A Model of Stress or Adaptation?

The effects of the dominance hierarchy on (chronic) stress, behavior, physiology, and neurobiology in Wild Type Groningen rats

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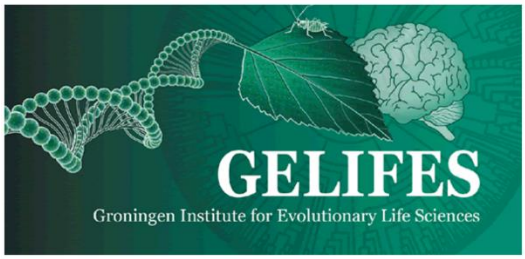

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Abstract:

Introduction: Throughout life we are exposed to stressful challenges, ranging from daily hassles to severe traumatic events. However, only a small percentage of individuals will develop stress-related disorders. Social rank is one of the factors influencing individual susceptibility to the development of psychopathology. An ethologically valid model to study the effects of social rank on behavior, physiology, and neurobiology is the Visible Burrow System (VBS), a model that mimics the natural environment of rats. Social stress causes structural and functional remodeling of neuronal dendrites and spines in brain areas involved with stress perception and adaptation, such as the hippocampal CA1 and CA3, the basolateral amygdala (BLA), and the medial prefrontal cortex (mPFC). The aim of this study was to enhance our understanding of the relationship between social rank, sex, and the associated physiological and neurobiological alterations. A second aim was to validate the Wild Type Groningen (WTG) rat strain as a good model for social stress studies, and the last aim was to see which one of two behavioral scoring methods is better for the determination of dominance hierarchies.

Method: 48 male and 48 female adult WTG rats of minimally 6 months old were divided over 12 VBS', each being comprised of 4 males and 4 females. They were housed together for 10 days in the VBS and all interactions were videotaped. The formation and maintenance of the social hierarchy was assessed by scoring agonistic interactions between all rats in two different manners, and validated by scoring their location preference (open area vs burrows). The consequences of social rank on the brain were assessed by a morphological analysis on the number of spines, using Golgi-Cox staining, for the CA1, CA3, BLA, and mPFC. Moreover, we looked at colony characteristics to see whether female dominance changes the stability or average aggression levels of the colony, and how changes in body weight and corticosterone levels are mediated by colony characteristics.

Results: Both scoring methods show very similar results, with only a difference in the number of colonies were a female becomes most dominant. There was a clear difference in agonistic behavior between dominant and subordinate individuals for both males and females, which was only validated by location preference in males. We found no clear differences for the stress-associated physiological variables, such as corticosterone levels, body weight, adrenal weight, thymus weight, testes weight, and seminal vesicle weight. Moreover, we found no difference in spine number for the CA1, CA3, and BLA, for both males and females, but we did find a strong difference in the mPFC. At last, we found that mPFC spines was significantly correlated with time spend in the open arena of the VBS.

Discussion: These findings suggest that subordinate and dominant individuals in this study experienced similar levels of stress during the VBS housing, which is in contrast to results found in VBS studies suing Long Evans (LE) rats. This suggests that whereas the high levels of aggression found in LE rats cause subordination to be very stressful, the lower and more variable levels of aggression in WTG rats allow them to adapt to the dominance hierarchy, with most individuals 'accepting' their position. Moreover, since we only found spine remodeling in the mPFC, we hypothesize that this neural difference existed (partially) prior to the colony housing, with rats that show a 'more developed' mPFC being more able to fight for and achieve higher dominance rank. As such, the WTG strain shows potential to be used to investigate (mal)adaptive behavior to psychosocial stressors. Although females are crucial for the establishment of a dominance hierarchy, they do not seem to influence dominance positions of males.

Abstract

Only few individuals develop stress-related disorders. It is thought that lasting subordination results in higher psychosocial stress than high-ranked males, but little is known for female rats. Subordination stress can be assessed by the Visible Burrow System (VBS), a model that mimics the natural environment of rats. The aim of this study is to investigate the relationship between dominance rank, sex, and behavior on stress-induced neuronal (mal) adaptation in Wilde Type Groningen (WTG) rats, and to compare two behavioral scoring methods. 48 male and 48 female adult WTG rats were divided over 12 VBS' (4 males + 4 females) and housed for 10 days in the VBS. All agonistic interactions were videotaped and scored (in two ways) to determine the dominance hierarchy. Consequences of social rank were assessed in body and organ weight changes, including corticosterone levels, as well as spine number changes in the basolateral amygdala (BLA), hippocampal fields (CA1 & CA3), and medial prefrontal cortex (mPFC), using Golgi-Cox staining. Both scoring methods showed very similar results, with only female dominance being different. Dominance rank showed a clear effect on agonistic behavior (with a clear dominance hierarchy after 3 days) and mPFC spines, but not on organ and body weight, corticosterone, or spines in the CA1, CA3, and BLA. These findings suggest that WTG subordinates and dominants experience similar stress in the VBS, contrasting previous studies using Long Evans rats. We hypothesized that lower aggression levels in WTG rats allow them to adapt to the dominance hierarchy, with mPFC spine number being associated with agonistic dominance behavior and not stress.

List of Abbreviations

SAM: sympathetic adrenomedullary system

HPA: hypothalamic-pituitary-adrenal axis

ForF: fight-or-flight response

CORT: corticosteroids

CRH: corticotropin-releasing hormone

ACTH: Adrenocorticotropic hormone

SES: socioeconomic status

UVS: unpredictable variable stress model

VBS: Visible Burrow system

WTG: Wilde Type Groningen rat

LE: Long-Evans rat

MDD: major depressive disorder

BD: bipolar disorder

mPFC: medial prefrontal cortex

PL: prelimbic cortex

IL: infralimbic cortex

CA1: cornu ammonius field 1

CA3: cornu ammonius field 3

BLA: basolateral complex of the amygdala

CeA: central nucleus of the amygdala

BNST: bed nucleus of the stria terminalis

ApD: apical dendrites

FAT: retroperitoneal fat

DOM: most dominant ranked rats (per colony)

SUB: most subordinate ranked rats (per colony)

INT: intermediate ranked rats

Curley's method: the scoring method for agonistic interactions as implemented by Curley, Lee and Williamson (2016)

Miguel's method: the scoring method for agonistic interactions as implemented by Escamilla et al. (unpublished data, 2020)

Table of Content

1. Introduction

1.1. What is stress?

Throughout life we are exposed to stressful challenges, ranging from daily hassles to severe traumatic events. Traditionally, stress is defined as the behavioral and physiological response of the body towards a non-specific noxious stimulus, and the external causes of stress are called 'stressors' (Selye, 1950). In the face of a perceived threat or direct physical challenge to homeostasis, the brain activates many neuronal circuits to respond adaptively to the situation with three main biological mechanisms being employed in the stress response to restore the body to homeostasis: the sympathetic adrenomedullary (SAM) system, an inflammatory response, and the hypothalamic-pituitary-adrenal (HPA) axis (McEwen, 2007). Within seconds after stress exposure, the SAM gets activated by the hypothalamus and signals to the adrenal medulla to produce adrenaline and noradrenaline, both contributing to the infamous fight-or-flight (ForF) response. By increasing the heart rate and blood pressure, the ForF response prepares the individual for action readiness, whether it is running away from a predator, fighting for limited resources, or asking for a raise from a tyrannical boss. Because of potential injury or infection during the ForF response, the brain also elicits an inflammation response to fight off possible pathogens (Morey, Boggero, Scott & Segerstrom, 2015).

In parallel, the HPA axis is activated, although much slower. Its end-product, corticosteroids (CORT's), are only in full swing after 10-15 minutes and slowly decline to normal pre-stress levels 60 minutes later (de Kloet, Joëls & Holsboer, 2005). Although having a very similar function, the main CORT for humans is cortisol and for rodents corticosterone. CORT sustains the organism to respond appropriately to the stressor by mobilizing metabolic resources to organs contributing to the ForF response, such as exercising muscle and increasing cardiovascular tone. The HPA response starts when neural pathways activate the secretion of corticotropin-releasing hormone (CRH) from the hypothalamus (specifically the paraventricular nucleus). CRH in turn activates the anterior pituitary to secrete adrenocorticotropic hormone (ACTH), which enters the blood circulation and activates the adrenal cortex. Upon activating the adrenal cortex, CORT's are synthesized and secreted into the circulation to reach every organ, allowing adaptive coordination of brain and body functions aimed at coping with the stressor, recovery, and adaptation (McEwen, 2007). In healthy individuals, CORT secretion goes back to homeostasis, either because the threat has passed or has been deemed undangerous. Moreover, the stress response resides due to negative feedback, since CORT inhibits the activity of the HPA axis at the level of the hypothalamus and the pituitary gland, but also inhibits the activation of the SAM system (de Kloet, Joëls & Holsboer, 2005).

Besides reacting to a direct challenge to homeostasis, most mammals can also anticipate that a challenge to homeostasis looms. Based on prior experience, animals can predict an upcoming stressor and prepare the body to optimally deal with the potential stressor. This adaptive process is referred to as 'allostasis', which is the mechanism of changing physiological parameters to the predicted level that would meet the anticipated demand (Sterling, 2012). Allostasis is essential to maintain homeostasis and is often summarized as 'stability through change'. However, when the stress response is chronically activated by prolonged (perceived) stressors, this adaptive allostasis comes with a price. When the systems involved with the stress response are overworked and fail to 'shut off', the changes in physiological parameters are also prolonged. If this physiological state, originally aimed to protect homeostasis, remains active for a prolonged time, it can actually 'wear out' and 'tear down' the body, bringing it further away from homeostasis. These long-term consequences of 'wear and tear' on the body are collectively referred to as the 'allostatic load', which is associated with an increased risk to develop cardiovascular, metabolic, and psychiatric diseases (McEwen, 1998).

Thus, for the short-term, the stress response allows the individual to adaptively cope with the stressor (real or implied) - allocating metabolic resources to organs contributing to the ForF response - but once the stressor chronically activates the stress response, metabolic resources are exhausted and the corresponding allostatic load accelerates a range of pathophysiological processes. A perfect example of the detrimental effects of allostatic load on the body is the immune system. Acute stress stimulates the immune system, whereas stressors that are active for prolonged times, with chronically increased levels of CORT in the blood circulation, causes the immune system to be suppressed and less adaptive to present demands (Dhabhar & McEwen, 1997).

At last, it should be noted that the physiological 'readouts' of the stress response are often similar to the physiological response seen in individuals anticipating appetitive and rewarding stimuli (Buwalda, de Boer, Coppens & Koolhaas, 2012). Moreover, whether a stimulus is perceived as a threat depends in part on the cognitive appraisal of the individual (Koolhaas, de Boer, Buwalda & Meerlo, 2017). Thus, what makes an aversive stimulus a stressor is not its physical nature perse, but rather the degree in which the individual can predict and/or control the stimulus. These caveats lead to a recent reformulation of the stress concept, including not only the behavioral and physiological response, but also the nature of the stimulus and the perceptual processing of the individual (Koolhaas et al., 2011; Koolhaas et al., 2017). As such, an individual is 'stressed' when the demands of the stressor exceed its natural regulatory capacity to cope with it.

Stressors can be psychosocial and arise from a social context. Since this usually includes the anticipation of a challenge to homeostasis, justified or not, and since it is difficult to directly investigate the relationship between neuropathology and social stress in humans, it is important to find reliable and consistent ways in which we can investigate social (perceived) stress in animal models. One such reliable framework, as will become clear below, is the use of dominance hierarchies.

1.2. Dominance Hierarchies

Most animal species, such as fish, birds, mammals, primates, and humans, spend most of their waking hours among conspecifics. Animals that live in social groups naturally form linear dominance hierarchies in both the wild and in laboratory settings, making dominance hierarchies one of the most well-studied forms of social organization (Chase & Seitz, 2011). Hierarchies are formed when there is competition for resources such as food, water, mates, and territory and each member tries to maximize its control over access to resources (Chase, Tovey, Spangler-Martin & Manfredonia, 2002). A dominance relationship is established between two individuals if one consistently shows aggressive behavior (like chasing, threatening, or biting) towards the other, with little to no aggression back. In most social species, the degree in which individuals are exposed to physical and psychosocial stressors depends on their dominance rank (Sapolsky, 2005). As such, dominance rank may function as a risk factor for the development of stress-related disorders in both animals and humans. Since each aggressive conflict brings a risk of injury, it is beneficial for members of the social group to recognize and adapt to their social rank once it is established (Chase & Seitz, 2011). When subordinate individuals show behavior aimed to de-escalate future aggression, a stable dominance hierarchy is formed (Drew, 1993). Generally, dominance hierarchies are considered to be evolved social structures for optimal survival, health, and reproduction, as evidenced by their species-wide occurrence and the fact that, when stable, the individuals within the dominance hierarchy usually show no clear signs of stress (Sapolsky, 2004).

However, most social groups are quite dynamic, and individuals must re-establish their dominance rank over time. Despite dominance hierarchies being evolutionary adaptive, dominance hierarchies can also be a great source of social stress, depending on the specific organization of the social group. Whether high- or low-ranking individuals experience the most stress depends on many

factors, such as the stability of social ranks, the ability of subordinates to avoid dominant individuals, and whether the maintenance of dominance is done through psychological intimidation or aggressive behavior (Sapolsky, 2005). Nevertheless, within most species it is typically the lower rank that experiences most physical and psychosocial stressors, which translates into lower survival rates and more severe stress-related pathologies (Sapolsky, 2005). In other words, the position one holds within a dominance hierarchy can potentially increase the vulnerability for the development of stress-related disorders.

The underlying mechanisms by which a linear dominance hierarchy is established are since long under debate. According to the '*Prior attribute hypothesis'*, dominance relationships arise due to intrinsic differences, or attributes, between the individuals (Hemelrijk, Wantia & Isler, 2008). Characteristics such as body weight, age, baseline aggression, or sex, determine the social rank one will obtain. For example, studies have found that higher plasma testosterone levels influence behavior and intermale aggression, increasing the likelihood of dominance (Albert, Jonik, Watson, Gorzalka & Walsh, 1990; Albert, Walsh, Gorzalka, Siemens & Louie, 1986). However, prior traits of aggressiveness do not always predict dominance status (Buwalda, Koolhaas & de Boer, 2017a). Accordingly, another hypothesis, called the *'Self-organization hypothesis'*, suggests that the formation of a dominance hierarchy results from self-reinforcing mechanisms of winning or losing fights, even in the absence of pre-existing differences (Theraulaz, Bonabeau & Deneubourg, 1995, 1999; Hemelrijk et al., 2008). Having lost a fight, an individual is more likely to also lose the next fight and vice versa for winning. This phenomenon is called the '*winner-loser'* effect and its causation is not certain. It may be the result of endocrine changes in the individual post-fight, as studies have shown that losing rats show increased levels of basal plasma CORT and decreased levels of testosterone (Bernstein, Gordon & Rose, 1983; Tamashiro et al., 2004; McKittrick, Blanchard, Hardy & Blanchard, 2009). However, it may also be caused by a change in perception of one's own fighting capabilities, as it has been shown that a losing animal that showed submission without first countering the attack from the dominant individual, showed much stronger behavioral and physiological consequences than a losing-animal that did fight back before subjugation (Meerlo et al., 1999; Wood et al., 2010; Rod et al., 2014).

At first sight, linear dominance hierarchies may seem irrelevant for studying social-induced stress pathologies in humans. Humans do not usually live in only one social group but belong to multiple hierarchies: at home, at work, with friends, or even on the internet. However, most westernized societies show that dominance rank, which in humans is most akin to socioeconomic status (SES), can predict patterns of disease. Compared to the high end of the SES gradient, the lower end is associated with increased percentages of people with somatic diseases and affective disorders (Adler & Ostrove, 1999). Importantly, these differential health patterns remain robust when differences in lifestyle (such as drinking and smoking), access to healthcare, and basic income are controlled for (Siegrist & Marmot, 2004; Pickett & Wilkinson, 2015). Interestingly, areas with higher perceived income inequality show higher incidences of stress-related diseases than areas with less income inequality, even when most people have objectively lower SES (Adler et al., 1999; Pickett & Wilkinson, 2015). In other words, subjective SES predicts health differences more accurately than objective SES does, suggesting that psychosocial factors are largely responsible for the SES gradient.

1.3. Towards a relevant model: The Visible Burrow System

Traditionally, stress research with animals has been done by experimentally exposing animals to non-social, noxious physical stimuli. These physical stressors include, among others, electrical foot shocks, forced swimming, or immobilization stress. Since rodents can habituate to predictable stimuli, the 'unpredictable variable stress' (UVS) model is often used that implements systematic exposure to multiple types of physical stressors at unpredictable times during the day and week. However, rather

than physically painful and unusual stimuli, most stimuli that humans experience, and that usually lead to stress-related psychopathologies, arise from a social context (Hidaka, 2012). Hence, during the last two decades, there has been an attentional shift away from traditional non-social stress models towards paradigms that make use of social defeat stress. Two commonly used social defeat models are the resident-intruder paradigm (Koolhaas et al., 2013), which involves physical fights between two animals and subsequent single housing of the defeated rat; and the sensory contact model (Kudryavtseva, 1991), where two rats are housed together with a transparent partition separating them and that at unpredictable times is briefly removed to allow for social defeat exposure.

Notwithstanding the usefulness of these social defeat models in investigating neural and endocrine effects of dominance and subordination, they are limited by the contextual and temporal situation as arranged by the investigator. These models still entail a certain degree of artificially induced procedures to cause stress, albeit social. As such, they do not allow for investigating the development of dominance relationships over time, how these relationships are modified by the larger context of the social group, and the associated neural consequences of dominance rank (Williamson, Lee & Curley, 2016). These caveats make these models less translatable to humans, who live in very complex and multiple stress-inducing hierarchies. It is likely that the more a social stress model resembles the typical stressors experienced in daily life, the better its biological relevance is for human stress-related psychopathologies. Fortunately, the use of social environments as a model to investigate social stress and its neural and physiological correlates has gained renewed interest in the last two decades. The role of social hierarchies as possible stressors in brain research became appreciated only after the marriage between the work of Bruce McEwen and Randall Sakai, investigating the effects of stress on the brain, and that of Bob and Caroline Blanchard, who introduced the world to the Visible Burrow System (VBS) paradigm.

In the Visible Burrow system (VBS) paradigm, a colony system that mirrors the natural environment in which wild rats live, rats seem to produce natural, stress-provoking hierarchies (Blanchard et al., 1995). Animals within the VBS are housed in an open arena with a diurnal photoperiod, which is connected to a system of tunnels and chambers in which rats are visible (e.g. via infrared camera's), even though the tunnels are continuously dark. The animals are not exposed to stressors that are at predictable times of the day, nor are they exposed to unusual physical stressors like inescapable shock stress, restraint stress, or forced swimming stress. They are only exposed to the presence of other members of the social group, with whom they try to fight for - and maintain - a certain dominance rank. Since the stressors within this model are much like the psychosocial stressors experienced by humans in their daily lives, the model shows high translational value to investigate the effects of social dominance and subordination on neural and endocrine correlates of chronic social stress (Blanchard, Sakai, McEwen, Weis & Blanchard, 1993; Blanchard et al., 1995; Spencer et al., 1996; McKittrick et al., 2000; Tamashiro et al., 2004). As such, The VBS seems to be a sound neuroethological animal model to study how neuroendocrine systems regulate stress and energy homeostasis.

Within a few days of VBS housing, males reliably form dominance hierarchies, with the dominant and subordinate rats showing rank-specific patterns of offensive and defensive behavior (Blanchard et al., 1993). Dominant individuals generally control access to resources such as water, food, and females by portraying aggressive behaviors aimed towards rats lower in dominance rank, whereas subordinate rats frequently show submissive behaviors and decreased social contact. These behavioral differences are generally translated into heightened mortality rates for subordinate rats, as compared to dominant rats and unstressed controls. Interestingly, this appears to be a consequence of stress-induced physiological alterations rather than lethal wounding (Barnett, 1958; Blanchard & Blanchard, 1990). Because of the heightened mortality, VBS studies generally do not last longer than two weeks.

During VBS housing, dominant individuals typically maintain their body weight, whereas subordinate rats show a significant amount of weight loss and increased plasma CORT levels (Blanchard et al., 1993; Blanchard et al., 1995; Spencer et al., 1996; McKittrick et al., 2000; Tamashiro et al., 2004). Higher CORT secretion into the bloodstream affects a host of organs and tissues in the body, which is in most cases rank dependent. Compared to dominant individuals and unstressed controls, subordinate males show lower plasma levels of testosterone, lower testes weight, lose more adipose tissue (but retain higher percentage of visceral fat), and showed more decreased thymus weight. Dominant males also often show an increase in plasma CORT and a decrease in bodyweight and thymus weight, albeit not as substantial as subordinate males. Usually, adrenal weight seems to be equally increased in dominant and subordinate males.

1.4. Females

Like wild rat colonies, a VBS colony generally consists of mixed sex groups. A VBS colony usually contains 4 males and 2 female Long-Evans rats, which is also known as the "STANDARD" colony (McEwen et al., 2015). The inclusion of females in VBS studies is usually seen as another resource for which males can compete. This is because in males-only colonies, males do not readily form a dominance hierarchy, nor do they show different behavioral and physiological profiles (assuming that there is plenty of food and water) (Flannelly & Lore, 1977; Tamashiro et al., 2004). Indeed, previous studies have shown that colony housing of males together with females increases aggressive behavior among males, but also between females and subordinate males, suggesting that the sex composition of the colony changes the degree of stress experienced by males (Barnett, 1958; Barnett, Evans & Stoddart, 1968; Barr, 1981). Still, however, females show relatively little aggression, and as a result, there is little research done that investigates the stress-induced behavioral and physiological consequences in VBS-housed females.

Since social stress-related psychopathologies are more common among women than men, it is unfortunate that social stress models have traditionally focused on only males (Takahashi, Flanigan, McEwen & Russo, 2018). The last decade has seen a surge of interest in social stress models that allow for the study of females, though most models do not fully capture the complexity of stress-induced mental illnesses (Kuske & Trainor, 2021). In a study that used the ethologically valid VBS paradigm, it was shown that females living in female-only colonies do not produce dominance hierarchies, with little to no agonistic behavior observed and no changes in body weight (Tamashiro et al., 2004). This seems to be consistent with reports from research done on other social animal species (Jones, Stoddart & Mallick, 1995), pointing towards the reluctance of females for agonistic dominance behavior. Furthermore, the offensive aggression of males in mixed colonies is mainly directed at males instead of females, and females do not show as fierce agonistic defense behavior to protect their territory as males do (Jones et al., 1995).

Therefore, in this study, we are also interested in examining the behavior, physiology and neurobiology of females in mixed-sex colonies, to see whether the VBS paradigm can be used as a social stress model for females. Towards this end, instead of using the STANDARD colony, we will implement VBS-colonies consisting of 4 males and 4 females. A recent study showed that female aggression levels not only rise during hierarchy formation, but also seem to rise again after day five (Zhou, Sandi & Hu, 2018). Usually, behavior is mainly analyzed during the first 3 days of VBS-housing, missing possible changes in the dominance hierarchy during the second half of VBS-housing. For these reasons, it is interesting to analyze the behavioral characteristics and hierarchy dynamics over time and to include the second half of VBS-housing.

1.5. Consequences of (social) stress

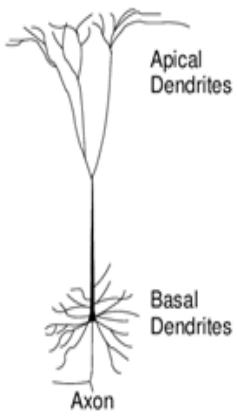
As previously mentioned, stress induced by adverse experiences may lead to long-term alterations in physiology and endocrinology. More importantly, stress is also known to affect, quite substantially, mechanisms of brain plasticity. Hormones that are fundamental in the stress-response, like glucocorticoids, are lipophilic in nature and easily pass the Blood-Brain-Barrier (BBB), either by diffusion or facilitated by a membrane transporter (Nahar, Rainville & Jeffrey, 2016). Once glucocorticoids pass the BBB, they bind to brain regions important for the regulation of stress, such as the limbic brain regions. Upon binding, properties within these limbic structures are altered to remember the experience and respond adaptively to the next stressful event. However, as previously mentioned, failed adaptation to the stressor (such as the inability to change the stressor or change the perception of the stressor), can lead to long-term stress-related psychopathologies (Von Frijtag et al., 2000).

Functional and structural neuroimaging studies in humans suffering from stress-related psychiatric disorders show three main brain structures that are especially affected by stress: namely the hippocampus, the medial prefrontal cortex (mPFC), and the amygdala (Chattarji et al. 2015). Each of these structures regulates the stress response by influencing the activity of the HPA axis. Importantly, the hippocampus and mPFC regulate the HPA axis by inhibiting its activity, whereas the amygdala enhances it (Ulrich-Lai & Herman, 2009). Interestingly, the manner and direction in which the hippocampus, mPFC and amygdala are affected by stress also appear to differ from each other, as will become apparent below. In short, there seems to be a general trend of dendritic atrophy and reduction in spine number in the hippocampus and mPFC because of stress, whereas we see the opposite pattern for the amygdala. Thus, the hippocampus, amygdala and mPFC show a different relationship with stress in two ways: how they regulated the stress response and how they are subsequently affected by the stress response.

These stress-induced alterations are manifested across a spectrum of organizations, at each level aimed to adapt to the stressor and mitigate the detrimental effects of the stress-induced allostatic load (McEwen, 2007). At one end of the spectrum we find behavioral changes, and via functional changes at the level of synaptic connectivity and structural remodeling of dendrites, we find at the other end changes in molecular levels (Hammels et al., 2015). The structural remodeling of the apical part of pyramidal dendrites is probably the most thoroughly studied stress-induced change in the brain and is thought to be especially constructive for behavioral and cognitive adaptation (Czéh & Fuchs, 2016; Cline, 2001) [see box for more information on pyramidal apical dendrites]. As of now, however, it is not yet possible to use therapeutic intervention to specifically target structural changes underlying psychopathologies, but the pace in which our understanding of the mechanisms involved is increasing holds much promise for the future of neuroplasticity-targeted therapeutics.

Pyramidal Apical Dendrties

Pyramidal cells form most neurons in the neocortex - including the mPFC, amygdala and hippocampus - and can be subdivided into apical and basal dendrites (see figure right). Whereas the basal dendrites are largely responsible for receiving synaptic inputs from local pyramidal cells and interneurons, APD receive synaptic inputs from subcortical projections and distant neurons of the cortex (Cline, 2001). It is through the modulation of apical dendritic number and number of spines (little mushroom-shaped extensions on dendrites that integrate synaptic inputs) that pyramidal neurons change their excitatory and inhibitory input and integration of information. Especially dendritic spine dynamics are considered crucial for synaptic plasticity and learning, as they undergo rapid changes in response to the physiological and external environment (Cline, 2001; Kirov et al., 2004; Zuo et al., 2005).



1.5.1. Stress and the hippocampus

Structural remodeling of pyramidal apical dendrites (ApD) was first demonstrated in the hippocampus (Watanabe, Gould & McEwen, 1992). The hippocampus is crucially involved in spatial processing and the formation of declarative and episodic memories (Eichenbaum, Otto & Cohen, 1992; Squire & Zola-Morgan, 1991). It plays a major role in learning how to navigate life and learning from past experiences, including past interactions with other, possibly more aggressive rats. Neuroimaging studies have consistently found a reduction in hippocampal volume in people with stress-related disorders, such as major depressive (MDD), anxiety, and bipolar (BD) disorders (Hastings et al., 2004; Spalletta et al., 2014; Logue et al., 2018). In line with these results, several animal models show that hippocampal impairments are associated with reduced spatial learning and working memory (Morris, 1984; Lassalle et al., 2000). Since the hippocampus is crucially involved with interpreting potential stressors, and therefore with eliciting an allostatic response, damage to the hippocampus may reduce the ability to discriminate between environmental cues that are safe or pose a threat (McEwen, 1998).

The hippocampus is an S-shaped, complex structure that is found in the medial temporal lobe of the cerebral cortex. Its macrostructure can be subdivided into four parts: the hippocampus proper (cornu ammonius, CA), dendate gyrus, subiculum, and the entorhinal area. The dendrites of the hippocampal pyramidal cells extend both from the apex and base, with the basal dendrites extending in the direction of the lateral ventricles, and the APD extending towards the dentate gyrus (Sigh Anand & Dhikav, 2012). Based on distant cytoarchitectural properties, the hippocampus can be further subdivided into CA fields (see figure …). The pyramidal cells closest to the subiculum are referred to as the CA1 field, whereas the cells within the hilus of the dentate gyrus are referred to as CA4. The CA3 field is fittingly located in between these fields and shows an additional feature: its axonal fibers (also known as Schaffer Collaterals) project back to the ApD of the CA1 field. CA3 neurons receive input from the EC, either via the perforant path (via the subiculum) or from axons of granule cells (also known as mossy fibers) coming via the dentate gyrus (Cherubini & Miles, 2015). Whereas the CA3 neurons are involved with associating spatial locations with an object or reward, CA1 neurons are crucial for information consolidation and retrieval of memories (Cherubini & Miles, 2015; Mueller, Chao, Berman & Weiner, 2011).

