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The Influence of Social Environment on Plasma Oxytocin Levels in New Zealand White Rabbits

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THE INFLUENCE OF SOCIAL ENVIRONMENT ON PLASMA OXYTOCIN LEVELS IN NEW ZEALAND WHITE RABBITS

By

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Previous research attests to the relationship between social support and positive health outcomes while linking social isolation or aggression/hostility with negative health outcomes. Several studies examining atherosclerosis with either genetic or behavioral origins, have reported decreased disease severity in socially supportive environments. In order to identify and understand the mechanism responsible for decreased disease, the current study examined physiological differences in New Zealand White rabbits within unstable, stable, and isolated social environments and observed whether functional hormonal changes were apparent over time and as a response to behavior characteristic of these environments. Results indicated that animals within the unstable condition displayed increased agonistic behavior, increased cortisol and epinephrine, decreased body weight, epididymal fat, and retroperitoneal fat, as well as larger spleens. Cortisol values positively correlated with measures of agonistic behavior for all animals, while the reverse relationship was found for affiliative behavior. The novel finding of an increase in oxytocin in animals in the unstable condition within the first ten minutes of pairing that was noticeably distinct from the other two groups suggests that plasma oxytocin levels are related to acute stress. Limitations and interpretations of these findings are discussed.

Future work is still needed to help further explain the physiological response to social stress and affiliation and to elucidate the mechanism by which a supportive social environment appears to protect against progression and severity of heart disease.

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Chapter 1: Introduction

Across most species, positive social interactions are vital for continued existence, whether for the intention of mating, mutual support, or protection (Cole & Young, 2008). Social support, provided by the presence of a bonding partner, has been shown to reduce the endocrine response to a stressful situation (Sachser, Dürschlag, & Hirzel, 1998). This "social buffering" has a positive influence on both short- and long-term stress (Hennessy, Kaiser, & Sachser, 2009, p. 470).

Additionally, previous research has implicated a lack of positive social interactions as well as behaviors such as aggression, anger, and hostility with negative health outcomes, including cardiovascular disease and increased risk of mortality (House, Landis, & Umberson, 1988; Knox & Uvnäs-Moberg, 1998; Smith, Glazer, Ruiz, & Gallo, 2004). Unclear at this point are the means by which a supportive environment offers its protective effects against stress. The purpose of the current study was to identify the biological characteristics of a socially supportive environment and to quantify the physiological mechanism that may be responsible for decreased disease outcomes.

Stress, Behavior, and Disease

Researchers have examined the mediating effects of socially stressful, stable, and isolated environments on rabbits displaying a genetic susceptibility to heart disease; the Watanabe Heritable Hyperlipidemic (WHHL) rabbit. McCabe and colleagues (2002) reported that rabbits housed in a stable environment, characterized by affiliative behaviors such as nuzzling or grooming their partner, were found to have significantly less area of atherosclerotic lesion than animals housed in an unstable environment or

housed alone. Our findings from this study highlight the profound role that social environment can play in the progression of disease, even after accounting for a strong genetic component.

A similar study by Kaplan and Manuck (1999) examined the influence of social environment within a dietary model of heart disease. Researchers found that dominant male cynomolgus monkeys fed a moderately atherogenic diet, differed in their development of the disease depending on their housing environment. Specifically, those housed in a socially unstable, and thereby stressful, environment exhibited twice the coronary atherosclerosis as dominant males housed in a socially stable environment. The authors suggested that risk of atherosclerosis was partly determined by activation of the sympatho-adrenal axis necessary to maintain dominance in an environment that was unstable.

Both aforementioned studies highlight the buffering effect that a stable social environment has on disease, regardless of genetic or dietary etiology. The question that arises, therefore, is what is the physiological mechanism that could account for these findings? One such proposed mechanism is the neuropeptide oxytocin.

Oxytocin, Social Interactions, and the Stress Response

Oxytocin has been thought to play a role in diminishing the stress response by decreasing activity of the hypothalamic-pituitary-adrenal (HPA) axis that regulates the stress hormone, cortisol, however, this action may be stressor specific (Bartz & Hollander, 2006; Neumann, 2002). Oxytocin is released into peripheral circulation by the posterior pituitary gland, which is stimulated by magnocellular neurons of the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus (Falke, 1989;

Landgraf, Neumann, & Schwarzberg, 1988; McCarthy & Altemus, 1997; Uvnäs-Moberg, 1998). Centrally, it is released by magnocellular and parvocellular neurons of the paraventricular nucleus (PVN), supraoptic nucleus (SON), and medial preoptic area of the hypothalamus, along with the medial amygdala and bed nucleus of the stria terminalis (BnST) (Cole & Young, 2008; Russell & Brunton, 2008). Levels in the periphery do not directly reflect levels in the central nervous system and as such, are considered independent (Cole & Young, 2008; Light, Grewen, & Amico, 2005; Lim, Bielsky, & Young, 2005).

Functions of peripherally released oxytocin include parturition and lactation, while centrally released oxytocin plays a role in social bonding, mating, and regulating maternal behavior (Cole & Young, 2008; Russell & Brunton, 2008). In light of the fact that stable social environments are typically characterized by pro-social behaviors, the aim of the current study was to examine whether oxytocin is the mechanism by which animals housed in stable environments exhibit less disease. Additionally, because peripheral levels are thought to reflect acute changes, we focused our attention on measuring how oxytocin changed over time and across differing social conditions.

Physical contact (e.g., warmth, touch, and olfactory cues) characteristic of positive social interactions have been found to stimulate the release of oxytocin and can also reduce sympatho-adrenal activity and increase parasympathetic-vagal activity (Uvnäs-Moberg, 1998). It has been proposed that at the hypothalamic level, oxytocin is related to the stress response short-term (via its activation of the sympatho-adrenal system) while long-term exposure integrates an anti-stress effect (via activation of the vagal nerve). The occurrence of short-term response versus long-term pattern may be

due to whether oxytocin is triggered by noxious or non-noxious stimuli (Uvnäs-Moberg, 1998). These findings may help to explain the protective effects of enduring socially supportive environments.

In rodents, oxytocin appears to function peripherally as a stress response to both psychological and physical distress, while in humans, it has been proposed that oxytocin functions in relieving symptoms of stress, such as anxiety (reviewed in McCarthy & Altemus, 1997). The intrinsic differences of social relationships across species may account for variations in oxytocin release, while at the same time providing an integrated view of its overall effects.

Response Patterns within Animal Studies

Hashiguchi, Hua Ye, Morris, and Alexander (1997) examined acute versus long-term stress on oxytocin levels. Rats exposed to a five-minute shaker stress test exhibited significantly increased plasma oxytocin over baseline, while exposure to the same stress test for 30 minutes did not significantly increase oxytocin over the five-minute value. Kalin, Gibbs, Barksdale, Shelton, and Carnes (1985) reported decreased plasma oxytocin levels in rhesus monkeys after a loud bell behavioral stressor. These studies demonstrate a variable response pattern of oxytocin to stress; which is apparently dependent on exposure time, and possibly by stimulus type as well as species.

Machatschke, Wallner, Schams, and Dittami (2004) observed plasma oxytocin and cortisol in cohabited versus isolated guinea pigs. Isolated guinea pigs demonstrated higher plasma cortisol than sexual-pairs when exposed to a noise stress test. Cohabited animals expressed higher mean oxytocin levels than isolated animals but this outcome was not significantly correlated with cortisol levels. As a group, animals benefited from

cohabitation as indicated by their decreased response to stress. However, individual responses to social support and stress may vary, and therefore, correlational analyses may not be the best approach to quantifying this relationship.

The previously described studies have elucidated the stark differences between stable versus stressful social environments, however, not all environments are necessarily this striking. While it appears that isolation can be as detrimental to health as socially stressful situations (McCabe et al., 2002), it is important to consider whether oxytocin can protect against the potentially harmful outcomes of an isolated social environment. A recent study, conducted using a rodent model of depression, examined administration of exogenous oxytocin as a potential buffer to the negative effects of isolation. In fact, isolated rodents receiving oxytocin did not exhibit the two characteristics for depression, decreased sucrose consumption and immobility, as demonstrated by the control group receiving saline (Grippo, Trahanas, Zimmerman II, Porges, & Carter, 2009).

Thus far, the studies mentioned have depicted differences in short and long-term exposure to stress, the benefits of a social partner, as well as the protective effects of exogenous oxytocin. However, all of these studies were conducted with animal models. While similarities between species may exist, especially within the necessity and significance of social relationships, it is important to examine oxytocin in light of characteristics specific to human interactions.

Human Response Patterns

Tops, Van Peer, Korf, Wijers, and Tucker (2007) examined the relationship between measures of attachment, anxiety, and plasma levels of cortisol and oxytocin in a group of healthy women. They found that attachment (which measured "the tendency to

express and share emotions and feelings with friends", p. 445) significantly predicted levels of oxytocin and cortisol (positively and negatively related, respectively). There was also a negative association between anxiety and oxytocin, which was mediated by attachment. In addition, they reported a positive relationship between oxytocin and cortisol, which was considered to reveal a cortisol-influenced oxytocin release.

These findings are important considering behaviors that may reflect stable versus unstable environments, such as feelings of attachment. The seemingly contradictory finding of a positive relationship between oxytocin and cortisol may reflect an immediate buffering effect that oxytocin has during times of stress while overall levels distinguish extent of attachment. At this point, we have seen that exogenous oxytocin can ameliorate symptoms of depression in an animal model, however, it remains to be seen whether exogenous oxytocin has similar effects in humans; and more specifically, whether exogenous administration offers the protective benefits of a socially supportive environment.

