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Oxytocin Mitigates the Negative Consequences of Chronic Social Isolation in Prairie Voles (Microtus ochrogaster)

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OXYTOCIN MITIGATES THE NEGATIVE CONSEQUENCES OF CHRONIC SOCIAL ISOLATION IN PRAIRIE VOLES (*MICROTUS OCHROGASTER*)
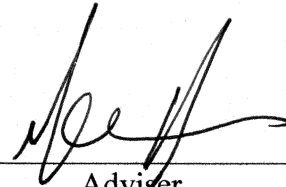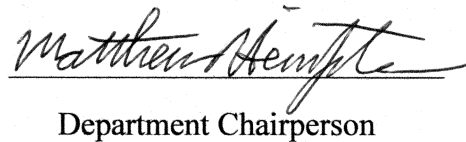
by

Elyse McMahon

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Elyse McMahon

<u>Table of Contents</u>

Abstract

Chronic stressors, such as chronic isolation in social mammals, can elevate glucocorticoids (CORT), which may affect cellular aging mechanisms such as increasing levels of oxidative stress and shortening telomere lengths. Prairie voles (Microtus ochrogaster) are a useful model species to study chronic isolation due to their social and pair-bonding behaviors. Recent work in prairie voles suggests that oxytocin and social support may mitigate some of the negative consequences of social isolation, possibly by reducing CORT levels. We investigated the influences of isolation, oxytocin or social support on stress physiology, behavior, and cellular aging. Voles were divided into six groups: isolated (I), paired (P), isolated (IV) and paired (PV) with daily vehicle injections, and isolated (IO) and paired (PO) with daily oxytocin injections. Blood samples were collected at the start of the study, then again after 3 and 6 weeks. Acute stress tests were conducted using the resident-intruder test (RIT) at 6 weeks to determine if treatment had any effect on stress responsiveness. Anhedonia, a behavioral index of depression, was measured using sucrose solution preference tests to determine depression-like symptoms throughout the study. We found that six weeks of chronic isolation lead to increased CORT levels, oxidative damage, telomere degradation and anhedonia. However, daily oxytocin injections in isolated individuals prevented these negative consequences as compared to those who were isolated with and without daily vehicle injections. During the RIT, I prairie voles had elevated CORT levels at baseline and stress-induced time points, which continued to increase during the recovery time point. IO prairie voles had a similar stress response in terms of CORT secretion to the P,

PO and tremetn groups. Oxidative damage markers (ROMs) were elevated in the I and IV

groups during the stress-induced time point of the RIT, but returned to baseline levels by

the recovery time point. However, baseline levels of ROMs were still significantly higher

in the I and IV treatment groups than all other treatments. IO prairie voles had no change

in ROMs during the RIT, similar to the paired groups. Antioxidant capacity stayed the

same for all groups until the recovery sample during the RIT when all treatment groups

significantly declined in TAC. This demonstrates that isolation caused elevated

biological aging, stress and depression-like behavior. This is the first study to link social

isolation with oxidative stress and telomere shortening.

Chapter 1-Thesis Introduction

Social isolation is a growing epidemic in today's society. It is 20% more prevalent today than it was in the 1980's (AARP, 2010). Loneliness or extended periods of social isolation are characterized as a chronic stressor leading to many negative behavioral and physiological consequences in the human body (Cacioppo et al 2015a,b; Holt-Lunstad et al 2015). Individuals that are living alone or isolated from society show signs of increased depression and anxiety (Heinrichs et al 2003; Cacioppo et al 2007; Cacioppo et al 2010). Further, chronic loneliness is linked to early death, obesity, gastrointestinal ulcers, elevated stress hormones, and decreased immunity (Surwit et al 1992; Rosmond and Bjorntorp 2000; Cacioppo et al 2002a; Hawkley et al 2006; Cole et al 2007; Hawkley et al 2010). Human studies are important to understand the current state of chronic isolation and loneliness, however animal models are more advantageous in order to understand the physiological consequences of chronic social stress. This allows us to examine the mechanisms and also possible treatments for these negative consequences. While there is a growing body of literature on the impacts of social stress, there is still much we do not know or understand. Studies ranging from mice and prairie voles through baboons and chimpanzees enable scientists to analyze social behavior. They are also able to apply these findings to human situations; conquering physical illnesses and mental health problems for humans lacking social support, and understanding why social support is beneficial.

<u>Stress and Physiological Adaptations</u>

While stress results in negative health consequences, it is a ubiquitous part of living, whether it is originating from social interactions or isolation. When organisms experience a stressor, one key physiological response is the activation of the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is comprised of the hypothalamic paraventricular nucleus (PVN), the anterior pituitary gland and the adrenal cortex. In response to a stressor, higher brain regions, including the hippocampus, perceive a stressor and stimulate the PVN causing the release of corticotrophin releasing hormone (CRH) which binds to cellular receptors on the anterior pituitary. The anterior pituitary releases adrenocorticotropic hormone (ACTH) into the systemic blood stream. This initiates the secretion of glucocorticoids from the adrenal cortex. These are a class of steroid hormones, including corticosterone and cortisol (CORT), which produces a variety of effects that center on glucose metabolism in response to both daily metabolic cyclic activities and stressful events. Specifically, during stressful situations, CORT acts to mobilize energy stores into the blood, inhibit unnecessary physiological functions, and control energy intake and expenditure (Munck et al 1984; Sapolsky et al 2000).