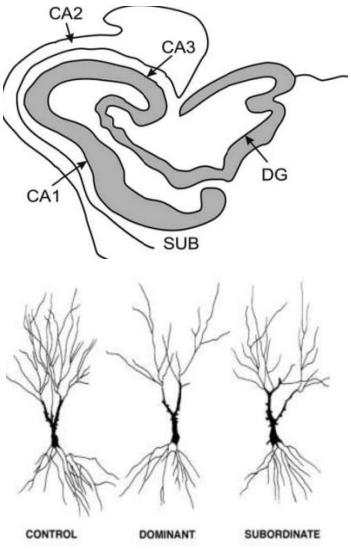


Figure 1. A. *Microstructure of hippocampus (Singh Anand & Dhikav, 2012).* **B**. *Dendritic remodeling in the apical dendrtic tree of CA3 pramidal neurons of both dominant and subordinate Long-Evans rats, compared to unstressed controls, in the VBS (McKittrick et al., 2000).*

Pioneering studies regarding stress-induced brain alterations found that repeated restraint (6 hr/d) stress over a period of 21 days substantially shortens and debranches the APD of CA3 pyramidal neurons (Watanabe, Gould & McEwen, 1992), likely mediated by high levels of glutamate and glucocorticoids (Conrad, LeDoux, Magarinos & McEwen, 1999). Repeated restraint stress causes apical dendritic atrophy in CA3 neurons in males, with a reversed pattern seen for females (Galea et al., 1997). Stress also causes a reduction in spine number in the APD of CA3 pyramidal neurons (Chen et al., 2010). Using social stress paradigms, it has been shown that chronic social stress leads to decreased dendritic branching points and total dendritic length in APD of both CA3 and CA1 neurons in subordinate animals as compared to unstressed controls (see figure …) (McKittrick et al., 2000; Herman & Tamashiro, 2017). Interestingly, dominants also showed a CA3 apical dendritic atrophy, surprisingly even stronger than subordinates, possibly indicating that they also experience stress in maintaining their dominance rank. The spine number of APD of CA3 pyramidal neurons is also decreased in mice experiencing chronic social defeat stress, which was not found in a resilient group (Qu et al., 2018). A study using the same rat strain as our study, WTG rats, also showed a decreased spine number in CA1

ApD (Patel, Anilkumar, Chattarji & Buwalda, 2018). Surprisingly, in another study of WTG rats that implemented the resident-intruder paradigm, both winners and losers showed a similar reduction in spine number in the APD of CA1 neurons (Patel, Anilkumar, Chattarji, de Boer & Buwalda, 2021).

1.5.2. Stress and the amygdala

The Amygdala is a structure that is crucially involved with fear (extinction), sexual behavior, defensive aggression and autobiographical memory (Markowitsch & Staniloiu, 2011). Neuroimaging studies have found that people with mood disorders show increased volume and activity of the amygdala (Drevets & Raichle, 1992; Bremner et al., 2000). Furthermore, rodents with lesions to the amygdala show impairment in the recognition of fearful stimuli, while electrical stimulation evokes fear responses in both humans and animals (Blanchard & Blanchard, 1972; LeDoux et al., 1990). It seems that without an amygdala, both animals and humans would not be able to assign emotional values to sensory information in a Pavlovian manner (Olucha-Bordonau et al., 2015). As such, the amygdala is seen as a key structure in managing emotional information.

The Amygdala is an almond-shaped structure that is nestled deep in the temporal cortex. It lies just anterior to the hippocampal formation. Despite being modest in size, it comprises approximately 13 interconnected nuclei (AbuHasan, Reddy & Siddiqui, 2021). For our present purpose, we will focus on the basolateral complex (BLA, further subdivided into lateral, basal and basomedial nuclei) and the central nucleus (CeA, further subdivided into lateral and medial nuclei). The BLA consists of glutamatergic principal neurons and inhibitory interneurons, whereas CeA neurons are mainly GABAergic. The BLA receives information from the thalamus and the sensory cortices and shows strong reciprocal connections with prefrontal and sensory association cortices, including the mPFC and the hippocampus (Janak & Tye, 2015). Unidirectional output from the BLA travels primarily to the striatum, the bed nucleus of the stria terminalis (BNST) and the CeA, which are considered to be primarily responsible for BLA behavioral output. However, the CeA is the major output nucleus of the Amygdala. It projects to the (lateral) hypothalamus and the brain stem and is as such responsible for the autonomic components of emotions, like heart rate and the processing of pain information (Gilpin, Herman & Roberto, 2015). Note that this is a simplified view of amygdala information flow.

Both primary and secondary branches of pyramidal neurons in the BLA have been found to increase in spine number because of chronic and acute restraint stress (Mitra, Jadhav, McEwen, Vyas & Chattarji, 2005). An increase in dendritic arborization and spine number was seen in BLA pyramidal neurons in rats exposed to chronic immobilization stress (Vyas, Mitra, Rao & Chattarji, 2002). These rats showed greater anxiety-like behavior and both the BLA neuronal hypertrophy and enhanced anxiety remained even after a recovery phase, suggesting that stress causes enhanced emotionality. Interestingly, a recent study showed that WTG rats that repeatedly win agonistic interactions in the resident-intruder parApDigm show an increased spine number in the APD of BLA neurons, specifically at the proximal segments (Deepika et al., 2021).

1.5.3. Stress and the medial Prefrontal Cortex

The mPFC is involved with higher-order executive functions that include decision-making and conflict monitoring (McKlveen et al., 2019). It is especially important for emotional processing and 'top-down' behavioral control, as it encodes context (socially) relevant information and uses this information to regulate other parts of the limbic system (Euston, Gruber & Mcnaughton, 2012). Neuroimaging studies in humans have shown that decreased mPFC activity is associated with maladaptive behavioral, cognitive and affective symptoms typically present in stress-related disorders (Newport & Nemeroff, 2000; Kimbrell et al., 2002).

In rodents, the mPFC is likewise associated with behavioral adaptation. The mPFC can be subdivided in the prelimbic (PL) and infralimbic (IL) cortices based on (although not always clear) distinct functions. The PL promotes appetitive behavior and PL inhibition increases the stress response to psychological stimuli, whereas the IL is involved with (stress) response inhibition (Tavares, Correa & Resstel, 2009; Radley, Arias & Sawchenko, 2006; Jessica, Scarpa & Moorman, 2019). Since we are primarily concerned with the activation of the stress response, for this study we will focus on the PL The prelimbic cortex (PL) is strongly connected with other structures of the limbic system, such as the hippocampus and amygdala, which together are sometimes referred to as the 'PL' circuit. The PL is mainly responsible for the integration of contextual and past information and is as such important for goal-directed behavior (Sierra-Mercado, Padilla-Coreano & Quirk, 2011; Capuzzo & Floresco, 2020).

Like the hippocampus, rodent studies suggest that pyramidal neurons within the mPFC are structurally, and reversibly, remodeled by chronic restraint stress (Cook & Wellman, 2004; Radley et al., 2004; Bloss, Janssen, McEwen & Morrison, 2010). Repeated restraint stress induces a decrease in the length and number of dendritic branches and apical dendritic spine number in layer II-III pyramidal neurons in males (Holmes & Wellman, 2009; Shansky, Hamo, Hof, McEwen & Morrison, 2009; Radley et al., 2006), while a reversed pattern is seen for females (Garrett & Wellman, 2009). Moreover, the mPFC shows dendritic reorganization as a consequence of daily CORT injections for 3 weeks (Wellman, 2004). In this study, the researchers demonstrated that the number and length of apical dendritic branches were reduced by 18 and 32%, but only in the distal dendritic branches. Basal dendrites were unaffected. These structural changes combined are estimated to decrease the synaptic input of the mPFC by 40% (Chatterji, 2015). It is possible that these cellular alterations may impair the capacity of the mPFC to inhibit the stress response, leading to the pathology found in stress-related disorders. Accordingly, a human postmortem study showed that patients with MDD had decreased expression of genes responsible for forming dendrites and spines (Kang et al., 2012).

1.6. Research objectives

There is already extensive data on behavioral, physiological and neurobiological correlates of dominance rank in VBS housed rats, but independent validation of the VBS paradigm is important to further explore its usefulness. We are as such interested in the temporal dynamics of dominance hierarchies in the VBS. However, the main objective of this study is, therefore, to investigate *how dominance rank affects exposure to socially induced (chronic) stress and how this translates into behavioral, physiological, and neurobiological alterations*. We are especially interested in the effect of social stress on the spine densities of ApD. In this thesis, we will mainly focus on the medial prefrontal cortex (the prelimbic cortex), although the amygdala (specifically the BLA) and hippocampus (both CA1 and CA3) will also be considered. Moreover, since social stress models have traditionally focused on only males, we are in this study also examining the behavior, physiology and neurobiology of females in mixed-sex colonies. We are also interested in investigating the role of females in dominance hierarchies, by looking at colony characteristics, such as female dominance. By analyzing these variables, we can investigate whether the VBS paradigm can be used as a social stress model for females.

Furthermore, this study aims to investigate the strength and possible weaknesses of the implemented methodology. First, two contrasting approaches to determining the dominance hierarchy will be compared and evaluated. The first scoring method is based on a method using Observer® XT software to observe the recorded videos of VBS colony housing, at multiple time points of 10 minutes, as implemented by Puentes-Escamilla, Buwalda and Hoppenreijs (unpublished data, 2020). The second scoring method is based on the approach as implemented by Curley, Lee and Williamson (2016), where behavioral observations were conducted for 1-4 hours per day without the use of computational software. We will compare the pros and cons of each method, basing our evaluation on the obtained behavioral and physiological data per method. Note that due to time constraints, we only obtained brain data based on the dominance hierarchy as determined by the approach of Escamilla et al. (unpublished data, 2020), and will only compare neuronal remodeling dynamics of groups ranked by Puentes-Escamilla.

At last, we want to investigate whether the Wild Type Groningen (WTG) rat suits as a useful rat strain within the VBS paradigm. Since WTG rats are not traditionally used for VBS studies, we firstly want to see whether WTG rats readily form a stable dominance hierarchy, which will be assessed by scoring antagonistic behavior and validated by location preference, in line with Patel et al. (2019). Furthermore, because of the differences in behavior and physiological parameters in different rat strains, it might be the case that these strains are differently affected by - and influence the - dominance hierarchy and have different patterns of (chronic) social subordination stress than other strains. It may be that WTG rats habituate and adapt to the dominance hierarchy after a certain amount of time, which would suggest that the VBS model is not actually a model that chronically induces social stress in WTG rats. All theoretical and methodological sub-questions are listed below, including the hypothesis that guide this study.

Theoretical:

- Is dominance rank associated with different stress-induced patterns of behavioral and physiological alterations?
    - *We expect that subordinates show lower relative body weight and thymus weight, but higher CORT levels and retroperitoneal fat, compared to dominants.*
- Is dominance rank associated with different stress-induced patterns of structural remodeling of neuronal spines?
    - *We expect that for pyramidal apical dendritic spines, subordinate WTG rats show an higher number in the BLA and lower number in the mPFC, CA1 and CA3 than dominant WTG rats.*
- Does dominance rank in female rats show a different association with social (chronic) stress than dominance rank in males?
    - *We expect that dominance rank in female rats show similar behavioral characteristics as for males, except for total body weight.*
- What are the temporal dynamics of the dominance hierarchy?
    - Do WTG rats readily form a dominance hierarchy?
        - *We expect that most colonies will form a dominance hierarchy within 3-5 days of VBS-housing.*
    - How stable is the dominance hierarchy over time?
        - *We expect that dominance hierarchies are stable after the first few days of VBS-housing. We expect that females may increase in dominance rank after day 5 of VBS-housing.*
    - What is the role of females in the dominance hierarchy?

Methodological:

- Which scoring method results in a dominance hierarchy that is better reflected by the physiological parameters associated with dominance rank?
    - *We expect that Curley's method will show higher dominance ranking for females than Miguel's method, as the former analyzes more days after day 5 (when female aggression rises) than the latter does*.
- Are WTG rats a useful strain to use in VBS studies to investigate (chronic) social subordination stress?
    - *We expect that subordinate WTG rats experience higher levels of social (chronic) stress than dominant WTG rats do, although not as high as seen in Long-Evans rats*.

2. Method & Materials

2.1. Animals and VBS-housing

For this project, 48 male and 48 female Wilde Type Groningen (WTG) rats were used. These were bred at the University of Groningen and were approximately 7 to 8 months old during the experiment. Compared to the more frequently used strain Long Evans rats, WTG rats show on average lower aggression levels, while still showing a large variation in aggression, ensuring a dominance hierarchy (de Boer et al, 2003). Rats were divided into 12 Visible Burrow Systems (VBS), each colony being comprised of 4 males and 4 females. These VBS' were based on the design by Blanchard et al. (1995) and constructed at the University of Groningen, with two extra chambers (nests) (see figure 2, or appendix A). Each VBS is comprised of a large open surface area with a feeding station in the open area and a dark burrow system with a tunnel and four nest boxes using Plexiglas. The open area showed a 12:12 hour light/dark cycle (lights turn on at 22:00), while the burrow system remained continuously dark using a black lid only transparent for infrared light (Perspex 962 IR). To score the behavior, the VBS' were recorded 24 hours per day using digital monochrome Basler GigE cameras (using infrared light), which were connected to a computer running Media Recorder (by Noldus). Because we only had four VBS at our disposal at the University of Groningen, we performed the experiment in three batches, each batch consisting of four VBS'. All experiments performed were approved by the Animal Ethics Committee of Groningen University.

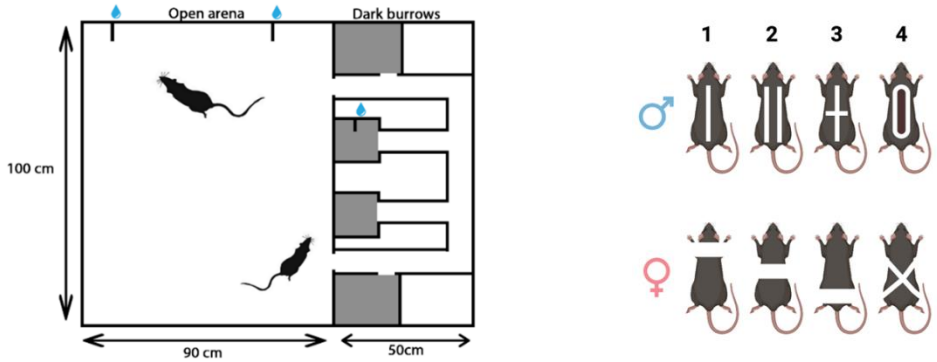


Figure 2. ***Left***. *Visible Burrow System.* ***Right***. *Dye patterns marked with Garnier Olia Super Blond B++*

2.2. Experimental procedure

Prior to colony housing in the VBS, all animals underwent one week of group-housing in same-sex house cages, each consisting of 4 animals, which was followed up by one week of pair housing (1 male + 1 female) in large home cages. We pair-housed the animals so that they could become acquainted with the other sex, so the time spent having sex during the initial phase of the colony housing would decrease and agonistic behaviors could be analyzed more abundantly. To prevent any pregnancies, all females were sterilized by surgical ligation of the oviduct before the start of the experiment. Prior to pair-housing, the fur of all animals was marked with dye (Garnier Olia Super Blond B++), enabling us to distinguish between the animals (see figure 2). The animals were put into colony housing for a total of 10 days, allowing enough time to observe possible temporal dynamics of the social hierarchy and for the biological consequences to become settled. The colonies were formed in such a way that all animals were unfamiliar to each other and did not share any prior interactions.

In between the different housing conditions, the animals were single-housed for one day to either dye their fur, measure their bodyweight and/or collect their feces for CORT measurements. The measurement of bodyweight and collection of feces were also obtained during colony housing for days 2, 5, 8, and 10. On these days we also counted the number of wounds each animal had obtained as a consequence of agonistic interactions. A schematic overview of all experimental procedures is shown below in figure 3.

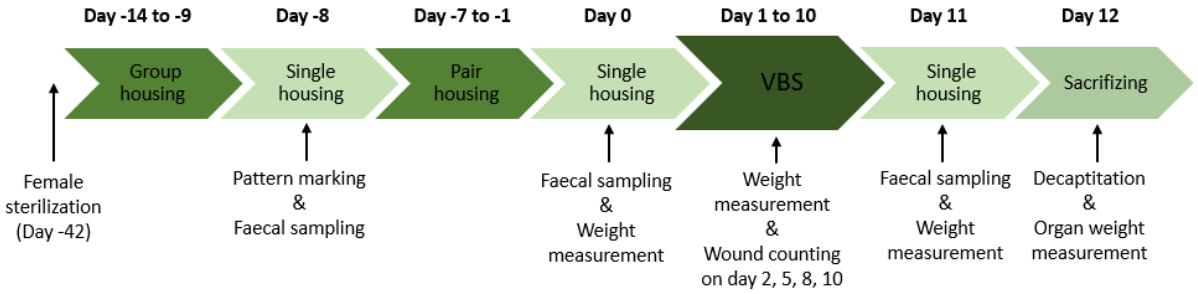


Figure 3. *Overview of the experimental procedure.*

2.3. Behavioral analysis

For the first objective, the formation and maintenance of the social hierarchy were assessed by scoring agonistic behaviors (offensive and defensive) between rats during the 10-day VBS housing (see table 1). Offensive behaviors in rats can be used as a proxy for social dominancy in colonies (Patel et al., 2019). For each behavioral event, we recorded the subject directing the behavior, the recipient receiving the behavior, the time and location of the interaction, and the types of behaviors that were involved (table 1). The winners of each recorded interaction were defined as the individual directing the fighting, chasing, mounting, and side-way lateral threat. Losers, on the other hand, showed types of behavior like subordinate posture or induced flee. The agonistic interaction was considered to have ended after both individuals separated and engaged in different behavior, such as self-grooming, drinking or eating, and social exploration of other individuals.

Table 1. Behavioral ethogram of the behaviors that were scored during day 1, 2, 3, 5, 8, and 10 of colony housing.

Agonistic behavior	Definition
Offensive act	An aggressive act towards an opponent, including clinch attack, chasing, keep down behavior (pinning), sideways lateral threat mounting (male to male), tunnel patrolling, and moving towards behavior. These behaviors are listed from most fierce to least fierce.
Defensive act	A self-protective act often (but not necessarily) in response to an offensive act, including upright posture (boxing), subordinate posture, induced flee, and moving away behavior

Since behavioral scoring is very time-consuming, but we still want to obtain insights into the temporal dynamics of the dominance hierarchy, the behavioral scoring was done during day 1, 2, 3, 5, 8 and 10 at multiple time points, scoring primarily during the dark phase as rats are more active at night. We implemented two contrasting approaches, each one coming with some advantages and possible pitfalls.

The first approach was based on the method implemented by Puentes-Escamilla, Buwalda and Hoppenreijs (unpublished data, 2020). In this approach, the recorded videos are observed using Observer® XT software (Noldus) at 7 time points of 10 minutes per day, with one during the light phase and six during the dark phase. The timestamps were 05:00, 07:00, 08:00, 12:00, 12:30, 14:00, 16:00, and 18:00. The timestamps were observed for days 1, 2, 5 and 10. During these time points, agonistic as well as affiliative behaviors were coded and registered. Although this approach registers all interactions within the time points, it does skip a lot of interactions between the timepoints, making the results possibly less reliable than the second approach. The second approach is based on the method implemented by Curley, Lee and Williamson (2016). In this approach, behavioral observations were conducted for 1-4 hours per day, usually starting at 12:00. We only looked for agonistic

interactions and could fast-forward the videotapes looking for these. This gives us the advantage of scoring more agonistic interactions per day without losing too much time. Since we didn't follow each individual throughout the time points of scoring, this approach might run the risk to miss nuanced interactions between individuals from a distance. From here on, the first approach is referred to as Miguel's method, and the latter as Curley's method.

2.3.1. Determination of dominance hierarchy:

For each colony a dominance hierarchy was determined by calculating the Average Dominance Index (ADI) for each individual (see appendix B1). The ADI is a measure of the average proportion of wins an individual has with every other interacted individual of the colony. The ADI ranges from 0 to 1, with higher values indicating higher dominance within the group. We also calculated the Female Dominance Index (FDI) for each colony, which is a measure of the relative dominance of females over males (appendix B2). It ranges from 0 to 1 and reflects the summed number of males showing a lower ADI than females, divided by the sum of potential number of males (always 4 in our study) showing a lower ADI than all individuals of the colony. Both ADI and FDI were automatically calculated from a dominance matrix using the excel extension MatrixTester version 3.0.1 (Hemelrijk, 2017). To gain insight into the temporal dynamics of dominance, we calculated ADI not only for each individual over all scored days, but also for each scored day separately. The dominance matrices and their ADI linear scales from all colonies (VBS1-12) can be found in appendix B3 and C. The resulting dominance ranking was validated by the number of wounds, and location preference as measured by the time spent in the open arena versus the burrows.

2.3.2. Colony characteristics:

For each colony we investigated the degree of female dominance (FemDom), stability, intensity of aggression and number of wounds. Stability was calculated by calculating Kendall's Tau correlation between the ADI ranking on day 3, when a stable dominance hierarchy is usually formed (Blanchard et al., 1996), with the ADI ranking on day 10 at the end of VBS housing. The intensity of aggression was calculated by dividing the proportion of fierce fights (defined by the presence of clinch attack and/or chasing) of the total fights per individual, followed by taking the average of these individual proportions per colony. At last, we included the total number of wounds as another aspect of a colony that indicates the intensity of aggression. All colony characteristics can be found in appendix D.

2.4. Data collection

To assess how dominance rank affects stress, we wanted to measure adrenocortical activity at multiple time points throughout the experiment. The most common method to obtain multiple read-outs of adrenocortical activity is by obtaining CORT samples through blood collection (Morton et al., 1993). However, blood sampling involves handling the rats and physical restraint, which rapidly affects circulating CORT levels, with highly elevated levels 10 minutes after sampling (Good, Khan & Lunch, 2003). Another method to obtain CORT measures is through feces sampling, which contains 80% of the CORT metabolites and does not disturb CORT levels during sampling (Touma, Palme & Sachser, 2004). Therefore, feces were collected before and after colony housing for each animal and stored at -80°C.

The last day on which we sampled the feces of all animals was the day after the colony housing, when all animals were single housed. On the next day all animals were sedated with CO_2 and rapidly decapitated. The brains were first collected and divided into two hemispheres. The right hemisphere was placed in Golgi-staining fixative, incubated for 15 days and later used for a morphological analysis, whereas the left hemisphere was frozen with liquid nitrogen and later used for molecular analysis (not

relevant for this thesis). Furthermore, to investigate the association of dominance rank and physiological parameters known to be affected by stress, we harvested organs such as the adrenal gland, the thymus, retroperitoneal fat (from now on referred to as just FAT), seminal vesicles, and the testes. All organs were weighted on an electric scale that measures in grams with a precision of 4 decimals. By measuring final organ weights, we wanted to gain a general understanding of the impact of colony housing on the animals, for both dominant and subordinate rats.

For the morphological analysis, the right hemisphere was removed and placed in a Golgi-Cox fixative, as used by Suvrathan et al. (2013). After 15 days of incubation at room temperature, they were cut into coronal brain sections of 100 µm using the vibratome (leica VT 1200S), collected on an object-glass and colorized with sodium carbonate. The brain slices were subsequently placed in absolute alcohol for dehydration and cleared in xylene before the coverslip was mounted on top of the slices. (Unfortunately, the microscope at the University of Groningen malfunctioned, so we send the slides to Deepika Patel, NCBS, Bangalore in India.) Per colony, we selected the most and least dominant males and females. Dendrites that branch off from the main shaft are called primary apical dendrites (ApD), which were used for this analysis. Only neurons that showed a consistent, dark color were selected from the primary neurons of the PL, the BLA, and the CA1/3 brain regions. For each individual, a total of 5-6 primary ApD per brain region were analyzed using NeuroLucida software attached on Olympus BX61 microscope (100X, 1.3 numerical aperture, Olympus BX61; Olympus, Shinjuku-Ku, Tokyo, Japan). The ApD were selected and cut into 8 segments of each 10 µm. All spines were counted and summed for each segment.

2.5. Statistics

For both the statistical analysis and visualization of the results we used SPSS software (version 22) and Graphpad Prism (version 9). For all variables, both a Shapiro-Wilk normality test and Levene's test was performed to determine whether the data were normally distributed and showed homogeneity of variances for the groups that were compared (appendix F). The analyzed physiological and behavioral variables include time spent in open arena (in percentage), bodyweight change and CORT levels change during the VBS housing, final retroperitoneal fat weight, adrenal gland weight, thymus weight, and for the males the weight of the seminal vesicles and the testes. To control for individual variances in total weight and make the data more representative, all organ weight variables were transformed such that their value represents the relative organ weight in milligram per gram body weight ((finalorganweight/finalbodyweight)*1000). The data on body weight change was also transformed into a value reflecting the percentage of change, with the start of the VBS as baseline ((finalweight/startweight)*100). Data of CORT levels were calculated by subtracting the pre-VBS CORT levels from the post-VBS CORT levels, times a 100 (((postVBScort-preVBScort)/postVBScort)*100). CORT data showed outliers that skewed the distribution substantially, which were filtered out for all further analysis including CORT (appendix F).

For analyzing effects of dominance rank and sex, the obtained data were grouped according to dominance rank (most dominant and most subordinate individual) and sex (male or female) for the analysis of all physiological, behavioral and neurobiological variables. All behavioral and physiological data was analyzed using both ranking methods (although the results based on Curley's method are covered in much more detail later), whereas the neurobiological variables were based on Miguel's ranking. Since both ranking methods showed similar results for physiological changes (see appendix X), we found this to be justified. Since ANOVA is quite robust against small violations of normality when sample sizes are equal, we performed a Two-Way ANOVA for almost all physiological variables. Only FAT weight, body weight, and the number of wounds showed such strong violations, that we used non-

parametric alternatives (e.g. bootstrapping) (see appendix G4.1). For post-hoc multiple comparisons, we used Sidak's test for all ANOVA's with a significant results.

Furthermore, we performed a two-way Repeated Measures (RM) ANOVA to analyze whether the effect of dominance rank on spine number was mediated by the distance of dendritic segments from the origin of the main shaft. For the Two-Way RM ANOVA we analyzed brain regions and sex separately, and also controlled for unequal variability of differences (we did not assume sphericity) by using a Geisser-Greenhouse correction.

To compare both scoring methods (Miguel's vs Curley's), we analyzed how well the ranking method would map onto physiological changes (and location preference). First, we correlated each ranking with all physiological variables using Kendall's Tau tests (appendix I1). Second, we inspected the variance within each dominance group to see whether they differed significantly, for which we also included all intermediate individuals to gain more insight (appendix I2). Last, we performed Kendall's Tau correlations among physiological variables (and location preference) within each dominance group, as determined by either scoring method, to see if one would result in stronger correlations between variables (appendix I3). Kendall's Tau correlations were also used to analyze colony characteristics, such as FemDom, stability, intensity of aggression and number of wounds. Since body weight change and CORT change reliable reflect the experience of stress (Blanchard et al., 1995), we further included these variables in the correlation tests. To assess whether the colonies showed, on average, stable hierarchies from day 3 onwards, we also calculated Fisher's combined probability test (FCP).

Each group will be graphed together, showing the mean and Standard Error of the Mean (SEM). Graphs comparing the two scoring methods will include the mean and Standard Deviation (SD), as we are interested in differences in variance (which possibly indicates the degree of accuracy of the method). All tests were pairwise comparisons and two-tailed. A probability level of $p \leq 0.05$ was considered significant.

3. Results

3.1. Influence of dominance rank and sex on behavior

3.1.1 behavioral characteristics

Based on Curley's method, for all colonies DOM individuals showed more offensive agonistic behavior than SUB individuals (see table 2). These behaviors included initiating clinch attack, chasing, lateral attack and keep down behavior. In contrast, SUB showed more defensive agonistic behavior, such as upright (boxing) posture, induced fleeing, moving away behavior, and assuming subordinate positions like lying on their back (under DOM), which is consistent with other VBS studies (Blanchard et al., 1995; Blanchard et al., 1996; McKittrick et al., 2000; Tamashiro et al., 2004). See appendix E for all raw agonistic demographics.

Table 2. Behavioral Characteristics. The average of total fights per group, the percentage of initiated fights per group, and the percentage of fights won. Data is presented as Means ± S.E.M.

	Total Fights	Fights Initiated (%)	Fights Won (%)
DOM males	44.00 ± 5.08	0.83 ± 0.03	0.82 ± 0.04
SUB males	23.50 ± 2.73	0.15 ± 0.03	0.15 ± 0.04
DOM females	26.67 ± 5.95	0.64 ± 0.03	0.61 ± 0.05
SUB females	19.17 ± 3.58	0.35 ± 0.17	0.30 ± 0.05

DOM individuals initiated and won significantly more antagonistic interactions than SUB individuals, for both males and females (all tests showed $p < 0.001$) (appendix G1). We only did not find a statistically significant difference between DOM and SUB females in the total number of fights ($p = 0.817$), nor did we find a difference between females and SUB males ($P > 0.100$). As expected, we saw a decrease of agonistic interactions during the VBS housing for both males and females (figure 4A).

Furthermore, for the time spend in the open-surface arena of the VBS, we found a statistically significant interaction effect between rank and sex ($F(1,44) = 26.330$, $p < 0.001$), as well as rank ($F(1,44) = 33.980$, $p < 0.001$) and sex ($F(1,44) = 29.480$, $p < 0.001$) separately (appendix G1). Post-hoc Sidak's multiple comparison test showed a significant difference between DOM and SUB males ($p < 0.001$), but not between DOM and SUB females ($p = 0.859$). Thus, SUB males prefer to spend most of their time in the dark burrow system, as opposed to DOM males and all females. SUB males showed more wounds than DOM males ($U = 30.000$, $p = 0.014$), with females showing little to no wounds (appendix G2.1).

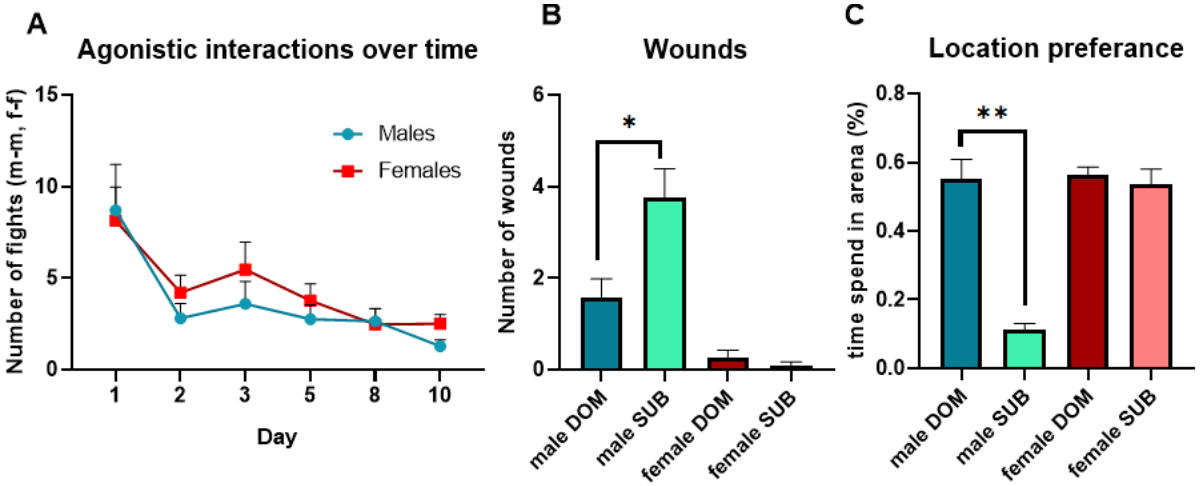


Figure 4. A. Agonistic interactions over time, of both males and females**. *B***. number of wounds, *for DOM (mean = 1.58 ± 0.40) and SUB (mean = 3.75 ± 0.64) males, and DOM (mean = 0.25 ± 0.18) and SUB (mean = 0.08 ± 0.08) females. All groups showed N = 12.* ***C.*** Location preference. *The percentage of time spend in the open arena during the VBS housing of DOM (mean = 0.55) and SUB (mean = 0.17) males, and DOM (mean = 0.56) and SUB (mean = 0.54) females. All groups showed N = 12. P-values; * < 0.05, ** < 0.01, *** < 0.001.*

3.1.2 Temporal dynamics of the dominance hierarchy

Fisher's combined probability test showed that the dominance hierarchies were stable from day 3 onwards ($p < 0.001$). After day 3, despite some females becoming more dominant than males, the ranking among males and the ranking among females remains stable. For more information on the temporal dynamics of the dominance hierarchy, see appendix D2.

