	28	37	46	52	58	62	65	72	82		
ladder:			Ladder											Ladder	
volume on gel (µl):	10	10	8	10	10	10	10	10	10	10	10	10	10	8	
tissue	dorsal hipp.	dorsal hipp.		dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.		
protein	proBDNF	proBDNF		proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF		

C.4.2.2 WB 2 Membrane





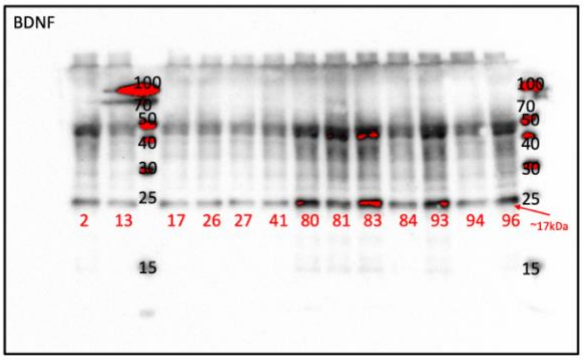
C.4.3.1 WB 3 Scheme

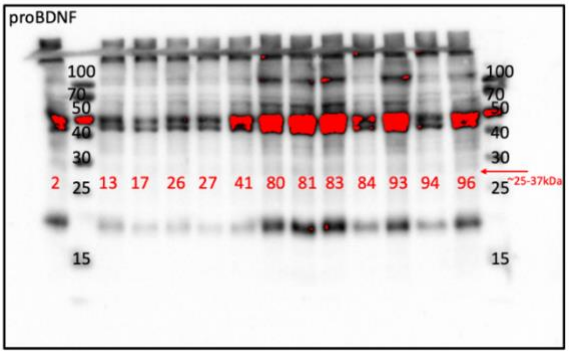
10-mei-22

lane nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
sample:	2		13	17	26	27	41	80	81	83	84	93	94	96	
ladder:		Ladder													Ladder
volume on gel (µl):	10	8	10	10	10	10	10	10	10	10	10	10	10	10	8
tissue	dorsal hipp.		dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	
protein	BDNF		BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	

lane nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
sample:	2	13		17	26	27	41	80	81	83	84	93	94	96	
ladder:			Ladder												Ladder
volume on gel (µl):	10	10	8	10	10	10	10	10	10	10	10	10	10	10	8
tissue	dorsal hipp.	dorsal hipp.		dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	
protein	proBDNF	proBDNF		proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	

C.4.3.1 WB 3 Scheme





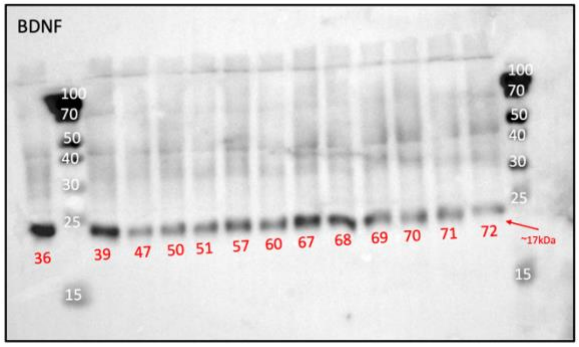
C.44.1 WB 4 Scheme

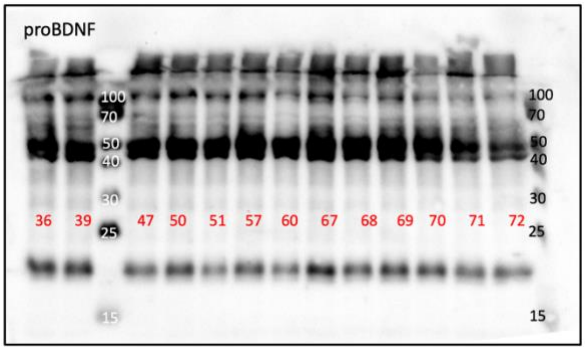
17-mei-22

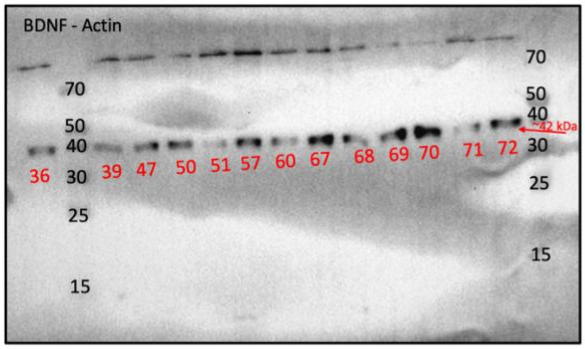
lane nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
sample:	36		39	47	50	51	57	60	67	68	69	70	71	72	
ladder:		Ladder													Ladder
volume on gel (µl):	10	8	10	10	10	10	10	10	10	10	10	10	10	10	8
tissue	dorsal hipp.		dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	
protein	BDNF		BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	

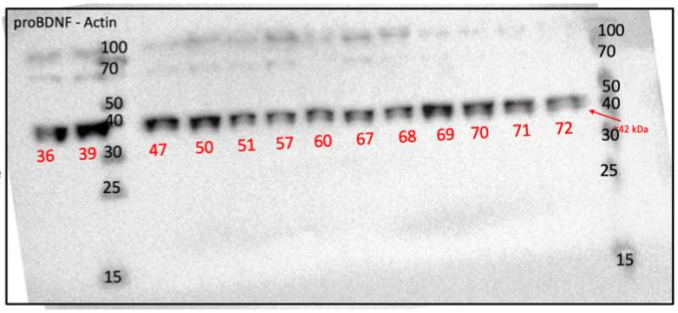
lane nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
sample:	36	39		47	50	51	57	60	67	68	69	70	71	72	
ladder:			Ladder												Ladder
volume on gel (µl):	10	10	8	10	10	10	10	10	10	10	10	10	10	10	8
tissue	dorsal hipp.	dorsal hipp.		dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	
protein	proBDNF	proBDNF		proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	

C.4.4.1 WB 4 Membrane









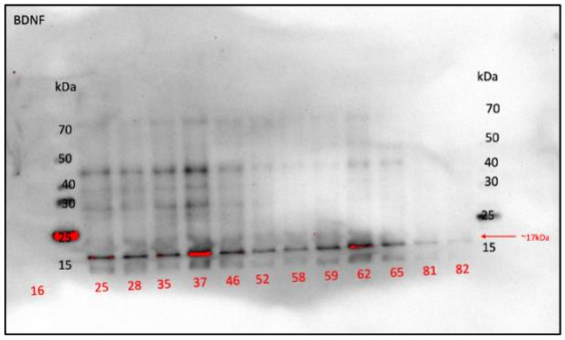
C.4.5.1 WB 5 Scheme

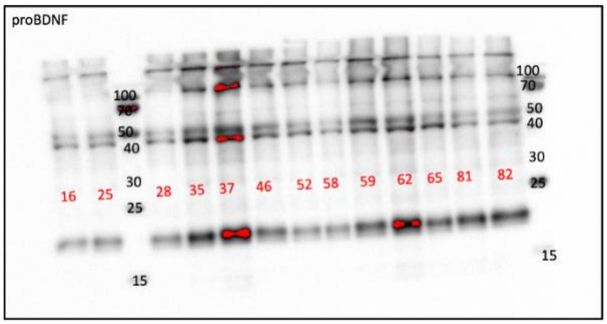
31-mei-22

lane nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
sample:	16		25	28	35	37	46	52	58	59	62	65	81	82	
ladder:		Ladder													Ladder
volume on gel (µl):	10	8	10	10	10	10	10	10	10	10	10	10	10	10	8
tissue	dorsal hipp.		dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	
protein	BDNF		BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	

lane nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
sample:	16	25		28	35	37	46	52	58	59	62	65	81	82	
ladder:			Ladder												Ladder
volume on gel (µl):	10	10	8	10	10	10	10	10	10	10	10	10	10	10	8
tissue	dorsal hipp.	dorsal hipp.		dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	
protein	proBDNF	proBDNF		proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	

C.4.5.2 WB 5 Membrane





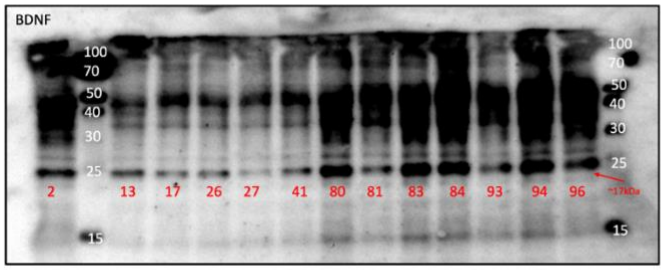
C.4.6.1 WB 6 Scheme

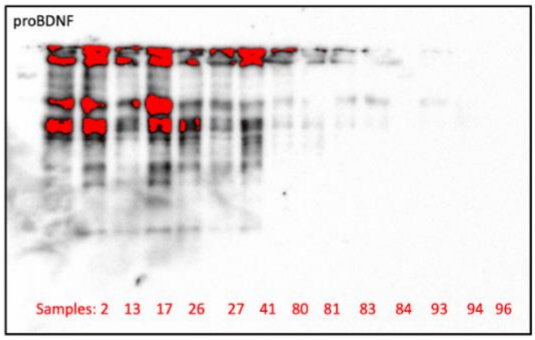
22-jun-22 same samples as 10-mei-22

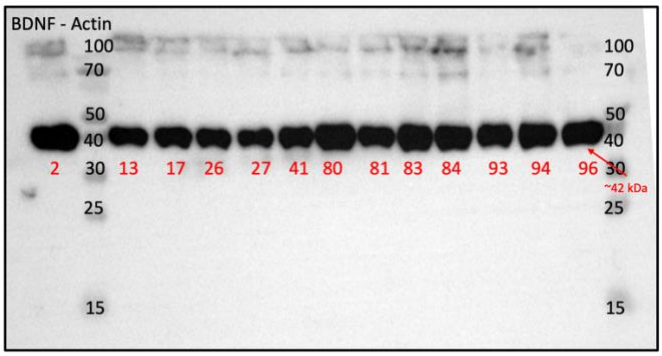
lane nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
sample:	2		13	17	26	27	41	80	81	83	84	93	94	96	
ladder:		Ladder													Ladder
volume on gel (µl):	10	8	10	10	10	10	10	10	10	10	10	10	10	10	8
tissue	dorsal hipp.		dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	
protein	BDNF		BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	BDNF	

lane nr.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
sample:	2	13		17	26	27	41	80	81	83	84	93	94	96	
ladder:			Ladder												Ladder
volume on gel (µl):	10	10	8	10	10	10	10	10	10	10	10	10	10	10	8
tissue	dorsal hipp.	dorsal hipp.		dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	dorsal hipp.	
protein	proBDNF	proBDNF		proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	proBDNF	