Upon release from the adrenal cortex, CORT can either become bound to a binding globulin or stay as a "free" or unbound hormone. Corticosterone Binding Globulin (CBG) is a protein that has a high affinity for CORT. The main physiological function of this binding is to transport CORT throughout the body. It also has the potential to modulate the organismal stress response by altering the potency and tissue specificity of CORT (Westphal 1983, Mendel et al 1989, Ekins 1990). Some hypothesize that only free

CORT is able to enter cells or be broken down and therefore is believed to be the only type that can cause biological effects (Pemberton et al 1988; reviewed in Breuner and Orchinik 2002). Further, it is thought that CBGs are able to lock up CORT in the circulatory system, rendering it biologically inactive (Mendel 1989 and Breuner and Orchinik, 2002).This theory, known as the "Free CORT Hypothesis," suggests that the measure of total CORT values are potentially unreliable indicators of the stress response (Mendel 1989). Other sources speculate CBG should be viewed as a transportation system for CORT and that this bound hormone may cause similar effects as free CORT (reviewed in Schoech et al 2013). The "Bound CORT Hypothesis" disagrees with the "Free CORT Hypothesis" stating that measuring total CORT is more useful in understanding the entire system and free CORT measures are inaccurate (Schoech et al 2013). Free CORT is very dynamic and able to bind and unbind to CBGs, transport molecules and plasma membranes (Hammond et al 1980). Moreover, approximately 5-10% of all CORT is unbound (Ekins 1990), suggesting that a measure of free CORT is not accurately depicting what is actually occurring. Further, free and total CORT function over different time periods, with free CORT acting in seconds to minutes while total CORT could function at larger time scales of minutes to hours (reviewed in Sapolsky et al 2000), resulting in a more accurate and comparable measure. Finally, and potentially most importantly, Petersen and colleagues (2006) suggest that CBG binding is essential for negative feedback on the HPA axis to downregulate hormone release. Within this study, we measure total CORT through a radioimmunoassay to further understand the

interactions of CORT within the system and more accurately analyze the negative feedback process.

Negative feedback of CORT to the rest of the HPA axis restores homeostasis once the stressor is effectively coped with. Specifically, circulating CORT binds to mineralcorticoid (MR) and glucocorticoid (GR) receptors to initiate the negative feedback process. These receptors are found throughout the body. However within the neuroendocrine system, CORT binds to MR primarily in the hippocampus and lateral septum and GR in the lateral septum, central amygdala, PVN and the pituitary gland (Reul and deKloet 1985; Sapolsky 1992; Joels 1997). It is important to note that MR have a ten-fold higher affinity for CORT when compared with GR (Reul and deKloet 1985). This means that during stress events, CORT will first bind to MRs in the hippocampus before binding to those in the PVN or pituitary. It should be noted that ACTH also assists in this negative feedback process in similar ways by binding to receptors in the hypothalamus, however, it is CORT that is causing most of the negative feedback process within this system. When CORT binds to these receptors after the stressor, it causes the hypothalamus and pituitary to diminish the release CRH and ACTH respectively to baseline levels. Once ACTH is no longer at elevated levels, it causes the adrenal to reduce the release of CORT and overtime, this will return to baseline levels, resulting in the return to homeostasis. This negative feedback process occurs on a daily basis and is important during the recovery of stressful situations.

Sympathoadrenal system

While the HPA axis is the focus of our study, the sympatho-adrenal system is a contributing factor to the stress response (Axelrod and Reisine 1984; Johnson et al 1994). Activation of the sympathetic nervous system results in the secretion of epinephrine (adrenaline) from the adrenal medulla and release of norepinephrine (noradrenaline) from sympathetic nerve terminals throughout the body (Axelrod and Reisine 1984). These hormones are released within seconds after a stressor. They play an important role in mobilizing energy and regulating energy balance along with cardiovascular function. This includes transport of oxygen and fuel to tissues in need of energy. Specifically, epinephrine causes increased heart rate, vasoconstriction and overall increased cardiac output. Norepinephrine causes increase in arterial pressure but also decreases heart rate. Factors resulting from stressors that vary in intensity, such as socioeconomic status (Steptoe et al 2010), anxiety and mood states (Wittstein et al 2005; Van der Kooy et al 2007) and lack of significant social support (De Vogli et al 2007) modulate and interfere with cardiovascular health in part due to the sympatho-adrenal system response. Moreover, Scotti and colleagues (2011) suggest that reducing parasympathetic output may enhance physical and depression-like responses to stressors, further showing the important correlation and potential downstream effects between these hormones and the stress response. While these hormones play crucial roles in the stress response, we were unable to measure them due to their quick outputs and inability to capture an accurate baseline reading before stress-induction.

Acute vs Chronic stress

Stressors vary in intensity and duration. Sudden, and typically short lived stressful events are referred to as acute stress. This response is an important and adaptive part of a typical fight or flight response (Wingfield et al 1998); for example the race of life and death between a gazelle and cheetah that plays out on the African savannah. This race is stressful for both predator and prey. During this acute stressor, higher brain regions including the hippocampus and prefrontal cortex perceive the prey or threat causing the activation of the neuroendocrine stress response and first leads to enhanced secretion of CRH and ACTH along with epinephrine and norepinephrine, from the sympathetic nervous system (Reviewed in Sapolsky et al 2000). This occurs within ten seconds of the initiation of the stressor (Reviewed in Sapolsky et al 2000). The eventual release of CORT follows minutes later. Lower CBG concentrations are found during acute stressors, leading to more CORT circulation (Breuner et al., 2013). CORT concentrations usually reach peaks within twenty to thirty minutes after the initiation of the stressor and return to baseline levels in as little as an hour (Sapolsky 1992; Wingfield and Romero 2001). During an acute stress, epinephrine increases ventilation and heart rate to increase blood flow. At the same time, CORT levels increase the chance of survival by mobilizing energy through increased blood glucose concentrations that will provide energy to rapidly metabolizing muscle cells. This causes downregulation of other nonessential systems during the stress event. These systems include the immune, digestive, growth and reproductive just to name a few. Taken together, the action of both of those hormones

help allow the gazelle to either escape the cheetah, or the cheetah to successfully catch its next meal.