3.2. Influence of dominance rank and sex on physiology

All analyses in this section are based on the dominance ranking as determined by Curley's method (appendix G2). Prior to VBS housing, there was no significant difference in absolute total body weight between dominants and subordinates for both males ($U = 68.000$, $p = 0.817$) and females ($U = 66.000$, $p = 0.729$). For body weight change during VBS housing, relative to the total weight of the rats at the start, we found no statistically significant difference between dominant and subordinate males ($U = 63.000$, $p = 0.603$) or females ($U = 49.000$, $p = 0.184$) (Figure 5A, last day). We did find a statistically significant interaction between the effects of sex and VBS-day on body weight change ($F(4,176) = 66.86$, $p < 0.001$), as well as significant effects of sex ($F(1, 44) = 105.7$, $p < 0.001$) and day ($F(1.900, 83.59) = 6.566$, $p = 0.0026$) separately.

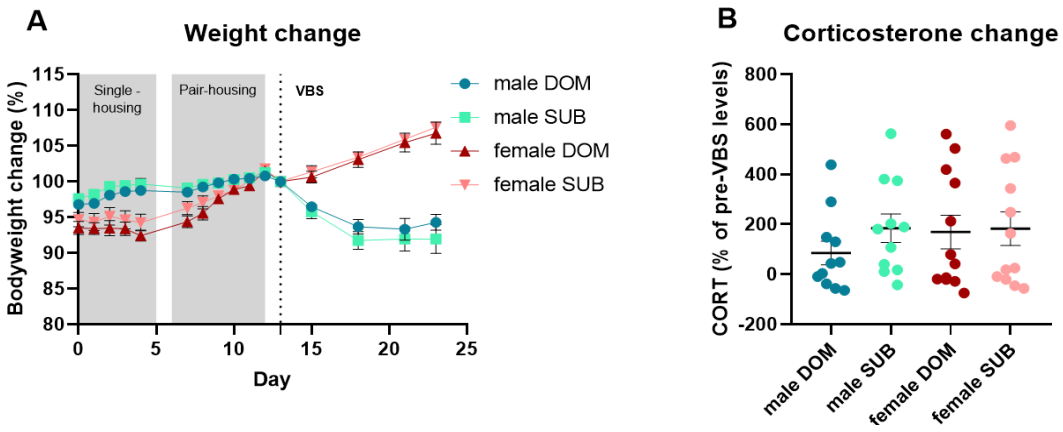


Figure 5. A. Bodyweight change of DOM and SUB males and females over the course of the entire experiment. *At the last day, body weight changes were DOM (median = 95.01 ± 1.12) and SUB (median = 93.16 ± 2.01) males, and DOM (median = 103.56 ± 1.57) and SUB (median = 107.59 ± 0.58) females. All groups had N = 12.* **B**. Corticosterone change (Post-VBS CORT levels minus Pre-VBS CORT levels) *of DOM males (mean = 85.11 ± 47.60, N = 11), SUB males (mean = 184.36 ± 56.74, N = 11), DOM females (mean = 169.28 ± 67.18, N = 12), and SUB females (mean = 183.11 ± 67.44, N = 12).*

The change in CORT during VBS housing showed no statistically significant interaction between rank and sex ($F(1, 42) = 0.489$, $p = 0.488$), nor for sex ($F(1, 42) = 0.461$, $p = 0.501$) and rank ($F(1, 42) = 0.856$, $p = 0.360$) separately (see figure 5B). Furthermore, adrenal weight, was not statistically significant affected by dominance rank ($F(1,44) = 0.316$, $p = 0.577$) or by the interaction of dominance rank and sex ($F(1,44) = 0.915$, $p = 0.344$). Only sex showed a statistically significant effect on adrenal weight ($F(1,44) = 62.92$, $p < 0.001$) (figure 6A). Likewise, the interaction between dominance rank and sex did not show a statistically significant effect on thymus weight ($F(1,43) = 0.394$, $p = 0.534$). However, both sex ($F(1,43) = 61.883$, $p < 0.001$) and rank ($F(1,43) = 5.817$, $p = 0.020$) separately show a strong statistically significant effect on thymus weight (figure 6B). Sidak's multiple comparison test did not show any statistically significant difference in thymus weight between dominance rank, although the difference between DOM males and SUB males is almost statistically significant ($p = 0.078$). For fat weight, we found no significant interaction between rank and sex ($F(1,44) = 1.769$, $p = 0.190$), nor for sex ($F(1,44) = 0.448$, $p = 0.507$) separately (figure 6C). The effect of dominance rank on fat weight almost showed significance ($F(1,44) = 4.016$, $p = 0.051$). Finally, we did not find a statistically significant difference between DOM males and SUB males for both testes weight ($t(22) = 0.700$, $p = 0.491$) and vesicle weight ($U = 75.000$, $p = 0.887$).

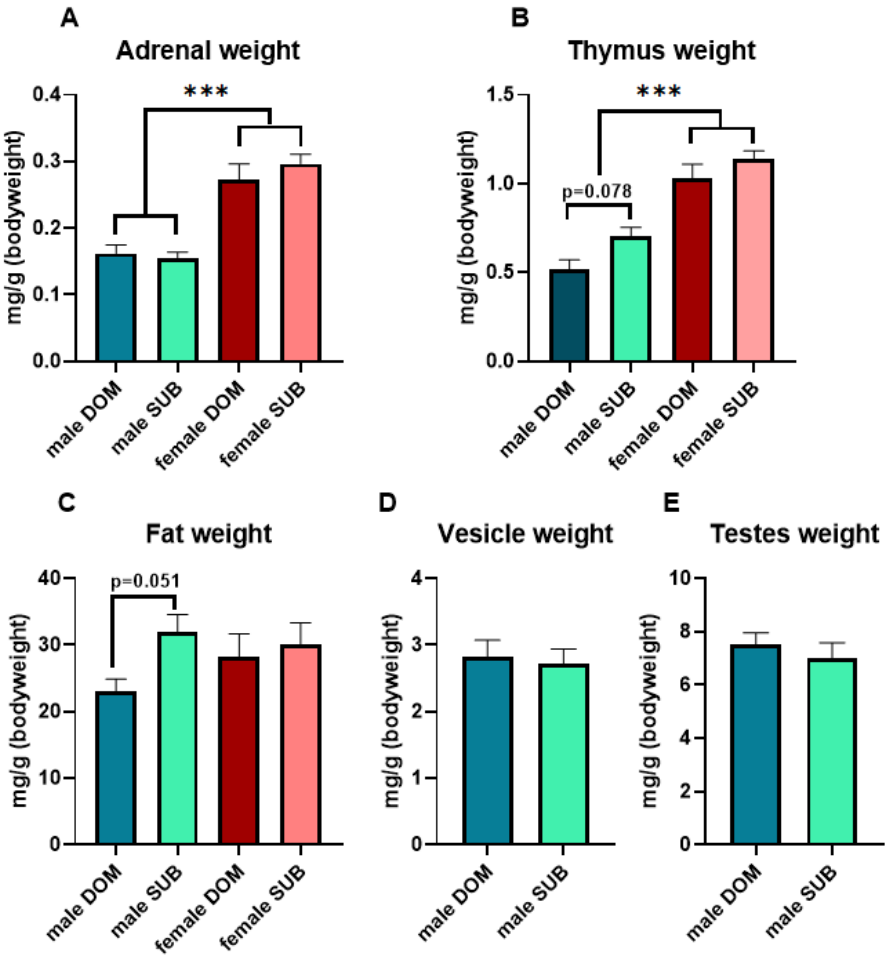


Figure 6. A. Adrenal weight, *with DOM males (mean = 0.16 ± 0.01), SUB males (mean = 0.16 ± 0.01), DOM females (mean = 0.27 ± 0.02), SUB females (mean = 0.30 ± 0.01). All groups had N = 12.* **B**. Thymus weight, *with DOM males (mean = 0.52 ± 0.05, N = 11), SUB males (mean = 0.70 ± 0.05, N = 12), DOM females (mean = 1.03 ± 0.08, N = 12, SUB females (mean = 1.14 ± 0.05, N = 12).* **C.** Fat weight, *with DOM males (median = 20.83 ± 1.83), SUB males (median = 30.85 ± 2.60), DOM females (median = 23.16 ± 3.52), SUB females (median = 25.61 ± 3.21).* **D**. Vesicle weight, *with DOM males (mean = 2.73 ± 0.22, N = 0.75), and SUB males (mean = 2.81 ± 0.26, N = 12).* ***E***. Testes weight, *with DOM males (mean = 7.52 ± 0.44, N = 12) and SUB males (mean = 7.00 ± 0.58, N = 12). P-values; * < 0.05, ** < 0.01, *** < 0.001.*

3.3. Comparison of two scoring methods: Miguel vs Curley

No difference was observed for the correlations between the physiological variables (including location preference) and the dominance ranking of either Miguel's or Curley's scoring method (see appendix I1). Both scoring methods showed similar center points and spread for each group for all physiological variables and preference of location (appendix I2). Moreover, we found no clear difference in correlations among these variables within each dominance group, where the dominance groups were based on either one of the scoring methods (appendix I3).

We only found a difference in dominance ranking between the two scoring methods in the number of colonies with a female being the most dominant individual (see appendix B4). Whereas the scoring method of Miguel resulted in female dominance in only 1 out of 12 colonies (0.08%), the scoring method of Curley resulted in female dominance in 5 colonies (0.42%). Moreover, whereas Miguel's method resulted in undecided ranks (two or more individuals sharing a rank position) in all colonies, with 6 colonies showing undecided ranks for three individuals or more (50%), Curley's method results in only 2 colonies with undecided ranks (0.17%).

3.4. Dominance rank, sex and spine remodeling

Note that all data on spine remodeling is based on the ranking method of Miguel. Because both methods showed very similar results per group for the behavioral and physiological variables, we assume that continuing with Miguel's ranking is justified. Data can be found in appendix G3.

3.4.1. Medial Prefrontal Cortex remodeling:

For the mPFC, the two-way ANOVA revealed that there is no statistically significant interaction between the effects of dominance rank and sex on spine number ($F(1,20) = 2.047$, $p = 0.1680$), nor did sex show a significant effect on spine number ($F(1,20) = 0.1077$, $p = 746$). Dominance rank, however, did show a statistically significant effect on spine number ($F(1,20) = 43.24$, $p < 0.001$). These results are graphed in figure 7A below.

For segmental analysis of the mPFC neurons in males, the two-way RM ANOVA revealed that there is a statistically significant interaction between the effects of dominance rank and distance from the main shaft ($F(7,70) = 2.982$, $p = 0.009$). The simple main effects analysis showed that dominance rank ($F(1,10) = 39.92$, $p < 0.001$), but not distance from the main shaft ($F(3.074,30.74) = 1.919$, $p = 0.146$), showed a statistically significant effect on spine number. Sidak's multiple comparisons test for dominance rank showed that dominant and subordinate individuals differed significantly in spine number mainly at the proximal segments (10 µm – 30 µm) of the dendrites (see figure 7B). For females, the two-way RM ANOVA showed no statistically significant interaction between the effects of dominance rank and distance from the main shaft ($F(7,70) = 1.307$, $p = 0.260$), but did show that dominance rank has a statistically significant effect on spine number ($p = 0.007$). Sidak's multiple comparisons test for dominance rank showed that dominant and subordinate individuals only differed statistically significant at 40 µm ($p = 0.042$).

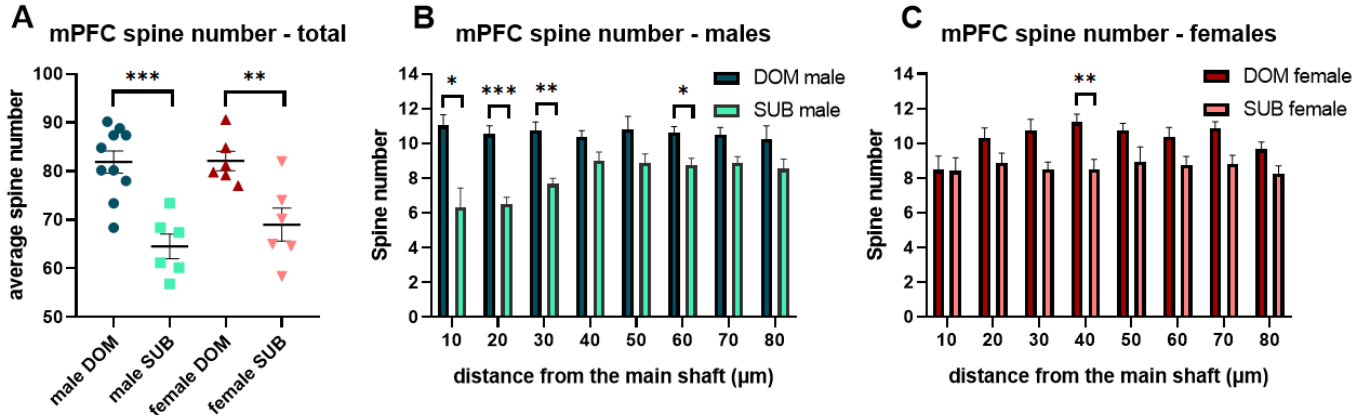


Figure 7. Data of spine numbers in the medial prefrontal cortex (mPFC) neurons. ***A**: average total spine number per group, with DOM (mean = 81.88 ± 2.26) and SUB (mean = 64.57 ± 2.26) males, and DOM (mean = 82.10 ± 2.00) and SUB (mean = 69.03 ± 3.38) females. All groups had N = 6. **B-C**: Segmental analysis for males and females seperately of the spine number per dominance rank per distance of dendritic segments from the origin of the main shaft. For males, the mPFC dendritic segments at distance 10 µm (p = 0.048), 20 µm, (p < 0.001), 30 µm (p = 0.004), 60 µm (p = 0.031), but not at 40 µm (p = 0.373), 50 µm (p = 0.368), 70 µm (p = 0.109), and 80 µm (p = 0.596) from the main shaft show statistical significance. P-values; * < 0.05, ** < 0.01, *** < 0.001.*

3.4.2. Hippocampus remodeling:

For the CA1 neurons, we did not find a statistically significant interaction between the effects of dominance rank and sex ($F(1,20) = 0.071$, $p = 0.793$), nor does dominance rank ($F(1,20) = 0.229$, $p = 0.638$) or sex ($F(1,20) = 0.590$, $p = 0.452$) alone show a statistically significant effect on spine number (figure 8A). Moreover, for CA3 we did not find a statistically significant interaction between dominance rank and sex ($F(1,20) = 0.04781$, $p = 0.829$), nor does dominance rank ($F(1,20) = 3.381$, $p = 0.081$) or sex ($F(1,20) = 0.143$, $p = 0.710$) alone show a statistically significant effect on spine number (figure 8D). Surprisingly, however, both DOM males and females showed a higher, although non-significant, spine number in CA3 ApD neurons than SUB males and females.

For segmental analysis of the CA1 neurons in males, the two-way RM ANOVA revealed that there is also not a statistically significant interaction between the effects of dominance rank and distance from the main shaft ($F(7,70) = 0.814$, $p = 0.579$), nor does dominance rank ($F(1,10) = 2.360$, $p = 0.156$) or distance from the main shaft ($F(3.901,39.01) = 2.209$, $p = 0.087$) alone show a statistically significant effect on spine number (figure 7B). However, it is noteworthy to point out that despite the small sample size per group (N=6), we already see for all segments that SUB males seem to have a higher number of spine numbers compared to DOM males, though it never reaches statistical significance. For females, there was no statistically significant effect of dominance rank ($F(1,10) = 0.016$, $p = 0.901$) and distance from the main shaft ($F(3.233,32.33) = 1.532$, $p = 0.223$) on spine number, nor did it reveal a significant interaction between these two ($F(7,70) = 1.514$, $p = 0.177$) on spine number (figure 8C).

For the CA3 neurons in males, there was a statistically significant effect of dominance rank on spine number ($F(1,10) = 6.203$, $p = 0.032$), but not for the effect of the distance from the main shaft ($F(3.975,39.75) = 1.673$, $p = 0.176$) or the interaction between dominance rank and distance from main shaft ($F(7,70) = 1.018$, $p = 0.427$). Sidak's multiple comparisons test for dominance rank showed that dominant and subordinate individuals differed significantly in spine number at 60 µm distance from the main shaft ($p = 0.014$). For females there was no statistically significant effect of dominance rank ($F(1, 10) = 0.9207$, $p = 0.360$), distance from the main shaft ($F(4.418,44.18) = 1.319$, $p = 0.276$) or an interaction effect on spine number ($F(7,70) = 1.317$, $p = 0.255$). See figure 8E-F.

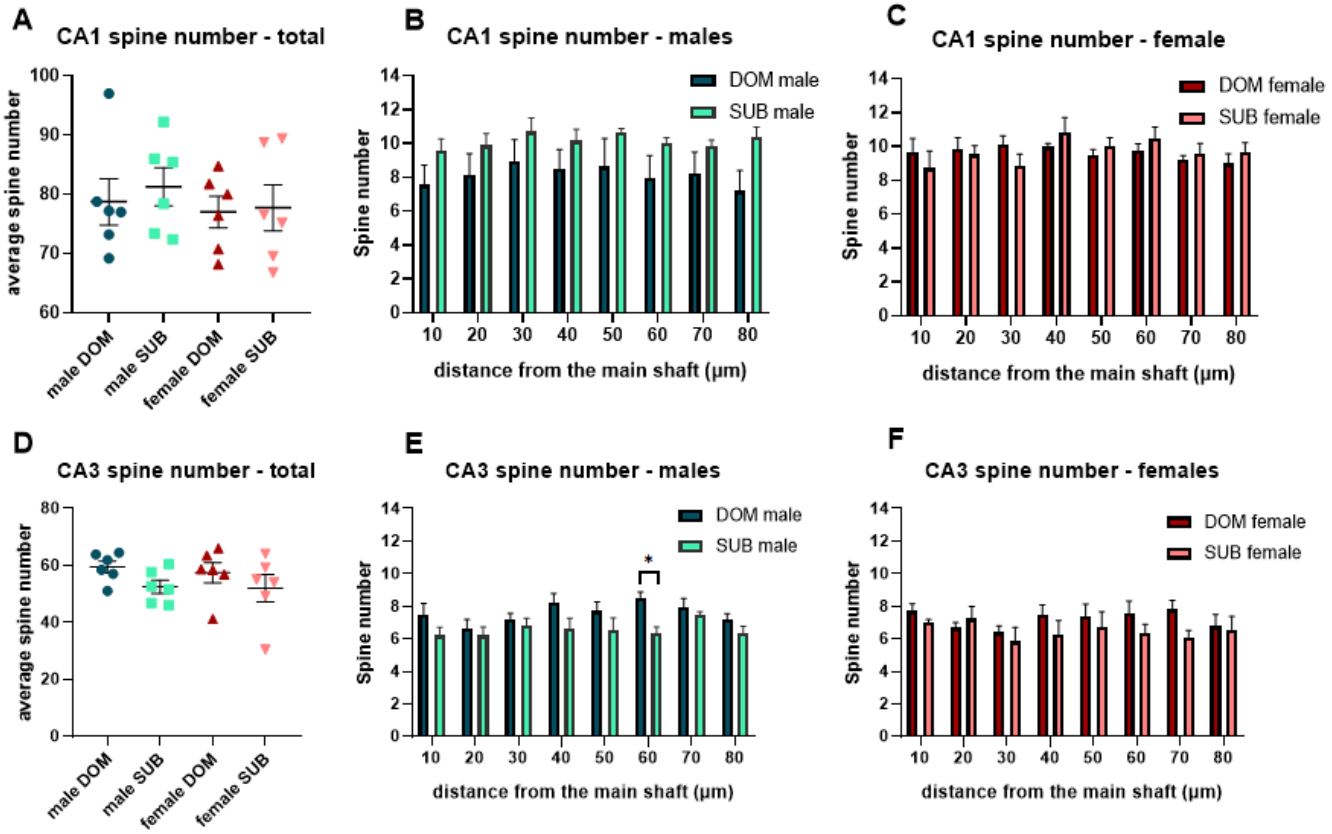


Figure 8. Data of spine numbers in the hippocampal CA1 and CA3 fields. ***A***. *Total CA1 spine number difference for DOM (mean = 78.73 ± 3.92) and SUB (mean = 81.90 ± 3.20) males, and DOM (mean = 77.00 ± 2.64) and SUB (mean = 77.73 ± 2.64) females. All groups had N = 6.* **B-C**. *Spine number remodeling per segment of the apical dendrite.* **D.** *Total CA3 spine number difference for DOM (mean = 59.40 ± 2.06) and SUB (mean = 52.50 ± 2.34) males, and DOM (mean = 57.40 ± 3.54) and SUB (mean = 51.97 ± 4.77) females.* **E-F.** *Segmental analysis for males and females separately of the spine number per dominance rank per distance of dendritic segments from the origin of the main shaft.*

3.4.3. Basolateral Amygdala remodeling:

For the basolateral amygdala (BLA) neurons, the two-way ANOVA revealed that there is not a statistically significant interaction between the effects of dominance rank and sex ($F(1,20) = 0.949$, $p = 0.342$), nor does dominance rank ($F(1,20) = 0.460$ $p = 0.506$) or sex ($F(1,20) = 0.108$, $p = 0.093$) alone show a statistically significant effect on spine number (figure 9A). Compared to DOM males, SUB males did show an increase in spines, although this did not achieve statistical significance.

For segmental analysis of the BLA neurons in males, the two-way RM ANOVA revealed that there is not a statistically significant interaction between dominance rank and distance from the main shaft on spine number ($F(7,70) = 1.816$, $p = 0.098$), nor does dominance rank alone show a statistically significant effect spine number ($F(1,10) = 3.701$, $p = 0.083$). However, the distance from the main shaft does show a statistically significant effect on spine number ($F(2.915,29.15) = 5.238$, $p = 0.006$). Sidak's multiple comparisons test for distance from the main shaft showed that for the SUB males there was a significant difference of spine number between 20 µm and 40 µm ($p = 0.037$), 20 µm and 70 µm ($p = 0.003$), 30 µm and 70 µm ($p = 0.027$), and 50 µm and 70 µm (0.033) distance. For females, the two-way RM ANOVA showed that there is also not a statistically significant interaction between the effects of dominance rank and distance from the main shaft ($F(7,70) = 0.589$, $p = 0.762$), nor does dominance rank ($F(1,10) = 0.052$, $p = 0.825$) or distance from the main shaft ($F(2.714,27.14) = 1.139$, $p = 0.348$) alone show a statistically significant effect on spine number. See figure 9B-C.

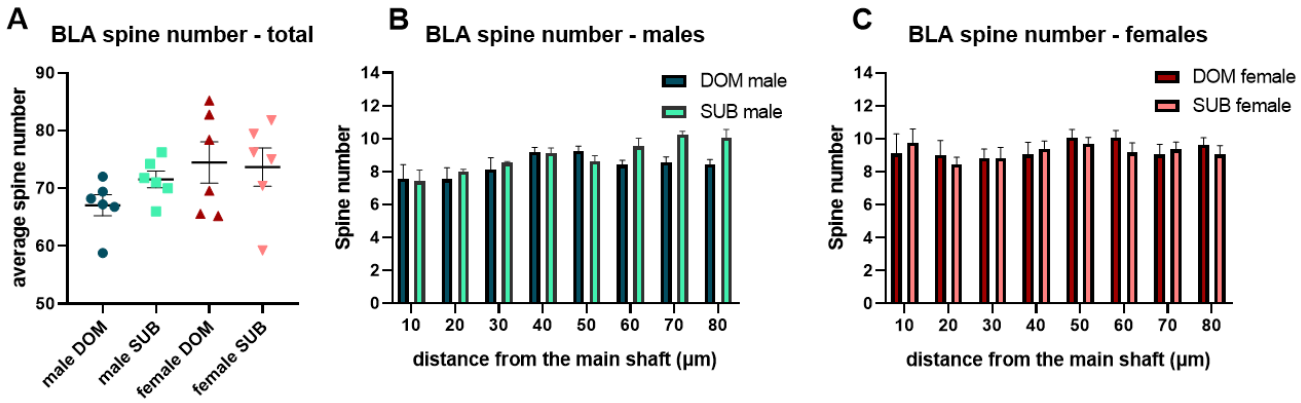


Figure 9. Data of spine numbers in the Basolateral Amygdala (BLA). A. *total spine number difference between DOM (mean = 67.07 ± 1.82) and SUB (mean = 71.53 ± 1.44) males, and DOM (mean = 74.47 ± 3.60) and SUB (mean = 73.67 ± 3.30) females.* ***B-C.*** *Spine number remodeling per segment of the apical dendrites.*

3.5. Correlations between spine number and physiological/behavioral variables:

Regardless of dominance ranking, Kendall's Tau correlations implicated some significant correlations between spine number remodeling and physiological and behavioral variables (see appendix H2 for all correlations). For males, there were no significant correlations between spine number and body weight, CORT change, adrenal weight, thymus weight, fat weight and testes weight (all $p > 0.05$), whereas for females, there were no significant correlations between spine number and number of wounds, location preference, and thymus weight ($p > 0.05$). In table 3 below, for the sake of space, only a subset of significant and relevant correlations is shown.

Table 3. Kendall's Tau correlations between spine number per region and selected behavioural / physiological variables. Left are Kendall's Tau values, right the P-values; * < 0.05, ** < 0.01.

		Location Preferance	CORT change	Body weight	Fat weight	Vesicle weight
Male	CA1	0.183, 0.431	-0.200, 0.392	-0.182, 0.411	0.121, 0.583	0.030, 0.891
	CA3	0.424, 0.055	-0.127, 0.586	0.061, 0.784	0.000, 1.000	-0.515, 0.020*
	BLA	0.116, 0.616	0.152, 0.493	0.152, 0.493	0.394, 0.075	-0.061, 0.784
	mPFC	-0.647, 0.005**	0.200, 0.392	0.303, 0.170	-0.364, 0.100	0.212, 0.337
Female	CA1	-0.061, 0.784	-0.515, 0.020*	-0.121, 0.583	0.455, 0.040*	-
	CA3	-0.030, 0.891	-0.303, 0.170	-0.455, 0.040*	0.545, 0.014*	-
	BLA	0.273, 0.217	-0.182, 0.411	-0.033, 0.131	0.545, 0.014*	-
	mPFC	-0.303, 0.171	-0.091, 0.681	-0.121, 0.583	0.212, 0.337	-

3.6. Colony Characteristics

We found no significant correlations for FemDom and stability with any other colony characteristic or bodyweight, CORT and number of wounds (see figure 10A-B). We did find a significant negative correlation between the number of wounds per colony and the average change in bodyweight for males ($r = -0.868$, $p < 0.001$) (not shown in figure). Furthermore, we found that the intensity of aggression showed a significant negative correlation with the change in CORT in SUB males ($r = -0.792$, $p = 0.019$) (figure 9C), but not in DOM males ($r = 0.091$, $p = 0.846$). See appendix D5 for all correlations between colony characteristics.

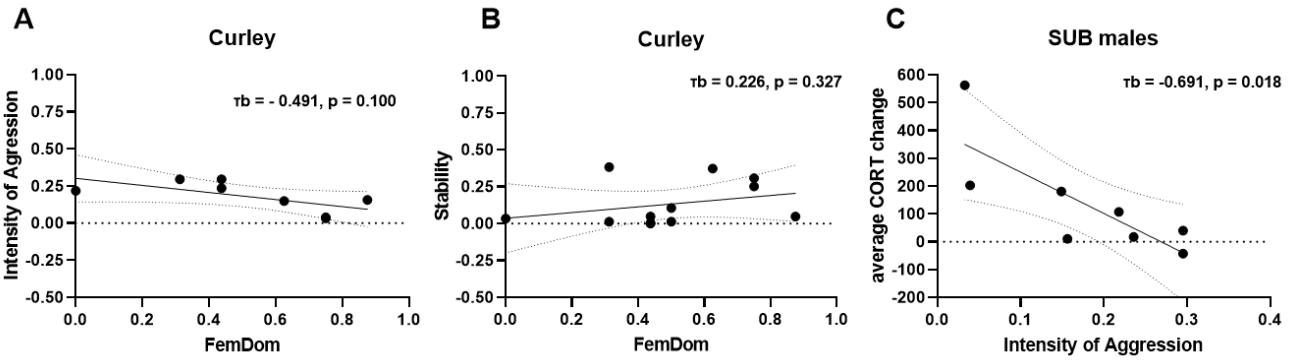


Figure 10. Scatterplots of selected colony characteristics. A. *Female Dominance (FemDom) with the average Intensity of Aggression per colony.* **B.** *FemDom with Stability per colony.* **C**. *intensity of aggression and average CORT change in SUB (but not for DOM males). For all correlations, Kendall's Tau was calculated.*

4. Discussion

4.1. Behavior

As expected, this study showed that WTG rats readily form a dominance hierarchy. Dominant males exhibited significantly more offensive behavior and spent more time in the open-arena than subordinate males, in line with previous results (Blanchard et al., 1993; Tamashiro et al., 2004; Patel et al., 2020). Interestingly, this behavioral difference in dominance rank for males was also seen in females. For both males and females, the number of agonistic interactions decreased over time, with most dominant males at day 3 remaining the most dominant male throughout the colony housing, and the subordinate male at day 3 staying subordinate, and vice versa for females. Moreover, most colonies showed a stable hierarchy from day 3 of VBS-housing onwards, which is in line with our expectations.