C.4.6.1 WB 6 Membrane

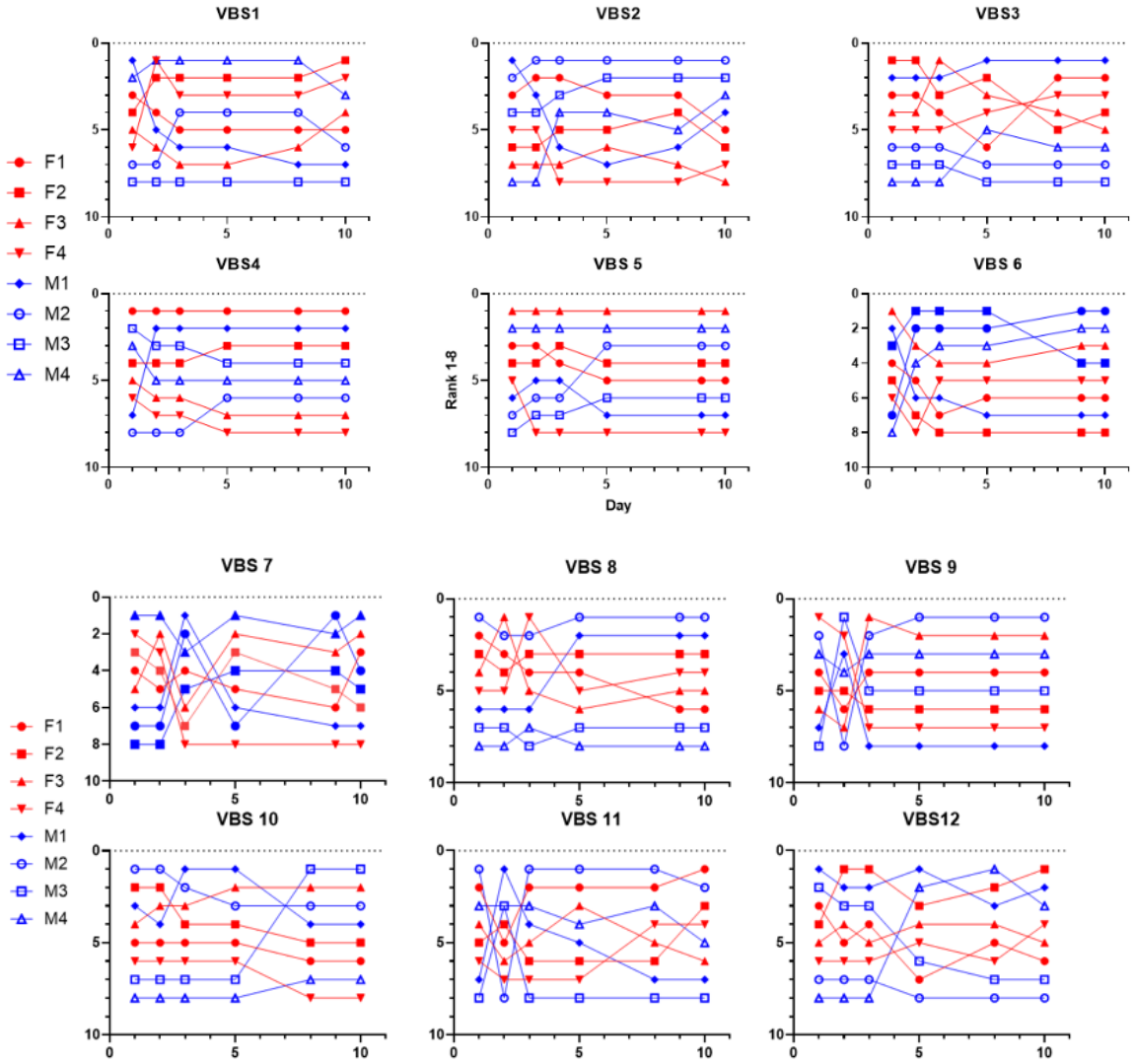





BDNF - Actin
100
70
50
40
30
25
15
2
13
17
26
27
41
80
81
83
84
93
94
96
~42 kDa

D. Matrices – new ranking method

D1. Dominance hierarchies throughout the VBS [Based on Curley Method]



D2. Most Dominant and Subordinate Males and Females based on Ranking by Miguel Puentes-Escamilla and James Curley

	Ranking based on Miguel Puentes				Ranking based on James Curley			
	DOM-F	DOM-M	SUB-F	SUB-M	DOM-F	DOM-M	SUB-F	SUB-M
VBS1	F4	M4	F3	M1	F2	M4	F1	M3
VBS2	F3	M2	F2	M1	F1	M2	F3	M1
VBS3	F4	M1	F3	M4	F1	M1	F3	M3
VBS4	F1	M1	F3	M4	F1	M1	F4	M2
VBS5	F4	M4	F1	M1	F3	M4	F4	M1
VBS6	F3	M2	F1	M3	F2	M2	F2	M1
VBS7	F3	M3	F2	M1	F3	M4	F4	M1
VBS8	F3	M2	F1	M1	F3	M2	F1	M4
VBS9	F4	M2	F1	M1	F2	M2	F4	M1
VBS10	F3	M1	F4	M2	F3	M3	F4	M4
VBS11	F1	M2	F2	M1	F1	M2	F3	M3
VBS12	F4	M4	F1	M2	F2	M1	F1	M2

D3. AvgDI and DomRank all days combined (based on Curley's method)

VBS1	All days										
	Row Labels	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank
	F1		5	6	3			1		0,733333	F2
	F2	1		1	3		0	1	2	0,65625	F4
	F3	5	1		0			1	1	0,620513	M4
	F4	5	0	4				1	0	0,590909	F3
	M1						0		1	0,550758	F1
	M2					1				0,5	M2
	M3								1	0,1	M1
	M4	1				4	13	12		0,015385	M3

VBS2	All days										
	Row Labels	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank
	F1		4	3	8	0				0,928571	M2
	F2	5		1	7	0	1		0	0,7	M3
	F3		2		2					0,666667	M4
	F4	3	3	5		0	0	0		0,583333	M1
	M1	1		1	4		0	1		0,361953	F1
	M2	2	1	1	2	12		10	15	0,348148	F2
	M3	2	1		4	1				0,214502	F4
	M4		2			1	0			0,190476	F3

VBS3	All days										
	Row Labels	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank
	F1		1	1			1	2		0,983333	M1
	F2			0	1	0	4	2	1	0,875	F1
	F3	1	5							0,75	F4
	F4		1	1						0,5	F2
	M1		2	1			16	15	11	0,375	F3
	M2	0				0				0,041667	M4
	M3					0				0	M2
	M4					1				0	M3

VBS4	All days										
	Row Labels	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank
	F1		28	9	20	0				0,988506	F1
	F2	1		3	9		1	0	2	0,915675	M1
	F3	0								0,806897	F2
	F4	0	0	0					0	0,714286	M3
	M1	0	0				15	7	17	0,208333	M4
	M2				0	1			1	0,140625	M2
	M3				1	0	2		6	0	F3
	M4	0	0	0	0	4	1	1		0	F4

VBS5	All days										
	Row Label	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank
	F1		1							1	F3
	F2	1								0,943791	M4
	F3	1	2		1					0,535714	M2
	F4									0,25	F2
	M1								1	0,166667	F1
	M2					1			2	0,0625	M3
	M3							1	1	0,045455	M1
	M4	1				10	26	15		0	F4

VBS 6	**All days**										
	Row Label	F1	F2	F3	F4	M1	M2	M3	M4	**AvgDI**	**DomRank**
	F1								1	0,861607	**M2**
	F2									0,761905	**M4**
	F3	1	3		1	1				0,583333	**F3**
	F4			1						0,5	**M3**
	M1						3			0,166667	**F4**
	M2	2		1	1	8			6	0,111111	**F1**
	M3						1			0,0625	**M1**
	M4	2		2	1	1	1			0	**F2**

VBS 7	**All days**										
	Row Label	F1	F2	F3	F4	M1	M2	M3	M4	**AvgDI**	**DomRank**
	F1		1	1	1		1		1	0,70974	**M4**
	F2	2		2	2	2		1		0,65	**F3**
	F3	2	3		2				2	0,583333	**F1**
	F4		2	1					1	0,547619	**M2**
	M1		1		1		2		2	0,533333	**M3**
	M2				1	12			4	0,455556	**F2**
	M3		1		2				1	0,414502	**M1**
	M4	3	4	1	1	9	8	9		0,190476	**F4**

VBS 8	**All days**										
	Row Label	F1	F2	F3	F4	M1	M2	M3	M4	**AvgDI**	**DomRank**
	F1		1		1	2			3	0,834465	**M2**
	F2			3	4	1			2	0,655844	**M1**
	F3	1	1		4			1	2	0,61	**F2**
	F4	3	1	3			1	2	4	0,554082	**F4**
	M1		1	1	1		2	2	4	0,498299	**F3**
	M2	1		4	1	20		4	27	0,475	**F1**
	M3						2		1	0,266667	**M3**
	M4	2		1			2			0,114614	**M4**