Heinrichs, Baumgartner, Kirschbaum, and Ehlert (2003) compared the benefits of social support versus exogenous oxytocin during a stressful situation, the Trier Social Stress Test (TSST; Kirschbaum, Pirke, & Hellhammer, 1993). Neuropeptides administered intranasally have been found to bypass the bloodstream and access the cerebrospinal fluid within 30 minutes (Born et al., 2002). Healthy men were grouped into either isolated or supportive groups and given intranasal infusions of either oxytocin or placebo. Those in the supportive group were told to bring their best friend to the session and that person was directed to give supportive comments during the speech preparation period (Heinrichs et al., 2003).

No significant increases in salivary cortisol were detected from baseline in either participants with social support plus oxytocin or in those with social support alone. In contrast, participants with no social support plus placebo exhibited the highest cortisol response. Psychological measures revealed that those who received social support, oxytocin, or both demonstrated decreased anxiety and increased calmness while participants without social support and placebo showed a decrease in calmness and an increase in anxiety. Additionally, oxytocin had a significant anxiolytic effect on pre and post levels of anxiety in the group given oxytocin but not social support (Heinrichs et al., 2003).

The aforementioned study highlights the protective benefit of positive social interactions with and without administration of exogenous oxytocin. Considering these interactions and the behaviors that typify them, the question of interest now is how peripheral levels respond to particular affiliative behaviors. Light et al. (2005) examined plasma levels of oxytocin in pre-menopausal women undergoing a speech stressor after warm partner contact. Participants completed questionnaires assessing emotional support and frequency of physical affection (ranging from holding hands, hugs, and similar warm touches). They found that greater frequency of partner hugs and massages were correlated with higher baseline oxytocin levels, while women who reported greater partner hugs demonstrated lower baseline blood pressure levels. In addition, greater partner hugs predicted lower heart rate levels during speech preparation and delivery, but not at baseline or recovery, which may reflect the protective effects of oxytocin during acute times of stress.

Effects of Oxytocin on the Heart

Thus far, we have seen that social support, alone and with the administration of oxytocin, can attenuate the cortisol response to a stressful situation (Heinrichs et al., 2003). Additionally, research has shown decreased severity of heart disease within stable environments (Kaplan & Manuck, 1999; McCabe et al., 2002). However, the mechanism by which oxytocin may mediate disease is unknown; it is unclear whether oxytocin acts to decrease the effects of sympathetic nervous system hyper-activation (by decreasing the deleterious effects of catecholamines and/or cortisol), or by way of a direct effect of oxytocin on the vascular system. Considering the results of the previous study showing that individuals with higher partner affection demonstrated higher oxytocin levels as well as lowered blood pressure, the next question is how oxytocin works on vascular cells *in vitro*.

A recent report from our laboratory examined the effects of oxytocin on measures of oxidative stress and inflammation in aortic endothelial and smooth muscle cells, THP-1 monocytes and macrophages (Szeto et al., 2008). Results indicated that oxytocin reduced NADPH oxidase activity in all of these cells. Additionally, it was found that oxytocin decreased IL-6 secretion in THP-1 macrophages and endothelial cells. This study is important as it potentially designates a direct vascular recipient of the positive effects of a stable social environment.

A recently released report examined the effects of oxytocin administration on myocardial infarction in a rat model. Jankowski and colleagues (2010) reported that oxytocin treated animals displayed decreased inflammation (reduced macrophages, neutrophils, and T-lymphocytes) as well as decreased expression of the pro-inflammatory

cytokines, IL-6 and TNF-α within the infarct, as compared to control rats. Additionally, they found a dose-dependent increase in oxytocin receptor expression within the damaged heart.

We recently found that oxytocin treatment in ApoE-/- mice produced significantly less area of thoracic atherosclerosis compared to non-treated controls (Nation et al., 2010). Moreover, after examining adipose tissue *ex vivo*, animals treated with oxytocin displayed significantly less IL-6 secretion than controls. The question now is whether the oxytocin acting on the vasculature is stemming from a pituitary-driven release or that which is locally produced by the heart. Indeed, research has demonstrated that not only are oxytocin receptors present in the heart, but also that the heart is able to produce its own source of oxytocin (Gutkowska, Jankowski, Mukaddam-Daher, & McCann, 2000; Jankowski et al., 1998; Jankowski et al., 2000)

Central Oxytocin and Social Environment

Up to this point, we have considered both animal and human literature suggesting an important role that peripheral oxytocin may play in decreasing the stress response, as well as the anti-inflammatory effects of oxytocin on vascular cells *in vitro*. However, because oxytocin is also released centrally (Cole & Young, 2008; Russell & Brunton, 2008), it is important to consider whether social environment affects central as well as peripheral levels. To answer this question, our lab used micro dialysis to collect oxytocin from the paraventricular nucleus of the hypothalamus in WHHL rabbits housed in unstable, stable, and isolated social conditions (Paredes et al., 2006). We found that oxytocin increased significantly over time in animals within the unstable condition as compared to animals within the stable condition as well as individually caged animals;

although acutely after a two-hour social encounter, oxytocin was not elevated in any of the three groups compared to baseline. Plasma oxytocin was measured, however, there were no significant group differences on day 1 or day 22, or over time within groups. Consistent with previous published work, the animals housed in the stable group displayed the least atherosclerotic lesion area in the aortic arch compared to the other two groups.

While we expected to find group differences in plasma oxytocin, it is possible that methodological concerns interfered with collection ability. On the two days of collection, it was necessary to place a clear barrier between the animals in order to accommodate micro dialysis collection, and therefore, animals did not have direct physical contact with each other. Subsequent research would be better advised to collect blood on a different day in order to avoid this problem. Thus, the current study was designed to capture an acute response by collecting blood before, during, and immediately after behavioral pairing.

If previous work has shown that plasma oxytocin appears to be reflective of the interactions occurring within a positive social environment, why would animals within the unstable group display such a dramatic increase (almost doubled from day 1 to day 22) of central oxytocin over time (Paredes et al., 2006)? This finding, in addition to the fact that animals in the stable group displayed less disease, may suggest that central oxytocin reflects the stress response experienced by the unstable animals. Additional work is needed within the same paradigm to examine whether an unstable environment is, in fact, characterized by increased central oxytocin, decreased peripheral oxytocin, and again, increased area of atherosclerotic lesion, and whether the inverse profile typifies

animals within a stable environment. In addition to the central versus peripheral question, it is also important to consider previously mentioned research proposing opposing functions of oxytocin in the short-term versus long-term, as well as whether there is a noxious or non-noxious stimulus trigger (Uvnäs-Moberg, 1998). Furthermore, it is essential to ascertain whether a clear response pattern exists that would explain the effects of acute versus chronic exposure to various social interactions.

Indeed, Ondrejcakova and colleagues (2010) reported an increase in plasma oxytocin in rats after both acute and chronic exposure to stressful stimuli. Additionally, they found an increase in plasma ACTH and corticosterone as well as an enlargement of the adrenal glands. Interestingly, this finding was more pronounced in animals that had been infused with oxytocin via osmotic mini-pumps, with the exception of absolute adrenal weight. Animals receiving the infusion displayed an increased concentration of plasma oxytocin, which would be expected. It would be interesting to repeat this experiment with the addition of a socially supportive group to compare endogenous versus exogenous oxytocin and whether there is an optimal basal level for protective effects.

Rationale

Positive social interactions are important for healthy relationships, psychological well being, dealing with stress, and good health in general. Research has elucidated the negative effects of social isolation while at the same time, demonstrated the positive, and even protective, function of socially supportive environments against disease severity, both in dietary as well as genetic studies (Kaplan & Manuck, 1999; Knox & Uvnäs, 1998; McCabe et al., 2002). What is not completely understood is the biological

mechanism that could account for this influence. Although the literature suggests that oxytocin may be playing an important role in social support, to our knowledge, no study has compared peripheral oxytocin levels between differing social environments.

The purpose of this study was to quantify physiological differences among three different social conditions and to observe functional changes in oxytocin over time and across distinctive types of behavior. In addition, we investigated the relationship between plasma oxytocin, plasma cortisol, urinary catecholamines (NE, Epi), and a marker of inflammation, C-reactive protein (CRP), as well as measures of agonistic and affiliative behavior. We hypothesized that oxytocin levels would be higher in the stable group, as compared to the unstable group and individually caged animals. Because cortisol is a marker of the stress response, we expected plasma cortisol to be higher in the unstable group as compared to the other two groups, and higher in the individually caged as compared to animals within the stable group. Also, we hypothesized that urinary catecholamines (NE, Epi) and CRP would be highest in the unstable group, and higher in the individual group compared to animals within the stable group. Additionally, we proposed that animals would spend the majority of their time in behaviors reflecting their assigned social conditions; agonistic behavior characterizing the unstable group and affiliative behavior distinguishing the stable group. Finally, in investigating response patterns over time, we hypothesized that oxytocin would increase as a function of social environment, both acutely as well as sustained over time.

Chapter 2: Method

Participants

Forty-two male New Zealand White (NZW) rabbits (12 weeks old, approximately 2 kg) were purchased through Covance Research Products, Inc. (Denver, PA) and were acclimated for one week prior to the study, which included familiarizing animals to the researchers as well as the equipment used throughout the study (e.g., rabbit restrainers). NZW rabbits were chosen because of ease of obtaining and inexpensive cost relative to WHHL rabbits, and also because disease outcome was not a component of the current study. Additionally, we have previously found comparable stress responses between these two strains of rabbits (Szeto et al., 2004). The rabbits were housed in individual cages (6 sq. ft.) on a 12-hour light/dark schedule (lights on at 7 AM). Standard rabbit chow (Purina, 2.5% fat, 0% cholesterol) and water were provided *ad libitum* and the rabbits were weighed weekly. All procedures were approved by the University of Miami Laboratory Animal Care and Use Committee.