However, the total number of agonistic interactions of each female was not affected by their dominance rank, whereas dominant males engaged in much more fights than subordinate males. Moreover, dominant and subordinate females did not differ in their location preference, nor did they differ in the number of wounds, as all females were seldomly harmed. This is in stark contrast with the pattern found for males, suggesting that females, despite showing clear participation in the dominance hierarchy (as reflected by clear differences in agonistic behavior per rank), likely experience subordination in a different manner than subordinate males do. Moreover, as female dominance did not show any correlation with the intensity of aggression, stability of the colony, or changes in body weight or CORT, this study suggests that females do not experience the dominance hierarchy the same as males do.

4.2. About physiological differences between dominant and subordinate rank

As mentioned in the introduction, a common response to VBS housing is an increased activation of the HPA axis in dominant rats, as compared to unstressed controls, with a further increase in subordinate rats compared to dominant rats. Increased HPA activity leads to higher glucocorticoid secretion into the bloodstream that affects a host of organs and tissues in the body. A sub-question within this study is whether we find similar changes in organ weight per dominance rank in the Wild Type Groningen (WTG) rat strain as compared to the Long-Evans rat strain. Additionally, we are interested in the effect of the dominance rank of females on organ and tissue changes.

For males, one of the most consistent consequences of VBS housing is that subordinate individuals lose a significant amount of total body weight and show a substantial elevation of basal CORT secretion, as compared to dominants, (Blanchard et al., 1993; Blanchard et al., 1995; McKittrick et al., 2000; Tamashiro et al., 2004). Usually, dominants also show increased basal CORT levels as compared to controls, but not as much as subordinates, which is likely the result of experiencing stress from efforts to protect their dominance rank. Whereas dominants usually lose little to no weight and healthy controls show increased body weight, subordinates often lose weight (largely lean body mass) until it is 85-90% of their original weight. However, this study found that body weight was reduced for both dominant (94%) and subordinate (91%) males, with no significant difference between them. Similarly, we did not replicate any difference between dominants and subordinates in their CORT metabolite levels. It should be noted that for batch 2, all CORT levels were increased, which has likely affected our finding in multiple ways (including an increase in variance, and therefore decreased likelihood of finding a statistically significant difference between groups), reducing its replicability.

Adipose tissue consists of visceral (between organs) and subcutaneous (under the skin) fat. Since visceral fat, like retroperitoneal fat, but not necessarily subcutaneous fat, is associated with negative health consequences such as metabolic syndrome (Hung et al., 2014), we analyzed the relative weight of retroperitoneal fat for investigating cardio-metabolic relationships. Despite both

dominants and subordinates losing subcutaneous fat in VBS studies, compared to controls, they both show an increased percentage of visceral fat, which is more pronounced in subordinates (Tamashiro et al., 2004; Tamashiro et al., 2007). Our finding that subordinate males show a slightly higher percentage of retroperitoneal fat than dominant males, although non-significant, is in line with previous studies.

With regards to adrenal weight, both subordinate and dominant individuals have been found to show a relative increase compared to unstressed controls, with no clear differences between the two (Blanchard et al., 1995; Tamashiro et al., 2004). Our finding that there is no difference between subordinate and dominant individuals in adrenal weight change confirms this pattern. Furthermore, previous research shows a bigger decrease in thymus weight in subordinate males as compared to dominant males, for both standard (2 females and 4 males) and female-biased colonies (4 females and 2 males) (Blanchard et al., 1995; Tamashiro et al., 2004). This study showed, however, a non-significant trend (($p = 0.078$) in the opposite direction, with the relative thymus weight of dominant males being lower than of subordinate males.

There is a lot of data that shows that the hypothalamic-pituitary-gonadal axis is suppressed as a response to chronic stress, resulting in lower sexual behavior and reproduction in males (Hardy et al., 2002). Males in the standard colonies show decreased levels of plasma testosterone and decreased testes weight, while they remain unaffected in dominant individuals, compared to controls (Blanchard et al., 1995; Hardy et al., 2002; Tamashiro et al., 2004). For female-biased colonies, however, the dominant individuals also showed decreased plasma testosterone levels, alongside even lower levels in subordinates (Tamashiro et al., 2004). This relationship between dominance rank and testes weight was, however, not found in this study, despite implementing colonies with 4 females. Nor did we find an effect of dominance rank on the relative weight of seminal vesicles, which are glands involved with semen production and male reproduction. There is to the best of our knowledge no data on seminal vesicles weight changes for the VBS paradigm, but it has been found that rats subjected to chronic immobilization and forced swimming stress show decreased height and secretion of the seminal vesicle (Lamsaard et al., 2021).

For females, we similarly found little differences between dominant and subordinate rank in relative organ weight. We did find, however, a difference between males and females for change in body weight, thymus weight, and adrenal weight – but not for fat weight and plasma CORT levels. Whereas males decreased in bodyweight, both dominant (103%) and subordinate (108%) females showed an increase in body weight during VBS housing. (Both are based on the median. If based on the mean it would be 107% and 108%, respectively.) However, this study showed that body weight for males was negatively correlated with adrenal weight and positively correlated with time spend in the open surface, whereas no such correlations where found for females. Since females also increased in body weight when paired with one male, prior to colony housing, it is likely that changes in body weight have a different meaning for females than for males.

The observation that females, as compared to males, showed a higher relative thymus weight, might suggest that females are less stressed than males. This seems, however, not reflected by the higher relative adrenal weight and similar levels of CORT of females compared to males. Nonetheless, higher relative adrenal weight does not translate directly into an increased stress experience in females. This is because plasma CORT is bounded to corticotrophin binding globulin (CBG) in much higher levels in females than in males, resulting in lower levels of biologically active (free) corticosteroids (Linthorst et al., 2009). An higher relative adrenal weight in females compared to males does not, therefor translate into higher levels of biological active CORT. Moreover, in this study, CORT levels were measured by obtaining CORT metabolites from feces, instead of CORT from plasma, as is usually done. It has been shown that female rodents excrete fewer CORT metabolites in their feces than males, with males secreting 73% of their circulating CORT metabolites and females only 53%

(Touma, Sachser, Möstl & Palme, 2003). Because of these factors, it is hard to directly compare CORT (metabolite) levels and relative adrenal weight between males and females. Future research is needed that take into account these factors in order to reliably compare male and female stress levels.

In summary, this study failed to replicate the differences in dominance rank for males for almost all physiological variables that were found to differ in previous studies. Only fat weight showed an almost significant difference between dominance rank, with the same pattern as found in the literature, while for thymus weight we even found a reversed, nonsignificant pattern. These physiological results suggest that subordinate and dominant individuals in this study experienced similar levels of stress during the VBS housing. It is likely that subordination showed similar stress levels as the dominant rats seen in other VBS studies than vice versa, since both did not lose any body weight after day 3 of VBS housing. The only significant differences we found were between males and females, irrespective of dominance rank. These sex differences included higher body and thymus weight for females. These differences might have nothing to do with the dominance hierarchy, however, as social stress in females is usually more evident during social instability and disruption than because of dominance rank (Haller, Fuchs, Halasz & Makara, 1999). As such the WTG rat does not seem to be a useful model within the VBS paradigm to investigate chronic stress-induced physiological differences between dominant and subordinate individuals, for both males and females.

4.3. Differences in methodology – a comparison of scoring method between Curley and Miguel

One objective of this study was to see which scoring method (Curley's or Miguel's) results in a dominance hierarchy that is better reflected by the physiological parameters associated with dominance rank. Curley's method produces very similar results as the scoring method of Miguel, showing similar center points and variances in physiological variables for each dominance group. Directly correlating the dominance ranking of each scoring method with the physiological variables also produces very similar, if not identical results. Curley's method is, however, seems more effective in ranking nuanced differences at the lower end of the dominance hierarchy. This difference in likely explained by the fact that Curley's method allows for scoring more agonistic interactions, thereby distinguishing rank even between individuals who barely fought at all.

Moreover, we found that Curley's method results in a higher number of colonies where a female obtains the highest dominance rank, with only 0.08% for Miguel's method and 0.42% for Curley's. This is likely explained by the fact that whereas Miguel's method only analyzed day 1, 2, 5, and 10, Curley's method also scored agonistic interactions on day 3 and 8. Since it has been shown that female, but not male, aggression rises again after day 5 (Zhou, Sandi & Hu, 2018), the inclusion of an extra day increases the relative weight of female agonistic interactions. Moreover, since average levels of aggression decreased over colony housing, and aggressive interactions often happen in bouts of activity, Miguel's approach of scoring short time periods of only 10 minutes, resulted in finding relatively little agonistic interactions during day 5 and day 10. This was less so the case for Curley's method.

4.4. Differences in methodology – Wild Type Groningen rats as animal model for VBS studies

One of the objectives of this study was to validate the VBS paradigm and to investigate whether the Wild Type Groningen (WTG) rat is a useful strain for VBS studies. As previously mentioned, this study failed to replicate most physiological and neurobiological findings found in other VBS studies, specifically regarding differences between dominant and subordinate rats. Most experimental data that comes from the VBS paradigm is based on the Long-Evans (LE) rat strain, which has been inbred and maintained at the University of Hawaii in the laboratory of the Blanchards and Sakai (Blachards et al., 1995). Interestingly, while our results did not replicate the findings found for LE rats, our results

are similar to those of the study of Patel et al. (2020), who also implemented WTG rats within the VBS paradigm. They found no discernable difference between subordinate and dominant males for bodyweight, adrenal weight, fat weight and seminal vesicle weight. Since their study included a control group, they additionally showed that both dominant and subordinate males decreased in bodyweight and fat but increased in adrenal weight. Interestingly, it seems that regardless of dominance rank, WTG rats decrease in bodyweight and increase in adrenal weight, a pattern usually only seen in LE subordinate rats in VBS studies.

As previously mentioned, LE rats, like WTG rats, reliably form a dominance hierarchy (Blachard et al., 1995; Tamashiro, 2007). LE rats usually show clear and strong differences in chronic stress levels between subordinate and dominant males and are therefore a useful model strain for VBS studies investigating the effects of rank-specific chronic social stress. Whereas LE rats show on average high levels of offensive aggression, WTG rats show a much larger variation in levels of offensive aggressive behavior (de Boer, van der Vegt & Koolhaas, 2003). It is likely that the differences in observed results for LE and WTG rats, in physiology and neurobiology, are possibly explained by differences in aggression levels between the two strains.

The relationship between average aggression levels and colony-induced stress levels per dominance rank was investigated by Buwalda, Koolhaas and de Boer (2017). In their study, different WTG colonies were formed for non-aggressive (NA), low-medium aggressive (LA-MA), and high-aggressive (HA) males, as selected by the resident-intruder paradigm before colony housing. Using the same LE rats as used in most VBS studies, they also formed colonies with only (3) LE rats, and mixed colonies with both LE and WTG rats. They found that especially colonies with HA WTG males and colonies with only LE rats, showed a strong reduction in subordinate body weight, but not as much for LA-MA and NA WTG colonies. Moreover, whereas for all WTG colonies body weight only decreased during the first few days of VBS housing (and increased again once the dominance hierarchy was established), the body weight in LE colonies continued to decrease throughout the experiment. This was also the case for subordinate LE rats in mixed WTG-LE colonies (with a WTG rat as dominant), but not for subordinate WTG rats. LE rats exhibited such a high number of bite wounds and decreased body weight that the authors stopped the colony housing for ethical considerations. In contrast, WTG rats only showed high levels of agonistic interactions and bite wounds during the first few days, which reduced significantly once the social hierarchy was established. It should be noted, however, that Buwalda et al. (2017) based their dominance ranking on differences in bodyweight changes and not on differences in agonistic behavior.

Thus, the study of Buwalda et al. (2017) suggests, like our study, that subordination is not necessarily experienced as a stressor in WTG rats, under the condition that the colony is not comprised of solely highly aggressive WTG rats. The level of aggression is an important component of the behavioral strategy to cope with environmental stressful demands. Whereas rats high in aggression generally cope proactively with stressors by trying to prevent them from happening, rats with low aggression levels show a tendency to passively react to the stressors, both being associated with different profiles of associated physiological and neurobiological determinants (de Boer, Buwalda & Koolhaas, 2017). Considering our results, this suggests that WTG rats seem to passively habituate to their rank, whereas LE rats will keep trying to prevent, or change, their subordination rank, causing them to experience substantially higher levels of stress than WTG rats. Accordingly, both in our study and in the study of Buwalda et al. (2017), subordinate and dominant WTG rats did not show, on average, a significant difference in long-term basal CORT levels.

In conclusion, WTG rats still experience a certain degree of chronic social stress, albeit without much difference per social rank and certainly not as severe as LE subordinate rats. It seems that whereas the high levels of aggression found in LE rat's cause subordination to be very stressful, the lower and more

variable levels of aggression in WTG rats allows them to adapt to the dominance hierarchy, with most individuals 'accepting' their position. The specific strain that is used therefore likely influences the perceived levels of stress and is as such an important consideration for VBS and stress studies. Future research should explore the possibility that, whereas WTG rats seem to show 'normal' variability in levels of aggression, LE rats may reflect pathologically high aggression and an inability to 'accept', or 'adapt', to the dominance hierarchy. Depending on the variables of interest and the human population that the study tries to mimic (normal aggression stress or pathological aggression stress; successful adaptation or failure to adapt), VBS studies should make use of the different colony characteristics of WTG and LE strains and choose accordingly. As both humans and animals show large individual variability in their response to stress and experience of long-term adverse consequences, the variability within and between these two rat strains increases the face validity of the VBS model.

4.5. About the spine remodeling results

Chronic stress can lead to behavioral and cognitive abnormalities, which is reflected by structural alterations on a cellular level. Exposure to stress hormones can lead to behavioral and cognitive abnormalities, eliciting a range of plasticity mechanisms in different regions of the brain (Chattarji et al. 2015). As mentioned in the introduction, it is thought that these structural alterations, especially within the limbic system, may encode a form of memory that allows the animal to respond adaptively to the stressor the next time it is encountered. However, these changes can also reflect a failure to adapt, leading to long-term stress-related psychopathologies (Von Frijtag et al., 2000). Whereas chronic stress usually decreases dendritic complexity and reduces the spine number in the hippocampus and mPFC, it enhances these in the BLA.

4.5.1. Medial Prefrontal Cortex remodeling

Repeated restraint stress has been shown to decrease dendritic length and spine number of APD of mPFC neurons, sometimes even up to 18% (Goldwater et al., 2009; Cook & Wellman, 2004; Radley et al., 2006). Our finding that the prelimbic cortex of the mPFC of subordinate males also shows a decreased spine number of the APD, as compared to dominant males, is in line with previous findings regarding the effects of chronic stress (see introduction). This suggests that despite our failure to see clear stress-induced physiological differences between dominants and subordinates, we do see differences at a neuronal structural level.

As stress enhances behavioral inflexibility and habit learning (Dias-Ferreira et al., 2009; Park, Lee & Chey, 2017), it may be that rats that obtain a subordinate position shift from goal-directive behavior to a reliance on habits. As previously mentioned, the mPFC (mainly the PL) is responsible for the integration of contextual and past information in goal-directed behavior (Sierra-Mercado, Padilla & Quirk, 2011; Capuzzo & Floresco, 2020). The reduction in ApD spine number in the mPFC might, therefore, indicate that subordination stress is associated with a decreased ability to integrate contextual information for effective goal-directed behavior. This idea is supported by the study of Franklin et al. (2017), who used the tube test to determine dominance. They showed that repeated social defeat led to the synaptic weakening of the projections from the medial dorsal thalamus (MDT) to the mPFC - projections that are critical for working memory and attentional control (Bolkan et al., 2017; Schmitt et al., 2017). In other words, social defeat stress reduces mPFC synaptic input. However, in our study, we failed to observe a difference between subordinates and dominants in experienced stress levels, as reflected by equal CORT (metabolite) levels, suggesting that the reduction in mPFC synaptic complexity in subordinates is mediated by a different mechanism than CORT signaling. We also did not find any correlation between mPFC spines and change in CORT, regardless of rank. It might be that spines in the mPFC are reduced when rats, habitually, reside in the burrow system, as is reflected by our study finding of a significant positive correlation between mPFC spine number and

time spent in the open surface arena in males. Regardless of the causal mechanism, whether this neuronal change reflects an adaptation or maladaptation of WTG rats to subordination rank merits further research.

Another possibility for the observed dendritic differences between dominant and subordinate rats is that subordinates already showed a lower spine density than dominants prior to VBS housing. The mPFC of rats that show a higher density in spine number probably receives more information from other brain structures and may therefore be better equipped to integrate contextual information with experience. Dominance rank is associated with increased access and control over resources, which are reasonable goals for humans and rats alike. Accordingly, it has been shown that optogenetic stimulation of the synaptic input from MDT to the mPFC directly enhances dominance behavior, as reflected by an increased likelihood of winning fights in a tube test (Zhou et al., 2018). In our study, dominants and subordinates were distinguished by differences in their dominance behavior, with dominants initiating and winning significantly more agonistic interactions. Therefore, in line with the prior-attribute hypothesis (see introduction), the reduced number of ApD spines in subordinates might indicate that they are less able to fight for and maintain a higher dominance position prior to colony housing, a characteristic that caused them to obtain a lower dominance rank. Furthermore, that these neuronal rank differences are not caused by stress and may already be present before VBS-housing, is also illustrated by the fact that female subordinates also show reduced ApD spine number in the mPFC, whereas in contrast, other studies have shown that stress increases ApD spine number in the mPFC of females (Garrett & Wellman, 2009). However, this is still speculation. Whether dominance behavior or neuronal change came first, and whether this change occurred prior to or after colony housing, form interesting questions for future research.

At last, our study found that the difference between dominants and subordinates in spine number was mainly at the proximal segments of ApD. As ApD are crucial for the integration of information from different brain areas, the segmental location may indicate input from different brain areas. Moreover, the receiving postsynaptic potential (PSP) from dendritic spines closer to the main shaft has less distance to travel from the site of generation to the site of action potential initiation (axon hillock), meaning that proximal segments may have a larger effect on neuronal excitability (Spruston, Stuart & Häusser, 2016). Although the different functions (and connections) of apical and basal dendrites are known, current knowledge about the functional consequences of spine site on APD remains elusive. Future research is needed to investigate the relationship between apical dendritic segments and synaptic integration dynamics.

Interestingly, however, a recent study of Patel et al. (2021) showed that WTG rats exposed to repeated social defeat stress similarly showed a reduction in spine number in proximal segments of APD. However, there are two important differences between their study and our study. First, despite both studies investigating the mPFC, this study analyzed the spine density of APD of the prelimbic cortex specifically, whereas Patel et al. (2021) analyzed the infralimbic cortex. Secondly, we found that subordinate males showed lower spine numbers as compared to dominant males, whereas they found that not only 'losers' decreased in spine number, but also 'winners'. instead, they found that both showed an equal decrease in the spine number of the proximal segments of mPFC ApD. Future research is needed to replicate these findings and explore their possible implications.

4.6.2. Basolateral Amygdala and hippocampal CA1 and CA3 fields

Previous studies have shown that different models of stress elicit structural remodeling in spine number, with a decrease in spines on ApD in hippocampal pyramidal CA1 and CA3 neurons (Watanabe, Gould & McEwen, 1992; McKittrick et al., 2000; Chen et al., 2010; Herman & Tamashiro, 2017), and an increase in spine number on ApD of the amygdala (Mitra, Jadhav, McEwen, Vyas & Chattarji, 2005). In

this study, however, we failed to replicate these findings. The observation that dominants and subordinates do not significantly differ in these brain regions suggests that subordination is not associated with an increased experience of stress for both males and females, just like the physiological observations did. Since dominant individuals initiated more fights than subordinates and are therefor more aggressive, these results are in line with a recent study by Patel et al. (2021a), who similarly failed to demonstrate a difference between high and low aggressive rats in ApD spine densities in the CA1 and BLA.

Interestingly, however, Patel et al., (2021b) demonstrated that, compared to an unstressed control group, the spine number of ApD of CA1 was decreased for both 'winners' and 'losers' of agonistic interactions between WTG rats, without any difference in rank. It may therefore be possible that both subordinate and dominant males in our study actually decreased in spine density, rather than both ranks showing no loss of spines. However, since our study did not include an unstressed control group, it is hard to make any conclusions.

It is worth mentioning that in this study we were only able to include 7 rats from the dominant and subordinate groups, for both sexes. It is possible that with the inclusion of the other 5 rats per group, the slight difference in rank for the ApD spine number in CA1 and CA3 becomes significant. Noted, this study showed a slight, although non-significant, increase in spines in the CA3 of DOM males and females, compared to SUB males and females, respectively. CA3 neurons are involved in associating spatial locations with rewards (Cherubini & Miles, 2015). This suggests that subordinate individuals are slightly deficient in associating spatial context with reward, complimenting the idea that subordinates are less able to integrate contextual information for goal-direct behavior, compared to dominants. Moreover, the number of spines for the CA1 showed a slight, but non-significant increase in SUB males and females, compared to DOM males and females respectively. This is a surprising finding, considering that previous studies usually found fewer spines in the CA1 of (stressed) subordinate individuals. Moreover, dendritic spines increase in the CA1 when rats are put into complex environments that promote spatial learning (Moser et al., 1994; Diamond et al., 2006), and is as such crucial for information consolidation. Our finding that subordinate males and females show a slightly higher (non-significant) number of spines in the CA1 is, therefore, highly speculative at best, and merits further research. At last, this study showed a slight, but non-significant increase in ApD spines for the BLA of SUB males, as compared to DOM males. Just like the slight difference per rank in CA1, this pattern, if it would become significant with the inclusion of the other 5 rats per group, is in line with previous studies regarding the effects of (chronic) stress on the BLA (see introduction).

In conclusion, this study found no differences between dominant and subordinate individuals for the ApD spines of pyramidal neurons of the CA1, CA3 and BLA neurons, and only found a rank difference for the mPFC. To the best of our knowledge, the observation that subordinate males and females show a reduced number of spines in the mPFC might be explained in two ways. On the one hand, colony-induced stress might cause a deficit in mPFC functioning, reducing its capacity to integrate contextual information in a goal-directed manner. On the other hand, since dominance rank was not associated with any difference in CORT levels, it may be that subordinates showed fewer mPFC spines prior to colony-housing, which made them less able to fight for and achieve higher dominance rank. The idea that subordinates only experienced mild stress, and that their stress levels did not differ with dominants, is further illustrated by our failure to observe any rank difference in CA1, CA3 and BLA spines. However, our failure to see a rank difference might be caused by the small sample size (6) per group. Future research is needed to elicit the molecular mechanisms underlying these results. It is especially interesting to investigate whether the dendritic complexity of the mPFC might be causally involved with achieving high dominance rank in WTG rats housed in the VBS.

4.6. Limitations

This study does have a few limitations. No control group was used, which for this study, should have consisted of weight- and age-matched male-female pairs kept in conventional cages. This made it impossible to reliably conclude whether the similar results in dominance rank were due to both subordinates and dominants experiencing little stress, or both experiencing a lot of stress. Moreover, due to time constraints, we did not obtain as much data on possible brain alterations as we initially had hoped. We only obtained brain data based on the ranking method of Miguel (Escamilla et al. unpublished data, 2020). Although our analysis showed that the scoring methods showed similar results for the physiological variables, it might be that we missed nuanced differences in the neurobiological data. Moreover, we were only able to obtain the neuronal data of 6 male and 6 female rats for each of the brain regions. It may very well be that the inclusion of the remaining 6 rats will alter the data. At last, time constraints and technological complications made it impossible to investigate the molecular mechanisms involved with structural and functional remodeling.

This study also had a few methodological limitations. We experienced some technical difficulties with obtaining the CORT metabolite levels. For all colonies of batch 2, the data shows a sudden increase in the average change in CORT levels over colony housing. Moreover, when only looking at CORT pre-colony levels, but not post-colony levels, we see a sudden increase in batch 3. Since we did not find these sudden increases to affect dominants and subordinates statistically different, especially after removing two extreme outliers, we belief the CORT levels in this study to still be (limited) use. Nonetheless, these technical issues reduce the reliability of the CORT data. Furthermore, whereas for batch 2 and 3 water was available in the open surface arena and in the burrow system from the start of colony housing, for batch 1, water was only given in the open surface arena after the first two days of colony housing. Only after the experimenters saw that the lack of water in the burrows caused the subordinates to not have free water availability (as they preferred to stay in the burrow system), did they add water availability to the burrow system. This might have skewed the data from batch 1 from social stress to homeostatic stress, affecting the reliability of those results.

At last, we only had two student that scored agonistic behavior of all 12 colonies using the method of Curley. As both students were new to scoring agonistic behavior, the data may not be very reliable. For example, to an untrained eye, it is sometimes hard to distinguish between real agonistic aggression of rats and rumble-tumble play behavior, as is reported in Pellis and Pellis (1987). Furthermore, whereas for VBS 1 to 4, and 9 to 12, the sum of wins was used to calculate the dominance hierarchies, the count of wins was used for VBS 5 to 8. Since these colonies were also scored by different persons, it should be noted that the dominance hierarchies among these VBS' not solely reflect differences in characteristics of the VBS, but might be (partially) caused by differences in methodology. For similar reasons, we did not obtain data on the intensity of aggression for VBS 5 to 8 and only used VBS1-4 and 9-12 for our calculations, further limiting this study.

4.7. Conclusion

In summary, despite a clear difference in agonistic behavior between dominant and subordinate individuals, for both males and females, we did not find that physiology was differently affected by dominance rank. This suggests that subordinate and dominant individuals in this study experienced similar levels of stress during the VBS housing, which contrasts our hypothesis. Moreover, most colonies showed a stable hierarchy from day 3 onwards, showing decreased agonistic interactions for both males and females, whereas for other VBS studies this was less the case. Our hypothesis were based on previous VBS studies using Long Evans rats instead of Wild Type Groningen rats, suggesting that the difference in results is due to the use of different rat strains. It seems that whereas the high levels of aggression found in LE rats cause subordination to be very stressful, the lower and more variable levels of aggression in WTG rats allow them to adapt to the dominance hierarchy, with most individuals 'accepting' their position. Moreover, our study showed a clear difference in the number of apical dendritic spines in the medial PFC, in both males and females, without a clear rank-specific difference in colony-induced stress and other stress-related brain regions. Therefore, we hypothesized that this neural difference of the medial PFC existed (partially) prior to the colony housing, with rats that have a 'more developed' medial PFC are more able to fight for and achieve higher dominance rank. Interestingly, this suggests that VBS studies using Long Evans rats are not necessarily investigating the neuronal and physiological effects of chronic subordination stress, but the effects of subordination stress when rats are unable to adapt to the dominance hierarchy. As such, instead of investigating subordination stress, the WTG strain shows potential for studying the neuronal underpinnings of (mal) adaptive responses to psychosocial stressors. At last, we did not find any relationship between female dominance with the stability of the dominance hierarchy, average aggression or stress levels, or body weight changes of the colony members, nor did we find any rank-specific differences in female physiology and neurobiology (except for the medial PFC, which is likely not a result of stress). Despite females showing similar agonistic behavior as males, this does not translate into clear rank differences in physiology or neurobiology. Therefore, this study suggests that the role of females in the establishment of the dominance hierarchy, and the usefulness of the VBS model to investigate socially induced (chronic) stress in females, is of negligible value.

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Appendix A – Visible Burrow System

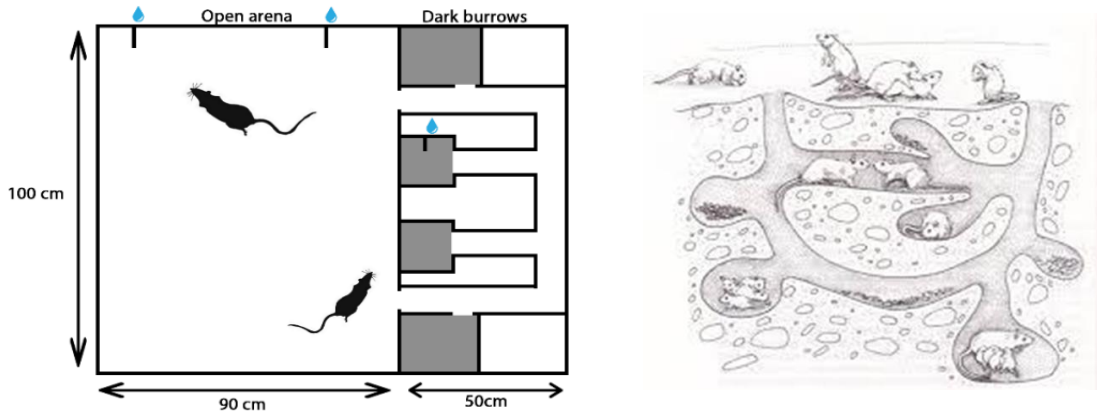


Figure Appendix A. **Visible Burrow System (VBS).** *Constructed at the University of Groningen, based on the design by Blanchard et al. (1995) with two extra chambers (nests). Left, the open surface arena with a imposed photoperiod of 12:12, is represented. At the droplet symbols, continuous water is available. Right, a representation of the natural environment in which wild rats live, and which the VBS tries to mimic.*

Appendix B – Dominance matrices

B1 - Average Dominance Index (ADI)

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

Figure Appendix C1. **Average Dominance Index**. *W_{ij} = dominance index with one specific interaction partner, j = individual for who the average dominance index is calculated, I = individual with whom one or more fight(s) have occurred*

The Dominance Index was calculated for each pair of individuals as the ratio of the number of conflicts won over a particular partner, divided by the total number of conflicts with that individual. We calculated an individual's average Dominance Index in relation to all group members, but whenever a pair did not interact at all it was excluded from the calculation of the average (Hemelrijk, Wantia & Gygax, 2005).

B2 – Female Dominance Index (FDI)

$$DOM_{femtot} = \frac{1}{N}\sum_{F} \frac{M_s}{M_t} \qquad DOM_{fem} = \frac{M_s}{M_t}$$

Figure Appendix C2. Female Dominance Index (FDI). *M_s = number of subordinate males M_t = total number of males, N = total number of females in the colony*

The Female Dominance Index (FDI) is a measure of the relative dominance of females over males. It ranges from 0 to 1 and reflects the summed number of males shower a lower ADI than females, divided by the sum of the potential number of males (always 4 in our study) showing a lower ADI than all individuals of the colony. This average percentage of males in a group dominated by each female was calculated by means of the standardized Mann-Whitney-U-Value.