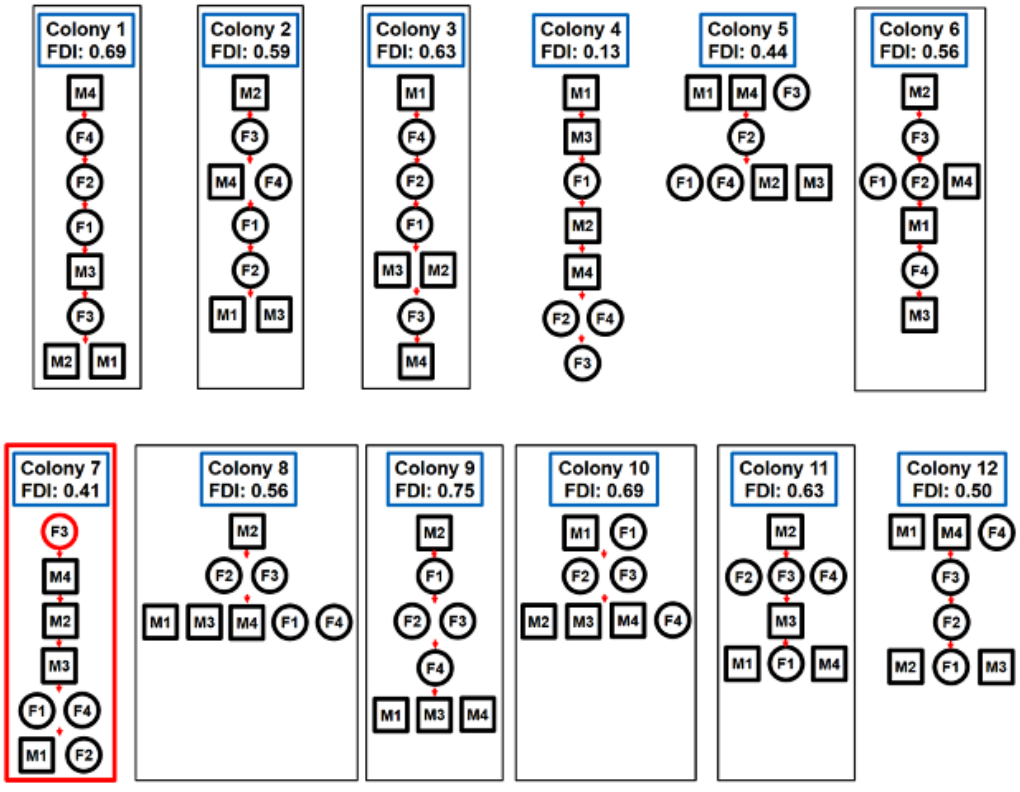
VBS9	**All days**										
	Row Labels	**F1**	**F2**	**F3**	**F4**	**M1**	**M2**	**M3**	**M4**	**AvgDI**	**DomRank**
	F1		5		5					1	**F3**
	F2	3		2	7		0			1	**M2**
	F3	1	5		1	0				0,666667	**M4**
	F4		2			0				0,458333	**F1**
	M1									0,333333	**M3**
	M2	1	1			9		5	7	0,193333	**F2**
	M3		1							0,066667	**F4**
	M4					1	0	2		0	**M1**

VBS10	**All days**										
	Row Label	**F1**	**F2**	**F3**	**F4**	**M1**	**M2**	**M3**	**M4**	**AvgDI**	**DomRank**
	F1		1	0	9					1	**M3**
	F2	3		3	2					0,908333	**F3**
	F3	2	5		22	1			1	0,625	**M2**
	F4	1		2		0				0,606481	**M1**
	M1		1		3		3		8	0,53125	**F2**
	M2	1				1		0	0	0,2875	**F1**
	M3					1	0			0,055556	**M4**
	M4			0		1	0	0		0,045833	**F4**

VBS11	All days										
	Row Labels	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank
	F1		3	5	1	1			0	0,986667	M2
	F2					3				0,958333	F1
	F3	1	1							0,533333	M4
	F4	0		1		1				0,5	F4
	M1							1		0,388889	F3
	M2		1		1	3		14	14	0,25	F2
	M3						0		0	0,2	M1
	M4						1	1		0	M3

VBS12	All days										
	Row Labels	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank
	F1			1				2		0,84375	M4
	F2			6	0		1	0		0,833333	F2
	F3	2	3		1		0	1	1	0,75	M1
	F4							1	0	0,583333	F3
	M1			1	2		4	6	0	0,416667	F1
	M2									0,333333	F4
	M3	2	0	0		2			1	0,175	M3
	M4			1	0	2	1	7		0	M2

D4. Current ranking, based on Miguel Puentes-Escamilla's method



[Miguel Puentes-Escamilla, 2021]

E. Tables & Statistics

All statistical tests were performed in SPSS unless mentioned otherwise

E1) Change in weight

To compare the means of the ranks and sexes per day in the VBS
E1a) Table

	Female Most Dominant		Female Most Subordinate		Male Most Dominant		Male Most Subordinate	
Day	Mean (%)	SEM	Mean (%)	SEM	Mean (%)	SEM	Mean (%)	SEM
0	0		0		0		0	
2	0,78	0,93	2,24620151	0,76990555	-4,2145623	0,73596445	-3,7199905	0,70473952
5	2,11659683	0,98668911	2,89888897	0,61186921	-8,8191604	1,45977795	-5,9636127	0,60786818
8	4,57360607	0,76271456	5,6884151	0,98219118	-9,289769	1,72380015	-5,9886201	0,82738229
10	5,9953469	1,09913813	7,46786872	0,94713504	-8,7350881	1,74408563	-5,989667	1,13949156

E1b) Independent T-Test with SPSS between F-DOM and F-SUB, M-DOM and M-SUB

Group Statistics

	1=DomFemale, 2=DomMale, 3=SubFemale, 4=SubMale, 5=DF Curley, 6=DM Curley, 7=SF Curley, 8=SM Curley	N	Mean	Std. Deviation	Std. Error Mean
Day10weight	1,00	12	5,9958	3,80693	1,09897
	3,00	12	7,4683	3,28185	,94739

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Significance One-Sided p	Significance Two-Sided p	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference Lower	95% Confidence Interval of the Difference Upper
Day10weight	Equal variances assumed	,172	,682	-1,015	22	,161	,321	-1,47250	1,45096	-4,48160	1,53660
	Equal variances not assumed			-1,015	21,533	,161	,321	-1,47250	1,45096	-4,48539	1,54039

Independent Samples Effect Sizes

		Standardizer[a]	Point Estimate	95% Confidence Interval Lower	95% Confidence Interval Upper
Day10weight	Cohen's d	3,55410	-,414	-1,219	,400
	Hedges' correction	3,68129	-,400	-1,177	,386
	Glass's delta	3,28185	-,449	-1,260	,382

a. The denominator used in estimating the effect sizes.
Cohen's d uses the pooled standard deviation.
Hedges' correction uses the pooled standard deviation, plus a correction factor.
Glass's delta uses the sample standard deviation of the control group.

Group Statistics

	1=DomFemale, 2=DomMale, 3=SubFemale, 4=SubMale, 5=DF Curley, 6=DM Curley, 7=SF Curley, 8=SM Curley	N	Mean	Std. Deviation	Std. Error Mean
Day10weight	2,00	12	-5,9892	3,94624	1,13918
	4,00	12	-8,7350	6,04211	1,74421

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means							
		F	Sig.	t	df	Significance One-Sided p	Significance Two-Sided p	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference Lower	95% Confidence Interval of the Difference Upper
Day10weight	Equal variances assumed	2,187	,153	1,318	22	,101	,201	2,74583	2,08326	-1,57459	7,06626
	Equal variances not assumed			1,318	18,940	,102	,203	2,74583	2,08326	-1,61543	7,10709

E2) Change in Weight ABSOLUTE

To compare the means of the weights of the ranks and sexes for all of the days, including before and during pair housing and the VBS
Unpaired T-Test in GraphPad for Last Day in VBS

First the means and SEM (stdev.s(x)/wortel(aantal(x)) (English: stdev.s (x)/sqrt(amount(x))) were calculated in Excel.

E2a) Table

		Male Most Dominant		Female Most Dominant		Male Most Subordinate		Female Most Subordinate	
	Day	Weight (grams)	SEM	Weight (grams)	SEM	Weight (grams)	SEM	Weight (grams)	SEM
	-13	447,8333333	17,3313664	217	5,78268052	448,75	13,0396395	218	7,57787849
	-12	448,8333333	17,3963743	217,5	4,88736776	450,8333333	12,8049667	216,4166667	8,26865048
	-11	455,0833333	17,5246399	218,9166667	5,05793456	455,25	12,9194034	218,3333333	8,51410001
	-10	457,0833333	17,6018501	218,1666667	4,90026798	456,3333333	12,4178106	219,6666667	8,39131345
	-9	456,25	15,0015782	218,25	4,52957453	457,0833333	12,6242062	218,8333333	8,20553521
Pair housing	-6	454,8333333	15,6417436	223,9166667	4,94738733	453,75	12,3399603	223,4166667	8,171883
	-5	458,25	15,1623162	225,1666667	5,19590942	456,1666667	11,9765132	223,1666667	8,13506564
	-4	460,3333333	15,5218972	230,9166667	4,5981853	458,5	11,9958958	227,25	7,29063846
	-3	462,8333333	15,2244985	234,3333333	4,5064488	461,5	12,8284131	230,5833333	6,78284192
	-2	462,6666667	14,7993311	235,8333333	4,45658065	462,9166667	12,5411192	232,5	6,6486499
	-1	463,9166667	14,8754722	240,1666667	5,13873191	465,4166667	13,2370339	235,1666667	6,803334
VBS	0	460,5	15,6889751	237,5	5,00378645	458,8333333	12,8091074	232,25	6,50888088
	2	443,25	14,9945066	239	3,80987553	439,75	13,5250189	237,5	7,06624499
	5	433	14,829843	242,3333333	4,67639678	418,25	13,1674098	239,1666667	7,52655233
	8	432,1666667	12,7996409	248,1666667	4,58725727	415,5	12,1221685	245,25	6,56335673
	10	432,25	13,5138117	251,5	4,76651833	417,5833333	10,7917325	249,4166667	6,79734611

E2b) Statistical test

Column C	Male Most Subordinate
vs.	vs.
Column A	Male Most Dominant
Unpaired t test	
P value	0.5168
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.6560, df=30

Column D	Female Most Subordinate
vs.	vs.
Column B	Female Most Dominant
Unpaired t test	
P value	0.6109
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.5142, df=30

E2c) Delta weight change

		FDOM	FSUB	MDOM	MSUB
Day	Day	Delta mean of weight			
-13	0	0	0	0	0
-12	1	0,5	-1,5833333	1	2,0833333
-11	2	1,9166667	0,3333333	7,25	6,5
-10	3	1,1666667	1,6666667	9,25	7,5833333
-9	4	1,25	0,8333333	8,4166667	8,3333333
-6	5	6,9166667	5,4166667	7	5
-5	6	8,1666667	5,1666667	10,4166667	7,4166667
-4	7	13,9166667	9,25	12,5	9,75
-3	8	17,3333333	12,5833333	15	12,75
-2	9	18,8333333	14,5	14,8333334	14,1666667
-1	10	23,1666667	17,1666667	16,0833334	16,6666667
0	11	20,5	14,25	12,6666667	10,0833333
2	12	22	19,5	-4,5833333	-9
5	13	25,3333333	21,1666667	-14,833333	-30,5
8	14	31,1666667	27,25	-15,666667	-33,25
10	15	34,5	31,4166667	-15,583333	-31,166667