While many understand the concept of predator-prey dynamics, human acute stressors are of more interest in this study. Many acute stressors exist in daily life. For example, taking an exam can be a stressful experience for many. Armario and colleagues (1996) observed elevated salivary CORT levels in female medical students prior to taking a medical examination. Further, Morgan and colleagues (2001) reported an increase in salivary and plasma CORT levels along with epinephrine and norepinephrine in active duty U.S. Navy personnel following 24 hours of survival school stressors. These levels returned to baseline relatively quickly once the stressor was effectively coped with, signifying an acute stress response.

Stressors which are repeated or extended for long periods are termed chronic stress. These are often characterized as a constant and inescapable exposure to a stressor. While these stress events cause a similar initial activation of the HPA axis, there is a significant alteration in the negative feedback process, altering future recoveries from other stressors. Examples include working in a highly stressful job (Xie and Johns 1995; Kivimaki et al 2006), a parent caring for a child with a life-threatening disease (Eccleston et al 2004; Raina et al 2005), and soldiers in war zones (Gray et al 2004; Lew et al 2009; Kline et al 2010). These chronic stress events result in continuous stimulation of the hypothalamus creating elevated levels of CRH which generates a cascade effect of higher ACTH and higher CORT secretion. Further, CBG concentrations tend to decline with continuous stressors leading to more CORT within the bloodstream allowing it to bind

more easily (Lynn et al 2003; Malisch et al 2010). This elevated CORT can then lead to enhanced reactivity to stressors throughout the entire body (Epel et al 2004; Aubert and Lansdorp 2008; Sapolsky et al 2000). Cacioppo and colleagues (2006) found that students who experience chronic loneliness are less likely to effectively cope with acute stressors. This may suggest that chronic stress is causing a dysregulation of the HPA axis and other stress response systems leading to both behavioral and physiological consequences in future situations. This is further implied by Ockenfels and colleagues (1995) who found that unemployed individuals had significantly elevated CORT levels when exposed to acute stressors when compared to individuals who are employed, suggesting that chronic stress may severely impact normal CORT circulation and reactions to stressors. During acute stressors, reactions can last for minutes to hours. However, during chronic stressors, physiological reactions can last for much longer durations leading to longer periods of suppression to other systems such as immune, digestive, cardiovascular and reproductive.

Moreover, methylation of the MR and GR promoters from continuously elevated CORT can lead to reduced CORT receptors throughout the neuroendocrine system (Love et al 2013) specifically in the hippocampus and amygdala (Hennessy et al 2009; Lin and Koleske 2010; Costantini et al 2011). This leads to a weaker negative feedback system of the HPA axis, which remains active for longer periods, allowing more CORT to be secreted (Sapolsky et al 1984; Eldridge et al 1989; Spencer et al 1991; Merman and Spencer 1998). Not only do these elevated CORT levels influence receptor numbers, it also acts directly on reproductive hormones. CORT directly inhibits testosterone,

progesterone and prolactin secretion, leading to suppressed reproductive behaviors (both sexual and parental) (Wiegers et al 1993; reviewed in Wingfield and Romero 2001; French et al 2007; MacDougall-Shackleton et al 2013). Increased hepatic gluconeogenesis and decreased fatty acids and hematocrit levels are other effects from elevated CORT levels (Wingfield and Romero 2001) that may result in downstream effects in other systems of the body including the digestive and cardiovascular systems.

Another system affected by chronically elevated CORT includes the immune system. Both the adaptive and innate immune responses are suppressed through mechanisms that involve suppression of leukocyte numbers, trafficking and function along with changes in cytokine balance (Fridovich, 1986; Halliwell 1999; Costantini et al 2011; Silva et al 2016). Sheridan and colleagues (2000) further supported these findings reporting that chronic restraint tests in mice resulted in elevated CORT levels causing a diminished recruitment of inflammatory cells after a virus was administered. Moreover, people facing chronic stress are more likely to suffer from exacerbated allergic or autoimmune symptoms and show accelerated progression of chronic diseases such as immunodeficiency syndrome and coronary heart disease (Wright et al 1998; Rozanski et al 1999). These individuals are also more susceptible to other diseases such as type two diabetes (Rosmond and Bjorntorp 2000; Anderson et al 2001; Surwit et al 2002; Kyrou and Tsigos 2006). These and other studies suggest that chronic stress suppresses the adaptive and innate immune response, which could lead to increased disease susceptibility in humans.

Chronic stress also causes negative consequences on psychological disorders, specifically depression. People who experience chronic stress are more likely to develop clinical depression (Monroe and Hadjiyannakis 2002; Miller and Cohen 2005). Others who develop post-traumatic stress disorder (PTSD) after a chronic stress exposure tend to have elevated baseline CORT levels compared to others who were not chronically stressed (Miller et al 2006). Further, animal studies demonstrated that many different forms of chronic stress cause increased depression-like behaviors. Social isolation from partners resulted in a decrease in seeking pleasurable stimuli (Grippo et al 2007a,b,d Grippo et al 2008; Grippo et al 2011), while repeated stressors such as shaking or immobilization cause other depression-like behaviors (Adell et al 1988; Smith et al 2013; Grippo et al 2014; Kelly and Goodson 2014; Smith and Wang 2014). This is potentially associated with elevated CORT levels and increased oxidative damage. The former will be discussed here while the latter will be discussed in later sections. The connection between elevated CORT and depression is still not well understood. However, many studies demonstrate a strong relationship between these two areas (Sheline et al 1996; Cacioppo et al 2000; Steptoe et al 2004; Pressman et al 2005; Doane and Adam 2010). Individuals with Cushing's disease, which causes elevated secretion of CORT, show unusually high rates of depression (Sonino and Fava 2002). Moreover, elevated CORT causes damage to the hippocampus when binding to MR impairing the regulation of CORT, which may then produce neuronal changes in several brain regions leading to altered behaviors such as depression (Sapolsky et al 1984; Sapolsky and McEwen 1989; Vyas et al 2003).