B3 - Dominance matrices over time

The determination and temporal dynamics of the Dominance hierarchies, as determined by Curley's ranking. All are based on the 'Sum of Wins'. The calculations can be found in excel file 'DATA by Sebastiaan', under the sheets titled 'VBS1-4 DomMatrix' and 'VBS9-12 DomMatrix'. Note, that only VBS 1-4 and 9-12 are incluced, as the other matrixces were calculated by Hannah Lise.

VBS 1

Day 1

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank
F1		1	5	1					0,6786	M1 1
F2			1						0,5	M4 2
F3	2								0,1429	F1 3
F4	0								0	F2 4
M1								1	1	F3 5
M2									0	F4 6
M3									0	M2 7
M4	1				0	2	2		0,75	M3 8

Day 1,2

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank
F1		1	5	1					0,5119	M4 1
F2			1	1		0			0,6667	F2 2
F3	2								0,0952	F4 3
F4	2		1					0	0,5556	F1 4
M1								1	0,3333	M1 5
M2									0	F3 6
M3									0	M2 7
M4	1				2	9	2		0,9167	M3 8

Day 1,2,3

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank
F1		2	5	1					0,3952	M4 1
F2	1		1	2		0			0,7778	F2 2
F3	2	0							0,0952	F4 3
F4	4	0	3					0	0,6	M2 4
M1						0		1	0,1667	F1 5
M2					1				0,5	M1 6
M3									0	F3 7
M4	1				2	9	3		0,9167	M3 8

Day 1,2,3,5

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank
F1		3	5	3					0,4732	M4 1
F2	1		1	2		0			0,75	F2 2
F3	2	0							0,0952	F4 3
F4	4	0	4					0	0,5238	M2 4
M1						0		1	0,1	F1 5
M2					1				0,5	M1 6
M3									0	F3 7
M4	1				4	11	4		0,95	M3 8

Day 1,2,3,5,8

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank
F1		4	5	3					0,4188	M4 1
F2	1		1	2		0			0,7333	F2 2
F3	5	0							0,1667	F4 3
F4	5	0	4					0	0,5417	M2 4
M1						0		1	0,1	F1 5
M2					1				0,5	F3 6
M3									0	M1 7
M4	1				4	11	10		0,95	M3 8

Day 1,2,3,5,8,10

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank
F1		5	6	3			1		0,5508	F2 1
F2	1		1	3		0	1	2	0,7333	F4 2
F3	5	1		0			1	1	0,5909	M4 3
F4	5	0	4				1	0	0,6563	F3 4
M1						0		1	0,1	F1 5
M2					1				0,5	M2 6
M3								1	0,0154	M1 7
M4	1				4	13	12		0,6205	M3 8

VBS 2

Day 1

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank
F1		1	1						0,5	M1 1
F2	0		0	4	0	0			0,3333	M2 2
F3		0							0	F1 3
F4	1	2	2			0			0,3889	M3 4
M1				2					1	F4 5
M2	1		1	1			3	3	1	F2 6
M3				1					0,5	F3 7
M4									0	M4 8

Day 1,2

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank
F1		1	1	0					0,5	M2 1
F2	0		0	5	0	0			0,3571	F1 2
F3		0							0	M1 3
F4	1	2	4			0			0,381	M3 4
M1				2					0,5	F4 5
M2	1		1	2	2		3	4	1	F2 6
M3				1					0,5	F3 7
M4									0	M4 8

Day 1,2,3

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank
F1		3	1	2					0,5417	M2 1
F2	3		0	6	0	0			0,3889	F1 2
F3		2		1					0,3	M3 3
F4	1	3	4		0	0			0,2444	M4 4
M1			0	4					0,3333	F2 5
M2	2		1	2	8		3	9	1	M1 6
M3				1					0,5	F3 7
M4					1	0			0,5	F4 8

Day 1,2,3,5

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank
F1		3	1	5	0				0,5833	M2 1
F2	3		0	6	0	1			0,4167	M3 2
F3		2		2					0,3333	F1 3
F4	1	3	4		0	0	0		0,1944	M4 4
M1			0	4					0,25	F2 5
M2	2	1	1	2	10		6	11	0,9286	F3 6
M3				3	1				0,6667	M1 7
M4					1	0			0,5	F4 8

Day 1,2,3,5,8

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank
F1		3	2	7	0				0,5759	M2 1
F2	4		1	6	0	1		0	0,5179	M3 2
F3		2		2					0,2381	F1 3
F4	1	3	5		0	0	0		0,1954	F2 4
M1			0	4		0			0,25	M4 5
M2	2	1	1	2	12		10	15	0,9286	M1 6
M3				3	1				0,6667	F3 7
M4					1	0			0,5	F4 8

Day 1,2,3,5,8,10

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank
F1		4	3	8	0				0,362	M2 1
F2	5		1	7	0	1		0	0,3481	M3 2
F3		2		2					0,1905	M4 3
F4	3	3	5		0	0	0		0,2145	M1 4
M1	1		1	4		0	1		0,5833	F1 5
M2	2	1	1	2	12		10	15	0,9286	F2 6
M3	2	1		4	1				0,7	F4 7
M4		2			1	0			0,6667	F3 8

VBS 3

Day 1

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1									0	F2	1
F2				0				1	1	M1	2
F3									0	F1	3
F4									0	F3	4
M1						4	2	3	1	F4	5
M2	0				0				0	M2	6
M3									0	M3	7
M4									0	M4	8

Day 1,2

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1									0	F2	1
F2				0				1	1	M1	2
F3									0	F1	3
F4									0	F3	4
M1						10	4	3	1	F4	5
M2	0				0				0	M2	6
M3									0	M3	7
M4									0	M4	8

Day 1,2,3

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1									0	F3	1
F2			0	0		1		1	0,5	M1	2
F3		5							1	F2	3
F4									0	F1	4
M1		1				13	9	4	1	F4	5
M2	0				0				0	M2	6
M3									0	M3	7
M4									0	M4	8

Day 1,2,3,5

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1									0	M1	1
F2			0	1		1		1	0,6	F2	2
F3		5							0,5	F3	3
F4			1						0,5	F4	4
M1		1				14	11	11	0,9792	M4	5
M2	0				0				0	F1	6
M3					0				0	M2	7
M4					1				0,0417	M3	8

Day 1,2,3,5,8

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		1	1			1			0,8333	M1	1
F2			0	1	0	2		1	0,4167	F1	2
F3	1	5							0,5	F4	3
F4		1	1						0,75	F3	4
M1		2				14	13	11	0,9792	F2	5
M2	0				0				0	M4	6
M3					0				0	M2	7
M4					1				0,0417	M3	8

Day 1,2,3,5,9,10

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		1	1			1	2		0,875	M1	1
F2			0	1	0	4	2	1	0,5	F1	2
F3	1	5							0,375	F4	3
F4		1	1						0,75	F2	4
M1		2	1			16	15	11	0,9833	F3	5
M2	0				0				0	M4	6
M3					0				0	M2	7
M4					1				0,0417	M3	8

VBS 4

Day 1

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		7	2	5	0				1	F1	1
F2									0	M3	2
F3									0	M4	3
F4									0	F2	4
M1							0		0	F3	5
M2									0	F4	6
M3					1	0	2	5	1	M1	7
M4	0				3	1	0		0,6667	M2	8

Day 1,2

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		12	2	9	0				1	F1	1
F2	0		2	1					0,6667	M1	2
F3	0								0	M3	3
F4	0								0	F2	4
M1							1	10	0,8571	M4	5
M2									0	F3	6
M3					1	0	2	5	0,75	F4	7
M4	0				4	1	0		0,4286	M2	8

Day 1,2,3

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		16	7	14	0				0,9804	F1	1
F2	1		2	3					0,6863	M1	2
F3	0								0	M3	3
F4	0	0							0	F2	4
M1						1	4	12	0,9167	M4	5
M2					0				0	F3	6
M3					1	0	2	6	0,75	F4	7
M4	0				4	1	0		0,4167	M2	8

Day 1,2,3,5

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		23	8	18	0				0,9861	F1	1
F2	1		2	3		1	0	2	0,8083	M1	2
F3	0								0	F2	3
F4	0	0							0	M3	4
M1	0					2	4	13	0,8105	M4	5
M2					1			1	0,2083	M2	6
M3					1	0	2	6	0,7143	F3	7
M4	0	0			4	1	1		0,2195	F4	8

Day 1,2,3,5,8

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		25	9	19	0				0,9872	F1	1
F2	1		3	3		1	0	2	0,8077	M1	2
F3	0								0	F2	3
F4	0	0	0						0	M3	4
M1	0					11	6	16	0,9056	M4	5
M2					1			1	0,1458	M2	6
M3					1	0	2	6	0,7143	F3	7
M4	0	0		0	4	1	1		0,2107	F4	8

Day 1,2,3,5,8,10

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		28	9	20	0				0,9885	F1	1
F2	1		3	9		1	0	2	0,8069	M1	2
F3	0								0	F2	3
F4	0	0	0					0	0	M3	4
M1	0	0				15	7	17	0,9157	M4	5
M2				0	1			1	0,1406	M2	6
M3				1	0	2		6	0,7143	F3	7
M4	0	0	0	0	4	1	1		0,2083	F4	8

VBS 9

Day 1

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1									0	F4	1
F2									0	M2	2
F3									0	M4	3
F4		1			0				1	F1	4
M1									0	F2	5
M2	1				1		2	4	1	F3	6
M3									0	M1	7
M4					1	0	1		0,6667	M3	8

Day 1,2

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		3		2					0	M3	1
F2			0						0,225	F4	2
F3	1	3		0					0	M1	3
F4		1			0				0,8	M4	4
M1									0,5	F2	5
M2	1				2		3	6	0	F1	6
M3		1							0,875	F3	7
M4					1	0	2		0,5	M2	8

Day 1,2,3

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		5		4					0,5	F3	1
F2	0		0	3					0,15	M2	2
F3	1	3		1	0				1	M4	3
F4		1			0				0,0833	F1	4
M1									0	M3	5
M2	1	1			5		5	6	1	F2	6
M3		1							0,3333	F4	7
M4					1	0	2		0,6667	M1	8

Day 1,2,3,5

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		5		4					0,4286	M2	1
F2	2		2	6					0,2871	F3	2
F3	1	3		1	0				0,8667	M4	3
F4		2			0				0,0833	F1	4
M1									0	M3	5
M2	1	1			5		5	6	1	F2	6
M3		1							0,3333	F4	7
M4					1	0	2		0,6667	M1	8

Day 1,2,3,5,8

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		5		4					0,4063	M2	1
F2	3		2	6					0,305	F3	2
F3	1	3		1	0				0,8667	M4	3
F4		2			0				0,0833	F1	4
M1									0	M3	5
M2	1	1			9		5	7	1	F2	6
M3		1							0,3333	F4	7
M4					1	0	2		0,6667	M1	8

Day 1,2,3,5,8,10

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		5		5					0,4063	M2	1
F2	3		2	7		0			0,2877	F3	2
F3	1	5		1	0				0,9048	M4	3
F4		2			0				0,0741	F1	4
M1									0	M3	5
M2	1	1			9		5	7	1	F2	6
M3		1							0,3333	F4	7
M4					1	0	2		0,6667	M1	8

VBS 10

Day 1

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		0							0	M2	1
F2			1	1					0,6667	F2	2
F3	0			6					0,5	M1	3
F4			0						0	F3	4
M1		1		2					0,6667	F1	5
M2	1				1		0	0	1	F4	6
M3					0	0			0	M3	7
M4						0	0		0	M4	8

Day 1,2

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		0		2					0,25	M2	1
F2	2		2	1					0,75	F2	2
F3	1			12	0				0,6667	F3	3
F4		0			0				0	M1	4
M1		1		2					0,6667	F1	5
M2	1				1		0	0	1	F4	6
M3					0	0			0	M3	7
M4						0	0		0	M4	8

Day 1,2,3

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		0		4					0,2	M1	1
F2	2		3	2					0,6875	M2	2
F3	1	1		15	0				0,7108	F3	3
F4	1		2		0				0,0794	F2	4
M1		1		2		1		1	0,875	F1	5
M2	1				1		0	0	0,75	F4	6
M3					0	0			0	M3	7
M4						0	0		0	M4	8

Day 1,2,3,5

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		1		5					0,2917	M1	1
F2	2		3	2					0,5667	F3	2
F3	1	2		19	0				0,7683	M2	3
F4	1		2		0				0,0655	F2	4
M1		1		2		2		2	0,9167	F1	5
M2	1				1		0	0	0,6667	F4	6
M3					0	0			0	M3	7
M4						0	0		0	M4	8

Day 1,2,3,5,8

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		1		7					0,2813	M3	1
F2	3		3	2					0,5625	F3	2
F3	1	3		20	1				0,8523	M2	3
F4	1		2		0				0,054	M1	4
M1		1		2		3		6	0,6012	F2	5
M2	1				1		0	0	0,625	F1	6
M3					1	0			1	M4	7
M4			0		1	0	0		0,1429	F4	8

Day 1,2,3,5,8,10

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		1	0	9					0,2875	M3	1
F2	3		3	2					0,5313	F3	2
F3	2	5		22	1			1	0,9083	M2	3
F4	1		2		0				0,0458	M1	4
M1		1		3		3		8	0,6065	F2	5
M2	1				1		0	0	0,625	F1	6
M3					1	0			1	M4	7
M4			0		1	0	0		0,0556	F4	8

VBS 11

Day 1

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1			3						0,75	M2	1
F2									0	F1	2
F3	1	0							0,25	M4	3
F4									0	F3	4
M1									0	F2	5
M2		1			1		2	5	0,9583	F4	6
M3								0	0	M1	7
M4						1	1		0,5833	M3	8

Day 1,2

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		2	5	1				0	0,9444	M2	1
F2									0	F1	2
F3	1	0							0,1667	M4	3
F4									0	F3	4
M1									0	F2	5
M2		1			1		4	9	0,975	F4	6
M3								0	0	M1	7
M4						1	1		0,55	M3	8

Day 1,2,3

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		2	5	1				0	0,9444	M2	1
F2									0	F1	2
F3	1	0							0,1667	M4	3
F4									0	M1	4
M1							1		0,5	F3	5
M2		1			1		9	14	0,9833	F2	6
M3								0	0	F4	7
M4						1	1		0,5333	M3	8

Day 1,2,3,5

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		3	5	1				0	0,9444	M2	1
F2									0	F1	2
F3	1	1							0,5833	F3	3
F4									0	M4	4
M1							1		0,5	M1	5
M2		1			1		12	14	0,9833	F2	6
M3						0		0	0	F4	7
M4						1	1		0,5333	M3	8

Day 1,2,3,5,8

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		3	5	1	1			0	0,9583	M2	1
F2					3				0,25	F1	2
F3	1	1							0,3889	M4	3
F4	0		1		1				0,5	F4	4
M1							1		0,2	F3	5
M2		1		1	3		14	14	0,9867	F2	6
M3						0		0	0	M1	7
M4						1	1		0,5333	M3	8

Day 1,2,3,5,8,10

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1		5	5	3	2		1	1	0,9246	F1	1
F2	2		1		4	1			0,5714	M2	2
F3	1	1		0					0,2222	F2	3
F4	0	0	3		1				0,5	F4	4
M1	0						1	0	0,1667	M4	5
M2		1		1	4		14	14	0,8867	F3	6
M3						0		1	0,0833	M1	7
M4					1	1	2		0,4333	M3	8

VBS 12

Day 1

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1									0	M1	1
F2									0	M3	2
F3									0	F1	3
F4									0	F2	4
M1				1	2		4	5	0	0,9583	F3	5
M2									0	F4	6
M3					1				0,1667	M2	7
M4					0				0	M4	8

Day 1,2

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1									0	F2	1
F2			4						1	M1	2
F3		0				0	1		0,3333	M3	3
F4									0	F3	4
M1			1	2		4	5	0	0,9583	F1	5
M2									0	F4	6
M3			0		1			1	0,3889	M2	7
M4					0				0	M4	8

Day 1,2,3

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1			1						0,5	F2	1
F2			4						1	M1	2
F3		0				0	1	1	0,4	M3	3
F4									0	F1	4
M1			1	2		4	6	0	0,9375	F3	5
M2									0	F4	6
M3	1		0		2			1	0,5625	M2	7
M4					0				0	M4	8

Day 1,2,3,5

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1			1						0,1667	M1	1
F2			5	0			0		0,625	M4	2
F3	2	3		1		0	1	1	0,5903	F2	3
F4							1	0	0,3333	F3	4
M1			1	2		4	6	0	0,75	F4	5
M2									0	M3	6
M3	1	0	0		2			1	0,3167	F1	7
M4			1	0	1		2		0,7222	M2	8

Day 1,2,3,5,8

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1			1				2		0,4167	M4	1
F2			6	0		1	0		0,8333	F2	2
F3	2	3		1		0	1	1	0,5833	M1	3
F4							1	0	0,3333	F3	4
M1			1	2		4	6	0	0,75	F1	5
M2									0	F4	6
M3	2	0	0		2			1	0,175	M3	7
M4			1	0	2	1	7		0,8438	M2	8

Day 1,2,3,5,8,10

	F1	F2	F3	F4	M1	M2	M3	M4	AvgDI	DomRank	
F1			1				2		0,4167	F2	1
F2			7	0		1	0		0,8182	M1	2
F3	2	4		1		0	1	1	0,5051	M4	3
F4			1				1	1	0,625	F4	4
M1			1	2		4	6	0	0,75	F3	5
M2									0	F1	6
M3	2	0	0		2	0		1	0,1682	M3	7
M4			1	0	3	5	10		0,6818	M2	8

B4 – Simplistic overview of dominance ranking: method of Miguel Puentes-Escamilla's & method of James Curley

In this overview, the female dominance index per colony is included.

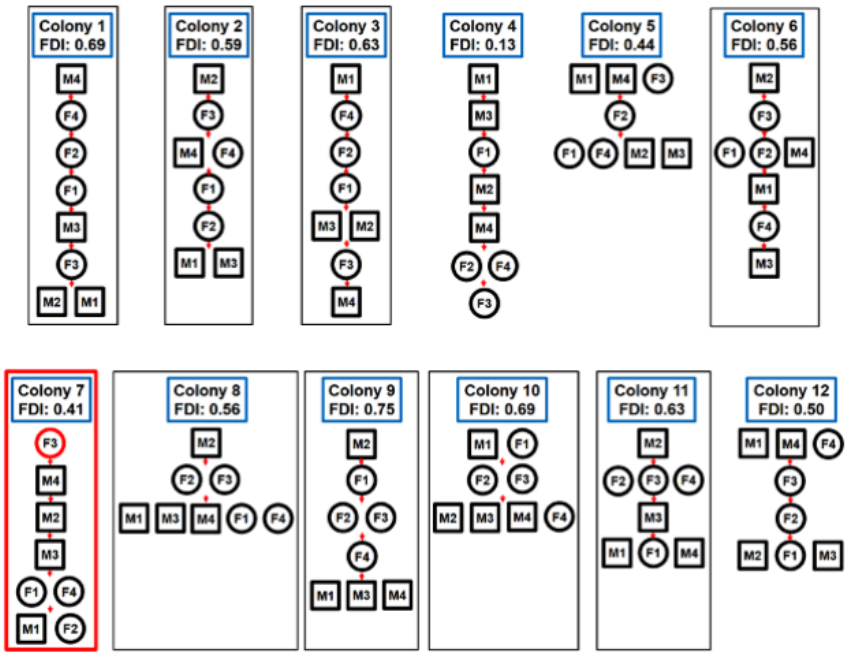


Figure Appendix C5.1. **Miguel Method**. *Based on the method by Puentes-Escamilla, Buwalda and Hoppenreijs (unpublished data, 2020). The red squares indicate female-dominant colonies.*

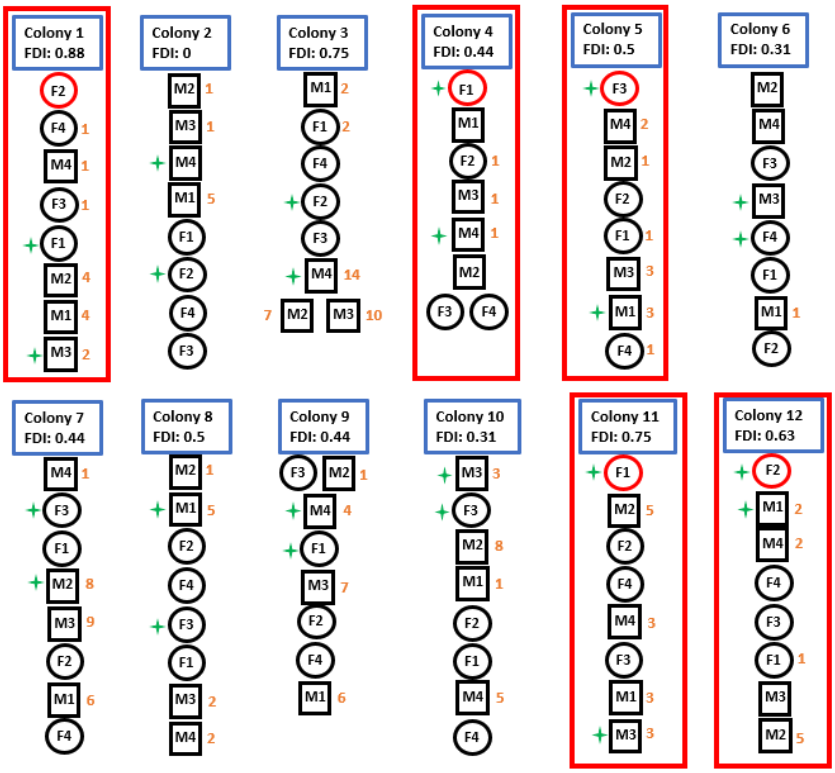
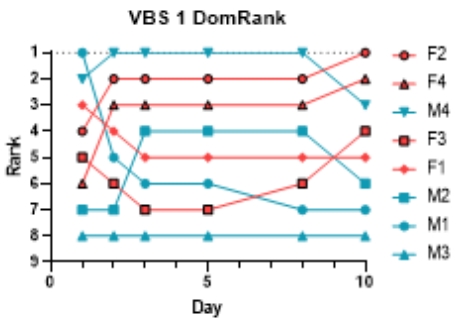
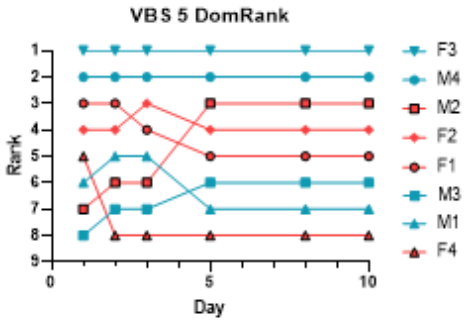


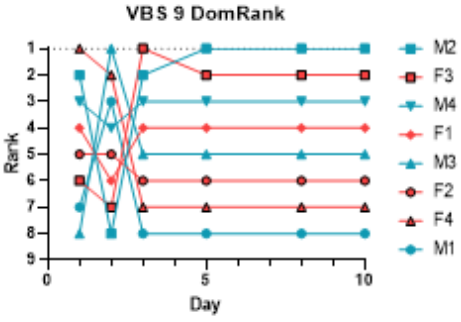
Figure Appendix C5.2. Curley Method. *Based on the method by Curley, Lee and Williamson (2016), implemented by Sebastiaan Legemaat and Hannah Lise Doosje. The red squares indicate female-dominant colonies. The number of wounds is indicated in orange, while rats that lost the most body weight for the males and females are indicated with a green star.*

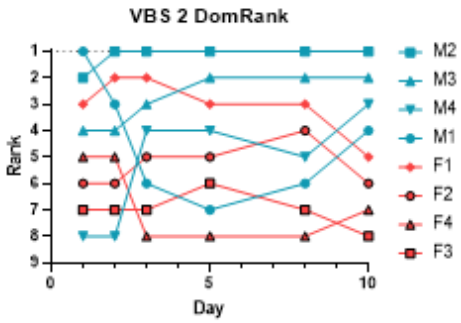
Appendix C – Temporal dynamics of Dominance rank per colony

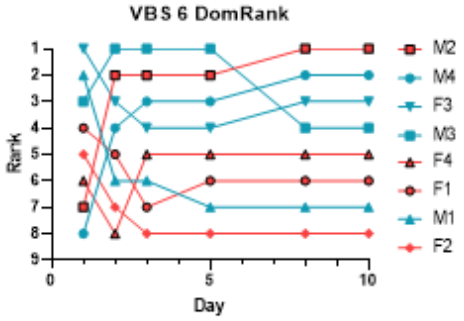
Batch 1 **Batch 2** **Batch 3**

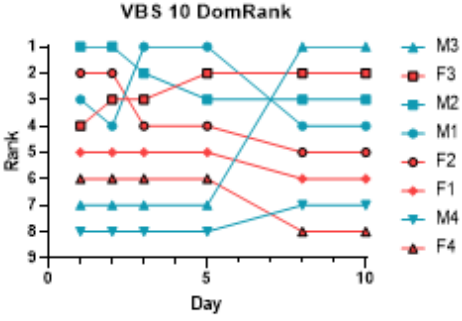


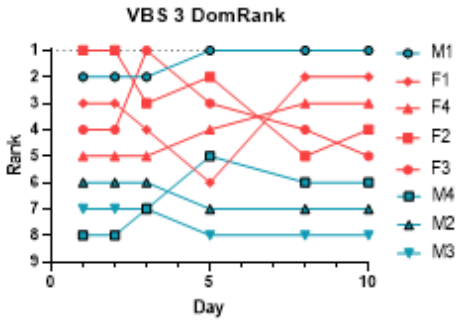


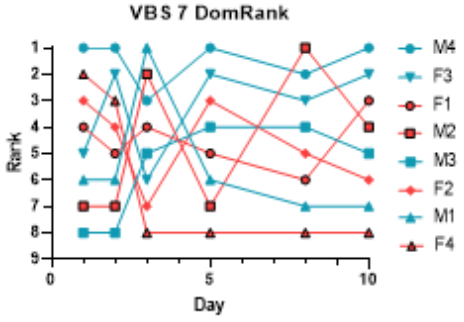


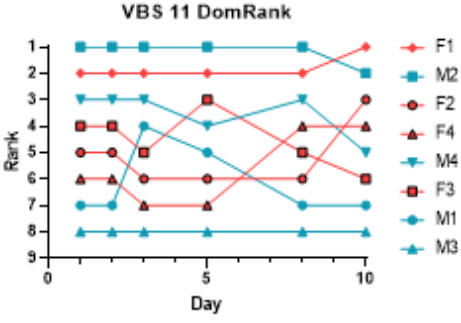


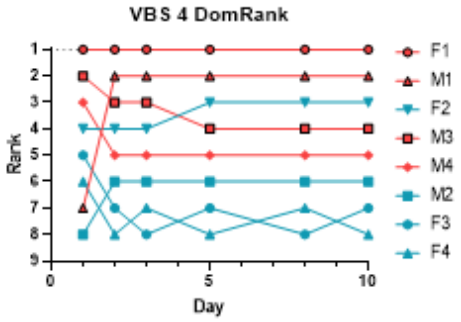


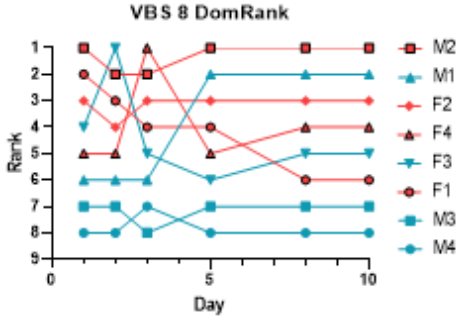


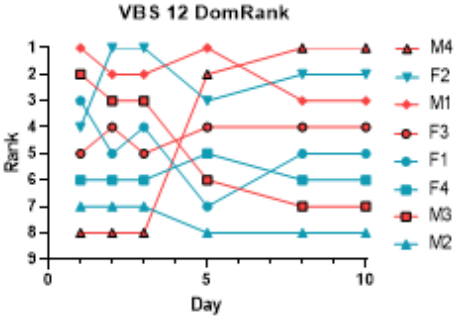












Appendix D – Colony Characteristics

All calculations of this appendix can be found in the excel file 'DATA by Sebastiaan', under the sheets titled 'Colony characteristics' (for an overview), 'IntensityAggressionCA&Ch', 'Number of wounds', and the sheets containing the dominance rankings per day per colony.

D1 - Female Dominance Index

Table Appendix F1. Female dominance.

Colony number	Female Dominance Index (FDI)
VBS 1	0.875
VBS 2	0
VBS 3	0.75
VBS 4	0.4375
VBS 5	0.5
VBS 6	0.3125
VBS 7	0.4375
VBS 8	0.5
VBS 9	0.4375
VBS 10	0.3125
VBS 11	0.75
VBS 12	0.625

The formula for female dominance index (FDI) can be found in appendix C2, and FDI calculations in the excel sheets titled 'Colony characteristics' 'VBS1-4 DomMatrix' and 'VBS912 DomMatrix'.

D2 - Stability

Table Appendix F2. Stability of colonies. *Correlations between the ranking of day 3 and day 10.*

Colony number	Kendall's Tau correlation	P-value	Natural logarithm
VBS 1	0,571	0,048	-3,0365543
VBS 2	0,618	0,034	-3,3813948
VBS 3	0,327	0,308	-1,1776555
VBS 4	0,885	0,003	-5,809143
VBS 5	0,714	0,013	-4,3428059
VBS 6	0,714	0,013	-4,3428059
VBS 7	0,571	0,048	-3,0365543
VBS 8	0,473	0,105	-2,2537949
VBS 9	0,982	0,001	-6,9077553
VBS 10	0,255	0,383	-0,9597203
VBS 11	0,34	0,252	-1,3783262
VBS 12	0,265	0,373	-0,9861769
		Fisher combined probability test	SUM: -37.6127 (75,225374)*-2 P = 3,441E-07

To calculate the stability of the colonies, we measured the correlation for the ranking after day 3 (when, usually, a stable hierarchy has formed) with the ranking after day 10. Note that each consecutive scored day includes the scored days of the previous days. To determine whether the colonies showed, on average, stable hierarchies from day 3 onwards, we also calculated the Fisher combined probability test (FCP) to assess the meta p-value of the stability p-values of all colonies together (see https://brainder.org/2012/05/11/the-logic-of-the-fisher-method-to-combine-p-values/). We took the sum of the natural logarithm of all p-values (of the stability correlations), multiplied that with '-2', and put the result in the excel function '=CHISQ.DIST.RT(result[75.2254 in our case];degrees of freedom[*24 in our case*])'. See the sheet titled 'Colony charecteristics'.