E2d) Body weight for every colony

		Base	2nd	5th	8th	10th
VBS1	M1	0	-5,6	-10,13	-8	-4,53
	M2	0	-6,31	-8,25	-5,83	-6,07
	M3	0	-5,82	-13,16	-11,39	-11,39
	M4	0	-2,93	-5,87	-4,65	-4,65
	F1	0	2,68	5,36	5,36	8,04
	F2	0	5,05	4,59	10,09	11,01
	F3	0	4,57	3,65	9,13	10,05
	F4	0	2,68	1,79	5,36	9,38
VBS2	M1	0	-1,99	-3,98	-1,99	-1
	M2	0	-2,13	-6,62	-4,96	-3,78
	M3	0	-3,67	-7,09	-4,89	-4,65
	M4	0	-1,57	-4,25	-4,03	-5,37
	F1	0	0,43	0,43	2,61	3,91
	F2	0	0	0	2,15	3
	F3	0	4,8	6,11	5,24	6,55
	F4	0	3,57	1,34	3,57	3,57
VBS3	M1	0	-6,24	-6,47	-6,71	-6,47
	M2	0	-4,91	-5,61	-3,27	-1,87
	M3	0	-10,69	-15,01	-13,49	-14,25
	M4	0	-8,01	-20,56	-23,16	-18,61
	F1	0	-0,41	9,5	14,46	17,36
	F2	0	2,87	5,74	9,84	9,84
	F3	0	0,8	4,8	9,6	10,4
	F4	0	2,61	2,61	5,65	9,13
VBS7	M1	0	-7,72	-16,49	-17,12	-16,08
	M2	0	-5,12	-6,46	-14,48	-20,71
	M3	0	-9,49	-11,34	-9,49	-14,81
	M4	0	-2,65	-3,76	-3,98	-3,1
	F1	0	2,8	1,87	6,54	9,35
	F2	0	5,5	4,59	8,72	10,09
	F3	0	3,17	4,07	4,98	3,17
	F4	0	2,63	3,51	7,89	6,14
VBS8	M1	0	-6,73	-9,48	-8,48	-9,23
	M2	0	-3,17	-4,76	-4,54	-4,08
	M3	0	-8,6	-4,88	-5,81	-6,28
	M4	0	-6,4	-6,4	-3,94	-1,97
	F1	0	1,52	2,03	5,08	7,61
	F2	0	-0,48	5,77	8,17	11,54
	F3	0	-1,4	-2,79	1,86	2,33
	F4	0	0,89	1,34	7,59	9,38
VBS9	M1	0	-1,8	-8,2	-8,8	-7,6
	M2	0	-5,09	-7,37	-11,23	-10,88
	M3	0	0	-5,42	-9,11	-7,38
	M4	0	-4,54	-5,72	-11,83	-11,24
	F1	0	1,57	1,96	-1,57	1,18
	F2	0	-2,21	0,88	3,1	5,31
	F3	0	2,13	2,48	-0,35	2,48
	F4	0	-4,2	0,38	2,67	4,2

VBS4	M1	0	-0,24	-3,07	-1,42	0
	M2	0	0	-3,09	-1,66	-1,19
	M3	0	-1,95	-5,84	-6,33	-2,68
	M4	0	-3,64	-6,21	-4,71	-3,85
	F1	0	1,17	0,78	1,95	2,73
	F2	0	4,26	4,26	3,88	4,65
	F3	0	4,91	6,67	5,26	8,07
	F4	0	2,88	0	4,53	6,17
VBS5	M1	0	-1,52	-9,52	-13,52	-19,05
	M2	0	-7,99	-12,7	-6,97	-6,97
	M3	0	-8,51	-11,49	-7,02	-11,06
	M4	0	-3,26	-6,74	-4,78	-6,74
	F1	0	6,36	2,27	6,82	8,64
	F2	0	4,85	4,85	7,49	7,49
	F3	0	-2,01	-0,4	4,82	5,22
	F4	0	2,1	3,36	5,46	7,56
VBS6	M1	0	-1,03	-4,33	-1,86	-3,09
	M2	0	-1,28	-3,85	-2,35	-1,5
	M3	0	-0,86	-4,51	-4,08	-3,43
	M4	0	0	-2,74	-0,68	-0,91
	F1	0	0,9	0,45	7,21	9,01
	F2	0	2,74	1,37	8,22	10,96
	F3	0	4,89	9,78	11,56	15,11
	F4	0	4,15	3,32	2,9	4,56
VBS10	M1	0	-4,22	-5,62	-7,56	-7,38
	M2	0	-5,81	-5,6	-8,71	-8,71
	M3	0	-6,87	-16,74	-21,03	-13,95
	M4	0	-6,23	-10,72	-8,23	-6,48
	F1	0	-1,23	2,47	2,06	4,12
	F2	0	-2,69	1,35	5,38	8,07
	F3	0	-3,73	-1,49	2,61	2,99
	F4	0	3,07	5,26	6,14	9,65
VBS11	M1	0	-4,7	-6,34	-6,13	-5,52
	M2	0	-4,22	-4,89	-5,56	-5,33
	M3	0	-11,29	-12,7	-18,15	-19,76
	M4	0	-9,3	-4,91	-8,53	-4,91
	F1	0	-3,2	0,8	2,8	3,2
	F2	0	-2,68	2,68	8,04	9,38
	F3	0	-0,83	1,65	5,37	6,2
	F4	0	0	1,66	3,73	5,39
VBS12	M1	0	-4,26	-5,97	-9,17	-8,1
	M2	0	-2,18	-4,8	-6,77	-7,21
	M3	0	-0,9	-3,6	-2,7	-2,03
	M4	0	-2,37	-4,96	-8,62	-6,25
	F1	0	-3,73	0,41	4,56	5,81
	F2	0	0,42	0,42	1,69	2,54
	F3	0	0,43	1,74	6,09	8,7
	F4	0	0,43	0	4,74	5,6

E3) Amount of wounds

To determine the most aggressive colonies

E3a) Amount of wounds per sex for every VBS

First the means of the wounds for every animal were calculated, then these means were tested for significancy with the independent T-test.

Table

	F1	F2	F3	F4	M1	M2	M3	M4
VBS1	0	0	1	1	4	4	2	1
VBS2	0	0	0	0	5	1	1	0
VBS3	2	0	0	0	2	7	10	14
VBS4	0	1	0	0	0	0	1	1
VBS5	1	0	0	1	3	1	3	2
VBS6	0	0	0	0	1	0	0	0
VBS7	0	0	0	0	6	8	9	1
VBS8	0	0	0	0	5	1	2	2
VBS9	0	0	0	0	6	1	7	4
VBS10	0	0	0	0	1	8	3	5
VBS11	0	0	0	0	3	5	3	3
VBS12	0	1	0	0	2	5	0	2
mean	0,25	0,16666667	0,08333333	0,16666667	3,16666667	3,41666667	3,41666667	2,91666667

E3b) Statistical test

Independent T-test between Male and Female

Group Statistics

	1=female,2=male	N	Mean	Std. Deviation	Std. Error Mean
wounds	1,00	4	,1667	,06804	,03402
	2,00	4	3,2292	,23936	,11968

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means					
						Significance			
		F	Sig.	t	df	One-Sided p	Two-Sided p	Mean Difference	Std. Error Difference
wounds	Equal variances assumed	6,682	,041	-24,614	6	<,001	<,001	-3,06250	,12442
	Equal variances not assumed			-24,614	3,482	<,001	<,001	-3,06250	,12442

Wounds male vs female

N= wounds

E.3c) Table

	Miguel's ranking			
	DOM-F	DOM-M	SUB-F	SUB-M
VBS1	1	1	1	4
VBS2	0	1	0	5
VBS3	0	2	0	14
VBS4	0	0	0	1
VBS5	1	2	1	3
VBS6	0	0	0	0
VBS7	0	9	0	6
VBS8	0	1	0	5
VBS9	0	1	0	6
VBS10	0	1	0	8
VBS11	0	5	0	3
VBS12	0	2	0	5
mean	0,16666667	2,08333333	0,16666667	5
sem	0,11236664	0,73297167	0,11236664	1,03718734

E3d) Statistical test between total wounds of female animals and male animals
One-Way ANOVA

ANOVA

n

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	184,417	3	61,472	12,474	<,001
Within Groups	216,833	44	4,928		
Total	401,250	47			

Multiple Comparisons

Dependent Variable: n
Bonferroni

(I) 1=DomFemale, 2=DomMale, 3=SubFemale, 4=SubMale	(J) 1=DomFemale, 2=DomMale, 3=SubFemale, 4=SubMale	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1,00	2,00	-1,83333	,90628	,295	-4,3372	,6705
	3,00	,08333	,90628	1,000	-2,4205	2,5872
	4,00	-4,75000*	,90628	<,001	-7,2539	-2,2461
2,00	1,00	1,83333	,90628	,295	-,6705	4,3372
	3,00	1,91667	,90628	,241	-,5872	4,4205
	4,00	-2,91667*	,90628	,015	-5,4205	-,4128
3,00	1,00	-,08333	,90628	1,000	-2,5872	2,4205
	2,00	-1,91667	,90628	,241	-4,4205	,5872
	4,00	-4,83333*	,90628	<,001	-7,3372	-2,3295
4,00	1,00	4,75000*	,90628	<,001	2,2461	7,2539
	2,00	2,91667*	,90628	,015	,4128	5,4205
	3,00	4,83333*	,90628	<,001	2,3295	7,3372

*. The mean difference is significant at the 0.05 level.

E3e) Correlation test
To determine if there is any correlation between the amount of wounds and corticosterone change the correlation tests was performed.

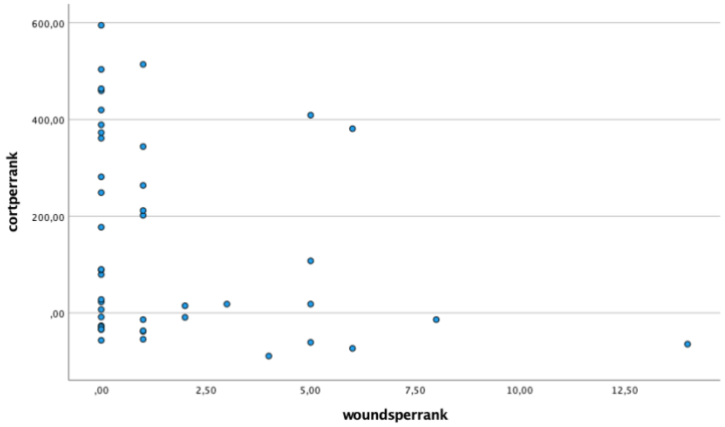
Table

Female Most Dominant		Male Most Dominant		Female Most Subordinate		Male Most Subordinate	
Wounds	CORT change (%)	Wounds	CORT change (%)	Wounds	CORT change (%)	Wounds	CORT change (%)
1	514	1		1	-36,89320388	4	-89,0562249
0	-8,366533865	1	-38,170347	0	389,1891892	5	107,6923077
0	-26,31578947	2	-9,108910891	0	463,6363636	14	-64,5021645
0	79,46428571	0	-56,59050967	0	248,8888889	1	-54,38596491
1	344,115977	2		1	263,7957598	3	
0	419,7837434	0	459,4510794	0	177,337924	0	281,5213241
0	503,647877	9		0	372,9927585	6	381,0316839
0	361,3491403	1	201,9277389	0	594,8651743	5	409,0474806
0	88,98010895	1	211,7648994	0	7,132511323	6	-73,23779807
0	-34,27803942	1	-13,75607175	0	22,77061709	8	-13,75607175
0	89,81297938	5	18,32851986	0	-33,86497565	3	18,32851986
0	-28,60266487	2	14,80626744	0	27,80485481	5	-60,75784209