Procedures

Social Manipulation

Rabbits were separated into three groups; stable, unstable, and individually caged, using littermate identification provided by the breeder. The individually caged group consisted of fourteen rabbits that did not have physical contact with other rabbits for the duration of the study. The stable group consisted of seven pairs of littermates. Rabbits were paired daily for four hours with one littermate for the duration of the study. The unstable group consisted of fourteen non-littermate rabbits paired for four hours a day. Pairings were systematically assigned so that rabbits in the unstable group were paired with a different

rabbit each week. Pairings for both the unstable and stable groups occurred in the home cage of one of the rabbits, the order of which was reversed each week. Changing the order of home cages ensured that each rabbit experienced pairings at home and as an intruder. Rabbits in the unstable group were housed in one room while rabbits in the individual and stable groups were housed in a separate room on the other side of the animal colony so as not to be influenced by the fighting behaviors characteristic of the unstable group. Two pairs from our stable group displayed excessive fighting behavior, and after multiple attempts to separate and reintroduce them, were removed from the study.

Behavioral Scoring

Three times a week all rabbits were observed and their behavior scored for the first ten minutes of the observation; the individual group during regular activity and stable/unstable groups during pairing. It was also noted whether a rabbit was a home rabbit or an intruder. Due to logistical constraints, only one lab member was responsible for all the scoring.

Scored behaviors included activities such as cage exploration, passive rest, self-grooming, immobility, chasing, mounting, biting, and combat. Twenty-three behaviors were scored and condensed into four types of behavior: agonistic, affiliative, other non-agonistic, and inactivity (Lockley, 1961; Grant & Mackintosh, 1963; Mykytowycz & Hesterman, 1975; Blanchard, Sakai, McEwen, Weiss, & Blanchard, 1993; Cirulli, Terranova, & Laviola, 1996). Percentage of time spent in each of the four types of behavior was then calculated for each rabbit; this is a more accurate representation of behavior than using actual number of occurrences because it accounts for the fact that

some behaviors are momentary while others are sustained. The initial behavior data was the average of the three behavior scores collected during the first week, while the midpoint and endpoint behavior data was the cumulative average collected up to that respective week.

Blood and Urine collection

For all blood draws, rabbits were placed in a plexiglass restrainer for approximately five minutes or less. Blood was collected from the marginal ear vein through a butterfly catheter into Vacutainer tubes, plasma tubes of which were stored on ice. Tubes were centrifuged at 4°C (2000 RPM for 30 minutes), the plasma or serum collected and stored in aliquots at -80 C until assay. See Figure 1 for a timeline of all blood draws.

Initial (week 0), midpoint (week 6), and endpoint (week 19) blood was collected to measure plasma hormones, oxytocin, and cytokines. Behavior scoring was suspended during the week for these three collection points. Our lab has previously shown that rabbits demonstrate a circadian rhythm for glucocorticoids (Szeto et al., 2004), and as such, blood was collected between 1 and 3 PM. In order to measure oxytocin and cytokines, rabbits were fasted overnight and their blood drawn at 8 AM. At each time point, a total amount of 2 ml was drawn for hormones and 8 ml for oxytocin and cytokines combined.

Non-fasted plasma oxytocin was measured at eight time points during the study, approximately every two weeks. We drew a pre-pairing measurement (3 ml) and rabbits were then paired and their behavior scored. Blood was drawn again after 10 minutes into the pairing (3 ml) and 2 hours (3 ml) into the pairing. Between blood draws, rabbits were returned to their pairings (with individual rabbits returning to their home cages). The

rabbit order was randomized to avoid a group or time effect. A hematocrit test was regularly performed to ensure that rabbits had not become anemic.

Urinary catecholamines were also measured at initial, midpoint, and endpoint. Rabbits were placed in a metabolic cage for 24 hours. Urine was collected on ice, acidified with hydrochloric acid, and stored in aliquots at -80 C until assay.

Heart Rate and Blood Pressure

Heart rate and resting systolic, mean, and diastolic blood pressure were measured at initial, midpoint, and endpoint data collections using an automated tail-cuff system (Model 29SSP, IITC, Inc., Woodland Hills, CA). Rabbits were placed in a plexiglass restrainer in a room warmed with a heat lamp to allow pulse detection.

A tail cuff that automatically inflates and deflates was placed on their previously shaved tails. Heart rate and blood pressure information were collected and stored in a MacLab data acquisition system. Three trials for each animal were collected and their mean calculated for each measure. Acquisition took approximately seven minutes per animal.

Oxytocin Extraction

Samples were extracted using 200 mg C18 Sep-Pak columns (Bachem, San Carlos, CA). Columns were equilibrated with 3 ml of acetonitrile, then twice with 3 ml of 0.1% trifluoroacetic acid (TFA). Up to 1 ml of plasma was mixed with an equal volume of 0.1% TFA, centrifuged at 14,000 g x 45 minutes at 4°C, and the acidified and clarified plasma loaded onto the column. The flow-through portion was discarded, columns were washed once with 3 ml of 0.1% TFA, then twice with 3 ml of water. Oxytocin was eluted with 3 ml of 60% acetonitrile. The solvent was evaporated under a stream of

nitrogen gas, frozen, and then lyophilized. Samples were reconstituted in 120 µl of assay buffer provided by the EIA kits.

Biochemical Assays

Oxytocin EIA (Assay Designs, Ann Arbor, MI), C-Reactive Protein EIA (Immunology Consultants Laboratory, Inc., Newberg, OR), and dual RIA for Epi/NE (Alpco Diagnostics, Salem, NH) assays were performed following the manufacturers' protocol. Catecholamine values were normalized by total volume of urine collected. Plasma cortisol was measured using an automated analyzer (Roche Diagnostics).

Chapter 3: Statistical Analysis

All values reported are mean and +/- SEM. Repeated measures ANOVA with a group by time mixed design was used to evaluate whether change over time differed as a function of group. When a test of the interaction was significant, we used *post-hoc* one way-ANOVA and LSD tests to assess specific group differences. If the interaction was not significant, we then examined the main effect; a significant result indicating group differences collapsed over time. LSD multiple comparisons were used as a follow-up to compare group pairs.

Although we attempted to acclimate the animals prior to initial collected measures, it appears that the time employed may have been insufficient, as elevated initial markers of stress (e.g., catecholamines, cortisol) relative to values collected later in the study indicated that the animals were still very stressed. It is important to note that while we acclimated the animals to the restrainer used during blood draws, initial collection was the first time the rabbits had their blood drawn, and the novelty and stress of which may have contributed to elevated levels.

Therefore, in order to control for this pre-existing elevation and examine whether a clear response was apparent, we analyzed difference scores from initial to midpoint and initial to endpoint for all measures collected at these three time points. For measures with elevated initial levels, we used one-way ANOVA and LSD tests to test group differences on the change from initial collection to midpoint and initial to endpoint. Behavior scores and weight were analyzed using the raw data from initial, midpoint, and endpoint collections in order to maintain consistency of comparisons between mean behavior collected throughout the study (from initial through endpoint) and mean behavior scores

collected during pairing. Also, in order to account for total body weight, all tissue weights were divided by endpoint weight prior to analyses.

Finally, because pairing oxytocin data essentially represented seven identical collection time points, we calculated mean values based on the number of observations available for each animal for pre-pairing levels as well as their 10-minute and 120-minute responses, after confirming no group by time interaction. We then compared group differences using one-way ANOVA and LSD tests.

We had some instances of missing data for some of the measures we collected, the proportion of which was relatively minor. For pairing oxytocin, out of 798 possible values, 2 percent were missing. For initial, midpoint, and endpoint oxytocin, out of 114 possible values, 7 percent were missing. This outcome was mainly due to limitations on the amount of blood we could draw at each time point (i.e., collected plasma levels less than minimum assay requirements), or by having values outside of the detection limits of the assay. For initial, midpoint, and endpoint measures of catecholamines, out of 456 possible values, 5.26 percent were missing. For adrenal weight, one value was missing. Given our small sample size, we did not exclude animals that had specific instances of missing data.

The approach we took was a variation of hot deck imputation where the missing value was replaced with a value derived from animals within the same group, as described next. Within each group, a prediction equation used data from all available time points, excluding outliers, to predict the missing data. Additionally, whenever possible, we subdivided groups into dominant and subordinate animals in order to obtain the most precise regression equation. Two instances with very small cell sizes yielded

implausible equations. In those cases, the lowest available value was substituted. Unfortunately, our initial sample sizes were too small to implement modern methods of handling missing data (FIML, multiple imputation). Degrees of freedom were adjusted to account for missing data.

On one of the pairing days, day number four, data collection was hindered by facilities work performed within the colony and an unusual change of animal care personnel, which objectively appeared to agitate the animals. After examining the behavior data, which proved to be consistent on all days except for this one, we excluded all data collected that day from further analyses (behavior and oxytocin).

Correlations were calculated among the following variables at all three main time points: cortisol, oxytocin, and affiliative and agonistic behavior. All analyses were run in SPSS. In repeated measures analyses, adjustments were made to degrees of freedom when sphericity assumption was violated. We did not apply any *p* value adjustment to *post-hoc* tests because of decreased power due to our initial sample size.

Chapter 4: Results

Behaviors Exhibited Within the Three Social Conditions

Initial, midpoint and endpoint. Means and standard error of the means for the following analyses are reported in Table 1. By definition, the individually caged animals did not have an opportunity to engage in affiliative or agonistic behavior, therefore, they were excluded from analyses involving these behaviors. Animals in the unstable condition spent less time in affiliative behavior collapsed over time and more time in agonistic behavior at midpoint and endpoint, $p < .05$ for all comparisons (see Figures 2 and 3, respectively). Group differences collapsed over time were found in other non-agonistic behavior and inactivity, such that individually caged animals spent more time in both of these types of behaviors than the other two groups, $p < .01$ for all comparisons (see Figures 4 and 5).