D3 - Intensity of Aggression (only for Batch 1 and 2)

Table Appendix F3. Intensity of Aggression. *The proportion of fights that involved clinch attack or chasing.*

Colony number	Mean Proportion of Fierce fights ± SEM
VBS 1	0.156 ± 0.066
VBS 2	0.218 ± 0.079
VBS 3	0.039 ± 0.021
VBS 4	0.236 ± 0.089
VBS 9	0.295 ± 0.122
VBS 10	0.295 ± 0.121
VBS 11	0.033 ± 0.022
VBS 12	0.149 ± 0.058

To calculate the proportion of fierce fights (defined as fights that included Clinch Attack or Chase), we first calculated the proportion of fierce fights of the total fights for each individual per colony, and then calculated the average proportion. For the calculations, see the sheet titled 'IntensityAggressionCA&Ch'. CA stands for 'Clinch Attack' and Ch stands for 'Chase'. Only VBS 1-4 and 9-12 are presented, as we did not have data on the types of agonistic interaction for VBS 5-8

D4 - Number of wounds

Table Appendix F4. Number of Wounds.

Colony number	Total number of Wounds	Mean ± SEM
VBS 1	13	1.625 ± 0.565
VBS 2	7	0.875 ± 0.611
VBS 3	26	4.375 ± 1.889
VBS 4	4	0.375 ± 0.183
VBS 5	11	1.375 ± 0.420
VBS 6	1	0.125 ± 0.125
VBS 7	24	3.000 ± 1.402
VBS 8	10	1.250 ± 0.620
VBS 9	20	2.250 ± 1.048
VBS 10	18	2.125 ± 1.060
VBS 11	14	1.750 ± 0.701
VBS 12	10	1.250 ± 0.620

See the excel sheet titled 'Number of wounds'.

D5 - Correlations between Colony Characteristics (including Corticosterone, Bodyweight, and Number of Wounds)

For an overview of the data used for these correlations, see excel file 'DATA by Sebastiaan', under the sheet titled 'Colony characteristics'. We used SPSS to check for normality and homogeneity, but also to calculate Kendall's Tau correlations.

Normality tests:

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Intensity of Agression	,203	7	,200*	,872	7	,195
Wounds	,132	7	,200*	,977	7	,944
FemDom	,161	7	,200*	,915	7	,435
Stability	,298	7	,059	,829	7	,078
Bodyweight Males	,291	7	,075	,875	7	,205
mSubWeight	,287	7	,084	,804	7	,045
mDomWeight	,114	7	,200*	,994	7	,998
Bodyweight Females	,338	7	,015	,809	7	,051
CORTMales	,233	7	,200*	,933	7	,576
CORTFemales	,311	7	,040	,860	7	,151
CORTsubMales	,258	7	,174	,847	7	,116
CORTdomMales	,215	7	,200*	,870	7	,185

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Note that this correlation matrix does not separate Bodyweight females into a DOM and SUB group.

Correlations

Note that the blue box in this correlation matrix means the significant correlation that it contains is logical, considering that SUB males are a subgroup of males. CORT males didn't show any significant correlations either.

Correlations between Colony characteristics (and CORT and Bodyweight)

		FemDom Miguel	FemDom Curley	Intensity of Agression	Wounds	Stability	Body weight Males	Body weight Submales	Body weight Dommales	Body weight Females	CORT Males	CORT Submales	CORT Dommales	CORT Females
FemDomMiguel	Correlation Coefficient	1,000	,163	,189	,313	,205	-,140	-,047	-,450*	,047	-,140	-,224	-,204	-,419
	Sig. (2-tailed)	.	,483	,527	,166	,367	,534	,836	,045	,836	,534	,346	,389	,062
	N	12	12	8	12	12	12	12	12	12	12	11	11	12
FemDom	Correlation Coefficient	,163	1,000	-,491	,272	,226	-,191	-,350	-,095	,318	-,032	,153	,058	-,127
	Sig. (2-tailed)	,483	.	,100	,235	,327	,403	,125	,676	,163	,889	,526	,812	,577
	N	12	12	8	12	12	12	12	12	12	12	11	11	12
Agression	Correlation Coefficient	,189	-,491	1,000	-,109	-,327	,109	,182	-,109	-,400	-,400	-,691*	-,293	-,036
	Sig. (2-tailed)	,527	,100	.	,708	,262	,708	,533	,708	,170	,170	,018	,362	,901
	N	8	8	8	8	8	8	8	8	8	8	8	7	8
Wounds	Correlation Coefficient	,313	,272	-,109	1,000	,295	-,779*	-,290	-,412	,107	-,046	,073	,147	-,137
	Sig. (2-tailed)	,166	,235	,708	.	,189	<,001	,192	,063	,630	,837	,755	,532	,536
	N	12	12	8	12	12	12	12	12	12	12	11	11	12
Stability	Correlation Coefficient	,205	,226	-,327	,295	1,000	-,215	-,031	-,431	,185	-,123	,037	-,220	-,062
	Sig. (2-tailed)	,367	,327	,262	,189	.	,335	,890	,054	,408	,582	,876	,349	,783
	N	12	12	8	12	12	12	12	12	12	12	11	11	12
BodyMales	Correlation Coefficient	-,140	-,191	,109	-,779**	-,215	1,000	,455*	,455*	-,121	-,152	-,127	-,273	-,061
	Sig. (2-tailed)	,534	,403	,708	<,001	,335	.	,040	,040	,583	,493	,586	,243	,784
	N	12	12	8	12	12	12	12	12	12	12	11	11	12
mSubWeight	Correlation Coefficient	-,047	-,350	,182	-,290	-,031	,455*	1,000	,212	,000	-,333	-,200	-,309	,182
	Sig. (2-tailed)	,836	,125	,533	,192	,890	,040	.	,337	1,000	,131	,392	,186	,411
	N	12	12	8	12	12	12	12	12	12	12	11	11	12
mDomWeight	Correlation Coefficient	-,450*	-,095	-,109	-,412	-,431	,455*	,212	1,000	,121	,152	,236	,018	,303
	Sig. (2-tailed)	,045	,676	,708	,063	,054	,040	,337	.	,583	,493	,312	,938	,170
	N	12	12	8	12	12	12	12	12	12	12	11	11	12
BodyFemales	Correlation Coefficient	,047	,318	-,400	,107	,185	-,121	,000	,121	1,000	,303	,200	,091	,333
	Sig. (2-tailed)	,836	,163	,170	,630	,408	,583	1,000	,583	.	,170	,392	,697	,131
	N	12	12	8	12	12	12	12	12	12	12	11	11	12
CORTMales	Correlation Coefficient	-,140	-,032	-,400	-,046	-,123	-,152	-,333	,152	,303	1,000	,491*	,673**	,242
	Sig. (2-tailed)	,534	,889	,170	,837	,582	,493	,131	,493	,170	.	,036	,004	,273
	N	12	12	8	12	12	12	12	12	12	12	11	11	12
CORTsub	Correlation Coefficient	-,224	,153	-,691*	,073	,037	-,127	-,200	,236	,200	,491*	1,000	,467	,200
	Sig. (2-tailed)	,346	,526	,018	,755	,876	,586	,392	,312	,392	,036	.	,060	,392
	N	11	11	8	11	11	11	11	11	11	11	11	10	11
CORTdom	Correlation Coefficient	-,204	,058	-,293	,147	-,220	-,273	-,309	,018	,091	,673**	,467	1,000	,382
	Sig. (2-tailed)	,389	,812	,362	,532	,349	,243	,186	,938	,697	,004	,060	.	,102
	N	11	11	7	11	11	11	11	11	11	11	10	11	11
CORTFemales	Correlation Coefficient	-,419	-,127	-,036	-,137	-,062	-,061	,182	,303	,333	,242	,200	,382	1,000
	Sig. (2-tailed)	,062	,577	,901	,536	,783	,784	,411	,170	,131	,273	,392	,102	.
	N	12	12	8	12	12	12	12	12	12	12	11	11	12

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix E – Agonistic (behaviorally) demographics

The data that is presented in table .. below comes from the excel document 'DATA by Sebastiaan', under the sheets titled 'DATAsebas', 'DATAhanne', and 'Behavioral Characteristics'.

Table. Appendix B.1 Demographics of antagonistic behaviors per colony, rank and sex. All variables are represented as the number of counts, with the percentage of 'initiated' and 'won' fights of the total number of fights per group between brackets. Total number of fights include all male-male, male-female, and female-female interactions.

VBS	Rank	Males			Females		
		Total	Initiated	Won	Total	Initiated	Won
1	**DOM**	41	35 (0.85%)	35 (0.85%)	30	12 (0.40%)	10 (0.33%)
	SUB	20	1 (0.05%)	4 (0.20%)	42	26 (0.62%)	22 (0.52%)
2	**DOM**	50	45 (0.90%)	46 (0.92%)	39	22 (0.56%)	16 (0.40%)
	SUB	30	10 (0.33%)	13 (0.43%)	26	7 (0.27%)	8 (0.31%)
3	**DOM**	50	45 (0.90%)	48 (0.96%)	10	6 (0.60%)	5 (0.96%)
	SUB	23	2 (0.09%)	0 (0.00%)	14	8 (0.57%)	7 (0.50%)
4	**DOM**	58	43 (0.74%)	43 (0.74%)	78	61 (0.78%)	72 (0.92%)
	SUB	25	4 (0.16%)	2 (0.08%)	20	3 (0.15%)	3 (0.15%)
5	**DOM**	66	62 (0.94%)	63 (0.95%)	6	4 (0.67%)	4 (0.67%)
	SUB	17	2 (0.12%)	2 (0.12%)	2	1 (0.50%)	1 (0.50%)
6	**DOM**	53	41 (0.77%)	42 (0.79%)	13	7 (0.54%)	8 (0.62%)
	SUB	24	5 (0.21%)	6 (0.25%)	10	2 (0.20%)	2 (0.20%)
7	**DOM**	50	38 (0.76%)	39 (0.78%)	17	11 (0.65%)	10 (0.59%)
	SUB	29	6 (0.21%)	6 (0.21%)	16	5 (0.65%)	5 (0.59%)
8	**DOM**	64	57 (0.89%)	56 (0.88%)	14	10 (0.71%)	10 (0.71%)
	SUB	48	5 (0.10%)	11 (0.23%)	14	7 (0.50%)	6 (0.43%)
9	**DOM**	26	24 (0.92%)	25 (0.96%)	14	9 (0.64%)	9 (0.64%)
	SUB	13	0.0 (0.00%)	0 (0.00%)	18	3 (0.17%)	2 (0.11%)
10	**DOM**	6	4 (0.67%)	3 (0.50%)	49	37 (0.76%)	37 (0.76%)
	SUB	16	5 (0.31%)	2 (0.13%)	43	6 (0.14%)	4 (0.09%)
11	**DOM**	38	35 (0.92%)	35 (0.92%)	31	23 (0.74%)	20 (0.65%)
	SUB	23	4 (0.17%)	2 (0.09%)	16	5 (0.31%)	2 (0.13%)
12	**DOM**	26	18 (0.69%)	15 (0.58%)	19	12 (0.63%)	10 (0.53%)
	SUB	14	0.0 (0.00%)	1 (0.07%)	9	4 (0.44%)	3 (0.33%)

The data that is presented in tables .. below comes from the excel document 'DATA by Sebastiaan', under the sheets titled 'DATAsebas' and 'Aggression over time'.

Table. **Appendix B.2 Hours analyzed of antagonistic interactions per day per colony**. Note that only data from VBS1-4 and 9-12 are used, as we did not know exactly how many hours were analyzed per day per colony for VBS 5-8.

VBS	Day						
	1	**2**	**3**	**5**	**8**	**10**	**Total**
1	1	1.5	1.5	1	1	3	9
2	1	1	1.5	1.5	3	3	11
3	1	3	1	3	4	4	16
4	1.5	2.5	3	2	3	3	15
9	2	3	4	3	4	3	19
10	2	2	4	3	3	3	17
11	2	3	3	4	4	3	19
12	2	3	3	2	4	4	18

Table. Appendix B.3 Demographics of total number of fights within each sex over time during the VBS colony housing. M-m, male-male agonistic interactions. F-f, female-female agonistic interactions. Between brackets, the total number of fights are normalized with the total time of scoring behavior, showing the number of total fights per hour. Note that only data from VBS1-4 and 9-12 are used, as we did not know exactly how many hours were analyzed per day per colony for VBS 5-8.

VBS	Sex	Day 1	2	3	5	8	10
1	m-m	8 (8)	9 (6)	2 (1.33)	5 (5)	6 (6)	6 (2)
	f-f	19 (19)	7 (4.67)	18 (12)	6 (6)	8 (8)	9 (3)
2	m-m	6 (6)	3 (3)	13 (8.67)	10 (6.67)	11 (3.67)	1 (0.33)
	f-f	23 (23)	8 (8)	15 (10)	6 (4)	8 (2.67)	9 (3)
3	m-m	10 (10)	8 (2.67)	9 (9)	10 (3.33)	2 (0.5)	4 (1)
	f-f	2 (2)	0 (0)	7 (7)	2 (0.67)	7 (1.75)	1 (0.25)
4	m-m	22 (14.67)	16 (6.40)	8 (2.67)	5 (2.50)	14 (4.67)	8 (2.67)
	f-f	16 (10.67)	14 (5.60)	22 (7.33)	16 (8)	8 (2.67)	10 (3.33)
9	m-m	26 (13)	5 (1.67)	5 (1.25)	0 (0)	5 (1.25)	0 (0)
	f-f	1 (0.5)	13 (4.33)	9 (2.25)	10 (3.33)	2 (0.50)	6 (2)
10	m-m	10 (5)	0 (0)	2 (0.5)	3 (1)	7 (2.33)	2 (0.67)
	f-f	12 (6)	14 (7)	14 (3.5)	8 (2.67)	8 (2.67)	9 (3)
11	m-m	11 (5.5)	6 (2)	13 (4.33)	4 (1)	4 (1)	4 (1.33)
	f-f	8 (4)	6 (2)	3 (1)	2 (0.50)	3 (0.75)	14 (4.67)
12	m-m	15 (7.5)	2 (0.67)	3 (1)	5 (2.5)	7 (1.75)	9 (2.25)
	f-f	0 (0)	6 (2)	2 (0.67)	10 (5)	3 (0.75)	3 (0.75)

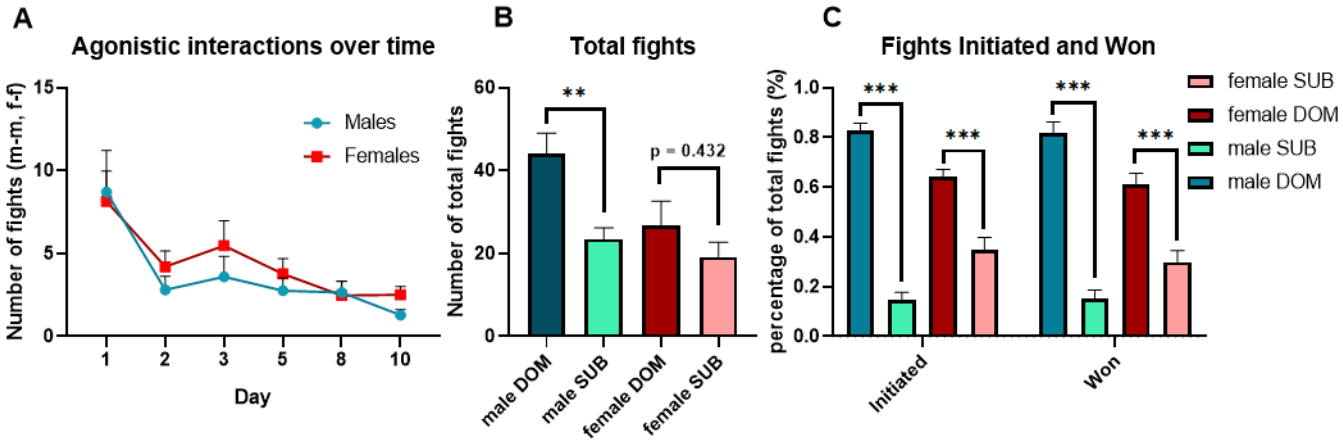
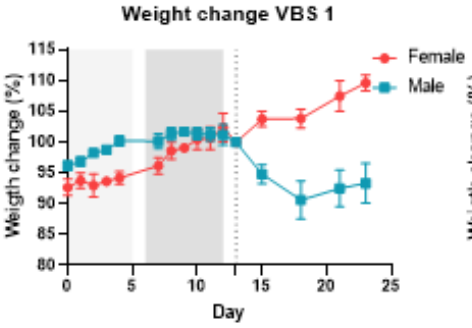
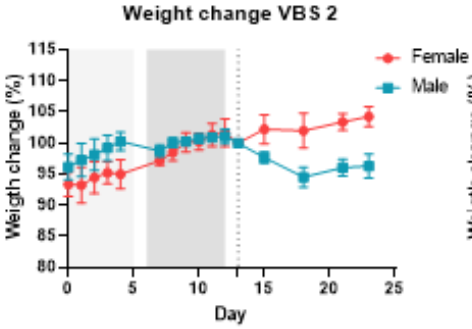


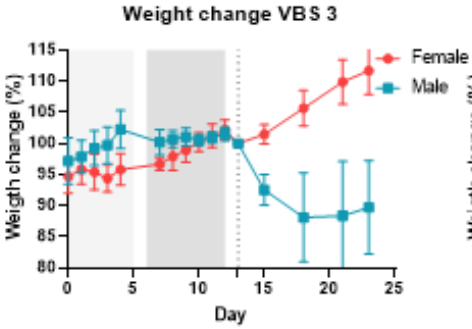
Figure Appendix B.1. Graphs of antagonistic data. ***A.*** *Agonistic interactions over time.* ***B****. Total fights.* ***C****. Fights initiated and won (in percentage).*

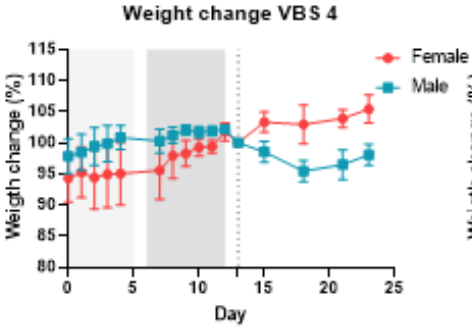
Appendix F – Temporal dynamics of Weight change

Batch 1









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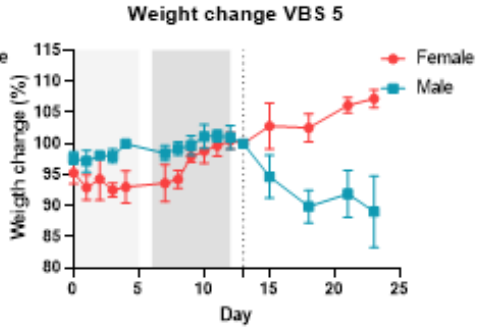

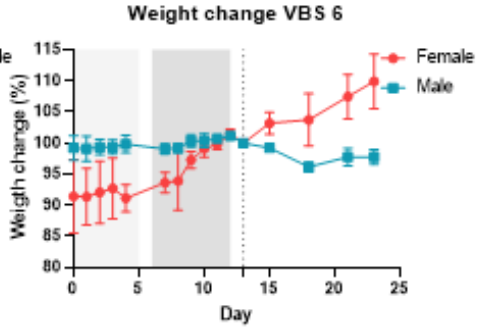

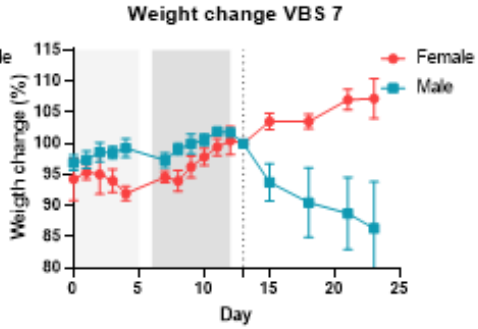

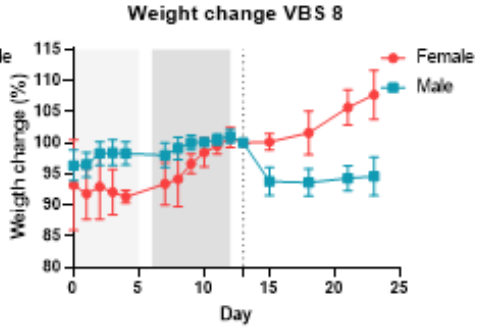

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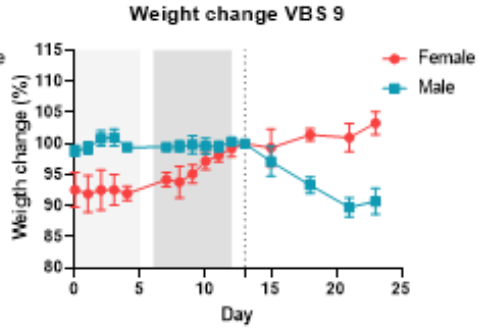
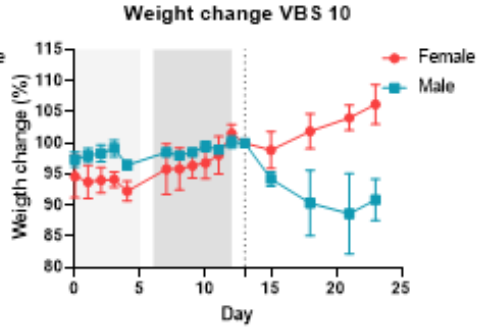
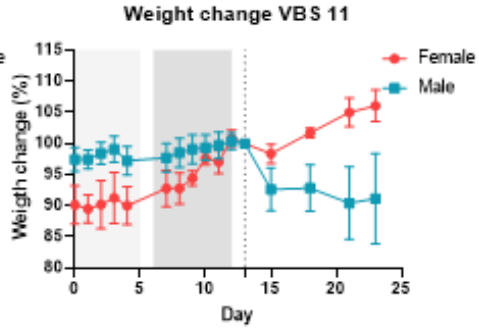
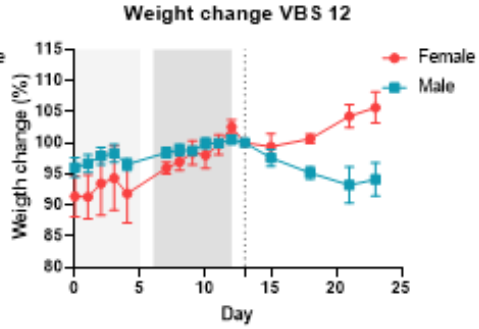








Appendix G – Statistics for ranked dominance groups

G1 - Behavioral variables - descriptive statistics and tests for normality and homogeneity

Table Appendix G1.1. Agonistic (behaviorally) demographics. This is based on the data represented in Appendix B. The P values of Levenes Test of homogeneity are presented for all four groups, but also for only the male groups (put in cursive).

Variable	Group	Mean (and size N) & median	SD & SEM	Skewness	Kurtosis	Normality p-value (S-W)	Homogeneity p-value (Levene)
Total fights	DOM-Male	44.00 (12), 50.00	17.596, 5.080	-0.881	0.469	0.354	Mean 0.087
	SUB-Male	23.500 (12), 23.00	9.472, 2.734	1.591	3.569	0.052	Median 0.340
	DOM-Female	26.67 (12), 18.00	20.628, 5.955	1.578	2.558	0.028	
	SUB-Female	19.17 (12), 16.00	12.408, 3.582	1.059	0.639	0.076	
Initiated fights	DOM-Male	0.830 (12), 0.872	0.108, 0.031	-0.523	-1.384	0.087	Mean 0.027
	SUB-Male	0.146 (12), 0.872	0.108, 0.031	0.361	-0.569	0.628	Median 0.118
	DOM-Female	0.640 (12), 0.645	0.107, 0.031	-0.862	1.049	0.575	
	SUB-Female	0.349 (12), 0.313	0.172, 0.050	0.250	-1.501	0.231	
Won fights	DOM-Male	0.820 (12), 0.864	0.151, 0.044	-1.170	0.557	0.045	Mean 0.711
	SUB-Male	0.150 (12), 0.121	0.122, 0.035	0.998	1.418	0.276	Median 0.735
	DOM-Female	0.610 (12), 0.629	0.157, 0.045	0.113	0.577	0.988	
	SUB-Female	0.299 (12), 0.310	0.162, 0.047	0.131	-1.610	0.148	

Table Appendix G1.2. Location preference. Curley's ranking and Miguel's ranking are separately presented, with for each group the presence of an outlier noted. The P values of Levenes Test of homogeneity are presented for all four groups, but also for only the male groups (put in cursive).

Location Preference	Group	Mean	SD & SEM	Skewness	Kurtosis	Normality p-value (S-W)	Homogeneity p-value (Levene)
Curley 1 outlier	DOM-Male	0.551, 0.542	0.202, 0.058	-0.665	1.653	0.388	Mean 0.452
1 outlier	SUB-Male	0.169, 0.110	0.208, 0.060	2.860	8.968	<0.001	Median 0.534
1 outlier	DOM-Female	0.563, 0.564	0.084, 0.024	0.735	1.009	0.628	*mean 0.060*
	SUB-Female	0.535, 0.531	0.158, 0.046	0.131	-0.583	0.913	
Miguel 1 outlier	DOM-Male	0.564, 0.564	0.206, 0.059	-0.880	1.735	0.404	Mean 0.108
1 outlier	SUB-Male	0.089, 0.066	0.071, 0.021	1.317	0.976	0.023	
1 outlier	DOM-Female	0.576, 0.544	0.106, 0.031	1.355	1.345	0.049	*mean 0.060*
	SUB-Female	0.569, 0.605	0.160, 0.046	-0.478	-0.426	0.586	

G2 - Physiological variables - descriptive statistics and tests for normality and homogeneity

G2.1 Using the approach of Curley

Table G.1.1. Overview of descriptive statistics based on the grouping of Curley's ranking. All values that may represent a violation of the assumptions are marked in red. The P values of Levenes Test of homogeneity are presented for all four groups, but also for only the male groups (put in cursive).

Variable	Group	Mean (and size N) & median	SD & SEM	Skewness	Kurtosis	Normality p-value (S-W)	Homogeneity p-value (Levene)
Bodyweight change 1 outlier DM R; Rank Trans-formed	DOM-Male	94.285(12), 95.012	3.894, 1.124	-0.773	0.623	0.759 R: 0.914	Mean 0.001 R mean: 0.002 Median 0.060
	SUB-Male	91.944(12), 93.156	6.964, 2.011	-0.716	-0.968	0.056 R: 0.051	
	DOM-Female	106.772(12), 103.557	5.440, 1.570	1.054	-0.478	0.005 R: 0.002	*Males mean 0.041* *Females mean >0.001*
	SUB-Female	107.599(12), 107.589	1.993, 0.575	0.229	-0.437	0.822 R: 0.319	
Adrenal weight	DOM-Male	0.162(12), 0.153	0.475, 0.013	0.558	-0.281	0.643	Mean 0.055 Median 0.090
	SUB-Male	0.155(12), 0.149	0.029, 0.008	0.368	-1.356	0.253	
	DOM-Female	0.272(12), 0.279	0.082, 0.024	-1.043	1.395	0.362	*mean 0.159* *median 0.242*
	SUB-Female	0.297(12), 0.316	0.050, 0.014	-0.208	-1.948	0.038	
CORT change Outliers are excluded, as they showed a >2000x change	DOM-Male	85.113(11), 43.599	157.868, 47.600	1.359	1.356	0.050	Mean 0.148 +outliers: 0.031 Median 0.414
	SUB-Male	184.356(11), 181.284	188.187 56.740	0.803	-0.062	0.328	
	DOM-Female	169.281(12), 60.583	232.715 67.179	0.655	-1.314	0.035	
	SUB-Female	183.113(12), 94.341	233.625 67.442	0.601	-1.240	0.065	
Fat weight 1 outlier R; Rank Trans-formed	DOM-Male	22.440(12), 20.832	6.342, 1.831	1.202	0.858	0.076 R: 0.059	Mean 0.032 R mean: 0.081 Median 0.282
	SUB-Male	31.990(12), 30.845	9.010, 2.601	0.460	-1.094	0.336 R: 0.315	
	DOM-Female	28.167(12), 23.156	12.199, 3.522	0.402	-1.635	0.057 R: 0.057	*Males mean 0.190* *Females mean 0.370*
	SUB-Female	30.098(12), 25.608	11.130, 3.210	1.001	-0.385	0.020 R: 0.369	
Thymus weight 1 outlier DM 1 outlier 1 outlier	DOM-Male	0.518(11), 0.501	0.175, 0.053	1.421	2.940	0.119	Mean 0.311 Median 0.320
	SUB-Male	0.701(12), 0.678	0.182, 0.052	0.210,	-1.533	0.253	
	DOM-Female	1.029(12), 1.060	0.277, 0.080	-0.771	0.784	0.703	*mean 0.468* *median 0.461*
	SUB-Female	1.136(12), 1.138	0.168, 0.049	-0.549	0.386	0.475	
Wounds 1 outlier DM 2 outliers 1 outlier	DOM-Male	1.583(12) 1.000	1.379 0.398	1.415	2.658	0.039	Mean <0.001 Median <0.001
	SUB-Male	3.750(12) 4.000	2.221 0.641	-0.220	-1.154	0.548	
	DOM-Female	0.250(12) 0.000	0.622 0.179	2.555	6.242	<0.001	*mean 0.039* *median 0.057*
	SUB-Female	0.083(12) 0.000	0.289 0.083	3.464	12.000	<0.001	
Testes weight	DOM-Male	7.516(12), 7.961	1.527, 0.441	-0.194	-0.800	0.461	Mean 0.147 Median 0.326
	SUB-Male	7.008(12), 7.689	1.998, 0.577	-0.119	-1.472	0.272	

Vesicle weight	DOM-Male	2.724(12), 2.624	0.754, 0.218	1.653	3.905	0.046	Mean 0.338 Median 0.350
	SUB-Male	2.814(12), 2.739	0.890, 0.257	0.680	-0.191	0.392	

All variables (besides bodyweight and wounds) are in mg/g. Bodyweight is in percentage of starting weight at the beginning of the VBS-housing.