E3f) Pearson Correlation for wounds every rank and corticosterone % change every rank

Correlations

		woundsperrank	cortperrank
woundsperrank	Pearson Correlation	1	-,261
	Sig. (2-tailed)		,087
	N	48	44
cortperrank	Pearson Correlation	-,261	1
	Sig. (2-tailed)	,087	
	N	44	44



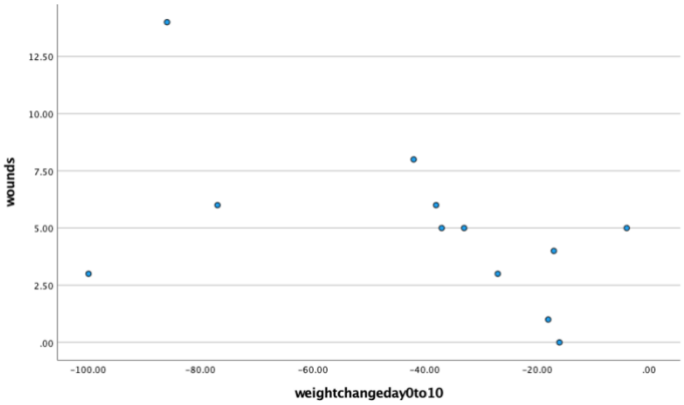
Wounds M-SUB	Weight change M-SUB	Wounds F-SUB	Weight change F-SUB	Wounds M-DOM	Weight change M-DOM	Wounds F-DOM	Weight change F-DOM
4.00	-17.00	1.00	22.00	2.00	-19.00	1.00	21.00
5.00	-4.00	.00	7.00	1.00	-33.00	.00	15.00
14.00	-86.00	.00	26.00	2.00	-27.00	.00	21.00
1.00	-18.00	.00	23.00	.00	-33.00	2.00	7.00
3.00	-100.00	1.00	19.00	2.00	-70.00	.00	18.00
.00	-16.00	.00	20.00	.00	-78.00	.00	34.00
6.00	-77.00	.00	22.00	9.00	-42.00	.00	7.00
5.00	-37.00	.00	15.00	1.00	-51.00	.00	5.00
6.00	-38.00	.00	3.00	1.00	-180.00	.00	11.00
8.00	-42.00	.00	22.00	1.00	-179.00	.00	8.00
3.00	-27.00	.00	21.00	5.00	-60.00	.00	8.00
5.00	-33.00	1.00	6.00	2.00	-74.00	.00	13.00

E3g) Pearson correlation for wounds rank separately and weight separately

Male Subordinate

Correlations

		VAR00002	VAR00003
Wounds M-SUB	Pearson Correlation	1	-.492
	Sig. (2-tailed)		.104
	N	12	12
Weight M-SUB	Pearson Correlation	-.492	1
	Sig. (2-tailed)	.104	
	N	12	12

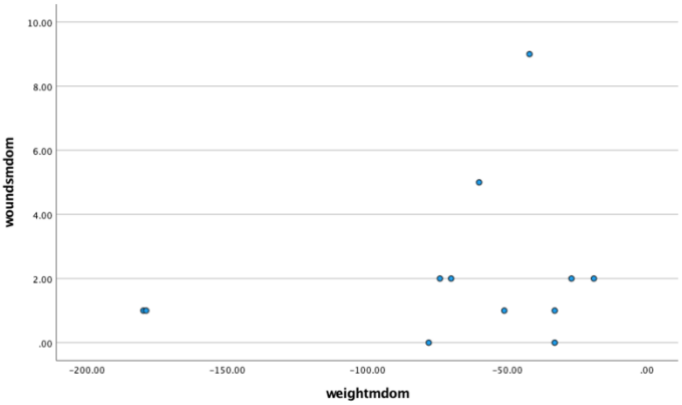


Male Dominant

Correlations

Correlations

		woundsmdom	weightmdom
Wounds M-DOM	Pearson Correlation	1	.221
	Sig. (2-tailed)		.491
	N	12	12
Weight M-DOM	Pearson Correlation	.221	1
	Sig. (2-tailed)	.491	
	N	12	12

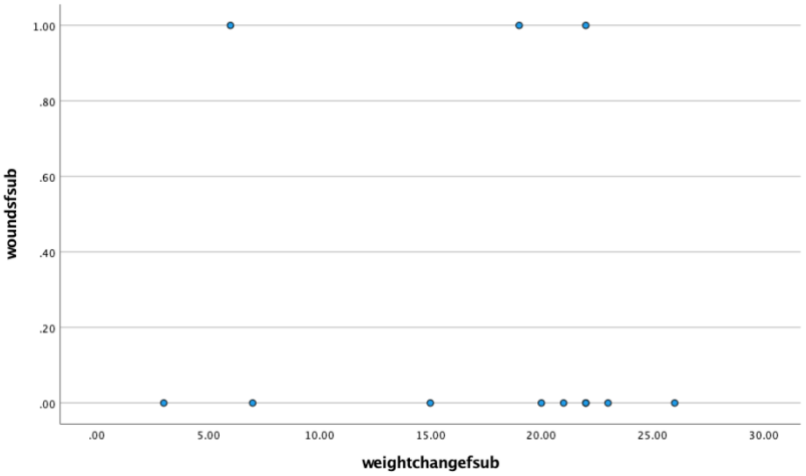


Female Subordinate

Correlations

Correlations

		woundsfsub	w
Wounds F-SUB	Pearson Correlation	1	-.118
	Sig. (2-tailed)		.714
	N	12	12
Weight F-SUB	Pearson Correlation	-.118	1
	Sig. (2-tailed)	.714	
	N	12	12

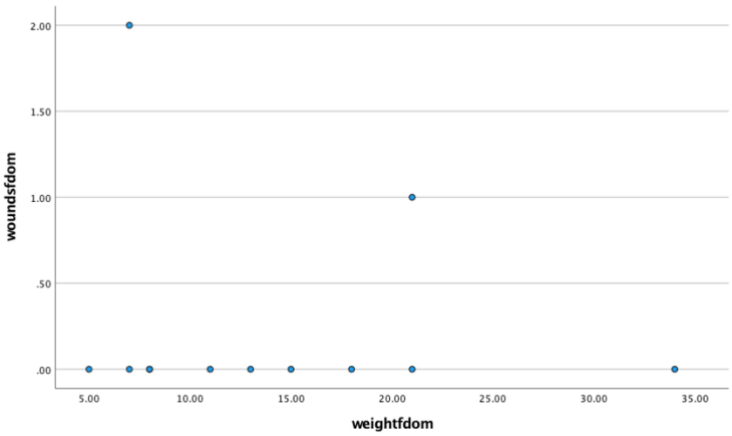


Female Dominant

Correlations

Correlations

		woundsfdom	weightfdom
Wounds F-DOM	Pearson Correlation	1	-.122
	Sig. (2-tailed)		.706
	N	12	12
Weight F-DOM	Pearson Correlation	-.122	1
	Sig. (2-tailed)	.706	
	N	12	12



E4) Change in organ weight for 100 gram body weight

To compare the means of the ranks and sexes for every organ

E4a) Data

The absolute body weights were calculated into factor to 100 gram body weight. All organ weights were then divided by this factor to obtain the results showed in the last columns (organ weight (g)/100g body weight).