Specific pairing days. There was a significant group difference in the change in agonistic behavior over time, $F(5.061,111.345) = 6.223$, $p < .01$, such that the animals in the unstable condition displayed higher agonistic behavior at all time points except the first pairing day, $p < .05$ for all comparisons. Unstable animals also displayed the least amount of affiliative behavior collapsed over time when compared to stable animals, $F(1, 22) = 30.909$, $p < .01$. Other non-agonistic behavior changed over time, as a function of group, $F(10.473, 183.281)$, $p < .01$; where individually caged animals consistently spent more time in this type of behavior at each time point than animals housed in stable or unstable conditions, $p < .05$ for all comparisons. In addition, animals in the stable group spent more time in other non-agonistic behavior than those in the unstable group at four time points (T3, T5, T7, and T8).

Finally, inactivity also changed over time, as a function of group, $F(11.125, 194.691) = 2.245$, $p < .05$; in that, individually caged animals engaged in more inactivity at each time point than animals housed in either of the other two conditions, $p < .01$ for all comparisons. See Table 2 for means and standard error of the means for all behaviors observed during pairing over weeks.

Physiological Characteristics of Supportive, Stressful, and Isolated Social Environments

Cortisol. We found a significant group difference in the change in cortisol from initial to midpoint collection, $F(2,35) = 3.317$, $p < .05$; where cortisol values remained significantly elevated in animals housed in the unstable condition compared to those in the stable condition, $p < .05$ (see Table 4, Figure 6). There was no significant difference between groups in the change from initial to endpoint collection, $p > .05$.

Catecholamines. There was a significant group difference in the change in epinephrine from initial to midpoint collection, $F(2,34) = 5.741$, $p < .01$, as well as from initial to endpoint, $F(2,30) = 3.902$, $p < .05$. At both time points, animals housed in the unstable condition had higher epinephrine compared to individually caged animals, $p < .05$ for both comparisons (see Table 4, Figures 7 and 8). There was no significant difference in the change of epinephrine at either time point between animals housed in an unstable versus stable condition or the stable condition versus individually caged animals. Additionally, there was no significant group difference in norepinephrine from initial to midpoint collection or from initial to endpoint, $p > .05$.

Blood Pressure/Heart Rate. We found a significant difference in the change from initial to endpoint collection in diastolic blood pressure, $F(2,35) = 5.667$, $p < .01$, mean arterial pressure, $F(2,35) = 5.100$, $p < .05$, and heart rate, $F(2,35) = 6.364$, $p < .01$;

such that individually caged animals displayed the least cardiovascular reactivity compared to those housed in the unstable and stable conditions, $p < .05$ for all comparisons (see Table 4). There was a similar trend for systolic blood pressure but this change was not significant, $p = .102$. We were unable to collect midpoint blood pressure or heart rate measurements due to equipment malfunction and subsequent replacement during this time.

Weight. There was a significant change in weight over time as a function of group, $F(3.617, 63.297) = 8.362$, $p < .01$. At midpoint, there was a significant difference between groups, $F(2,35) = 5.352$, $p < .01$, such that animals housed in the unstable condition weighed less than both animals housed in the stable condition and individually caged animals, $p < .05$ for both comparisons. A difference was found at endpoint, $F(2,35) = 5.799$, $p < .01$, where animals housed in the unstable condition weighed less than those housed in the stable condition, $p < .01$ (see Table 5 for all weight values).

Contrary to our hypotheses, we did not find significant group differences in the change from initial to midpoint collection or initial to endpoint collection in either oxytocin or C-reactive protein, $p > .05$.

Acute Effects of Social Condition on Oxytocin Immunoreactivity. Analyses of mean values at each time point indicated a significant difference between groups for the ten minute response, $F(2,35) = 3.256$, $p < .05$, such that the animals housed in the unstable condition displayed significantly higher values than the those housed in the stable condition as well as individually caged animals, $p < .05$ for both comparisons (see Table 3, Figure 9). There was no significant group difference in mean pre-pairing level or mean two-hour level, $p > .05$.

Chronic Effects of Social Condition on Tissues, Controlling for Body Weight

After controlling for body weight, we still found a significant difference between groups in epididymal fat, $F(2,35) = 3.584$, $p < .05$, as well as retroperitoneal fat, $F(2,35) = 21.288$, $p < .01$; such that animals housed in the stable condition were found to have the most epididymal fat compared to those housed in the unstable condition, $p < .05$, while both animals housed in the stable condition as well as individually caged animals were found to have increased retroperitoneal fat compared to those in the unstable condition, $p < .01$ for both comparisons (see Table 5 and Figures 10 and 11, respectively). Additionally, there was a significant difference between groups in spleen weight, $F(2,35) = 4.782$, $p < .05$. We found that animals housed in the unstable condition had larger spleens compared to the other two groups, $p < .05$ for both comparisons (see Table 5, Figure 12). There were no significant group differences in adrenal glands, testicular weight, or liver weight, $p > .05$.

Relationship between Behavior, Cortisol, and Oxytocin

Table 6 displays correlations among behavior, cortisol, and oxytocin at initial, midpoint, and endpoint collections. Cortisol measured at midpoint was significantly related to agonistic behavior at midpoint, $r(24) = .503$, $p < .05$, and agonistic behavior at endpoint, $r(24)=.618$, $p < .01$. We found a negative relationship between cortisol measured at midpoint and affiliative behavior at midpoint, $r(24) = -.592$, $p < .01$, as well as affiliative behavior at endpoint, $r(24) = -.642$, $p < .01$. There was no significant relationship between oxytocin and affiliative or agonistic behavior at any time point.

Chapter 5: Discussion

As predicted, animals spent the majority of their time in behaviors corresponding with their social placement; animals housed in the unstable condition spent the most time in agonistic behaviors and the least time in affiliative behaviors, while those housed in the stable condition displayed the reverse pattern. Additionally, individually caged animals spent more time in other non-agonistic behaviors and inactivity than the other two groups.

We failed to find significant differences between groups in the change of oxytocin from initial collection to midpoint or initial to endpoint, which would have supported our original hypothesis stating that oxytocin increases over time in response to a socially supportive environment, and that this neuropeptide may be one of the means by which animals in a stable condition display less disease. The pairing blood draws were designed to capture the immediate response to pairing (after the first 10 min) and also to examine whether this response was stable over time (2 hours into the pairings). We found that animals in the unstable condition displayed a significantly higher average oxytocin response within the first ten minutes of the pairing. The fact that we did not find significant differences between groups in average pre-pairing levels or 2 hours into the pairing implies that peripheral oxytocin is acting as an acute stress response and that elevated levels subside within a short period of time.

We found significant group differences in the change in cortisol levels from initial collection to midpoint, such that levels in animals within the unstable condition remained significantly elevated compared to those in the stable condition, concurrent with the stress experienced within an unstable social environment. The fact that we did not find

differences between animals in the unstable condition and individually caged animals may reflect that early on, the individually caged animals were equally stressed by being isolated. However, we did not find differences between animals in the stable condition and individually caged animals at this time point, which would have supported this observation.

Additionally, we failed to find significant differences in cortisol between any of the groups in the change from initial collection to endpoint; the reason of which is unclear. Perhaps by this point, the most aggressive animals had already established a clear dominance pattern, and thus, the midpoint change reflected the most volatile time in the study, while at endpoint overall aggression may have leveled out. Another consideration for midpoint differences is that this was around the time the animals were going through puberty. However, it is unlikely that increased aggression can account for these findings, because after the first pairing, animals in the unstable condition consistently displayed the highest agonistic behavior throughout the study.

As predicted, there were significant differences in the change in twenty-four hour urinary catecholamine levels from initial collection to midpoint and initial to endpoint. At both time points, animals in the unstable condition demonstrated the highest increase in epinephrine levels compared to individually caged animals. This finding further supports the idea that chronic stress characterizes an unstable environment. We predicted, but did not find differences between animals in the unstable and stable conditions. In a previous study measuring catecholamines in plasma (Paredes et al., 2006), we found that rabbits housed in an unstable condition had the highest mean change of epinephrine when compared to those housed in a stable condition; while

displaying the highest mean change of norepinephrine compared to the individually caged animals. It is unclear why the current study was not able to replicate previous findings. More recently, though, we found individually caged animals to have the highest levels of urinary norepinephrine compared to animals in unstable and stable conditions; while epinephrine levels were higher in individually caged animals compared to those in an unstable condition (Nation et al., 2008). Although this finding is the reverse of what we found in the current study, it is possible that NZW rabbits are more aggressive in unstable environments than the WHHL rabbits used in the previous study.

We were unable to find significant differences in CRP, a marker of systemic inflammation, between groups for the change from initial collection to midpoint or from initial to endpoint. This outcome may also be due to the fact that we used NZW instead of WHHL rabbits as in our previous behavioral study. On one hand, this outcome is unsurprising because NZW rabbits are not predisposed to developing atherosclerosis, and therefore may not display elevated systemic inflammation. On the other hand, recent work has shown a dose dependent relationship between social integration and CRP concentration; however, this finding was only significant in men over the age of 60 (Ford, Loucks, & Berkman, 2006). Future work is needed to determine how varying degrees and types of social interaction affect the inflammatory response, and whether this response is constant across gender and species.

We found significant differences in the change from initial collection to endpoint in diastolic blood pressure, mean arterial pressure, and heart rate; such that individually caged animals displayed the least cardiovascular reactivity compared to the other two groups. Additionally, we found a trend suggesting a similar response for systolic blood

pressure. The present study was longer in duration than preceding work and the current findings appear to reflect that over time, the individually caged animals adapted to isolation.

Animals in the stable condition weighed more than those in the unstable condition at midpoint and endpoint, and more than individually caged animals at midpoint. Previously we found no differences between groups in body weight (McCabe et al., 2002), however, due to increased immobility, we would expect individually caged animals to be the heaviest when compared to the other groups. Although the individually caged animals did, in fact, spend the majority of time in immobility or other non-agonistic behaviors, perhaps the increase in pro-social behaviors found in the animals within the stable condition contributed to an increased appetite. Future research interested in this question may examine whether time spent in a particular behavior is related to overall food consumption. What is noteworthy is that even after controlling for body weight, animals in the stable condition had the most epididymal fat compared to animals within the unstable condition, while both animals within the stable condition as well as individually caged animals had greater amounts of retroperitoneal fat when compared to those in the unstable condition.