Social Stress

In this study, we focus on social isolation as a chronic stressor, which is becoming more prevalent in today's society. House and colleagues (1982) were one of the first groups to determine that social isolation can lead to major risk factors associated with mortality. Social isolation can be categorized as "objective" where individuals are separated or isolated from friends and family or it can be categorized as "subjective" where it is a perceived loneliness (Cacioppo 2002). Both of these forms are chronic stressors (Cacioppo 2002; Cole et al 2007). It should be noted that it is unclear whether the consequences of social isolation originate predominantly from the withdrawal of social support or from the biological response from perceived threats that result in social isolation, instigating a return to social groupings (Cacioppo 2002). Objective social isolation is more prevalent for elderly living alone and having limited social interactions (Shepard 2006; Sayer 2010). This type of social isolation exacerbates the negative consequences of daily stress through higher reactivity to acute stressors (Cacioppo et al 2003), and increased cardiovascular morbidity (Hedblad et al 1992; Woloshin et al 1997) and mortality (House et al 1982; Kaplan et al 1988; Welin et al 1992).

Subjective social isolation is found in all ages and ethnicities and is also termed perceived isolation or loneliness. In this situation, individuals may be surrounded by others, but do not feel included or that they belong within these social settings. Recent evidence suggests that social media including Facebook, Twitter, YouTube and Flicker are reshaping socialization roles (Van Dijck 2013). While these media sources are providing a platform for sharing and a sense belonging through friendship and

connections, many are finding that adolescents and youth are predominantly feeling excluded and isolated because a screen can not replace the importance of human contact and experiences (Kraut et al, 1998; McPherson et al, 2006; Juvonen and Gross 2008; Milani et al, 2009). There is considerable evidence that suggests a negative correlation between social media practices and wellbeing (Van den Eijnden et al, 2008; O'Dea and Campbell, 2011; Devine and Lloyd, 2012; Pantic et al, 2012; Koles and Nagy, 2012). For example, a new phenomenon is occurring with many adolescents today called "Facebook Depression" where preteens and teenagers who spend large amounts of time on social media, such as Facebook, exhibit classic symptoms of depression (O'Keefe and Pearson 2011). The exact cause is thought to be a combination of lack of real physical contact and experiences and perceived friendships that lack affection and familiarity (Davila et al 2009; Selfhout et al 2009; Melville et al 2010). However, there is evidence suggesting that social media boosts positive behaviors and decreases depression in individuals with high and positive social contact but has no beneficial qualities to those who are subjectively isolated or lonely (Ko and Kuo, 2009; Maarten et al, 2009; Davis, 2012).

Moreover, subjective isolation or loneliness predicts elevated blood pressure (Hawkley et al 2006; Hawkley et al 2010), rise in cortisol (Adam et al 2006), greater vascular resistance (Cacioppo et al 2002a), decreased immune response (Cole et al 2007), increased depression (Cacioppo et al 2007; Cacioppo et al 2010), and decreased sleep quality (Hawkley et al 2010; Cacioppo et al 2002b). It is hypothesized that the feeling of loneliness evolved from a neuronal signal indicating that social connections are weakened and its purpose is to motivate the repair of these connection, which are needed

for our health and well being (Cacioppo 2002a; Cacioppo and Patrick 2008). In general,

loneliness is associated with poorer mental as well as physical health (Perkins, 1991;

Gupta and Korte, 1994; Ernst and Cacioppo, 1999).

Lonely individuals are also less likely to actively cope and more likely to feel

depressed, anxious and threatened by their environment and the people around them.

Studies using the Center for Epidemiologic Studies Depression Scale (CES-D) reported

high correlations between loneliness and symptoms of depression (Radloff, 1977;

Anderson & Arnoult, 1985; Levin & Stokes, 1986; Steffick, 2000; Cacioppo et al 2006).

This may be caused by elevated activation of the HPA axis (Seeman and McEwen 1996),

leading to elevated CORT secretion (Cacioppo et al 2000; Steptoe et al 2004; Pressman et

al 2005; Adam et al 2006 and Doane and Adam, 2010). As previously mentioned, the

connection between CORT and depression is not well characterized. However, many

studies demonstrate a strong correlation between chronically elevated CORT from

isolation and severe depression (Sheline et al 1996; Cacioppo et al 2000; Steptoe et al

2004; Pressman et al 2005; Adam et al 2006; Doane and Adam 2010). These negative

behavioral consequences, especially increased depressive symptoms, increase the risk for

many problems including functional impairments (Mehta et al 2002), cardiovascular

disease (Barefoot and Schroll 1996; Barth et al 2004; Carney et al 2004), weakened

immune response (Hawkley et al 2006; Hawkley et al 2010) and overall poor

performance in careers (Broadhead et al 1990; Ettner et al 1997).

In social isolation, animals are completely removed from all individuals within the

population or their partner, effectively disrupting social bonds. This negative social

environment results in elevated CORT levels and is demonstrated as a chronic stressor. McNeal and colleagues (2014) found that even after 5 days of isolation both male and female prairie voles showed increased CORT levels after acute stress tests. Pournajafi-Nazarloo and colleagues (2011) found that isolation for one hour or repeated isolation treatments caused significant increases in plasma CORT levels in prairie voles. Further, disruption of social bonds in Siberian hamsters resulted in a persistent increase in basal CORT concentrations (Castro and Matt 1997; Crawley 1984) and slower wound healing (Detillion et al 2003). Social isolation can also induce both behavioral and neuroendocrine disturbances that are relevant to depression (Grippo, et al 2007a,b,c; Grippo et al 2011; Djordjevic et al 2010; Lieberwirth et al 2012; Zlatkovic et al 2014). Depression in both human and animal models is associated with altered HPA axis function including altered CRH release and compromised feedback loops causing elevated ACTH and CORT levels throughout the system (Froger et al 2004; Maier and Watkins 2005; Grippo et al 2005; Grippo et al 2007b).