			per 100 gram		weight of organ in grams					organ weight (g) / 100 g body weight				
		absolute weight last day in VBS	factor	gram body weight	adrenal	thymus	fat	vesicle	testes	adrenal	thymus	fat	vesicle	testes
DOM-M	M 3.5	390	3,9	100	0,0715	N/A	7,878	1,832	3,406	0,018333333	#WAARDE!	2,02	0,46974359	0,87333333
	M 2.1	407	4,07	100	0,0609	0,186	8,815	0,838	3,25	0,014963145	0,04570025	2,16584767	0,20589681	0,7985258
	M 1.4	390	3,9	100	0,0612	0,213	6,711	1,062	3,14	0,015692308	0,05461538	1,72076923	0,27230769	0,80512821
	M 1.2	423	4,23	100	0,0435	0,212	8,009	1,213	3,19	0,010283688	0,0501182	1,89338061	0,28676123	0,75413712
	M 4.2	429	4,29	100	0,0639	0,1765	8,7452	1,1769	2,4152	0,014895105	0,04114219	2,03850816	0,27433566	0,56298368
	M 2.2	461	4,61	100	0,0535	0,4355	11,3803	1,1161	2,3754	0,011605206	0,09446855	2,46861171	0,24210412	0,51527115
	M 3.4	368	3,68	100	0,0712	0,1276	5,3356	0,4556	1,3760	0,019347826	0,03467391	1,4498913	0,12380435	0,37391304
	M 2.3	423	4,23	100	0,0460	0,2504	6,9798	1,3799	2,6961	0,010874704	0,05919622	1,65007092	0,32621749	0,63737589
	M 2.4	508	5,08	100	0,0962	0,1688	18,2004	0,9501	4,0323	0,018937008	0,03322835	3,58275591	0,18702756	0,79375984
	M 1.4	527	5,27	100	0,0875	0,0952	15,7414	0,9153	3,5620	0,016603416	0,01806452	2,98698292	0,17368121	0,67590133
	M 2.2	426	4,26	100	0,0940	0,2395	9,0650	1,3127	4,3001	0,022065728	0,05622066	2,12793427	0,30814554	1,00941315
	M 4.4	435	4,35	100	0,0693	0,1892	13,8026	1,4586	3,5771	0,015931034	0,04349425	3,17301149	0,33531034	0,82232184
DOM-F	F 4.3	245	2,45	100	0,0858	0,38	3,104			0,035020408	0,15510204	1,26693878		
	F 3.1	244	2,44	100	0,0626	0,261	6,328			0,025655738	0,10696721	2,59344262		
	F 4.1	251	2,51	100	0,0867	0,409	3,587			0,034541833	0,16294821	1,42908367		
	F 1.1	263	2,63	100	0,0638	0,261	4,4			0,024258555	0,09923954	1,6730038		
	F 4.3	256	2,56	100	0,0598	0,3003	5,8089			0,023359375	0,11730469	2,26910156		
	F 3.3	259	2,59	100	0,0521	0,2463	4,9581			0,02011583	0,09509653	1,91432432		
	F 3.4	228	2,28	100	0,0470	0,2561	5,2572			0,020614035	0,11232456	2,30578947		
	F 3.1	220	2,2	100	0,0502	0,2148	3,0032			0,022818182	0,09763636	1,36509091		
	F 4.4	273	2,73	100	0,0847	0,3727	12,2892			0,031025641	0,13652015	4,50153846		
	F 3.2	276	2,76	100	0,0968	0,2442	12,1155			0,035072464	0,08847826	4,38967391		
	F 1.1	258	2,58	100	0,0735	0,1715	11,6490			0,028488372	0,06647287	4,51511628		
	F 4.3	245	2,45	100	0,0956	0,2521	5,7515			0,039020408	0,10289796	2,34755102		
SUB-M	M 1.3	358	3,58	100	0,0468	0,214	7,124	1,445	3,32	0,013072626	0,05977654	1,98994413	0,40363128	0,9273743
	M 1.1	398	3,98	100	0,0526	0,352	10,32	0,857	3,05	0,01321608	0,08844221	2,59296482	0,21532663	0,76633166
	M 4.1	376	3,76	100	0,109	0,093	8,881	0,456	2,988	0,028989362	0,02473404	2,36196809	0,1212766	0,79468085
	M 4.2	449	4,49	100	0,05	0,337	16,512	0,778	3,6	0,011135857	0,07505568	3,67750557	0,17327394	0,80178174
	M 1.2	425	4,25	100	0,0710	0,4062	18,7069	0,9131	2,2250	0,016705882	0,09557647	4,40162353	0,21484706	0,52352941
	M 3.1	450	4,5	100	0,0484	0,1669	15,7946	0,8988	2,0845	0,010755556	0,03708889	3,50991111	0,19973333	0,46322222
	M 1.3	402	4,02	100	0,0600	0,3426	19,1612	1,0751	1,8618	0,014925373	0,08522388	4,76646766	0,26743781	0,46313433
	M 1.4	364	3,64	100	0,0719	0,2409	10,8156	1,0804	2,6523	0,019752747	0,06618132	2,97131868	0,29681319	0,72865385
	M 1.1	462	4,62	100	0,0928	0,2177	19,0532	0,8635	2,3781	0,02008658	0,04712121	4,12406926	0,18690476	0,51474026
	M 2.1	440	4,4	100	0,0825	0,2232	9,8142	1,3351	3,9197	0,01875	0,05072727	2,2305	0,30343182	0,89084091
	M 1.3	462	4,62	100	0,0681	0,2501	18,3030	0,9498	3,4084	0,01474026	0,0541342	3,96168831	0,20558442	0,73774892
	M 2.3	425	4,25	100	0,0787	0,3292	14,4352	1,5596	3,3366	0,018517647	0,07745882	3,39651765	0,36696471	0,78508235
SUB-F	F 3.4	241	2,41	100	0,0612	0,339	7,141			0,025394191	0,1406639	2,96307054		
	F 2.4	240	2,4	100	0,0788	0,219	5,126			0,032833333	0,09125	2,13583333		
	F 3.3	276	2,76	100	0,0888	0,363	6,193			0,032173913	0,13152174	2,24384058		
	F 3.2	308	3,08	100	0,108	0,365	8,641			0,035064935	0,11850649	2,80551948		
	F 1.4	239	2,39	100	0,0667	0,2135	4,4110			0,02790795	0,08933054	1,84560669		
	F 1.1	242	2,42	100	0,0628	0,2460	4,4366			0,025950413	0,10165289	1,83330579		
	F 2.3	240	2,4	100	0,0630	0,1471	3,7962			0,02625	0,06129167	1,58175		
	F 1.3	212	2,12	100	0,0513	0,2366	3,4404			0,024198113	0,11160377	1,62283019		
	F 1.2	258	2,58	100	0,0916	0,1429	9,0412			0,035503876	0,0553876	3,50434109		
	F 4.1	250	2,5	100	0,0896	0,2291	7,4332			0,03584	0,09164	2,97328		
	F 2.2	245	2,45	100	0,0875	0,2899	5,9615			0,035714286	0,11832653	2,43326531		
	F 2.4	242	2,42	100	0,0778	0,2754	10,8411			0,03214876	0,11380165	4,47979339		

E4b) Statistical test

To determine any significancy between sex and rank, the independent T-test is performed.

Adrenal gland weight Female Dominant vs Female Subordinate

Group Statistics

	1=DomFemale, 2=DomMale, 3=SubFemale, 4=SubMale, 5=DF Curley, 6=DM Curley, 7=SF Curley, 8=SM Curley	N	Mean	Std. Deviation	Std. Error Mean
adrenal100	1,00	12	,0283	,00644	,00186
	3,00	12	,0307	,00450	,00130

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means					
		F	Sig.	t	df	Significance One-Sided p	Significance Two-Sided p	Mean Difference	Std. Error Difference
adrenal100	Equal variances assumed	2,553	,124	-1,065	22	,149	,298	-,00242	,00227
	Equal variances not assumed			-1,065	19,682	,150	,300	-,00242	,00227

Adrenal gland weight Male Dominant vs Male Subordinate

Group Statistics

	1=DomFemale, 2=DomMale, 3=SubFemale, 4=SubMale, 5=DF Curley, 6=DM Curley, 7=SF Curley, 8=SM Curley	N	Mean	Std. Deviation	Std. Error Mean
adrenal100	2,00	12	,0158	,00360	,00104
	4,00	12	,0167	,00503	,00145

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means					
		F	Sig.	t	df	Significance One-Sided p	Significance Two-Sided p	Mean Difference	Std. Error Difference
adrenal100	Equal variances assumed	,828	,373	-,519	22	,304	,609	-,00093	,00178
	Equal variances not assumed			-,519	19,935	,305	,609	-,00093	,00178

Thymus and fat weight Dominant Female vs Subordinate Female

Group Statistics

	1=DomFemale, 2=DomMale, 3=SubFemale, 4=SubMale, 5=DF Curley, 6=DM Curley, 7=SF Curley, 8=SM Curley	N	Mean	Std. Deviation	Std. Error Mean
thymus100	1,00	12	,1117	,02775	,00801
	3,00	12	,1021	,02585	,00746
fat100	1,00	12	2,5476	1,23273	,35586
	3,00	12	2,5352	,85987	,24822

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means					
		F	Sig.	t	df	Significance One-Sided p	Significance Two-Sided p	Mean Difference	Std. Error Difference
thymus100	Equal variances assumed	,008	,929	,883	22	,193	,387	,00967	,01095
	Equal variances not assumed			,883	21,890	,193	,387	,00967	,01095
fat100	Equal variances assumed	1,396	,250	,028	22	,489	,978	,01235	,43388
	Equal variances not assumed			,028	19,655	,489	,978	,01235	,43388

Thymus and fat weight Dominant Male vs Subordinate Male

Group Statistics

	1=DomFemale, 2=DomMale, 3=SubFemale, 4=SubMale, 5=DF Curley, 6=DM Curley, 7=SF Curley, 8=SM Curley	N	Mean	Std. Deviation	Std. Error Mean
thymus100	2,00	11	,0483	,01940	,00585
	4,00	12	,0635	,02173	,00627
fat100	2,00	12	2,2731	,65641	,18949
	4,00	12	3,3320	,90516	,26130

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means					
		F	Sig.	t	df	Significance One-Sided p	Significance Two-Sided p	Mean Difference	Std. Error Difference
thymus100	Equal variances assumed	,782	,387	-1,763	21	,046	,093	-,01519	,00862
	Equal variances not assumed			-1,772	20,990	,045	,091	-,01519	,00858
fat100	Equal variances assumed	1,918	,180	-3,281	22	,002	,003	-1,05889	,32277
	Equal variances not assumed			-3,281	20,063	,002	,004	-1,05889	,32277

Vesicle and testes weight Dominant Male vs Subordinate Male

Group Statistics

	1=DomFemale, 2=DomMale, 3=SubFemale, 4=SubMale, 5=DF Curley, 6=DM Curley, 7=SF Curley, 8=SM Curley	N	Mean	Std. Deviation	Std. Error Mean
vesicle100	2,00	12	,2671	,09116	,02631
	4,00	12	,2463	,08303	,02397
testes100	2,00	12	,7185	,17395	,05022
	4,00	12	,6998	,16486	,04759

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means					
		F	Sig.	t	df	Significance One-Sided p	Significance Two-Sided p	Mean Difference	Std. Error Difference
vesicle100	Equal variances assumed	,001	,976	,586	22	,282	,564	,02084	,03560
	Equal variances not assumed			,586	21,811	,282	,564	,02084	,03560
testes100	Equal variances assumed	,001	,973	,271	22	,394	,789	,01875	,06918
	Equal variances not assumed			,271	21,937	,394	,789	,01875	,06918

E5) Changed corticosterone Pre VBS > Post VBS

E5a) Data

To compare the means of changed corticosterone levels for the different ranks and sexes

	Female Most Dominant	Male Most Dominant	Female Most Subordinate	Male Most Subordinate
VBS	CORT change (%)	CORT change (%)	CORT change (%)	CORT change (%)
1	514		-36,89320388	-89,0562249
2	-8,366533865	-38,170347	389,1891892	107,6923077
3	-26,31578947	-9,108910891	463,6363636	-64,5021645
4	79,46428571	-56,59050967	248,8888889	-54,38596491
5	344,115977	7218,681772	263,7957598	7281,681772
6	419,7837434	459,4510794	177,337924	281,5213241
7	503,647877	2223,843284	372,9927585	381,0316839
8	361,3491403	201,9277389	594,8651743	409,0474806
9	88,98010895	211,7648994	7,132511323	-73,23779807
10	-34,27803942	-13,75607175	22,77061709	-13,75607175
11	89,81297938	18,32851986	-33,86497565	18,32851986
12	-28,60266487	14,80626744	27,80485481	-60,75784209

Outliers are indicated with orange, which were excluded from the analysis.