G2.2 Using the approach of Miguel

Table G.1.2. Overview of describtive statistics based on the grouping of Miguel's ranking. All values that may represent a violation of the assumptions are marked in red. The P values of Levenes Test of homogeneity are presented for all four groups, but also for only the male groups (put in cursive).

Variable	Group	Mean (and size N) & median	SD & SEM	Skewness	Kurtosis	Normality p-value (S-W)	Homogeneity p-value (Levene)
Bodyweight change 1 outlier DM	DOM-Male	93.856(12), 95.096	3.994, 1.153	-0.701	0.992	0.816	Mean 0.169 Median 0.291
	SUB-Male	91.137(12), 92.597	5.960, 1.721	-0.783	-0.533	0.116	
1 outlier	DOM-Female	105.893(12), 105.265	3.750, 1.083	1.485	2.274	0.029	*mean 0.254* *Median 0.378*
1 outlier	SUB-Female	107.740(12), 108.823	2.954, 0.853	-1.430	1.149	0.015	
Adrenal weight 1 outlier	DOM-Male	0.160(12), 0.161	0.037, 0.012	-0.208	-0.797	0.624	Mean 0.299 Median 0.391
	SUB-Male	0.167(12), 0.158	0.051, 0.015	1.216	2.162	0.159	
	DOM-Female	0.285(12), 0.279	0.062, 0.018	0.287	-1.124	0.466	*mean 0.479* *Median 0.488*
	SUB-Female	0.309(12), 0.325	0.046, 0.013	-0.309	-1.861	0.040	
CORT change **Strangely,** the outliers here increases homogeneity	DOM-Male	61.840(11), 43.590	102.819, 31.001	1.077	1.020	0.213	Mean < 0.001 +outliers: 0.027 Median 0.079 +outliers: 0.429
	SUB-Male	152.673(11), 127.802	172.356, 51.967	0.174	-1.422	0.381	
	DOM-Female	203.445(12), 63.246	244.161, 70.483	0.458	-1.878	0.011	
	SUB-Female	197.534(12), 213.095	225.973, 65.233	0.332	-1.241	0.168	
Fat weight 2 outliers 1 outlier	DOM-Male	22.845(12), 20.832	6.744, 1.947	0.890	-0.247	0.273	Mean 0.750 Median 0.711
	SUB-Male	33.712(12), 34.532	8.503, 2.455	0.119	-1.266	0.580	
	DOM-Female	23.449(12), 21.101	10.781, 3.112	1.335	0.974	0.016	*mean 0.410* *Median 0.360*
	SUB-Female	25.593(12), 23.386	9.212, 2.659	1.280	1.866	0.119	
Thymus weight 1 outlier DM	DOM-Male	0.472(11), 0.457	0.200, 0.060	1.500	2.561	0.286	Mean 0.373 Median 0.604
	SUB-Male	0.621(12), 0.602	0.225, 0.065	-0.052	-1.181	0.802	
	DOM-Female	1.145(12), 1.049	0.297, 0.086	0.348	-0.864	0.358	*mean 0.318* *Median 0.314*
	SUB-Female	1.015(12), 1.045	0.256, 0.074	-0.451	-0.200	0.734	
Wounds 2 outliers 1 outlier 1 outlier	DOM-Male	2.083(12), 1.000	2.539, 0.733	2.208	5.087	<0.001	Mean 0.004 Median 0.009
	SUB-Male	5.000(12), 5.000	3.592, 1.037	1.298	3.113	0.131	
	DOM-Female	0.083(12), 0.000	0.289, 0.083	3.464	12.000	<0.001	*Males mean 0.554*

1 outlier	SUB-Female	0.167(12), 0.000	0.389, 0.112	2.055	2.640	<0.001	*Females mean 0.084*
Testes weight	DOM-Male	7.182(12), 7.739	1.737, 0.501	-0.465	0.186	0.848	Mean 0.858
	SUB-Male	6.737(12), 7.332	1.496, 0.432	-0.312	-1.502	0.119	Median 0.842
Vesicle weight	DOM-Male	2.597(12), 2.591	0.887, 0.256	0.940	2.090	0.492	Mean 0.609
	SUB-Male	2.333(12), 2.151	0.668, 0.193	0.451	0.225	0.921	Median 0.539

All variables (besides bodyweight and wounds) are in mg/g. Bodyweight is in percentage of starting weight at the beginning of the VBS-housing.

G3 - Neurobiological variables - descriptive statistics and tests for normality and homogeneity

Note that due to time constraints, we were not able to obtain neurobiological from the ranking method of Curley. As such, all variables are based on the scoring method of Miguel.

Table G.3. Overview of descriptive statistics based on the grouping of Miguel's ranking. All values that may represent a violation of the assumptions are marked in red. The P values of Levenes Test of homogeneity are presented for all four groups, but also for only the male groups (put in cursive).

Variable	Group	Mean	SD & SEM	Skewness	Kurtosis	Normality p-value (S-W)	Homogeneity p-value (Levene)
CA1	DOM-Male	78.73(6), 77.10	9.597, 3.918	1.714	3.697	0.093	Mean 0.849
	SUB-Male	81.30(6), 81.90	7.845, 3.203	0.153	-1.607	0.553	Median 0.910
	DOM-Female	77.00(6), 78.20	6.467, 2.640	-0.342	-1.533	0.723	*mean 0.886*
	SUB-Female	77.73(6), 75.90	9.508, 3.881	0.377	-1.775	0.313	*median 0.780*
CA3	DOM-Male	59.40(6), 60.10	5.049, 2.061	-0.894	0.265	0.501	Mean 0.378
	SUB-Male	52.50(6), 52.10	5.727, 2.338	0.264	-1.426	0.615	Median 0.626
	DOM-Female	57.40(6), 58.50	8.663, 3.536	-1.568	3.153	0.146	*mean 0.801*
	SUB-Female	51.97(6), 54.50	11.690, 4.772	-1.483	2.755	0.262	*median 0.805*
BLA	DOM-Male	67.07(6), 67.70	4.461, 1.821	-1.456	3.109	0.227	Mean 0.044
	SUB-Male	71.53(6), 71.40	3.527, 1.440	-0.360	0.387	0.951	Median 0.091
	DOM-Female	74.47(6), 74.00	8.812, 3.598	0.118	-2.484	0.260	*mean 0.848*
	SUB-Female	73.67(6), 75.60	8.089, 3.302	-1.313	1.829	0.376	*median 0.872*
mPFC	DOM-Male	81.88(6), 82.50	7.156, 2.263	-0.704	-0.391	0.408	Mean 0.446
	SUB-Male	64.57(6), 64.30	6.188, 2.526	0.226	-1.238	0.780	Median 0.481
	DOM-Female	82.10(6), 80.50	4.900, 2.000	1.183	1.094	0.422	*mean 0.389*
	SUB-Female	69.03(6), 67.60	8.284, 3.382	0.501	0.039	0.911	*median 0.421*

All variables (besides Location preference) refer to the number of spines. Location preference refers to the time spent in the open arena as a percentage of the total time. The p-values from the Levene's test put in cursive are calculated without the female groups.

G4 - Statistical tests & P values

Table Appendix G4.1. Statistical tests and P values. The grouping is based on Curley's ranking. All significant p values are marked in red. Sidak's multiple comparison test scores were calculated using Graphpad Prism.

Type of variable	Variable	Statistical Test	P values Rank	Sex	Rank*Sex	Pairwise comparisons *Sidak's (post-hoc) test*
Behavioural	Total fights	Two-Way ANOVA	0.003	0.021	0.157	Male DOM-SUB: 0.015 Fem DOM-SUB:0.817
	Initiated fights	Two-Way ANOVA	>0.001	0.859	>0.001	Male DOM-SUB: >0.001 Fem DOM-SUB: >0.001
	Won fights	Two-Way ANOVA	>0.001	0.483	>0.001	Male DOM-SUB: >0.001 Fem DOM-SUB: >0.001
	Location Pref	Two-Way ANOVA	>0.001	>0.001	>0.001	Male DOM-SUB: >0.001 Fem DOM-SUB: 0.859
	Wounds - Males	Mann-Whitney U	0.014			
	- Females	Mann-Whitney U	0.514			
Physiological	Body weight - Males	Mann-Whitney U	0.603	-	-	-
	- Females	Mann-Whitney U	0.184	-	-	-
	Adrenal weight	Two-Way ANOVA	0.577	>0.001	0.344	
	CORT change	Two-Way ANOVA	0.360	0.501	0.488	-
	Fat weight	Bootstrapped Two-Way ANOVA	0.051	0.507	0.190	Male DOM-SUB: 0.064 Fem DOM-SUB: 0.507
	Thymus weight	Two-Way ANOVA	0.020	>0.001	0.534	Male DOM-SUB: 0.077 Fem DOM-SUB: 0.374
	Wounds	Bootstrapped Two-Way ANOVA	0.014	>0.001	0.005	Male DOM-SUB: 0.014 Fem DOM-SUB: 0.514
	Testes weight	Independent Samples T Test	0.491	-	-	-
	Vesicle weight	Mann-Whitney U	0.887	-	-	-
Neurobiological	CA1	Two-Way ANOVA	0.638	0.452	0.793	
	CA3	Two-Way ANOVA	0.081	0.710	0.829	
	BLA	Two-Way ANOVA	0.506	0.093	0.342	
	mPFC	Two-Way ANOVA	>0.001	0.746	0.168	Male DOM-SUB: >0.001 Fem DOM-SUB: 0.003

Table Appendix G4.2. Statistical tests and P values for repeated measures designs.

Type of variable	Variable	Statistical test	Main effects	P values
Physiology	**Body weight change**	Three-Way RM ANOVA	Sex Day Rank Sex*Day	>0.001 >0.003 0.637 >0.001
Neurobiology	**CA1 – males**	Two-Way RM ANOVA	Segment Rank Segment*Rank	0.087 0.156 0.579
	CA1 - females	Two-Way RM ANOVA	Segment Rank Segment*Rank	0.053 0.249 0.251
	CA3 – males	Two-Way RM ANOVA	Segment Rank Segment*Rank	0.176 0.032 0.427
	CA3 – females	Two-Way RM ANOVA	Segment Rank Segment*Rank	0.276 0.360 0.255

	BLA – males	Two-Way RM ANOVA	Segment Rank Segment*Rank	0.006 0.083 0.098
	BLA – females	Two-Way RM ANOVA	Segment Rank Segment*Rank	0.348 0.825 0.762
	mPFC – males	Two-Way RM ANOVA	Segment Rank Segment*Rank	0.146 >0.001 0.009
	mPFC - females	Two-Way RM ANOVA	Segment Rank Segment*Rank	0.090 0.007 0.260

G4.1 - Rational behind chosen statistical tests:

Rational for the Two-Way ANOVA & Mann Whitney U Test

Since we are interested in investigating the effects of sex and dominance rank on a multitude of behavioral, physiological and neurobiological (including Location preference) variables, together and separately, the most straightforward choice is to implement Two-Way ANOVA's. One caveat of using a Two-Way ANOVA is that it does not has a robust, often used non-parametric alternative. Luckily, ANOVA is quite robust against small violations of normality when sample sizes are equal. One-Way ANOVA's would also not give us any insight into a sex effect, and would treat males and females as replaceable (which, as most of our results show, would be quite strange to do). Because of these reasons, we performed a Two-Way ANOVA for almost all variables. Moreover, for the post-hoc testing, we invariably used Sidak's multiple comparison test, as it adjusts the significance level for multiple comparisons and provides tighter bounds than the Bonferroni and Scheffe test. For all data on the means, medians, group sizes, standard deviations, standard error of the means, skewness, kurtosis, normality p-values, and homogeneity p-values, see appendix G1, G2, and G3.

There were three variables that showed very big violations of the assumptions: namely FAT weight, body weight and number of wounds. We tried to rank-transform these variables (the normality & homogeneity p-values that came from ranked data are included in appendix G1), but that did not really change anything. A possible approach a bootstrapped Two-Way ANOVA, which has been found to be a good non-parametric alternative if the assumption of equal variances is violated, but does not work well if the assumption of normality is substantially violated (Xu, Yang, Abula & Qin, 2013). According to these guidelines, we only performed a bootstrapped Two-Way ANOVA for FAT weight. The variables number of wounds and body weight did not meet these prerequisites. Since the literature indicates clear sex differences in body weight, we analyzed the body weight for males and females separately. Both males and females show, however, still a violation of homogeneity in body weight data, which is why we chose to perform the non-parametric Mann Whitney U test. The data on number of wounds violates the assumptions in such a way, that any approach to look for statistically significant differences would be inappropriate, including bootstrapping, a rank-transformation, and treating males and females separately using a Mann Whitney U test. However, for the purpose of this thesis, a visual graph representing the differences in wound number per group is still interesting, and probably sufficient. Just to compare two different approaches, we included both the bootstrapped Two-Way ANOVA and Mann Whitney U test approach in appendix G4 above, although these results should not be taken too seriously.

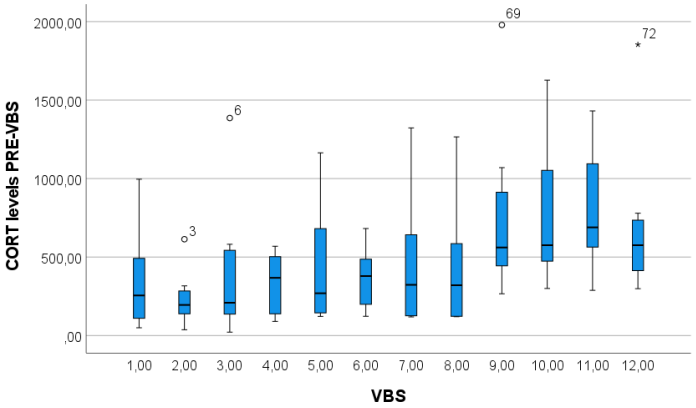
Rational behind the Two-Way an Three-Way Repeated Measures ANOVA

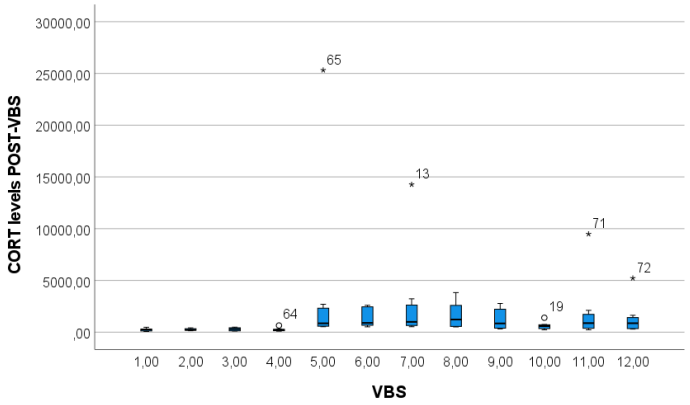
Besides a Mann Whitney U test on the total body weight change of males and females sepreately, we also performed a Three-way Repeated Measures (RM) ANOVA to analyze whether the effect of dominance rank on body weight change is mediated by both sex (Male vs Female) and the duration of colony housing (Day). Since the data was not normal, these results merit caution. However, there exist no non-parametric alternative for a Three-Way RM ANOVA, nor is a non-paramteric Kruskal Wallis test appropriate if we would have treated males and females also here separately.

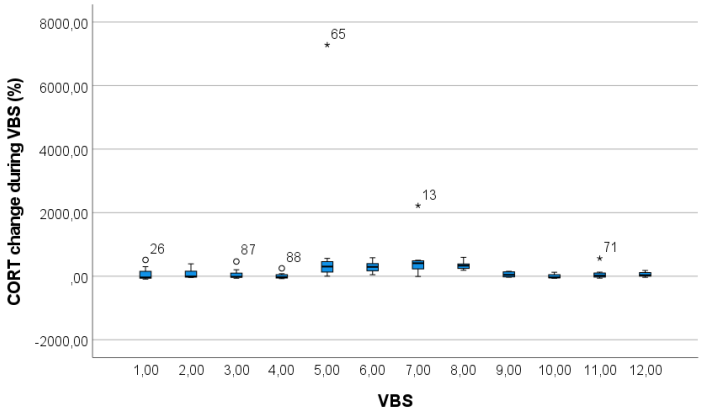
We performed a Two-way Repeated Measures (RM) ANOVA to analyze whether the effect of dominance rank on spine number was mediated by the distance of dendritic segments from the origin of the main shaft. In order to analysis this, we analyzed males and females seperately and also controlled for unequal variability of differences (we did not assume sphericity) by using a Geisser-Greenhouse correction. Even though the data measurements on spine number was not in a strict sense 'repeated' over time, it is repeated over distance. Considering the fact that besides this point, everything of the statistical design is similar to a typical repeated measures design, it is justified to perform this test.

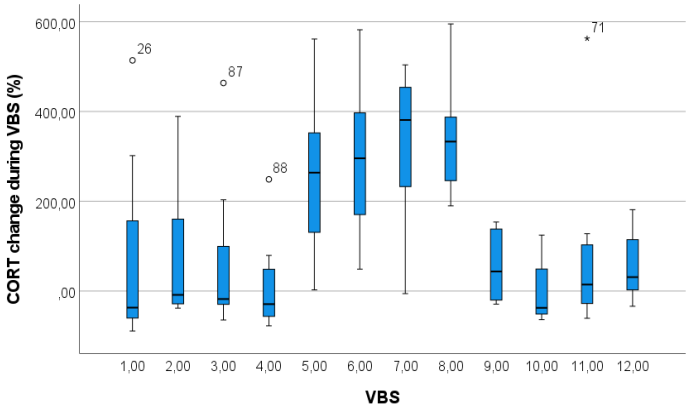
G4.2 CORT levels – sudden jump in batch 2

When looking at the Pre-VBS CORT levels for all animals per colony, there is a sudden increase in batch 3 when compared to Batch 1 and 2. And for the Post-VBS CORT levels, there is clear increase in Batch 2 compared to Batch 1 (See figures below). This makes it difficult to use all batches within the same analysis of CORT change as a consequence of colony housing. Note that the y-axis shows a substantial increase in magnitude in post-VBS CORT levels (right) compared to pre-VBS CORT levels (left).









On the left picture above (lower corner), it seems that only the samples 65 and 13, with ID 34 and 40 are methodological outliers, as it is hard to imagine that CORT levels can change with more than 2000%. It is more reasonable that the outliers are the result of technological issues. Therefore, these samples (that refer to ID 34 and 40, both males from batch 2) are excluded from further analysis on the CORT data, giving us the variance of CORT change per colony on the right picture above. The sudden increase in CORT levels is still there. However, in for this thesis, both DOM and SUB individuals were equally affected by this sudden increase. Since we are interested in the difference between DOM and SUB, and not the absolute levels of CORT, we concluded that these CORT caveats are not too much of a problem.

Appendix H – Statistics for correlations between all variables (without ranking)

Since most behavioral and physiological variables violate a normal distribution, we will use Kendall's Tau for all correlations.

H1 - Physiology: correlations among physiological and behavioral variables

Males:

Tests of Normality for Males[a]

	0=M, 1=F	Kolmogorov-Smirnov[b] Statistic	df	Sig.	Shapiro-Wilk Statistic	df	Sig.
LocationPref	,00	,219	44	<,001	,817	44	<,001
CORT change VBS	,00	,122	44	,098	,911	44	,002
Body Weight change	,00	,144	44	,022	,925	44	,007
Adrenal Weight	,00	,097	44	,200*	,930	44	,011
Thymus Weight	,00	,076	44	,200*	,982	44	,696
Fat Weight	,00	,134	44	,045	,895	44	<,001
Vesicle Weight	,00	,101	44	,200*	,980	44	,620
Testes Weight	,00	,140	44	,031	,952	44	,064

*. This is a lower bound of the true significance.

a. 0=M, 1=F = ,00

b. Lilliefors Significance Correction

Correlations for Males[a]

			LocationPref	CORT change VBS	Body Weight change	Adrenal Weight	Thymus Weight	Fat Weight	Vesicle Weight	Testes Weight
Kendall's tau_b	LocationPref	Correlation Coefficient	1,000	,034	,329*	-,152	-,172	-,208*	,057	-,015
		Sig. (2-tailed)	.	,746	,001	,137	,096	,042	,576	,887
		N	46	44	46	46	45	46	46	46
	CORT change VBS	Correlation Coefficient	,034	1,000	,032	-,178	,125	,258*	,032	-,423**
		Sig. (2-tailed)	,746	.	,762	,089	,233	,014	,762	<,001
		N	44	44	44	44	44	44	44	44
	Body Weight change	Correlation Coefficient	,329**	,032	1,000	-,431**	,190	-,126	,116	-,106
		Sig. (2-tailed)	,001	,762	.	<,001	,066	,218	,256	,298
		N	46	44	46	46	45	46	46	46
	Adrenal Weight	Correlation Coefficient	-,152	-,178	-,431**	1,000	-,133	-,014	-,024	,300*
		Sig. (2-tailed)	,137	,089	<,001	.	,197	,887	,813	,003
		N	46	44	46	46	45	46	46	46
	Thymus Weight	Correlation Coefficient	-,172	,125	,190	-,133	1,000	-,016	,200	-,079
		Sig. (2-tailed)	,096	,233	,066	,197	.	,876	,053	,445
		N	45	44	45	45	45	45	45	45
	Fat Weight	Correlation Coefficient	-,208*	,258*	-,126	-,014	-,016	1,000	-,246*	-,169
		Sig. (2-tailed)	,042	,014	,218	,887	,876	.	,016	,098
		N	46	44	46	46	45	46	46	46
	Vesicle Weight	Correlation Coefficient	,057	,032	,116	-,024	,200	-,246*	1,000	,289**
		Sig. (2-tailed)	,576	,762	,256	,813	,053	,016	.	,005
		N	46	44	46	46	45	46	46	46
	Testes Weight	Correlation Coefficient	-,015	-,423**	-,106	,300**	-,079	-,169	,289**	1,000
		Sig. (2-tailed)	,887	<,001	,298	,003	,445	,098	,005	.
		N	46	44	46	46	45	46	46	46

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

a. 0=M, 1=F = ,00

Females:

For Females:

Tests of Normality for Females[a]

	0=M, 1=F	Kolmogorov-Smirnov[b] Statistic	df	Sig.	Shapiro-Wilk Statistic	df	Sig.
LocationPref	1,00	,078	48	,200*	,986	48	,817
CORT change VBS	1,00	,222	48	<,001	,839	48	<,001
Body Weight change	1,00	,074	48	,200*	,954	48	,058
Adrenal Weight	1,00	,117	48	,101	,925	48	,004
Thymus Weight	1,00	,079	48	,200*	,980	48	,569
Fat Weight	1,00	,157	48	,005	,924	48	,004

*. This is a lower bound of the true significance.

a. 0=M, 1=F = 1,00

b. Lilliefors Significance Correction

Correlations for Females[a]

			LocationPref	CORT change VBS	Body Weight change	Adrenal Weight	Thymus Weight	Fat Weight	Vesicle Weight	Testes Weight
Kendall's tau_b	LocationPref	Correlation Coefficient	1,000	-,047	,020	,026	,043	-,154	.	.
		Sig. (2-tailed)	.	,638	,845	,797	,663	,124	.	.
		N	48	48	48	48	48	48	0	0
	CORT change VBS	Correlation Coefficient	-,047	1,000	,049	-,404**	-,020	-,259**	.	.
		Sig. (2-tailed)	,638	.	,625	<,001	,845	,009	.	.
		N	48	48	48	48	48	48	0	0
	Body Weight change	Correlation Coefficient	,020	,049	1,000	-,047	,052	-,115	.	.
		Sig. (2-tailed)	,845	,625	.	,638	,600	,251	.	.
		N	48	48	48	48	48	48	0	0
	Adrenal Weight	Correlation Coefficient	,026	-,404**	-,047	1,000	,076	,177	.	.
		Sig. (2-tailed)	,797	<,001	,638	.	,445	,075	.	.
		N	48	48	48	48	48	48	0	0
	Thymus Weight	Correlation Coefficient	,043	-,020	,052	,076	1,000	,005	.	.
		Sig. (2-tailed)	,663	,845	,600	,445	.	,957	.	.
		N	48	48	48	48	48	48	0	0
	Fat Weight	Correlation Coefficient	-,154	-,259**	-,115	,177	,005	1,000	.	.
		Sig. (2-tailed)	,124	,009	,251	,075	,957	.	.	.
		N	48	48	48	48	48	48	0	0
	Vesicle Weight	Correlation Coefficient	.	.	.	.	.	.	.	.
		Sig. (2-tailed)	.	.	.	.	.	.	.	.
		N	0	0	0	0	0	0	0	0
	Testes Weight	Correlation Coefficient	.	.	.	.	.	.	.	.
		Sig. (2-tailed)	.	.	.	.	.	.	.	.
		N	0	0	0	0	0	0	0	0

**. Correlation is significant at the 0.01 level (2-tailed).

a. 0=M, 1=F = 1,00

H2 - Neurobiology: Correlations between neurobiological and physiological/behavioral variables

Males

Tests of Normality for Males[a]

	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
HIPspinesCA1	,224	12	,099	,919	12	,280
HIPspinesCA3	,150	12	,200*	,941	12	,508
BLAspines	,157	12	,200*	,935	12	,438
mPFCspines	,135	12	,200*	,928	12	,357

*. This is a lower bound of the true significance.

a. 0=M, 1=F = ,00

b. Lilliefors Significance Correction

Correlations for neurobiological variables[a]

		Wounds	LocationPref	Body Weight change	CORT change VBS	Adrenal Weight	Thymus Weight	Fat Weight	Vesicle Weight	Testes Weight
Kendall's tau_b	HIPspinesCA1	,183	-,182	-,182	-,200	,242	-,091	,121	,030	,242
		,431	,411	,411	,392	,273	,697	,583	,891	,273
		12	12	12	11	12	11	12	12	12
	HIPspinesCA3	-,415	,424	,061	-,127	,121	-,455	,000	-,515	,061
		,073	,055	,784	,586	,583	,052	1,000	,020	,784
		12	12	12	11	12	11	12	12	12
	BLAspines	,116	-,091	,152	,091	-,152	,127	,394	-,061	-,212
		,616	,681	,493	,697	,493	,586	,075	,784	,337
		12	12	12	11	12	11	12	12	12
	mPFCspines	-,647	,485	,303	,200	,000	-,127	-,364	,212	,121
		,005	,028	,170	,392	1,000	,586	,100	,337	,583
		12	12	12	11	12	11	12	12	12

a. 0=M, 1=F = ,00

Females

Tests of Normality for Females[a]

	Kolmogorov-Smirnov[b]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
HIPspinesCA1	,135	12	,200*	,942	12	,524
HIPspinesCA3	,223	12	,101	,871	12	,067
BLAspines	,129	12	,200*	,958	12	,758
mPFCspines	,150	12	,200*	,965	12	,854

*. This is a lower bound of the true significance.

a. 0=M, 1=F = 1,00

b. Lilliefors Significance Correction

Correlations for neurobiological variables[a]

		Wounds	LocationPref	Body Weight change	CORT change VBS	Adrenal Weight	Thymus Weight	Fat Weight	Vesicle Weight	Testes Weight
Kendall's tau_b	HIPspinesCA1	,037	-,061	-,121	-,515	,515	,030	,455	.	.
		,885	,784	,583	,020	,020	,891	,040	.	.
		12	12	12	12	12	12	12	0	0
	HIPspinesCA3	-,186	-,030	-,455	-,303	,364	-,121	,545	.	.
		,469	,891	,040	,170	,100	,583	,014	.	.
		12	12	12	12	12	12	12	0	0
	BLAspines	,260	,273	-,333	-,182	-,061	,121	,545	.	.
		,311	,217	,131	,411	,784	,583	,014	.	.
		12	12	12	12	12	12	12	0	0
	mPFCspines	-,111	-,303	-,121	-,091	,030	,091	,212	.	.
		,664	,170	,583	,681	,891	,681	,337	.	.
		12	12	12	12	12	12	12	0	0

a. 0=M, 1=F = 1,00

H3 - Overview: Correlations between neurobiological, physiological and behavioral variables

Curley & Miguel

Sex	Correlation	Kendall's Tau, p value
Males	Location / Body	0.329, 0.001
	Location / Fat	-0.208, 0.042
	CORT / Fat	0.258, 0.014
	CORT / Testes	-0.423, <0.001
	Adrenal / Testes	0.300, 0.003
	Adrenal / Body	-0.431, <0.001
	Thymus / Body	*0.190, 0.066*
	Thymus / Vesicle	*0.200, 0.053*
	Fat / Vesicle	-0.246, 0.016
	Testes / Vesicle	0.289, 0.005
	mPFC / Wounds	-0.647, 0.005
	mPFC / Location	0.485, 0.028
	CA3 / Vesicle	-0.515, 0.020
Females	CORT / Adrenal	-0.404, <0.001
	CORT / Fat	-0.259, 0.009
	CA1 / CORT	-0.515, 0.020
	CA1 / Adrenal	0.515, 0.020
	CA1 / Fat	0.455, 0.040
	CA3 / Fat	0.545, 0.014
	BLA / Fat	0.545, 0.014

Appendix I – Comparison between Miguel's & Curley's Method

To compare both scoring approaches, we thought of multiple ways to see investigate their differences. These approaches are:

- Correlations of the ranking with all physiological variables, see appendix H1
- Visual inspection of the variances between ranked groups of all physiological variables, see appendix H2
- Correlations among physiological variales within each group for each ranking method, see appendix H3.
- Two-sample t-test (or the non-parametric Mann-Whitney U test) between scored groups from both approaches. Each test gives us the difference in means, but also the homogeneity of variances value, which possibly indicates the accuracy of the used method.
    - Noted, the groups that are compared are not independent, making such a test inappropriate.