We failed to find significant differences between groups for adrenal glands, testicular weight, or liver weight; however, animals in the unstable condition had larger spleens compared to the other two groups. Azpiroz and collegues (1999) found a significant increase in spleen mononuclear cell proliferative response in mice after chronic mild stress (CMS), indicating an enhanced immune response after both 4 and 7 weeks of chronic stress when compared to controls. Nevertheless, it is unknown whether

this response is related to overall spleen weight. Future work would do well to examine whether size of the organ affects enhancement or suppression of immune response while future behavioral studies could easily incorporate a test of immune function/responsivity.

Correlational analyses indicated a significant positive relationship between cortisol and agonistic behavior, and a significant negative relationship between cortisol and affiliative behavior, which would be expected. We expected, but did not find, a significant relationship between affiliative behavior and oxytocin. Although our current findings suggest that increased acute oxytocin levels are related to stress, we did not find a significant relationship between agonistic behavior and oxytocin. Future work can re-examine this relationship with a larger sample size.

The goal of this study was to quantify the physiological characteristics of social conditions responsible for the decreased and increased disease outcomes we have previously reported in stable and unstable/isolated social environments, respectively. The mechanisms that induce these outcomes are, as of yet, still unknown. In light of an abundance of research linking the neuropeptide oxytocin to positive social interactions, the primary purpose of this study was to examine whether groups differed in overall peripheral oxytocin levels and to measure changes in oxytocin as a function of time, both acutely and chronically. We focused on peripheral oxytocin for the purpose of capturing both an acute and chronic response to specific types of social behaviors, and also due to the fact that prior research has not shown a conclusive direct relationship between peripheral levels and central levels (Light, Grewen, & Amico, 2005; Lim, Bielsky, & Young, 2005). Additionally, by evaluating variation between groups in catecholamines and cortisol, we sought to uncover potential alternative or co-occurring mechanisms

responsible for change in disease outcomes within our original WHHL model, rabbits displaying a genetic susceptibility to heart disease.

We expected, but failed to find a comprehensible acute and chronic oxytocinergic response pattern explaining the protective effects of a supportive social environment. However, our novel finding that the animals in the unstable condition showed a marked increase in oxytocin within the first ten minutes of pairing may serve to shed light on the crucial purpose of the role of peripheral oxytocin. Considering that we did not find significant group differences in initial, midpoint, endpoint, average pre-pairing, or average 2-hour levels, it is safe to conclude that peripheral levels may be context specific and not a good indicator of chronic social conditions. Indeed, several studies have suggested that this response to stress appears to vary, depending on exposure time and possibly stimulus type and species (Hashiguichi et al., 1997; Kalin et al., 1985), and that short and long-term response patterns may be due to stimulus type (Uvnäs-Moberg, 1998). While human studies have reported that exogenous oxytocin, with and without the combination of a supportive partner, suppressed the cortisol response to stress (Heinrichs et al., 2003), again, it is necessary to examine whether this is also an acute response and thus, not a reliable indicator of constant supportive environments.

An important concern was recently raised regarding the lack of consensus in techniques used for measuring plasma oxytocin. Commercial kits presently available include enzyme immunoassay (EIA) and radioimmunoassay (RIA). We compared the two kits in extracted versus unextracted human plasma samples and found a 100-fold increase in unextracted versus extracted plasma when using the EIA kit, with no correlation between the two (Szeto et al., 2011). Additionally, we found the RIA kit was

not sensitive enough to detect most samples, both with and without extraction. These findings raise serious concerns when comparing outcomes across studies.

Given the results of the current study, it is possible that the mechanism by which a socially supportive environment appears to mediate the severity of heart disease may not be peripheral oxytocin. An alternative possibility is that oxytocin released by the pituitary gland may serve a different function within the stress response and may not be directly related to the progression of atherosclerosis, while locally produced oxytocin within the heart may be what is responsible for attenuating disease, and therefore, peripheral levels may not be the best indicator of local action. Further research is needed to elucidate factors that may influence local release within the vasculature. Finally, it is also important to consider other potential pathways that may modulate progression of the disease.

DeVries, Glasper, and Detillion (2003) reviewed research concerning the influence that social environment has on HPA activity. They found that while the primary response is adaptive in terms of stressor, over time chronic stress, and therefore, chronic activation can lead to persistently elevated concentrations of glucocorticoids, while positive social interactions appear to protect against over activation of the HPA axis in some animal species (DeVries, 2002; DeVries et al., 2003; McEwen, 2000; Sachser, Durschlag, & Hirzel, 1998; Sapolsky, 1992). Additionally, we have already seen that in humans, subjective measures of attachment negatively predicted cortisol levels (Tops et al., 2007). In the same study, however, results indicated a positive relationship between oxytocin and cortisol, possibly reflecting a buffering role of oxytocin during times of stress.

Taken together, these reported outcomes may help to explain why the animals in an unstable condition exhibited the most severe atherosclerotic lesions in our previous study. Perhaps HPA over activation found in an unstable social environment is what is responsible for exacerbating disease, while the decreased number of agonistic encounters is what provides a protective benefit to those housed in a stable environment. While this is a plausible explanation for the unstable and stable social conditions, what can explain the increased total area of disease found in individually caged animals (McCabe et al., 2002)?

In the previously mentioned study it was found that individually caged animals were hyperinsulinemic and had increased body weight compared to the other two groups. It has already been shown that social isolation can lead to negative heath outcomes (House, Landis, & Umberson, 1988; Knox & Uvnas-Moberg, 1998), however, over activation of the HPA axis may not be what contributes to this outcome. In the current study, isolated animals did not appear to be stressed, as indicated by decreased levels of catecholamines, lack of increased cortisol response, and decreased cardiovascular reactivity. Perhaps the presence or absence of social stress is what contributes to increased or decreased severity of disease, while other factors, including metabolic syndrome, drive disease progression within an environment characterized by social isolation.

An alternative consideration is that increased glucocorticoids, as well as catecholamines, may act on oxytocin receptors (OTR), thereby making them more sensitive to circulating, and/or locally produced oxytocin. Liberzon and Young (1997) examined the effects of acute versus chronic stress on OTR within rat brains. They found

that both stress conditions increased OTR binding within the ventral hippocampus compared to controls. If this pattern persists within the vasculature, it may help to explain the inconsistency of increased oxytocin levels found within stressful conditions (Hashiguichi et al., 1997; Kalin et al., 1985; Machatschke et al., 2004) compared to the anti-inflammatory effect and suppressed pro-inflammatory cytokine production reported recently (Szeto et al., 2008; Jankowski et al., 2010).

This idea is important, especially when considering peripheral levels of oxytocin to be an indicator of the degree of affiliative pro-social behavior within positive social relationships. It may be that peripheral levels reflect, simply, a response to a particular acute stressor, and therefore, are not the mechanism that leads to decreased severity of atherosclerosis. If central levels are a reflection of consistently stable or unstable social environments, further research is needed to dissect the possible reciprocal nature of peripheral and central response patterns over time. Furthermore, it is also important to resolve the nature of feedback systems from the periphery to the brain oxytocinergic system, how circulating levels of glucocorticoids may accelerate or inhibit production and release, and whether this system is context dependent.

However, the goal of the current study was to begin by focusing on acute peripheral changes in oxytocin and other circulating hormones during social interactions, examine whether these changes are stable or increase over time, and to determine whether the nature of this response varies across groups. We found that animals housed in an unstable environment were indeed more stressed, as indicated by increased agonistic behavior, increased hormonal responses (cortisol and epinephrine), and decreased body weight. We also found an increase in oxytocin in animals within the first

ten minutes of an unstable behavioral pairing that was notably distinct from animals within a stable pairing or animals housed alone. While this outcome was contradictory to our original hypothesis, this finding may underscore the direction for future work examining the oxytocinergic response within behavioral paradigms, and thus, help to further explain the physiological response to social stress and affiliation. Additionally, we found that the oxytocin response, at least peripherally, is acute and not stable over time, which is consistent with previous work reported from our lab (Paredes et al., 2006).

This study is an important first component of a bigger picture, that is, what is the biological component interacting with or being expressed during stable social environments that produces decreased severity of atherosclerosis in a genetically susceptible animal. The current findings support the idea that peripheral oxytocin is acting acutely as a stress response, however, further work is needed to clarify the nature of peripheral oxytocin; specifically, sites and means of action, as well as possible clinical implications (i.e., whether receptivity of these sites can be exogenously suppressed or stimulated). Additionally, future research may focus on specific oxytocin changes within the vasculature, and thus, potentially identify the elusive determinant responsible for the established outcome of diminished disease found within stable social environments.

Table 1

Summary of behavior data observed at initial, midpoint, and endpoint collections for unstable (n = 14), stable (n = 10), and individually caged animals (n = 14). Data is reported as mean (SEM) and represents the percentage of time spent in each behavior during the first ten minutes of pairing.

	Initial	Midpoint	Endpoint
Agonistic behavior			
Unstable	0.38 (0.07)	0.37 (0.03)	0.43 (0.03)
Stable	0.34 (0.04)	0.22 (0.05)	0.21 (0.04)
Affiliative behavior			
Unstable	0.14 (0.02)	0.17 (0.02)	0.17 (0.01)
Stable	0.22 (0.02)	0.26 (0.03)	0.28 (0.02)
Other non-agonistic behavior			
Unstable	0.18 (0.03)	0.20 (0.02)	0.19 (0.02)
Stable	0.17 (0.03)	0.26 (0.03)	0.28 (0.02)
Individual	0.41 (0.01)	0.44 (0.02)	0.46 (0.02)
Inactivity			
Unstable	0.30 (0.04)	0.26 (0.01)	0.21 (0.01)
Stable	0.26 (0.02)	0.26 (0.01)	0.24 (0.01)
Individual	0.59 (0.01)	0.56 (0.02)	0.54 (0.02)

Table 2

Summary of behavioral data collected during animal pairings, as observed over time (Time 1 - Time 8). Data is reported as mean (SEM) and represents the percentage of time spent in each behavior during the first ten minutes of pairing.