The behavioral index of depression in animal models can be measured in several ways including forced swim test (FST), changes in appetite or weight gain, and anhedonia. The FST measures how long animals actively swim in water compared to how long they float, which represents despair or learned helplessness (Cryan et al 2002). This method was used in several studies to measure depression-like behavior, however many times it is conducted in parallel with other measures of depression. This is potentially because measuring locomotor activity in a swimming situation may not be an accurate depiction of depression and locomotor activity in the animal's cage may confound data

(Slattery and Cryan 2012). Increased appetite or weight gain is another index of possible depression. In human studies, many who are depressed overeat or consume foods that do not comprise a balanced diet (Greeno and Wing 1994; Goossens et al 2008). However, in animal models, increased or decreased food consumption may signify independent factors such as boredom or sickness. There is also the reduced responsiveness to pleasurable stimuli (anhedonia), which is a critical feature of depression in humans (Willner et al 1987; American Psychiatric Association 2000). Anhedonia is used in many animal models including rats (Rygula et al 2005; Dwyer et al 2013; Rossetti et al 2016), mice (Strkalova and Steinbush 2010; Cline et al 2015; Erburu et al 2015) and prairie voles (Grippo et al 2007a,b,c; Grippo et al 2011). While these procedures slightly disturb the natural daily activities of animals, it is an accurate measure of a behavior that is associated with depression (Carter and Shieh 2015). Hedonic behaviors are associated with the reward circuit within the ventral tegmental area and the nucleus accumbens, which are altered in depressed patients. Specifically, increased stress causes an elevation in cAMP response element binding protein (CREB) in the nucleus accumbens (Dudman et al 2003; Conti and Blendy 2004; Carlezon et al 2005), which is associated with a decreased pleasure or reward seeking behavior (reviewed in Nestler and Carlezon 2005). This was further supported by Newton and colleagues (2002) along with McClung and Nestler (2003) who showed elevated CREB in the nucleus accumbens increased depression-like behavior in mice and reduced the rewarding effects of cocaine, morphine and sucrose. In our study we utilized anhedonia testing because we thought it would be the most effective and encompassing measure of depression-like behaviors in our study

model. It is important to note that there are many symptoms of depression and that some are easily studied in animal models while others can only be studied in humans.

<u>Stress and Aging</u>

Regardless of whether the stressors are acute or chronic, both cause elevated CORT levels. Recently, a growing body of work shows that elevated CORT is associated with an increase in oxidative stress (Sapolsky et al 2000; Agostinho et al 2010; Bjelkovic et al 2010; Haussmann and Marchetto, 2010; reviewed in Costantini et al 2011; Haussmann et al 2011; Fletcher et al 2015). And more interestingly, chronic stress and the subsequent chronically elevated CORT levels cause an increase in oxidative stress (Costantini et al 2011; Breuner et al 2013). Oxidative stress is defined as the imbalance of oxidizing molecules and antioxidant defense, specifically by an increase in oxidative damage and a decrease in antioxidant capacity (Halliwell and Gutteridge 2007). It is implicated in a variety of diseases including cancer, cardiac disease, diabetes, vascular disease and neurodegenerative diseases (Finkel et al 2000; Hulbert et al 2007; Monaghan et al 2009).

Oxidative damage results from Reactive Oxygen Species (ROS) (Gerschman et al 1954; Harman 1956), which are a free radical produced during normal energy metabolism (Valko et al 2006; reviewed in Valko et al 2007). During metabolism in the electron transport chain, electrons from NADH and FADH2 are transported through redox reactions leading to the production of adenosine triphosphate (ATP). During this process, a small percentage of electrons do not complete the whole process and directly leak to unstable oxygen molecules resulting in the formation of free radicals. Some

molecules that are produced include hydroxyl and nitric oxide, which are reactive and unstable causing a chain reaction in which reactivity is passed along to even more damaging compounds. These molecules only last for a short time, but their effects can cause deleterious consequences on other molecules. Hydrogen peroxide and hypochlorous acid are also produced and can persist for longer periods and can also cause oxidative damage to biomolecules if not removed from the system.

The key biological molecules notably affected include proteins, lipids and nucleic acids. Oxidative damage causes a change in the structure of these molecules, making them ineffectual (Siems et al 1995; Stadtman 2004). Oxidation of proteins produces reversible disulphide bridges, changes their formation and structures and eventually impairs function (Monaghan et al 2009). Damage to lipid structure can result in major consequences on membrane structure and function (Halliwell and Gutteridge 2007; Hulbert et al 2007; reviewed in Monaghan et al 2009). Finally, mitochondrial DNA is particularly vulnerable due to its proximity to ROS generation (Finkel and Holbrook 2000; Balaban et al. 2005; Falnes et al. 2007) and cellular DNA degradation will be discussed later.

While the specific connection between CORT and oxidative damage is not elucidated, many studies report a correlation between these two areas (Sapolsky et al 2000; Agostinho et al 2010; Bjelkovic et al 2010; Haussmann and Marchetto, 2010; reviewed in Costantini et al 2011; Haussmann et al 2011; Fletcher et al 2015). It was suggested that in causing glucose mobilization, CORT increases energy metabolism, which may elevate free radical production leading to dysregulation of macromolecules

such as lipids, proteins and nucleic acids (Sapolsky 1985; Rebuffe-Scrives et al 1991; Sapolsky et al 2000; Lin et al 2004; Le et al 2005; Lin et al 2006; Karatsoreos et al 2011). It is also suggested that elevated CORT may impact mitochondrial function through increased receptor binding during stress events, leading to altered mitochondrial gene expression and formation (Manoli et al 2007). This then results in elevated oxidative damage production (Picard et al 2014). You and colleagues (2009) demonstrated that blocking the binding of CORT to its receptors caused decreased ROS production, suggesting a positive correlation between CORT and oxidative damage. Further, Yi and colleagues (2016) found in cell culture studies that dexamethasone, a synthetic glucocorticoid, caused oxidative damage in thymocytes by inhibiting antioxidants and enhancing lipid peroxidation. These studies demonstrate that while we may not know the exact mechanisms connecting elevated CORT and oxidative damage, we know there is a significant correlation between the two. These studies and future research will assist us in better understanding the causes of oxidative damage.