E5b) One-way ANOVA with Benferoni

ANOVA

cortchangeperc

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	155425,750	3	51808,583	1,272	,297
Within Groups	1628938,731	40	40723,468		
Total	1784364,481	43			

E6) Most aggressive and stable colonies

VBS3	M1	M2	M3	M4	F1	F2	F3	F4
adrenal gland	0,0612	0,0626	0,088	0,109	0,0765	0,0945	0,0888	0,0867
thymus	0,213	0,2	0,17	0,093	0,342	0,234	0,363	0,409
retroperitoneal fat	6,711	10,775	7,399	8,881	11,04	5,965	6,193	3,587
seminal vesicle	1,062	1,951	0,908	0,456				
testes	3,14	3,24	3,24	2,988				
CORT change (%)	-9,1	203,3	-31,4	-64,5	-27,9	-4,2	463,6	-26,3
VBS7	M1	M2	M3	M4	F1	F2	F3	F4
adrenal gland	0,06	0,0588	0,0712	0,0566	0,0522	0,063	0,047	0,0597
thymus	0,3426	0,1732	0,1276	0,2769	0,1948	0,1471	0,2561	0,2729
retroperitoneal fat	19,1612	11,0013	5,3356	6,5971	3,5032	3,7962	5,2572	5,6318
seminal vesicle	1,0751	0,8689	0,4556	1,106				
testes	1,8618	1,9203	1,376	2,4328				
CORT change (%)	381,031684		-5,6968547	438,885121	92,6514098	372,992759	503,647877	469,088078
VBS2	M1	M2	M3	M4	F1	F2	F3	F4
adrenal gland	0,0526	0,0609	0,0726	0,0502	0,0735	0,0788	0,0626	0,0703
thymus	0,352	0,186	0,315	0,109	0,238	0,219	0,261	0,389
retroperitoneal fat	10,32	8,815	7,019	15,133	2,955	5,126	6,328	2,982
seminal vesicle	0,857	0,838	1,523	1,358				
testes	3,05	3,25	3,414	3,56				
CORT change (%)	-25,7	107,7	-38,2	-31,1		212,6	389,2	-8,4
VBS6	M1	M2	M3	M4	F1	F2	F3	F4
adrenal gland	0,0574	0,0535	0,0484	0,0613	0,0628	0,057	0,0521	0,0547
thymus	0,2515	0,4355	0,1669	0,369	0,246	0,1923	0,2463	0,316
retroperitoneal fat	17,7114	11,3803	15,7946	13,3486	4,4366	6,1434	4,9581	8,1669
seminal vesicle	0,8112	1,1161	0,8988	0,8658				
testes	1,8669	2,3754	2,0845	2,1802				
CORT change (%)	374,558629	48,7393411	281,521324	459,451079	177,337924	163,747734	419,783743	310,558274

E7) Comparison of the dominance scoring Miguel Puentes vs James Curley

E7a) Tables

weight in grams

	Most Dominant and Subordinate animals by Miguel Puentes' method				Most Dominant and Subordinate animals by James Curley's method			
Adrenal gland weight	Most dominant Female	Most subordinate Female	Most dominant Male	Most subordinate Male	Most dominant Female	Most subordinate Female	Most dominant Male	Most subordinate Male
	0,0858	0,0612	0,0715	0,0468	0,0906	0,0781	0,0715	0,0443
	0,0626	0,0788	0,0609	0,0526	0,0735	0,0626	0,0609	0,0526
	0,0867	0,0888	0,0612	0,109	0,0765	0,0888	0,0612	0,088
	0,0638	0,108	0,0435	0,05	0,0638	0,079	0,0435	0,0581
	0,0598	0,0667	0,0639	0,071	0,0714	0,0598	0,0639	0,071
	0,0521	0,0628	0,0535	0,0484	0,057	0,057	0,0535	0,0574
	0,047	0,063	0,0712	0,06	0,047	0,0597	0,0566	0,06
	0,0502	0,0513	0,046	0,0719	0,0502	0,0513	0,046	0,0479
	0,0847	0,0916	0,0962	0,0928	0,0682	0,0847	0,0962	0,0928
	0,0968	0,0896	0,0875	0,0825	0,0968	0,0896	0,101	0,0659
	0,0735	0,0875	0,094	0,0681	0,0735	0,0881	0,094	0,0787
	0,0956	0,0778	0,0693	0,0787	0,0778	0,0862	0,079	0,0787

	Most Dominant and Subordinate animals by Miguel Puentes' method				Most Dominant and Subordinate animals by James Curley's method			
Thymus weight	Most dominant Female	Most subordinate Female	Most dominant Male	Most subordinate Male	Most dominant Female	Most subordinate Female	Most dominant Male	Most subordinate Male
	0,38	0,339	N/A	0,214	0,308	0,278	N/A	0,226
	0,261	0,219	0,186	0,352	0,238	0,261	0,186	0,352
	0,409	0,363	0,213	0,093	0,342	0,363	0,213	0,17
	0,261	0,365	0,212	0,337	0,261	0,457	0,212	0,229
	0,3003	0,2135	0,1765	0,4062	0,3819	0,3003	0,1765	0,4062
	0,2463	0,246	0,4355	0,1669	0,1923	0,1923	0,4355	0,2515
	0,2561	0,1471	0,1276	0,3426	0,2561	0,2729	0,2769	0,3426
	0,2148	0,2366	0,2504	0,2409	0,2148	0,2366	0,2504	0,3829
	0,3727	0,1429	0,1688	0,2177	0,3348	0,3727	0,1688	0,2177
	0,2442	0,2291	0,0952	0,2232	0,2442	0,2291	0,1601	0,2662
	0,1715	0,2899	0,2395	0,2501	0,1715	0,3479	0,2395	0,2361
	0,2521	0,2754	0,1892	0,3292	0,2754	0,2736	0,1379	0,3292

	Most Dominant and Subordinate animals by Miguel Puentes' method				Most Dominant and Subordinate animals by James Curley's method			
Retroperitoneal	Most dominant Female	Most subordinate Female	Most dominant Male	Most subordinate Male	Most dominant Female	Most subordinate Female	Most dominant Male	Most subordinate Male
	3,104	7,141	7,878	7,124	4,581	5,977	7,878	7,583
	6,328	5,126	8,815	10,32	2,955	6,328	8,815	10,32
	3,587	6,193	6,711	8,881	11,04	6,193	6,711	7,399
	4,4	8,641	8,009	16,512	4,4	4,734	8,009	12,45
	5,8089	4,411	8,7452	18,7069	4,9144	5,8089	8,7452	18,7069
	4,9581	4,4366	11,3803	15,7946	6,1434	6,1434	11,3803	17,7114
	5,2572	3,7962	5,3356	19,1612	5,2572	5,6318	6,5971	19,1612
	3,0032	3,4404	6,9798	10,8156	3,0032	3,4404	6,9798	8,5886
	12,2892	9,0412	18,2004	19,0532	5,8614	12,2892	18,2004	19,0532
	12,1155	7,4332	15,7414	9,8142	12,1155	7,4332	9,8006	8,547
	11,649	5,9615	9,065	18,303	11,649	12,8858	9,065	12,6411
	5,7515	10,8411	13,8026	14,4352	10,8411	12,161	14,2647	14,4352

Most Dominant and Subordinate animals by Miguel Puentes' method			Most Dominant and Subordinate animals by James Curley's method	
Seminal vesicle	Most dominant Male M	Most subordinate Male M	Most dominant Male C	Most subordinate Male C
	3,41	3,32	3,406	3,276
	3,25	3,05	3,25	3,05
	3,14	2,99	3,14	3,24
	3,19	3,6	3,19	3,29
	2,42	2,23	2,4152	2,225
	2,38	2,08	2,3754	1,8669
	1,38	1,86	2,4328	1,8618
	2,7	2,65	2,6961	2,2398
	4,03	2,38	4,0323	2,3781
	3,56	3,92	3,5958	3,6867
	4,3	3,41	4,3001	3,643
	3,58	3,34	3,5215	3,3366

Most Dominant and Subordinate animals by Miguel Puentes' method			Most Dominant and Subordinate animals by James Curley's method	
Testes weight	Most dominant Male M	Most subordinate Male M	Most dominant Male C	Most subordinate Male C
	1,83	1,45	1,832	1,26
	0,84	0,86	0,838	0,857
	1,06	0,46	1,062	0,908
	1,21	0,78	1,213	0,855
	1,18	0,91	1,1769	0,9131
	1,12	0,9	1,1161	0,8112
	0,46	1,08	1,106	1,0751
	1,38	1,08	1,3799	1,3814
	0,95	0,86	0,9501	0,8635
	0,92	1,34	0,7937	1,0511
	1,31	0,95	1,3127	1,1744
	1,46	1,56	1,0602	1,5596

E7b) Statistics

To determine if there is any significant difference between the weights of the determined most dominant and subordinate animals of both scoring methods, an independent T-test is performed.

Puentes' method Female Most Dominant vs Curley's method Female Most Dominant

Group Statistics

	1=DomFemale, 2=DomMale, 3=SubFemale, 4=SubMale, 5=DF Curley, 6=DM Curley, 7=SF Curley, 8=SM Curley	N	Mean	Std. Deviation	Std. Error Mean
adrenal	1.00	12	.0716	.01794	.00518
	5.00	12	.0705	.01477	.00426
thymus	1.00	12	.2808	.07145	.02063
	5.00	12	.2683	.06331	.01828
fat	1.00	12	6.5210	3.47983	1.00454
	5.00	12	6.8968	3.47843	1.00414
vesicle	1.00	0[a]	.	.	.
	5.00	0[a]	.	.	.
testes	1.00	0[a]	.	.	.
	5.00	0[a]	.	.	.

a. t cannot be computed because at least one of the groups is empty.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means					
						Significance			
		F	Sig.	t	df	One-Sided p	Two-Sided p	Mean Difference	Std. Error Difference
adrenal	Equal variances assumed	1.787	.195	.153	22	.440	.880	.00102	.00671
	Equal variances not assumed			.153	21.219	.440	.880	.00102	.00671
thymus	Equal variances assumed	.171	.683	.451	22	.328	.657	.01242	.02756
	Equal variances not assumed			.451	21.686	.328	.657	.01242	.02756
fat	Equal variances assumed	.135	.717	-.265	22	.397	.794	-.37580	1.42035
	Equal variances not assumed			-.265	22.000	.397	.794	-.37580	1.42035

Puentes' method Male Most Dominant vs Curley's method Male Most Dominant

Group Statistics

	1=DomFemale, 2=DomMale, 3=SubFemale, 4=SubMale, 5=DF Curley, 6=DM Curley, 7=SF Curley, 8=SM Curley	N	Mean	Std. Deviation	Std. Error Mean
adrenal	2.00	12	.0682	.01728	.00499
	6.00	12	.0689	.01959	.00565
thymus	2.00	11	.2085	.08783	.02648
	6.00	11	.2233	.08184	.02467
fat	2.00	12	10.0553	3.93763	1.13670
	6.00	12	9.7038	3.43995	.99303
vesicle	2.00	12	1.1425	.34735	.10027
	6.00	12	1.1534	.27505	.07940
testes	2.00	12	3.1100	.79798	.23036
	6.00	12	3.1963	.62884	.18153