	T1	T2	T3	T5	T6	T7	T8
Agonistic behavior							
Unstable	0.43 (0.07)	0.48 (0.07)	0.48 (0.04)	0.48 (0.06)	0.57 (0.08)	0.62 (0.06)	0.64 (0.04)
Stable	0.53 (0.05)	0.20 (0.08)	0.20 (0.07)	0.14 (0.04)	0.27 (0.07)	0.18 (0.05)	0.17 (0.04)
Affiliative behavior							
Unstable	0.08 (0.01)	0.18 (0.03)	0.12 (0.02)	0.12 (0.02)	0.15 (0.04)	0.19 (0.04)	0.16 (0.03)
Stable	0.14 (0.03)	0.31 (0.04)	0.27 (0.04)	0.26 (0.04)	0.29 (0.04)	0.37 (0.05)	0.33 (0.04)
Other non-agonistic behavior							
Unstable	0.11 (0.03)	0.13 (0.03)	0.10 (0.03)	0.11 (0.03)	0.10 (0.02)	0.13 (0.03)	0.07 (0.01)
Stable	0.06 (0.02)	0.23 (0.04)	0.27 (0.05)	0.24 (0.04)	0.22 (0.05)	0.37 (0.04)	0.31 (0.02)
Individual	0.39 (0.03)	0.40 (0.05)	0.56 (0.03)	0.44 (0.03)	0.44 (0.05)	0.65 (0.06)	0.45 (0.07)
Inactivity							
Unstable	0.37 (0.06)	0.20 (0.04)	0.31 (0.02)	0.29 (0.03)	0.18 (0.04)	0.06 (0.02)	0.12 (0.03)
Stable	0.28 (0.04)	0.26 (0.04)	0.25 (0.04)	0.36 (0.03)	0.23 (0.02)	0.09 (0.02)	0.19 (0.04)
Individual	0.61 (0.03)	0.60 (0.05)	0.44 (0.03)	0.56 (0.03)	0.56 (0.05)	0.35 (0.06)	0.55 (0.07)

Table 3

Summary of oxytocin data (pg/ml) collected during animal pairings, as observed over time (Time 1 - Time 8). Data is reported as mean (SEM).

	T1	T2	T3	T5	T6	T7	T8
Oxytocin - Pre-pairing							
Unstable	8.76 (1.52)	21.05 (12.95)	5.04 (0.57)	2.74 (0.45)	10.18 (2.53)	15.47 (4.07)	15.56 (3.27)
Stable	10.36 (2.41)	12.10 (5.71)	10.43 (1.59)	6.00 (2.68)	9.84 (2.90)	9.69 (3.46)	15.87 (2.81)
Individual	37.89 (6.65)	19.85 (10.55)	9.92 (1.64)	3.27 (0.97)	7.12 (0.99)	10.61 (1.89)	21.90 (5.61)
Average Unstable	11.26 (7.79)						
Average Stable	10.61 (6.60)						
Average Individual	15.73 (9.11)						
Oxytocin - 10 min post-pairing							
Unstable	46.87 (13.62)	47.01 (24.61)	28.58 (8.65)	12.97 (3.50)	18.40 (4.83)	28.15 (7.60)	45.93 (8.11)
Stable	50.69 (10.04)	18.13 (7.51)	8.23 (1.73)	3.63 (1.72)	13.19 (5.96)	10.51 (1.64)	17.48 (3.94)
Individual	42.25 (8.02)	11.00 (2.94)	26.52 (18.31)	1.66 (0.47)	7.77 (0.96)	11.49 (2.76)	23.02 (4.82)
Average Unstable	32.46 (25.61)						
Average Stable	17.41 (7.30)						
Average Individual	17.02 (12.67)						
Oxytocin - 120 min post-pairing							
Unstable	24.90 (4.94)	19.02 (3.85)	28.34 (12.67)	2.70 (0.43)	12.06 (2.15)	55.19 (31.94)	25.48 (5.33)
Stable	21.61 (7.30)	45.36 (21.35)	19.55 (12.47)	1.70 (0.62)	7.63 (1.68)	10.73 (2.79)	8.37 (2.42)
Individual	47.99 (13.08)	22.16 (4.16)	17.38 (6.58)	1.59 (0.39)	9.54 (1.52)	27.06 (11.02)	12.44 (2.87)
Average Unstable	23.86 (19.98)						
Average Stable	16.84 (11.78)						
Average Individual	19.70 (12.10)						

Table 4

Summary of cortisol (μg/ml), catecholamines (ng/ml) and blood pressure/heart rate (mmHG and beats per minute, respectively) data collected at initial, midpoint, and endpoint for unstable (n = 14), stable (n = 10), and individually caged animals (n = 14). Data is reported as mean (SEM).

		Initial	Midpoint	Endpoint
Cortisol				
	Unstable	0.35 (0.05)	0.30 (0.05)	0.22 (0.05)
	Stable	0.35 (0.05)	0.05 (0.01)	0.30 (0.08)
	Individual	0.32 (0.06)	0.12 (0.03)	0.15 (0.03)
Epinephrine/TV				
	Unstable	54.46 (8.14)	102.90 (21.36)	116.11 (16.78)
	Stable	49.16 (7.19)	55.86 (16.75)	91.99 (12.37)
	Individual	110.21 (22.26)	53.91 (10.55)	86.12 (18.51)
Norepinephrine/TV				
	Unstable	1447.23 (274.69)	1416.15 (206.41)	1686.08 (342.34)
	Stable	1298.47 (136.89)	832.81 (371.63)	1370.31 (224.69)
	Individual	2098.92 (218.73)	1179.98 (282.52)	1743.72 (440.46)
Systolic BP				
	Unstable	102.42 (2.54)		82.26 (2.41)
	Stable	104.73 (2.46)		83.02 (2.89)
	Individual	90.49 (2.33)		78.06 (1.88)
Diastolic BP				
	Unstable	77.12 (1.88)		63.82 (2.53)
	Stable	79.93 (3.14)		60.58 (2.27)
	Individual	62.46 (1.92)		59.32 (2.20)
Mean Arterial Pressure				
	Unstable	85.75 (1.82)		69.98 (2.42)
	Stable	88.05 (2.79)		67.83 (2.35)
	Individual	71.92 (1.91)		65.57 (2.01)
Heart Rate				
	Unstable	236.00 (6.05)		206.57 (4.13)
	Stable	238.20 (6.20)		213.00 (6.35)
	Individual	238.29 (6.13)		231.00 (5.58)

Table 5

Summary of weight (kg) over time and tissue weights (g) collected at necropsy, adjusted by endpoint weight (kg).
Data is reported as mean (SEM).

	Initial weight	Midpoint weight	Endpoint weight	Epididymal fat	Retroperitoneal fat	Spleen
Unstable	2.50 (0.04)	3.15 (0.07)	3.94 (0.10)	3.79 (0.38)	84.75 (8.85)	1.45 (0.13)
Stable	2.52 (0.07)	3.44 (0.07)	4.43 (0.12)	6.02 (0.69)	175.63 (14.98)	1.24 (0.10)
Individual	2.53 (0.04)	3.38 (0.06)	4.15 (0.09)	4.62 (0.31)	163.29 (11.05)	1.13 (0.06)

Table 6

Summary of correlational analyses between percent of time spent in specific behaviors and measures of plasma oxytocin (pg/ml) and cortisol (μg/ml). * $p < .05$. ** $p < .01$

		Initial		Midpoint		Endpoint	
		Oxytocin	Cortisol	Oxytocin	Cortisol	Oxytocin	Cortisol
Initial	Agonistic Behavior	.020	-.104	-.084	-.104	.220	-.178
	Affiliative Behavior	.149	.006	.163	-.325	-.019	.152
Midpoint	Agonistic Behavior	-.230	-.093	-.318	.503 *	-.019	-.079
	Affiliative Behavior	.212	-.148	.187	-.592 **	.010	-.099
Endpoint	Agonistic Behavior	-.281	-.078	-.325	.618 **	-.087	-.164
	Affiliative Behavior	.248	-.063	.191	-.642 **	.018	.013

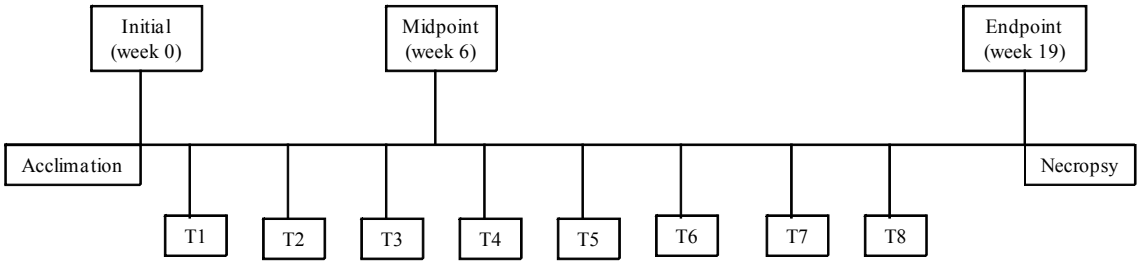


Figure 1. Graphical timeline of blood draws collected throughout the study; initial, midpoint, and endpoint, along with specific pairing blood draws (time points 1-8).