I1 - Correlations with ranking method: Curley vs Miguel

Males:

Correlations for Males[a]

		LocationPref	CORT change VBS	Body Weight change	Adrenal Weight	Thymus Weight	Fat Weight	Vesicle Weight	Testes Weight
Kendall's tau_b	Curley's Method 1=SUB, 2=MID, 3=MID, 4=DOM	,322	-,121	,066	,059	-,205	-,328	-,019	,067
		,003	,279	,546	,589	,063	,003	,862	,540
		48	46	48	48	47	48	48	48
	Miguel's Method 1=SUB, 2=MID, 3=MID, 4=DOM	,459	-,120	,043	-,042	-,217	-,282	,060	,098
		,000	,283	,693	,700	,049	,010	,582	,369
		48	46	48	48	47	48	48	48

a. 0=M, 1=F = ,00

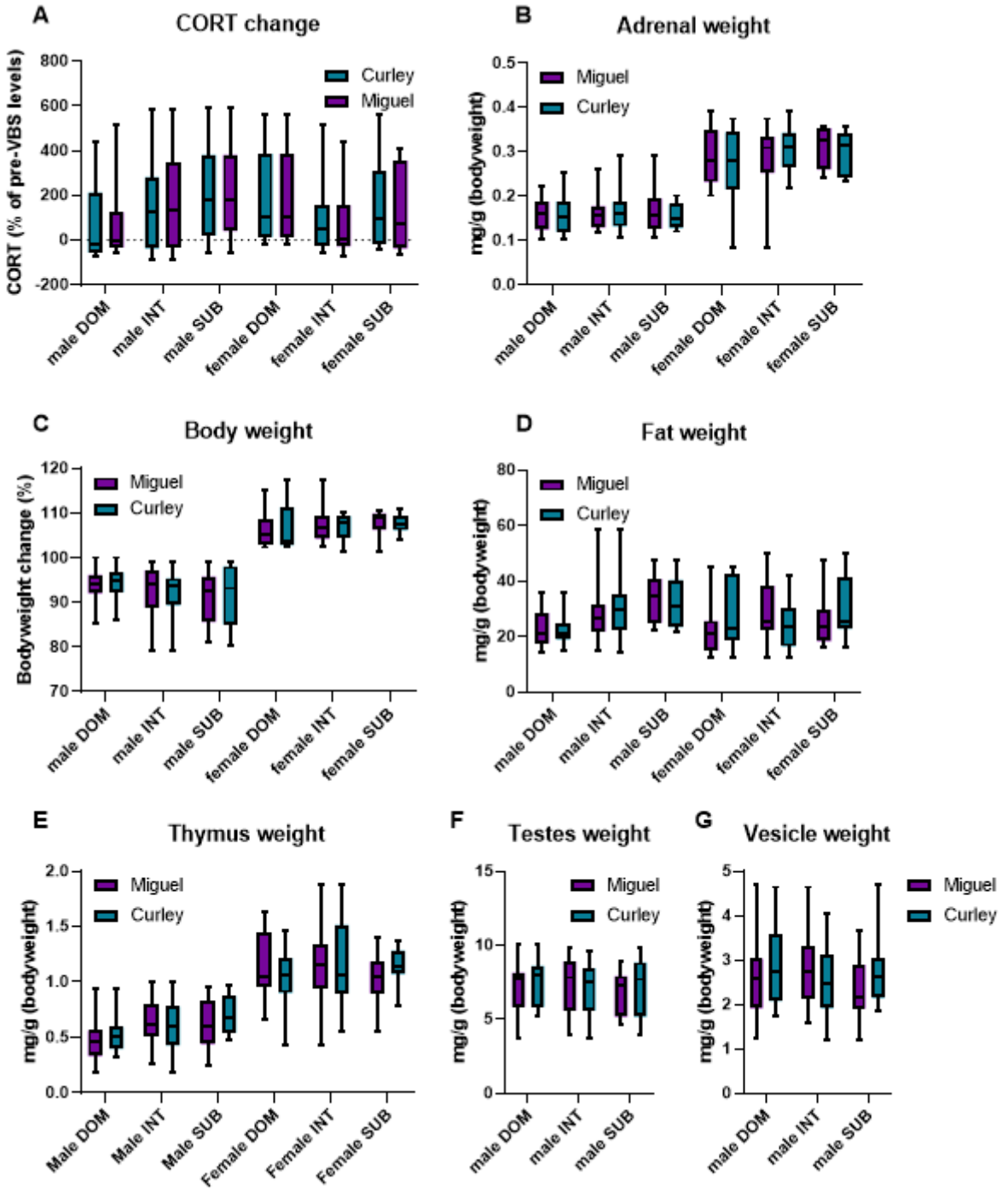
Females:

Correlations for Females[a]

		LocationPref	CORT change VBS	Body Weight change	Adrenal Weight	Thymus Weight	Fat Weight	Vesicle Weight	Testes Weight
Kendall's tau_b	Curley's Method 1=SUB, 2=MID, 3=MID, 4=DOM	,051	-,022	-,175	,012	-,044	-,099	.	.
		,641	,841	,107	,913	,687	,360	.	.
		48	48	48	48	48	48	0	0
	Miguel's Method 1=SUB, 2=MID, 3=MID, 4=DOM	-,037	,037	-,167	-,126	,037	-,110	.	.
		,735	,735	,124	,245	,735	,310	.	.
		48	48	48	48	48	48	0	0

a. 0=M, 1=F = 1,00

I2 - Graphs of all physiological variables



I3 - Correlations Rank and Sex: Miguel vs Curley – Kendall's Tau

I3.1 Overview

Green indicates that both methods show the correlation, blue indicates that only that method show the correlation. Correlations put in bold are significant in both DOM and SUB, correlations in orange are almost significant. Correlations are presented as Tau value, p value.

Curley's Method

- Males DOM
  - **Location / Body: 0.545, 0.014**
  - Location / Adrenal: -0.545, 0.014
  - Location / Testes: -0.424, 0.055
  - Thymus / Fat: -0.527, 0.024
  - Body / Adrenal: -0.636, 0.004
  - Body / Thymus: 0.600, 0.010
  - Adrenal / Testes: 0.636, 0.004
  - Fat / Vesicle: -0.424, 0.055
- Males SUB
  - **Location / Body: 0.515, 0.020**
  - CORT / Testes: -0.424, 0.055
  - Body / Adrenal: -0.424, 0.055
  - Fat / Testes: -0.424, 0.055
- Females DOM
  - Location / Fat: -0.485
  - CORT / Adrenal: -0.455, 0.040
- Females SUB
  - Adrenal / Fat: 0.485, 0.028

- For males:
  - Curley's rank /
    - Location: 0.322, 0.003
    - Fat: -0.328, 0.003
    - Thymus: -0.205, 0.063
- **For females**:
  - Non

Miguel's Method

- Males DOM
  - **Location / Body: 0.545, 0.014**
  - **Location / Adrenal: -0.545, 0.014**
  - Body / Adrenal: -0.576,
  - Thymus / Vesicle: 0.491, 0.036
  - Body / Thymus: 0.527, 0.024
  - Body / Vesicle: 0.424, 0.055
- Males SUB
  - **Location / Body: 0.697, 0.002**
  - **Location / Adrenal: -0.606, 0.006**
  - CORT / Testes: -0.485, 0.028
  - Body / Adrenal: -0.545, 0.014
  - Fat / Testes: -0.485, 0.028
- Females DOM
  - **CORT / Adrenal: -0.424, 0.055**
- Females SUB
  - CORT / Fat: -0.424, 0.055
  - **CORT / Adrenal: -0.455, 0.040**
  - Adrenal / Fat: 0.485, 0.028

- For males:
  - Miguel's rank /
    - Location: 0.459, 0.000
    - Fat: -0.282, 0.010
    - Thymus: -0.217, 0.049
- **For Females:**
  - Non

I3.2 Based on the Curley Method: four groups

For males DOM:

Correlations for DOM males[a]

			LocationPref	CORT change VBS	Body Weight change	Adrenal Weight	Thymus Weight	Fat Weight	Vesicle Weight	Testes Weight
Kendall's tau_b	LocationPref	Correlation Coefficient	1,000	-,055	,545*	-,545*	,236	-,091	,303	-,424
		Sig. (2-tailed)	.	,815	,014	,014	,312	,681	,170	,055
		N	12	11	12	12	11	12	12	12
	CORT change VBS	Correlation Coefficient	-,055	1,000	,018	-,200	,273	-,164	,127	-,382
		Sig. (2-tailed)	,815	.	,938	,392	,243	,484	,586	,102
		N	11	11	11	11	11	11	11	11
	Body Weight change	Correlation Coefficient	,545*	,018	1,000	-,636**	,600	-,364	,273	-,394
		Sig. (2-tailed)	,014	,938	.	,004	,010	,100	,217	,075
		N	12	11	12	12	11	12	12	12
	Adrenal Weight	Correlation Coefficient	-,545*	-,200	-,636**	1,000	-,345	,364	-,212	,636**
		Sig. (2-tailed)	,014	,392	,004	.	,139	,100	,337	,004
		N	12	11	12	12	11	12	12	12
	Thymus Weight	Correlation Coefficient	,236	,273	,600*	-,345	1,000	-,527*	,345	-,382
		Sig. (2-tailed)	,312	,243	,010	,139	.	,024	,139	,102
		N	11	11	11	11	11	11	11	11
	Fat Weight	Correlation Coefficient	-,091	-,164	-,364	,364	-,527*	1,000	-,424	,182
		Sig. (2-tailed)	,681	,484	,100	,100	,024	.	,055	,411
		N	12	11	12	12	11	12	12	12
	Vesicle Weight	Correlation Coefficient	,303	,127	,273	-,212	,345	-,424	1,000	,091
		Sig. (2-tailed)	,170	,586	,217	,337	,139	,055	.	,681
		N	12	11	12	12	11	12	12	12
	Testes Weight	Correlation Coefficient	-,424	-,382	-,394	,636**	-,382	,182	,091	1,000
		Sig. (2-tailed)	,055	,102	,075	,004	,102	,411	,681	.
		N	12	11	12	12	11	12	12	12

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

a. 1=mDOM, 2=mSUB, 3=fDOM, 4=fSUB = 1,00

For males SUB:

Correlations for SUB males[a]

			LocationPref	CORT change VBS	Body Weight change	Adrenal Weight	Thymus Weight	Fat Weight	Vesicle Weight	Testes Weight
Kendall's tau_b	LocationPref	Correlation Coefficient	1,000	,061	,515*	-,182	-,121	-,364	,182	,152
		Sig. (2-tailed)	.	,784	,020	,411	,583	,100	,411	,493
		N	12	12	12	12	12	12	12	12
	CORT change VBS	Correlation Coefficient	,061	1,000	-,303	,091	,152	,394	-,030	-,424
		Sig. (2-tailed)	,784	.	,170	,681	,493	,075	,891	,055
		N	12	12	12	12	12	12	12	12
	Body Weight change	Correlation Coefficient	,515*	-,303	1,000	-,424	-,061	-,303	,000	,030
		Sig. (2-tailed)	,020	,170	.	,055	,784	,170	1,000	,891
		N	12	12	12	12	12	12	12	12
	Adrenal Weight	Correlation Coefficient	-,182	,091	-,424	1,000	-,212	,333	,030	,121
		Sig. (2-tailed)	,411	,681	,055	.	,337	,131	,891	,583
		N	12	12	12	12	12	12	12	12
	Thymus Weight	Correlation Coefficient	-,121	,152	-,061	-,212	1,000	-,091	,091	,000
		Sig. (2-tailed)	,583	,493	,784	,337	.	,681	,681	1,000
		N	12	12	12	12	12	12	12	12
	Fat Weight	Correlation Coefficient	-,364	,394	-,303	,333	-,091	1,000	-,333	-,424
		Sig. (2-tailed)	,100	,075	,170	,131	,681	.	,131	,055
		N	12	12	12	12	12	12	12	12
	Vesicle Weight	Correlation Coefficient	,182	-,030	,000	,030	,091	-,333	1,000	,364
		Sig. (2-tailed)	,411	,891	1,000	,891	,681	,131	.	,100
		N	12	12	12	12	12	12	12	12
	Testes Weight	Correlation Coefficient	,152	-,424	,030	,121	,000	-,424	,364	1,000
		Sig. (2-tailed)	,493	,055	,891	,583	1,000	,055	,100	.
		N	12	12	12	12	12	12	12	12

*. Correlation is significant at the 0.05 level (2-tailed).

a. 1=mDOM, 2=mSUB, 3=fDOM, 4=fSUB = 2,00

For females DOM:

Correlations for DOM females[a]

			LocationPref	CORT change VBS	Body Weight change	Adrenal Weight	Thymus Weight	Fat Weight	Vesicle Weight	Testes Weight
Kendall's tau_b	LocationPref	Correlation Coefficient	1,000	,091	-,091	,273	,333	-,485*	.	.
		Sig. (2-tailed)	.	,681	,681	,217	,131	,028	.	.
		N	12	12	12	12	12	12	0	0
	CORT change VBS	Correlation Coefficient	,091	1,000	,152	-,455*	-,152	-,303	.	.
		Sig. (2-tailed)	,681	.	,493	,040	,493	,170	.	.
		N	12	12	12	12	12	12	0	0
	Body Weight change	Correlation Coefficient	-,091	,152	1,000	-,333	-,091	-,061	.	.
		Sig. (2-tailed)	,681	,493	.	,131	,681	,784	.	.
		N	12	12	12	12	12	12	0	0
	Adrenal Weight	Correlation Coefficient	,273	-,455*	-,333	1,000	,394	,061	.	.
		Sig. (2-tailed)	,217	,040	,131	.	,075	,784	.	.
		N	12	12	12	12	12	12	0	0
	Thymus Weight	Correlation Coefficient	,333	-,152	-,091	,394	1,000	-,242	.	.
		Sig. (2-tailed)	,131	,493	,681	,075	.	,273	.	.
		N	12	12	12	12	12	12	0	0
	Fat Weight	Correlation Coefficient	-,485*	-,303	-,061	,061	-,242	1,000	.	.
		Sig. (2-tailed)	,028	,170	,784	,784	,273	.	.	.
		N	12	12	12	12	12	12	0	0
	Vesicle Weight	Correlation Coefficient	.	.	.	.	.	.	.	.
		Sig. (2-tailed)	.	.	.	.	.	.	.	.
		N	0	0	0	0	0	0	0	0
	Testes Weight	Correlation Coefficient	.	.	.	.	.	.	.	.
		Sig. (2-tailed)	.	.	.	.	.	.	.	.
		N	0	0	0	0	0	0	0	0

*. Correlation is significant at the 0.05 level (2-tailed).

a. 1=mDOM, 2=mSUB, 3=fDOM, 4=fSUB = 3,00

For females SUB:

Correlations for SUB females[a]

			LocationPref	CORT change VBS	Body Weight change	Adrenal Weight	Thymus Weight	Fat Weight	Vesicle Weight	Testes Weight
Kendall's tau_b	LocationPref	Correlation Coefficient	1,000	-,182	,030	-,212	-,091	,061	.	.
		Sig. (2-tailed)	.	,411	,891	,337	,681	,784	.	.
		N	12	12	12	12	12	12	0	0
	CORT change VBS	Correlation Coefficient	-,182	1,000	,061	-,364	,061	-,455*	.	.
		Sig. (2-tailed)	,411	.	,784	,100	,784	,040	.	.
		N	12	12	12	12	12	12	0	0
	Body Weight change	Correlation Coefficient	,030	,061	1,000	,030	-,212	-,242	.	.
		Sig. (2-tailed)	,891	,784	.	,891	,337	,273	.	.
		N	12	12	12	12	12	12	0	0
	Adrenal Weight	Correlation Coefficient	-,212	-,364	,030	1,000	,091	,485*	.	.
		Sig. (2-tailed)	,337	,100	,891	.	,681	,028	.	.
		N	12	12	12	12	12	12	0	0
	Thymus Weight	Correlation Coefficient	-,091	,061	-,212	,091	1,000	,061	.	.
		Sig. (2-tailed)	,681	,784	,337	,681	.	,784	.	.
		N	12	12	12	12	12	12	0	0
	Fat Weight	Correlation Coefficient	,061	-,455*	-,242	,485*	,061	1,000	.	.
		Sig. (2-tailed)	,784	,040	,273	,028	,784	.	.	.
		N	12	12	12	12	12	12	0	0
	Vesicle Weight	Correlation Coefficient	.	.	.	.	.	.	.	.
		Sig. (2-tailed)	.	.	.	.	.	.	.	.
		N	0	0	0	0	0	0	0	0
	Testes Weight	Correlation Coefficient	.	.	.	.	.	.	.	.
		Sig. (2-tailed)	.	.	.	.	.	.	.	.
		N	0	0	0	0	0	0	0	0

*. Correlation is significant at the 0.05 level (2-tailed).

a. 1=mDOM, 2=mSUB, 3=fDOM, 4=fSUB = 4,00

I3.3 Based on the Miguel Method: four groups

For males DOM:

Correlations for DOM males[a]

			LocationPref	CORT change VBS	Body Weight change	Adrenal Weight	Thymus Weight	Fat Weight	Vesicle Weight	Testes Weight
Kendall's tau_b	LocationPref	Correlation Coefficient	1,000	-,018	,545*	-,545*	,164	,091	,152	-,303
		Sig. (2-tailed)	.	,938	,014	,014	,484	,681	,493	,170
		N	12	11	12	12	11	12	12	12
	CORT change VBS	Correlation Coefficient	-,018	1,000	-,236	,018	-,055	,200	,018	-,127
		Sig. (2-tailed)	,938	.	,312	,938	,815	,392	,938	,586
		N	11	11	11	11	11	11	11	11
	Body Weight change	Correlation Coefficient	,545*	-,236	1,000	-,576**	,527*	-,182	,424	,030
		Sig. (2-tailed)	,014	,312	.	,009	,024	,411	,055	,891
		N	12	11	12	12	11	12	12	12
	Adrenal Weight	Correlation Coefficient	-,545*	,018	-,576**	1,000	-,236	,182	-,242	,333
		Sig. (2-tailed)	,014	,938	,009	.	,312	,411	,273	,131
		N	12	11	12	12	11	12	12	12
	Thymus Weight	Correlation Coefficient	,164	-,055	,527*	-,236	1,000	-,273	,491*	-,018
		Sig. (2-tailed)	,484	,815	,024	,312	.	,243	,036	,938
		N	11	11	11	11	11	11	11	11
	Fat Weight	Correlation Coefficient	,091	,200	-,182	,182	-,273	1,000	-,273	,182
		Sig. (2-tailed)	,681	,392	,411	,411	,243	.	,217	,411
		N	12	11	12	12	11	12	12	12
	Vesicle Weight	Correlation Coefficient	,152	,018	,424	-,242	,491*	-,273	1,000	,364
		Sig. (2-tailed)	,493	,938	,055	,273	,036	,217	.	,100
		N	12	11	12	12	11	12	12	12
	Testes Weight	Correlation Coefficient	-,303	-,127	,030	,333	-,018	,182	,364	1,000
		Sig. (2-tailed)	,170	,586	,891	,131	,938	,411	,100	.
		N	12	11	12	12	11	12	12	12

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

a. 1=mDOM, 2=mSUB, 3=fDOM, 4=fSUB = 1,00

For males SUB:

Correlations for SUB males[a]

			LocationPref	CORT change VBS	Body Weight change	Adrenal Weight	Thymus Weight	Fat Weight	Vesicle Weight	Testes Weight
Kendall's tau_b	LocationPref	Correlation Coefficient	1,000	-,121	,697**	-,606**	,091	-,121	,000	,212
		Sig. (2-tailed)	.	,583	,002	,006	,681	,583	1,000	,337
		N	12	12	12	12	12	12	12	12
	CORT change VBS	Correlation Coefficient	-,121	1,000	-,182	-,091	,303	,273	,394	-,485*
		Sig. (2-tailed)	,583	.	,411	,681	,170	,217	,075	,028
		N	12	12	12	12	12	12	12	12
	Body Weight change	Correlation Coefficient	,697**	-,182	1,000	-,545*	-,091	-,121	-,061	,091
		Sig. (2-tailed)	,002	,411	.	,014	,681	,583	,784	,681
		N	12	12	12	12	12	12	12	12
	Adrenal Weight	Correlation Coefficient	-,606**	-,091	-,545*	1,000	-,121	-,091	,091	,121
		Sig. (2-tailed)	,006	,681	,014	.	,583	,681	,681	,583
		N	12	12	12	12	12	12	12	12
	Thymus Weight	Correlation Coefficient	,091	,303	-,091	-,121	1,000	,303	,242	-,030
		Sig. (2-tailed)	,681	,170	,681	,583	.	,170	,273	,891
		N	12	12	12	12	12	12	12	12
	Fat Weight	Correlation Coefficient	-,121	,273	-,121	-,091	,303	1,000	-,091	-,485*
		Sig. (2-tailed)	,583	,217	,583	,681	,170	.	,681	,028
		N	12	12	12	12	12	12	12	12
	Vesicle Weight	Correlation Coefficient	,000	,394	-,061	,091	,242	-,091	1,000	,061
		Sig. (2-tailed)	1,000	,075	,784	,681	,273	,681	.	,784
		N	12	12	12	12	12	12	12	12
	Testes Weight	Correlation Coefficient	,212	-,485*	,091	,121	-,030	-,485*	,061	1,000
		Sig. (2-tailed)	,337	,028	,681	,583	,891	,028	,784	.
		N	12	12	12	12	12	12	12	12

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

a. 1=mDOM, 2=mSUB, 3=fDOM, 4=fSUB = 2,00

For females DOM:

Correlations for DOM females[a]

			LocationPref	CORT change VBS	Body Weight change	Adrenal Weight	Thymus Weight	Fat Weight	Vesicle Weight	Testes Weight
Kendall's tau_b	LocationPref	Correlation Coefficient	1,000	,242	,242	,030	,333	-,030	.	.
		Sig. (2-tailed)	.	,273	,273	,891	,131	,891	.	.
		N	12	12	12	12	12	12	0	0
	CORT change VBS	Correlation Coefficient	,242	1,000	-,091	-,424	,182	-,364	.	.
		Sig. (2-tailed)	,273	.	,681	,055	,411	,100	.	.
		N	12	12	12	12	12	12	0	0
	Body Weight change	Correlation Coefficient	,242	-,091	1,000	,182	,303	-,061	.	.
		Sig. (2-tailed)	,273	,681	.	,411	,170	,784	.	.
		N	12	12	12	12	12	12	0	0
	Adrenal Weight	Correlation Coefficient	,030	-,424	,182	1,000	,212	,091	.	.
		Sig. (2-tailed)	,891	,055	,411	.	,337	,681	.	.
		N	12	12	12	12	12	12	0	0
	Thymus Weight	Correlation Coefficient	,333	,182	,303	,212	1,000	-,394	.	.
		Sig. (2-tailed)	,131	,411	,170	,337	.	,075	.	.
		N	12	12	12	12	12	12	0	0
	Fat Weight	Correlation Coefficient	-,030	-,364	-,061	,091	-,394	1,000	.	.
		Sig. (2-tailed)	,891	,100	,784	,681	,075	.	.	.
		N	12	12	12	12	12	12	0	0
	Vesicle Weight	Correlation Coefficient	.	.	.	.	.	.	.	.
		Sig. (2-tailed)	.	.	.	.	.	.	.	.
		N	0	0	0	0	0	0	0	0
	Testes Weight	Correlation Coefficient	.	.	.	.	.	.	.	.
		Sig. (2-tailed)	.	.	.	.	.	.	.	.
		N	0	0	0	0	0	0	0	0

a. 1=mDOM, 2=mSUB, 3=fDOM, 4=fSUB = 3,00

For females SUB:

Correlations for SUB females[a]

			LocationPref	CORT change VBS	Body Weight change	Adrenal Weight	Thymus Weight	Fat Weight	Vesicle Weight	Testes Weight
Kendall's tau_b	LocationPref	Correlation Coefficient	1,000	-,091	-,030	-,212	-,121	-,061	.	.
		Sig. (2-tailed)	.	,681	,891	,337	,583	,784	.	.
		N	12	12	12	12	12	12	0	0
	CORT change VBS	Correlation Coefficient	-,091	1,000	-,030	-,455*	,000	-,424	.	.
		Sig. (2-tailed)	,681	.	,891	,040	1,000	,055	.	.
		N	12	12	12	12	12	12	0	0
	Body Weight change	Correlation Coefficient	-,030	-,030	1,000	-,091	,303	-,182	.	.
		Sig. (2-tailed)	,891	,891	.	,681	,170	,411	.	.
		N	12	12	12	12	12	12	0	0
	Adrenal Weight	Correlation Coefficient	-,212	-,455*	-,091	1,000	-,121	,485*	.	.
		Sig. (2-tailed)	,337	,040	,681	.	,583	,028	.	.
		N	12	12	12	12	12	12	0	0
	Thymus Weight	Correlation Coefficient	-,121	,000	,303	-,121	1,000	,152	.	.
		Sig. (2-tailed)	,583	1,000	,170	,583	.	,493	.	.
		N	12	12	12	12	12	12	0	0
	Fat Weight	Correlation Coefficient	-,061	-,424	-,182	,485*	,152	1,000	.	.
		Sig. (2-tailed)	,784	,055	,411	,028	,493	.	.	.
		N	12	12	12	12	12	12	0	0
	Vesicle Weight	Correlation Coefficient	.	.	.	.	.	.	.	.
		Sig. (2-tailed)	.	.	.	.	.	.	.	.
		N	0	0	0	0	0	0	0	0
	Testes Weight	Correlation Coefficient	.	.	.	.	.	.	.	.
		Sig. (2-tailed)	.	.	.	.	.	.	.	.
		N	0	0	0	0	0	0	0	0

*. Correlation is significant at the 0.05 level (2-tailed).

a. 1=mDOM, 2=mSUB, 3=fDOM, 4=fSUB = 4,00

Appendix J – Performed labwork (unsuccesfull)

J1 - Three different RNA extraction approaches

In order to obtain as much brain tissue as we could fathom, before diving into the important sample tissue, we performed three different RNA extractions methods were on unimportant samples. The first and second method followed the guidance of a RNA extraction kid with and without metal beads (lysing the cell tissue). The third, slightly longer method followed the protocol previously used by Jocelien Oliver. This protocol makes use of toxic TRIzol which maintains RNA integrity while simultaneously breaking down the cell and its components, yielding higher (<50%) RNA extraction (Ahmed et al., 2015).

J2 - Measuring gene expression of BDNF

For the molecular analysis in this project, we are mainly interested in the levels of pro-BDNF and mature-BDNF. We first planned to perform Western Blotting for both the hippocampus and the prelimbic cortex (mPFC), but unfortunately, there was not enough brain tissue left (approximately 1 mg) to perform a Western Blot for the mPFC. To still reap reasonable data on BDNF levels from the mPRL, we switched to quantitative PCR, another analytic tool that can work effectively with very little brain tissue. However, using qPCR allows us only to investigate levels of gene expression and not gain insight into the different status of the BDNF protein (pre- vs mature-). As I became sick during the experiment, and since others working on this project were with holiday, the RNA samples lost their quality. We decided to let the molecular analysis for what it is, and not include that part in this thesis. The procedure, which was only partially executed, is as follows.

Objective: The performed procedures to obtain RNA levels of Brain-Derived-Neurotrophic Factor (BDNF) within the medial prefrontal cortex (mPFC).

RNA-isolation:
We isolated total RNA for all samples that were ranked as either the most dominant or most subordinate within a colony, for both males and females. The amount of sample approximated 1 mg and were stored in 2 ml tubes. First, we added 500µL TRIzol (…) and homogenized the samples with metal beads, using a Tissuelyser 2 (Qiagen) 2 times for 2:00 at 30 Hz. The samples were incubated at room temperature for 5:00, centrifuged at 12.000 x g and 4°C for 10:00, and the supernatant transferred to a new RNAase-free 2 ml tube without disturbing the pellet. Then, 100 µL Chloroform was added and the samples were shortly shaking vigorously by hand before they were incubated at room temperature for 2:00-3:00. Samples were centrifuged at 12.000 x g and 4°C for 15:00 and all of the upper aqueous phase was transferred to a new RNA-free 1.5 ml tube. Then, 6.7 µL GlycoBlue and 250 µL 100% isopropanol was added to the aqueous phase, mixed by inverting 10x and incubated at room temperature for 10:00. The samples were centrifuged at 12.000 x g and 18°C for 10:00. The supernatant was removed by decanting the tube and to the pellet 500 µL 75% ethanol was added, mixed by inverting 10x and centrifuged at 12.000 x g and 18°C for 5:00. These last steps, from removing the supernatant to centrifuging the samples, were repeated 2 times. To remove ethanol as much as possible, samples were shortly spun and the collected ethanol removed by carefully pipetting. The pellet was air-dried for approximately 10:00 before adding 27 µL UltraPure water, after which the samples were incubated at 55°C for 15:00 and put on ice. The concentration and purity of the RNA was determined using 2 µL (of the 27 µL) of the samples for the Nanodrop 200 (). For the full protocol, see appendix …

Complementary Deoxyribonucleic acid (cDNA) synthesis:
For cDNA synthesis, we first prepared per sample a mix in 0.2ml 8 tube strips. Each sample contained 0.5 Oligo(dT)$_{18}$ (100µM), 1000ng RNA, and depending on the concentration of the RNA per sample, we added Ultrapure water up to a total of 13.5 µL mix. All samples were incubated at 65°C for 05:00 in a thermocycler and afterward immediately transferred to ice (to 'shock' the samples) and incubated for 1 min. Then, 4.0 µL 5x Reaction Buffer, 1.0 µL dNTP (10 mM each), 1.0 µL RevertAid H minus RT (200U/µL), and 0.5 µL RiboLock RNase inhibitor (40U/µL) were added to each RNA/oligo(dT)$_{18}$ mix up to a total of 20 µL per sample. Each mix was gently vortexed and spun for several seconds before being put into a thermocycler with the following program: 25°C for 10:00, 45°C for 60:00, 70°C for 15:00. All cDNA samples, after measuring their concentrations and purity using the Nanodrop, were stored at -20°C before being used for qPCR.

Quantitative PCR:
Before qPCR, all samples were diluted to a cDNA concentration of 10ng/µL. The PCR was performed using TaqMan probes (Thermo Fisher Scientific / Applied Biosystems) for the genes encoding Bdnf (Rn02531967_s1) and GAPDH (Rn01775763_g1). A mastermix was prepared for 67 wells containing a total of 18 µL for each well: 10 µL iTaq mastermix, 7 µL UltraPure H_2O and 1 µL Taqman probe (Bdnf or GAPDH). Then, 2 µL cDNA was added to each well and the plates were sealed and spun. The qPCR machine was run in the following program: 02:00 at 95°C; 40 cycles of 15 sec at 95°C, 01:00 at 60°C.