Much of this molecular damage can be repaired by antioxidants and other repair mechanisms that are specific to each macromolecule (Monaghan et al 2009). Antioxidants are defined here as molecules, whether endogenous or exogenous, that inhibit or alter the oxidation of other molecules. Endogenous antioxidants are the primary defense against ROS and include molecules such as superoxide dismutase, glutathione peroxidase, catalase, nicotanamide adenine diphosphate, glutathione transferase and glutathione reductase. Exogenous antioxidants are a secondary defense through the diet and include molecules such as vitamin E (alphatocopherol), vitamin C

(ascorbic acid), β-carotene, minerals, and trace elements like zinc. These exogenous

antioxidants can produce differing impacts to combat ROS and act in different cell

compartments. For example, vitamin C acts within the cytoplasm while vitamin E acts in

membranes (Catoni et al 2008). The body can immediately respond to increased ROS

production by increasing antioxidant enzymes in tissues and mobilizing dietary

antioxidants and then decrease back to baseline levels within hours after the stressor is

alleviated (Aguilo et al 2005).

Antioxidants can produce many positive effects during normal energy metabolism

and stress events. Interestingly, von Zglinicki (2002) found that antioxidants not only

reversed accelerated DNA shortening induced by increased oxidative damage, but some

antioxidants even prolonged the replicative lifespan and impeded this shortening. The

impact of chronic stress or chronically elevated CORT on antioxidants is not as clear in

the literature. Some studies reported that elevated CORT from chronic stress results in

decreased antioxidant levels (Zlatovic et al 2013; Zlatovic et al 2014) suggesting that

antioxidants are utilized to neutralize oxidative damage. Yoshikoka and colleagues

(1995) reported the opposite effect showing that antioxidants increased from CORT

suggesting that they may be upregulated during chronic stress.

In the past decade, oxidative stress was implicated in the pathogenesis of various

neurodegenerative and neuropsychiatric disorders, including depression (Bilici et al 2001;

Young 2001; Takuma et al 2004; Lombard 2010; Colaianna et al 2013). However studies

report that antioxidants may neutralize these deleterious effects and possess

antidepressant properties. For example, Alpha-lipoic-acid (ALA) is an an endogenous

antioxidant measured in both animal (Silva et al 2016) and human clinical trials (Eren et al 2007) showing preventative effects on oxidative damage within the brain and positive social behaviors respectively. It is possible that this molecule and others can stop some forms of depression and its symptoms from occurring.

In addition to its impacts on proteins and lipids, oxidative damage also affects telomeres. Telomeres are DNA caps found at the ends of chromosomes and are defined as non-coding highly structured regions with the repeating sequence of TTAGGG in vertebrates (Moyzis et al 1988). This repeating G segment folds back onto the duplex telomeric DNA forming a T-loop structure (Griffith et al 1999). Telomeres play important roles in protecting chromosome integrity (Aubert and Lansdorp 2008). During cell division and chromosome replication, DNA polymerase is unable to completely replicate the terminal ends of one strand of the chromosome. This causes telomeric DNA to shorten with each replication and eventually leads to replicative senescence (Muller 1938; McClintock 1939; Blackburn 1991; von Zglinicki, 2002). Replicative senescence is defined as the number of times a cell can replicate before the DNA shortens to the point where it can no longer fully replicate and serve its purpose. It is also termed the Hayflick Limit. This so-called end-replication problem eventually leads to cell senescence also known as cell death (von Zglinicki, 2002; Campisi 2003; Hornsby 2003; Patil et al 2005; Capper et al 2007; Aubert and Lansdrop 2008), which causes physiological aging of the organism.

Interestingly, there are biological mechanisms that can regenerate telomeres growth and decline. Since DNA replication can only proceed from the 5' to 3' direction,

telomeres can not be replicated through DNA polymerase. However, telomerase is an enzyme that is capable of rebuilding and maintaining telomeres (Greider et al 1985). Telomerase is a ribonucleoprotein consisting of reverse transcriptase protein (TERT) and a RNA template component (TERC). Ribonucleoproteins are proteins containing RNA and act directly with nucleic acids. TERT uses reverse transcriptase, which is similar to retrovirus RNA production. TERT is able to create cDNA using a template from TERC as an RNA guide. Using TERC as a template allows for the synthesis of this repeated telomeric structure and eventually, a completed telomere sequence on both strands of the DNA. Interestingly, Jaskelioff and colleagues (2011) reported that mice engineered to block telomerase aged at a much faster rate, dying at six months, instead of reaching the average mouse lifespan, about three years, showing the significance of telomerase. Minamino and colleagues (2001) also reported that administering TERT into human vascular smooth muscle cells extends the lifespan of the cell and preserves a young phenotype, suggesting that telomerase has vast implications on aging. However, telomerase is also implicated in cancer, showing that newly formed tumors are caused by elevated telomerase activity. When examined in comparison to normal somatic cells, immortal cells or tumor cells show no loss of telomere length over time (Counter et al 1992; Counter et al 1994) suggesting that during cell division, telomerase is able to prevent cell senescence if continuously activated (Kim et al 1994). In most cases, telomerase activity isn't sufficient to prevent telomere degradation (Engelhardt et al 1997; Rubio et al. 2005; Lansdorp 2005; Houben et al. 2008; Haussmann and Mauck 2010; Haussmann et al 2012), causing telomeres to shorten with age. Telomerase is

suppressed in most normal adult somatic tissues probably as a mechanism to prevent tumor growth (Taylor et al 2000; Parwaresch et al 2002).