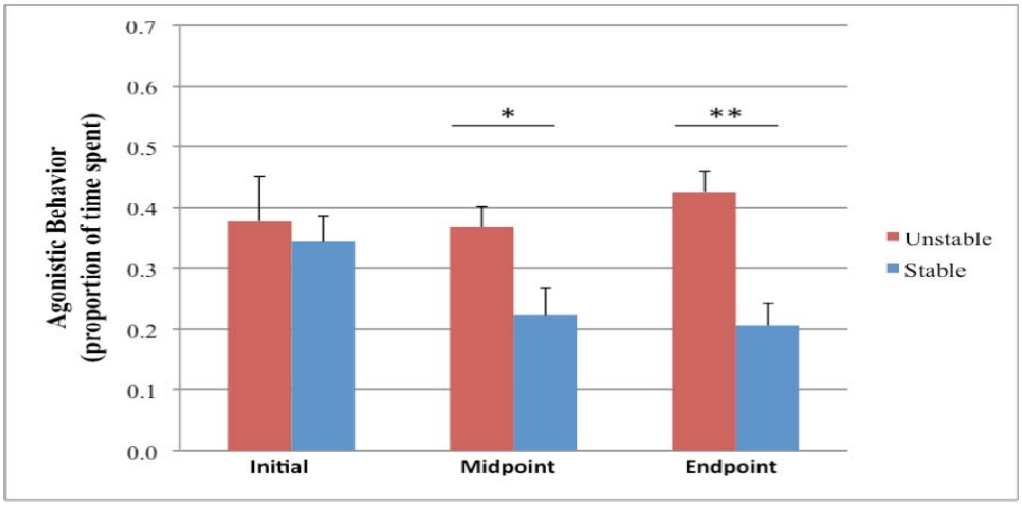


Figure 2. **Percentage of time spent in agonistic behavior over time.**
*** $p < .05$. ** $p < .01$.**

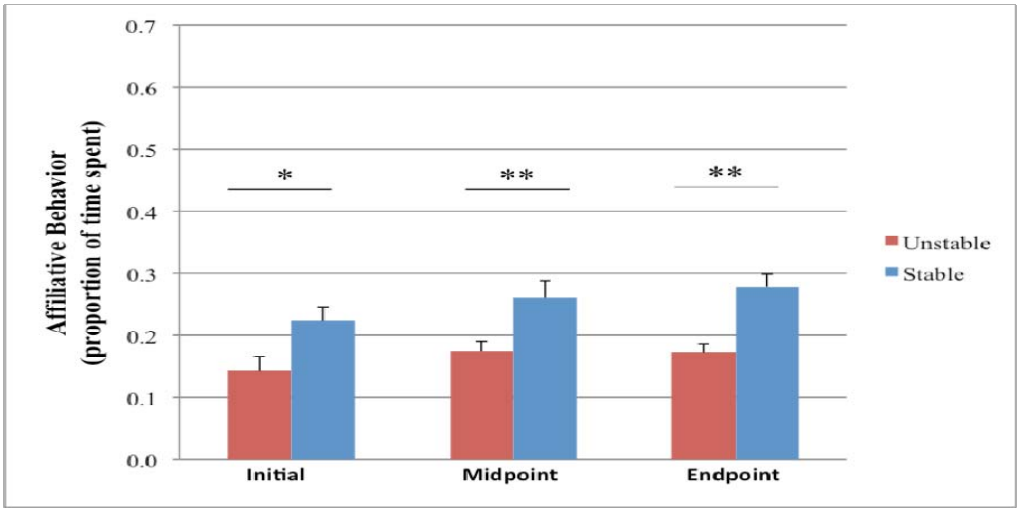


Figure 3. Percentage of time spent in affiliative behavior over time.
* $p < .05$. ** $p < .01$.

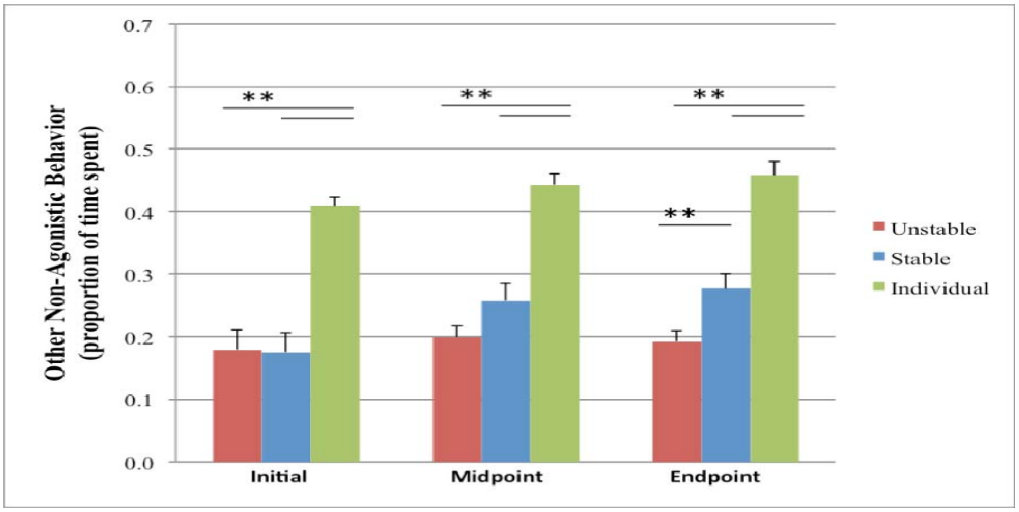


Figure 4. Percentage of time spent in other non-agonistic behavior over time.
* $p < .05$. ** $p < .01$.

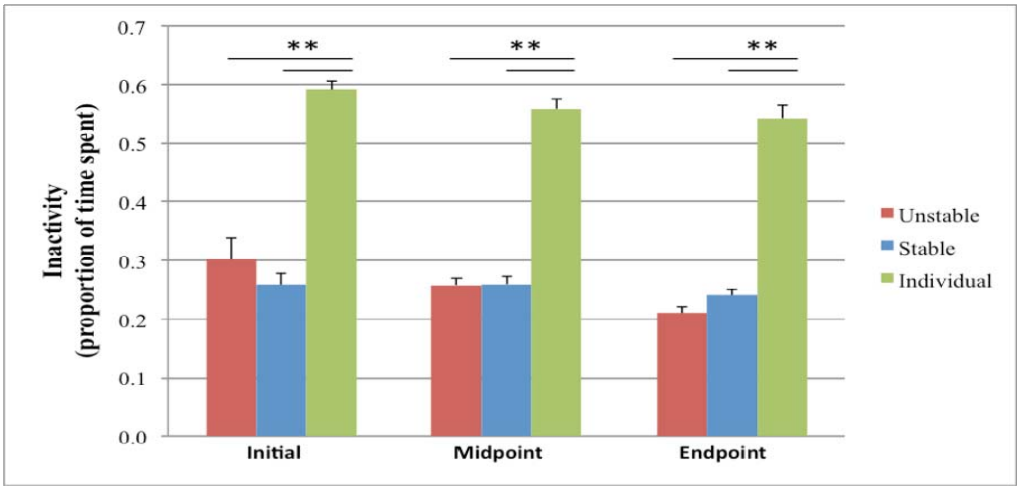


Figure 5. Percentage of time spent in inactivity over time.
* $p < .05$. ** $p < .01$.

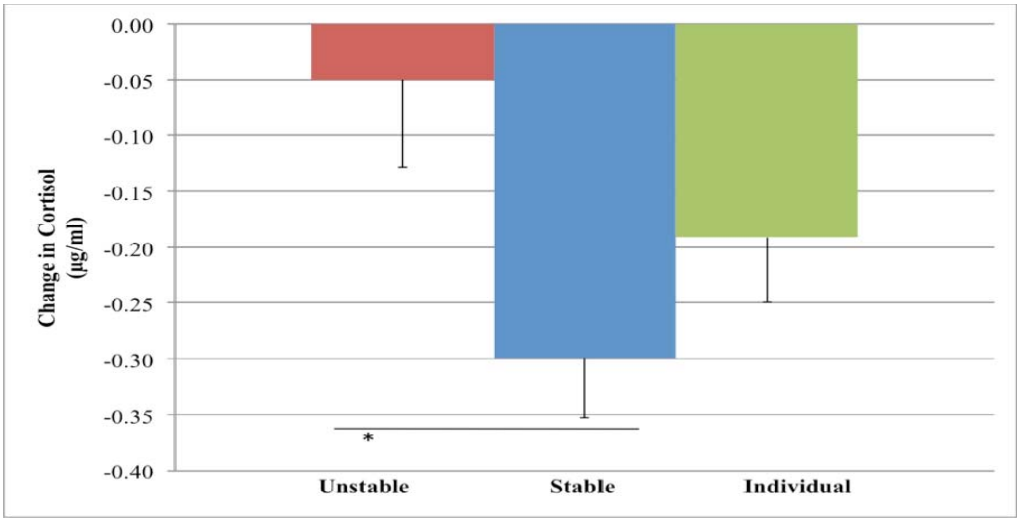
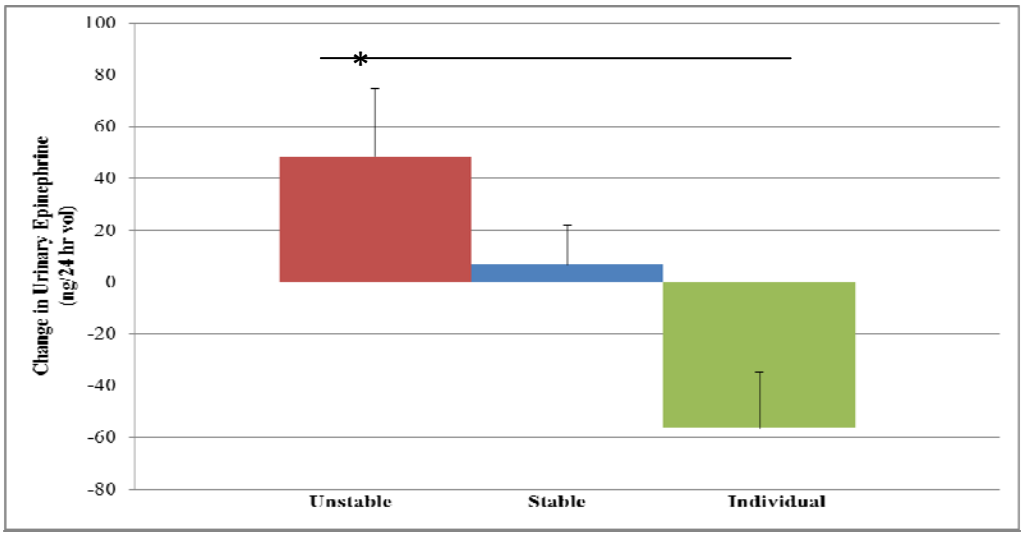


Figure 6. Change in cortisol from initial to midpoint collection.
* $p < .05$. ** $p < .01$.