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means					
		F	Sig.	t	df	Significance One-Sided p	Significance Two-Sided p	Mean Difference	Std. Error Difference
adrenal	Equal variances assumed	.458	.505	-.095	22	.463	.925	-.00072	.00754
	Equal variances not assumed			-.095	21.662	.463	.925	-.00072	.00754
thymus	Equal variances assumed	.000	.994	-.409	20	.343	.687	-.01481	.03620
	Equal variances not assumed			-.409	19.901	.343	.687	-.01481	.03620
fat	Equal variances assumed	.562	.462	.233	22	.409	.818	.35143	1.50936
	Equal variances not assumed			.233	21.610	.409	.818	.35143	1.50936
vesicle	Equal variances assumed	.528	.475	-.085	22	.467	.933	-.01087	.12790
	Equal variances not assumed			-.085	20.902	.467	.933	-.01087	.12790
testes	Equal variances assumed	.365	.552	-.294	22	.386	.771	-.08625	.29329
	Equal variances not assumed			-.294	20.860	.386	.772	-.08625	.29329

Puentes' method Female Most Subordinate vs Curley's method Female Most Subordinate

Group Statistics

	1=DomFemale, 2=DomMale, 3=SubFemale, 4=SubMale, 5=DF Curley, 6=DM Curley, 7=SF Curley, 8=SM Curley	N	Mean	Std. Deviation	Std. Error Mean
adrenal	3.00	12	.0773	.01651	.00477
	7.00	12	.0737	.01448	.00418
thymus	3.00	12	.2555	.07433	.02146
	7.00	12	.2987	.07401	.02137
fat	3.00	12	6.3718	2.30684	.66593
	7.00	12	7.4188	3.18114	.91832
vesicle	3.00	0[a]	.	.	.
	7.00	0[a]	.	.	.
testes	3.00	0[a]	.	.	.
	7.00	0[a]	.	.	.

a. t cannot be computed because at least one of the groups is empty.

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means					
		F	Sig.	t	df	Significance One-Sided p	Significance Two-Sided p	Mean Difference	Std. Error Difference
adrenal	Equal variances assumed	.031	.862	.555	22	.292	.585	.00352	.00634
	Equal variances not assumed			.555	21.630	.292	.585	.00352	.00634
thymus	Equal variances assumed	.005	.945	-1.425	22	.084	.168	-.04316	.03028
	Equal variances not assumed			-1.425	22.000	.084	.168	-.04316	.03028
fat	Equal variances assumed	1.052	.316	-.923	22	.183	.366	-1.04696	1.13436
	Equal variances not assumed			-.923	20.063	.183	.367	-1.04696	1.13436

Puentes' method Male Most Subordinate vs Curley's method Male Most Subordinate

Group Statistics

	1=DomFemale, 2=DomMale, 3=SubFemale, 4=SubMale, 5=DF Curley, 6=DM Curley, 7=SF Curley, 8=SM Curley	N	Mean	Std. Deviation	Std. Error Mean
adrenal	4.00	12	.0693	.01927	.00556
	8.00	12	.0663	.01566	.00452
thymus	4.00	12	.2644	.09016	.02603
	8.00	12	.2841	.07523	.02172
fat	4.00	12	14.0767	4.44338	1.28269
	8.00	12	13.0497	4.66141	1.34563
vesicle	4.00	12	1.0176	.30696	.08861
	8.00	12	1.0591	.24021	.06934
testes	4.00	12	2.9020	.65489	.18905
	8.00	12	2.8412	.67827	.19580

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means					
						Significance			
		F	Sig.	t	df	One-Sided p	Two-Sided p	Mean Difference	Std. Error Difference
adrenal	Equal variances assumed	.265	.612	.423	22	.338	.676	.00303	.00717
	Equal variances not assumed			.423	21.121	.338	.676	.00303	.00717
thymus	Equal variances assumed	.296	.592	-.582	22	.283	.567	-.01972	.03390
	Equal variances not assumed			-.582	21.316	.283	.567	-.01972	.03390
fat	Equal variances assumed	.007	.936	.552	22	.293	.586	1.02703	1.85904
	Equal variances not assumed			.552	21.950	.293	.586	1.02703	1.85904
vesicle	Equal variances assumed	.412	.528	-.369	22	.358	.716	-.04150	.11252
	Equal variances not assumed			-.369	20.798	.358	.716	-.04150	.11252
testes	Equal variances assumed	.225	.640	.224	22	.413	.825	.06088	.27217
	Equal variances not assumed			.224	21.973	.413	.825	.06088	.27217

E8) Spine density

To compare the means of the ranks and sexes of the different distances (10-80 µm)

E8a) Table – amount of spines (mean) per µm

	SUB-M CA1								DOM-M CA1							
Animal↓/Distance (µm)→	10	20	30	40	50	60	70	80	10	20	30	40	50	60	70	80
1	10,2	10,8	9,8	8,8	10,2	10,2	8,6	9,8	9,2	9,6	11,2	10	10,4	9,4	10,4	8,6
2	8,8	10,2	12,2	12,6	11,6	9,6	10,8	9,6	10,4	10,6	9,4	9,8	10,2	9,4	9	8,4
3	8,4	8,6	9,2	8,4	10,4	9,2	10,2	9	7,4	9,8	10,2	11,4	11,6	9,8	9	7,8
4	8,2	7,4	8	9,2	10,6	10,6	8,8	9,6	7	8,6	9,8	8,6	8,4	9,2	9	8,6
5	9	10,2	13	10,8	11	9,8	10,4	11,8	9	8,2	10,4	7,8	10,6	8,6	10	8,6
6	12,8	12,2	12	11,2	10,2	11	10,2	12,6	13,6	12,8	13,4	11,2	11,4	12	11	11,6
	F-DOM CA1								F-SUB CA1							
1	9,8	12,2	10,2	10,4	9,4	10,2	9,2	11,2	5,4	10,2	7,2	12,8	9	9,2	7	8,8
2	9,4	8,8	12	9,8	9,4	9,8	8,2	9	11,2	9,6	6,6	7,6	9,6	9,6	10,6	10,4
3	8,4	9,4	9,6	10,2	8,4	8,8	8,6	7,4	7	8	9,5	10	11,5	11	9,25	8,75
4	7,2	7,6	8,2	9,2	8,8	9	10	8,2	8	8,5	8,5	10,5	8,5	8,5	8	8
5	10,8	10,8	10,2	10,2	10,8	9,4	9,6	8,2	8,2	8,6	10,2	13	10,4	10,4	11,6	9,4
6	12,6	10,4	10,6	10,2	10	11,4	9,6	10	11	11,4	10,8	10,4	10,8	13,4	10	11,6

E8b) Statistics – one way ANOVA to determine if there is any difference in the number of spines between the distances (µm)

One-way ANOVA with Benferoni

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
ca1domf	Between Groups	6,463	7	,923	,620	,737
	Within Groups	59,613	40	1,490		
	Total	66,077	47			
ca1domm	Between Groups	13,023	7	1,860	,839	,562
	Within Groups	88,733	40	2,218		
	Total	101,757	47			
ca1subf	Between Groups	23,747	7	3,392	1,211	,320
	Within Groups	112,093	40	2,802		
	Total	135,840	47			
ca1subm	Between Groups	6,846	7	,978	,487	,838
	Within Groups	80,327	40	2,008		
	Total	87,173	47			
bladomf	Between Groups	10,379	7	1,483	,461	,856
	Within Groups	128,540	40	3,214		
	Total	138,919	47			
bladomm	Between Groups	17,267	7	2,467	1,500	,195
	Within Groups	65,760	40	1,644		
	Total	83,027	47			
blasubf	Between Groups	8,490	7	1,213	,644	,717
	Within Groups	75,347	40	1,884		
	Total	83,837	47			
blasubm	Between Groups	41,077	7	5,868	6,317	<,001
	Within Groups	37,160	40	,929		
	Total	78,237	47			
ca3fdom	Between Groups	11,490	7	1,641	,751	,631
	Within Groups	87,467	40	2,187		
	Total	98,957	47			
ca3mdom	Between Groups	15,739	7	2,248	1,376	,242
	Within Groups	65,340	40	1,634		
	Total	81,079	47			
ca3fsub	Between Groups	9,206	7	1,315	,426	,880
	Within Groups	123,353	40	3,084		
	Total	132,559	47			
ca3msub	Between Groups	7,419	7	1,060	,676	,691
	Within Groups	62,713	40	1,568		
	Total	70,133	47			

blasubm	10,00	20,00	-,60000	,55648	1,000	-2,4627	1,2627
		30,00	-1,13333	,55648	1,000	-2,9960	,7294
		40,00	-1,73333	,55648	,095	-3,5960	,1294
		50,00	-1,23333	,55648	,908	-3,0960	,6294
		60,00	-2,13333*	,55648	,012	-3,9960	-,2706
		70,00	-2,83333*	,55648	<,001	-4,6960	-,9706
		80,00	-2,66667*	,55648	<,001	-4,5294	-,8040
	20,00	10,00	,60000	,55648	1,000	-1,2627	2,4627
		30,00	-,53333	,55648	1,000	-2,3960	1,3294
		40,00	-1,13333	,55648	1,000	-2,9960	,7294
		50,00	-,63333	,55648	1,000	-2,4960	1,2294
		60,00	-1,53333	,55648	,246	-3,3960	,3294
		70,00	-2,23333*	,55648	,007	-4,0960	-,3706
		80,00	-2,06667*	,55648	,017	-3,9294	-,2040

E8c) To compare the most dominant and subordinate animals of the CA1, BLA and CA3 regions.

Data- mean of spines total per animal, per rank and sex

	animal	SUB-M	DOM-M	SUB-F	DOM-F
CA1	1	78,4	78,8	69,6	81,8
	2	85,4	77,2	75,2	76,4
	3	73,4	77	76,6	70,8
	4	72,4	69,2	66,8	68,2
	5	86	73,2	88,8	80
	6	92,2	97	89,4	84,8
BLA	1	76,2	58,8	76,2	69,6
	2	71,8	67,2	75	65,6
	3	66	72,6	81,8	82,8
	4	70	68,2	59,2	65,2
	5	74,2	71	79,4	78,4
	6	71	67,0666667	70,4	85,2
CA3	1	57,6	58,4	55	56,8
	2	52,6	61,8	49,2	58,4
	3	60,4	51	54	58,6
	4	46,8	57	30,4	41,2
	5	51,6	64,4	64	63,4
	6	46	63,8	59,2	66

E8d) One-way ANOVA with Benferoni

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Ca1	Between Groups	63,512	3	21,171	,296	,828
	Within Groups	1429,367	20	71,468		
	Total	1492,878	23			
bla	Between Groups	176,085	3	58,695	1,315	,297
	Within Groups	892,395	20	44,620		
	Total	1068,480	23			
ca3	Between Groups	241,020	3	80,340	1,190	,339
	Within Groups	1349,853	20	67,493		
	Total	1590,873	23			