The end-replication problem isn't the only cause of telomere loss. It is estimated that telomeres shorten by 50-300 base pairs per cell division (Harley et al 1990), but only a minimum of 10 base pair of this degradation is thought to be caused by the end replication problem (von Ziglinicki 2002). It is believed that the remaining loss of base pairs is from increased oxidative stress because telomeres are particularly vulnerable to attack (Rubio et al 2005; Houben et al 2008; Haussmann and Marchetto 2010). Oxidative stress also dramatically decreases TERT activity (Borras et al 2004; Kurz et al 2004) and therefore oxidative stress not only hastens telomere shortening by direct damage to telomeres but also by inhibiting telomere restoration. While indirect effects of CORT on telomere dynamics through oxidative damage are well established, the direct effects of CORT on telomere dynamics are still being studied. CORT may shorten telomeres by increasing cell proliferation causing more rapid end-replication shortening (Epel et al 2006; Haussmann et al 2010). In addition, CORT downregulates telomerase activity by decreasing the catalytic component of telomerase (Choi et al., 2008). Furthermore, the amount of unrepaired telomere damage influences the next replication, worsening telomere loss in future cell replication (von Zglinicki, 2002). Shortened telomeres whether from end replication problem or oxidative stress are risk factors for many diseases including cardiovascular disease (Samani et al 2001), diabetes (Valdes et al 2005), and Alzheimer's disease (Honig et al 2006).

Epel and colleagues (2004) reported that life stress leads to shorter telomeres and that this shortening rate correlates to the chronicity of the stressor. Specifically, they showed that individuals suffering from psychological stress such as caring for a chronically ill child tend to have shorter telomeres and accelerated telomere degradation due to elevated oxidative damage. These results demonstrate a significant correlation between perceived social stressors and accelerated aging. In addition to perceived stressors, major depressive disorder (MDD) is associated with shorter telomeres. This type of depression is described as a dysregulated activation of the stress response (McEwen 2003). Several studies correlated elevated morbidity and mortality with major depressive disorder (Evans et al 2005; Gump et al 2005). However, it was Simon and colleagues (2006) that were the first to correlate depression with shortened telomeres. They reported significantly lower telomere lengths in individuals with three different types of depressive disorders compared with nondepressed controls. This area of study was enhanced by other studies finding similar results. Lung and colleagues (2007) reported that major depressive disorder is a mediating factor of gene regulation of several proteins and enzymes potentially causing increased telomere degradation. Further, Hartmann and colleagues (2010) reported that while major depressive disorder is associated with shortened telomeres, there was no influence on applied therapies, duration of the illness or the severity of the depression, suggesting that other factors may be contributing. Hoen and colleagues (2011) reported that individuals with major depressive disorder did have shorter telomeres at the start of a five year study, but that over time, the rate telomere degradation did not significantly alter from control

participants. This study suggested that while depression may originally cause a decrease in telomere length, it did not alter the rate of decline. However, Verhoeven and colleagues (2014) reported that the more severe and the longer the depression, the more accelerated cellular aging in those individuals was. These groups, while differing in results still demonstrate a strong correlation to MDD and telomere degradation. However this area must be further studied to understand the mechanisms connecting them along with determining the possible additive effects between MDD and telomere degradation.

Social support

While physiological repair mechanisms can diminish these negative consequences, social factors can also help mitigate these ramifications. Numerous studies indicate that people with social support in the form of spouses, friends, and family members are healthier and have a more positive attitude than those with fewer supportive social contacts (Broadhead et al 1983; Leavy 1983). Cohen and Willis (1985) suggest that there are two models of social support. The first is a buffering model suggesting that social support is protective primarily during the stressful encounter. For example, having social support during a thesis defense is more beneficial to the presenter than having that support after the completion of the event. The second suggests that social support is beneficial regardless of stress events. An example of this would be having positive social support from advisers and friends throughout the entire thesis defense: before, during and after.

Cohen and Willis (1985) hypothesized that social buffering of stressful situations may occur at two key points. The first is during the stress event. Friends' or family's

perceptions of the event may result in the perception of the stressor not being as severe or significant. The second is after the event is processed and emotions and behaviors are adapted. During this time, social support may assist in preventing negative emotional and behavioral adaptations. Interestingly, individuals with social support display an improvement in recovery from health conditions including cardiovascular disease (Thorsteinsson and James 1999; Grace et al 2002) cancer (Spiegel and Sephton 2001) and chronic back pain (Guzman et al 2002). Social buffering reduced cortisol responses and subjective anxiety reactions following a social stressor in men (Heinrichs et al 2003). House (1982) suggested that having social support post-stressor may assuage the neuroendocrine system, causing downstream alleviation on other systems. However, the mechanisms through which social support improves health and immune function are not well known.

Regardless, social support is suggested to result in a positive influence on health by increasing an individual's self-esteem, control, and overall health (Uchino et al 1999). Longitudinal studies report that social support is correlated with longer life (Berkman and Syme 1979; House et al 1982; Blazer 1982) and positive mental health (Henderson et al 1981; Aneshensel and Frerichs 1982; Billings and Moos 1982; Adams et al 2004). Others report that social support plays a role in scholastic success, demonstrating that support from peers, teachers and family result in decreased failure rates and enhance coping strategies during stressful times such as exams (Hamre and Pianta 2005; Holmes et al 2015; Tennant et al 2015). However, Mossakowski and Zhang (2014) reported the opposite effect, showing that perceived emotional support from family during any level

of discrimination, a chronic stressor for minorities, is not a significant buffer for behavioral and emotional reactions. This may suggest that there are different variations of social support and it may be limited in its positive influences when relating to depression.