* $p < .05$. ** $p < .01$.*Figure 7*. Change in epinephrine from initial to midpoint collection.
* $p < .05$. ** $p < .01$.

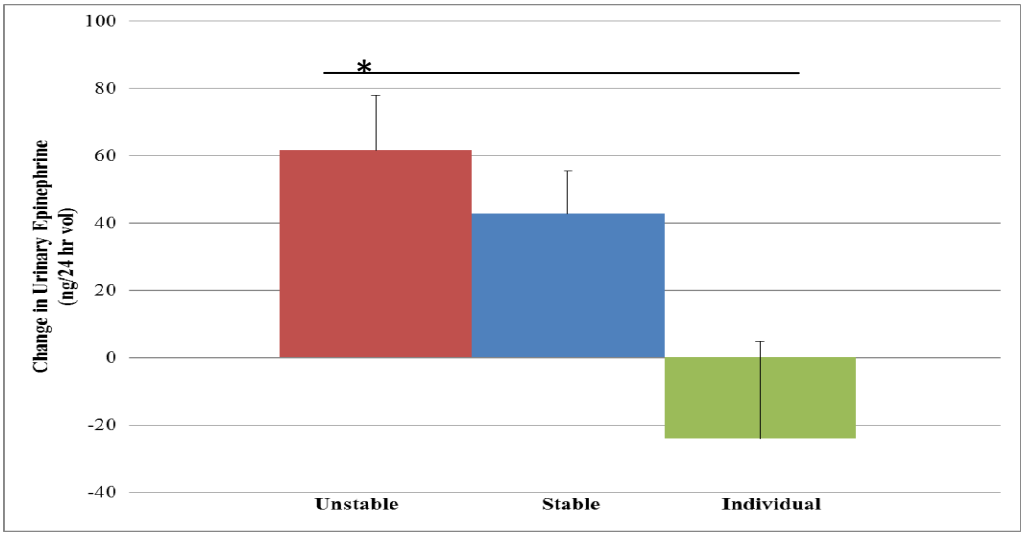


Figure 8. Change in epinephrine from initial to endpoint collection.

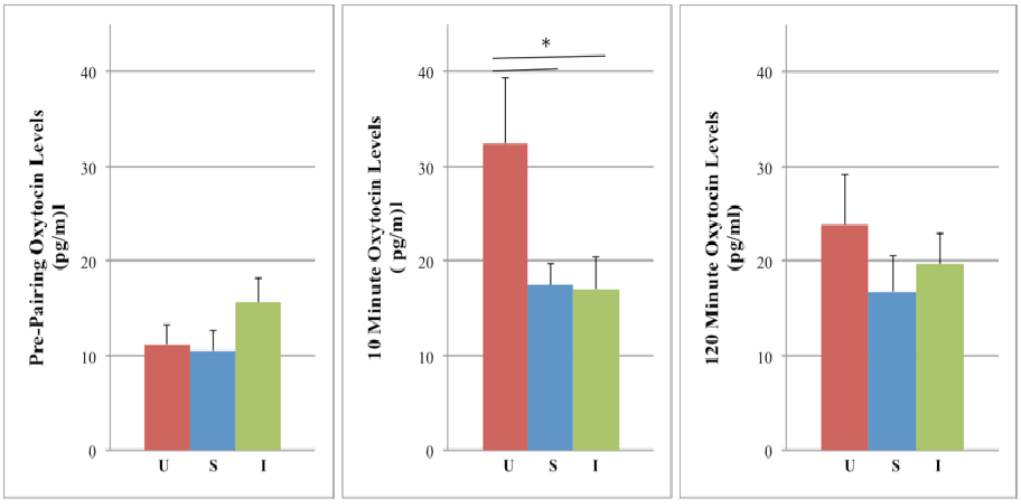


Figure 9. Mean oxytocin response over seven pairings.
* $p < .05$. ** $p < .01$.

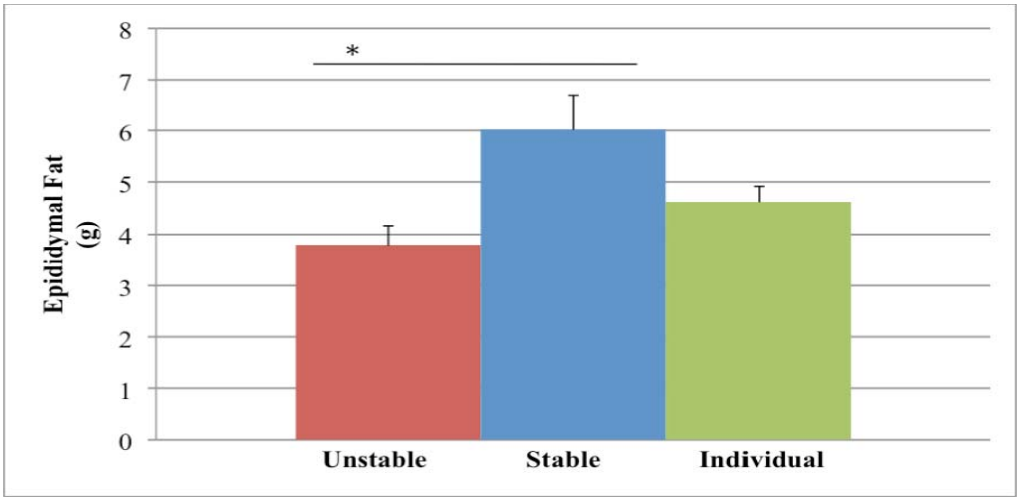


Figure 10. Epididymal fat adjusted by body weight.
* $p < .05$. ** $p < .01$.

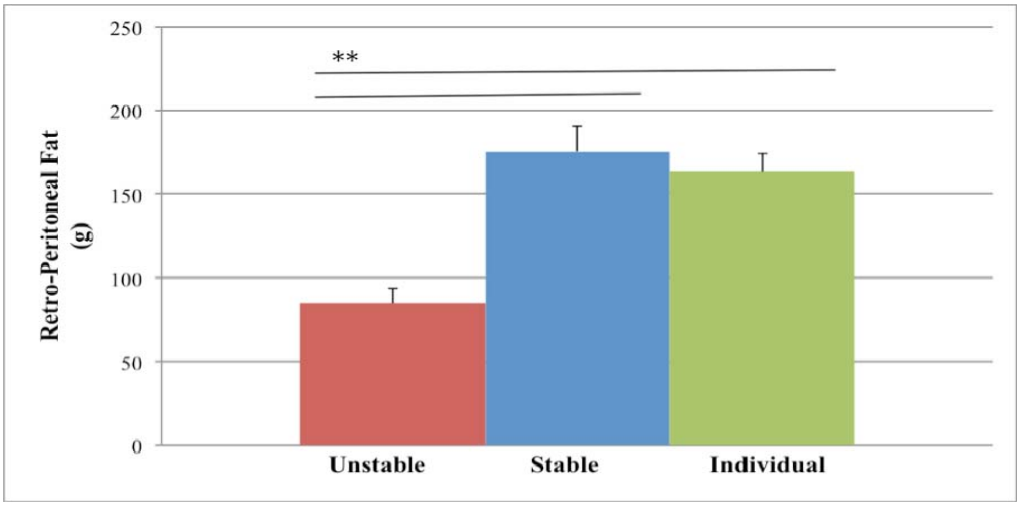


Figure 11. Retro-peritoneal fat adjusted by body weight.
* $p < .05$. ** $p < .01$.

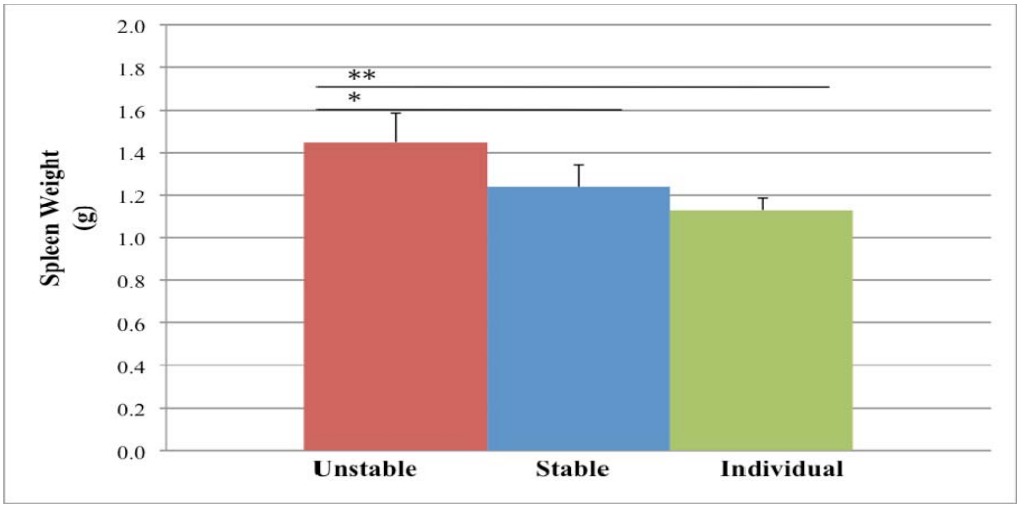


Figure 12. Spleen weight adjusted by body weight.
* $p < .05$. ** $p < .01$.

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