Animal models of social support and buffering show the positive effects of friendly social interactions on both physiology and behavior. Specifically, social animals, spanning from rodents through non-human primates, recover faster from negative experiences when with their partners. Davitz and Mason (1955) reported that social support, reduced fearful behavior in isolated rats that were subjected to shock mazes resulting in a case of social facilitation. Social facilitation is defined as an improvement in behaviors or performance to a task when in the presence of a partner or group. Moreover, social housing decreased HPA reactivity to restraint stress and improved wound healing in Siberian hamsters (Detillion et al 2004). Further, socially monogamous prairie voles that were paired with siblings did not have as severe of an increase in CORT levels or elevated CRH binding in brain regions following an acute stressor when compared to those that were chronically isolated (Grippo et al 2007b,d; McNeal et al 2014). Grippo and colleagues (2007c) further reported that social support in prairie voles reduced the amount of CRH binding in brain regions following an acute stressor and protected against depression-like behaviors. Moreover, Grippo and colleagues (2007a) also reported that social support protects against increased resting heart rate and exaggerated cardiac responses during acute stress testing. Zlatkovic and colleagues (2014) reported decreased depression- and anxiety-like behaviors following mild acute stressors in paired male Wistar rats when compared with chronically isolated

individuals. Balasubramaniam and colleagues (2016) recently reported that rhesus

macaques that participate in more direct connections in grooming and huddling in social

networks were less susceptible to infectious agents compared with those in isolation. This

extends the social buffering hypothesis to immune-function benefits, suggesting that

positive social interactions may assist in direct resistance to infectious diseases.

Interestingly, when kept with their partner, titi monkeys and rhesus monkeys have a

dampened stress response when faced with unfamiliar conspecifics (Hennessy et al

2009). These studies contribute to the social support hypothesis showing that positive

social interactions do in fact alleviate stress encounters or completely prevent chronic

stress effects.

<u>Oxytocin</u>

A likely mechanism through which social support influences the responsiveness to

stress is the release of oxytocin during positive social interactions. Oxytocin is a

neuropeptide that is released during labor and lactation (McNeilly and Ducker 1972). It is

also released in response to physical contact (Uvnas-Moberg 1997; Gimpl and

Fahrenholz 2001) and has a positive correlation with positive social behaviors including

acute or long term relaxation (Uvnäs-Moberg 1996), enhanced social bonds (Williams et

al 1992; Grewen et al 2005), parenting behaviors (Keverne and Kendrick 1992; Keverne

et al. 1997; Uvnas-Moberg 1997; Carter 1998; Perry et al 2014), trust (Zak et al 2005)

and lower levels of anxiety in patients with depression (Scantamburlo et al 2007). In

addition to its behavioral results, oxytocin affects the autonomic nervous system and

neuroendocrine system through its natural release during social encounters (Verbalis et al 1995; Porges 1998; Porges 2001).

Oxytocin is synthesized in the magnocellular and parvocellular neurons in the hypothalamic supraoptic nucleus and paraventricular nucleus. It is transported along the axonal projections to the posterior lobe of the pituitary, where it is stored and released into the periphery (Higuchi et al 1986). Oxytocin receptors are found throughout the body including the reproductive system, kidney, mammary glands, heart, vascular smooth muscle and thymus (Gimpl and Fahrenholz 2001). In addition, there is release of oxytocin through dendrites into the extracellular space moving through the brain by diffusion rather than acting strictly across a synapse or requiring transport by the circulatory system (Moos et al 1989; Neumann et al 1993). This allows it to spread farther and have a powerful impact on the central nervous system (Landgraf and Neumann 2004). Furthermore, smaller neurons in the PVN also produce oxytocin and project it directly to other brain regions (Ludwig and Leng 2006; reviewed in Meyer-Lindenberg et al 2011). Within the brain, oxytocin works in many areas including the lateral septum, subiculum, shell of the accumbens, bed nucleus of the stria terminalis (Caffe et al 1989; Loup et al 1991; Huber et al 2005; Boccia et al 2013; Ferris et al 2015).

Interestingly, a variety of stressors that stimulate the HPA axis induce peripheral (Grippo et al 2007a,b) and central (Wotjak et al 1996; McCarthy and Altemus 1997; Windle et al 1997; Nishioka et al 1998; Wigger and Neumann 1998; Neumann 2002; Grippo et al 2007b; Babygirija et al 2010) oxytocin secretion through the hypothalamo-neurohypophyseal tract (Benarroch et al 2013). It is possible that oxytocin

is a component of a homeostatic process that assists mammals in coping with stressful experiences (Legros 2001; Kramer et al 2003). Oxytocin may reduce fear and assuage the sympathetic responses to stressful stimuli, including those associated with social behavior (Carter et al 2008) and regulate parasympathetic functions (Higa et al 2009). Elevated endogenous oxytocin is suggested to decrease endocrine and autonomic reactivity to stressors (Lightman and Young 1989; Chiodera et al 1991; Porges 1998; Gimpl and Fahrenholz 2001; Porges 2007). Specifically, Pournajafi-Nazarloo and colleagues (2013) found that chronic isolation in prairie voles downregulated oxytocin receptor expression, but increased plasma oxytocin in females. Neumann and colleagues (2000) reported a significant rise in ACTH and CORT levels following an acute stressor after central administration of an oxytocin antagonist. This demonstrates that oxytocin may exert inhibitory effects on the basal and stimulated activity of the HPA axis and decrease the release of CORT. It is possible the release of oxytocin under stressful situations may protect animals against both the physical and behavioral effects of stress, which is particularly important for females who can be especially vulnerable to these consequences.

While endogenous oxytocin may regulate these systems, exogenous oxytocin may boost endogenous oxytocin's effects (Insel 1992; Theodosis 2002; Bowen et al 2011). There are different methods of oxytocin administration, but it is thought that central administration is the most efficient compared to peripheral administration (Ludwig et al 2013; Ferris et al 2015). There is speculation on the effectiveness of peripheral administration due to the theory that only about 0.2% crosses the blood-brain barrier and

may not be at levels sufficient to engage oxytocin receptors (Jones and Robinson, 1982; Ermishc et al 1985; De Wied et al 1993; Churchland and Winkielman 2012). However, peripheral oxytocin can reduce anxiety-like behaviors (Uvas-Moberg et al 1994; McCarthy et al 1997; Klenerova et al 2009) and promotes prosocial behaviors (Ramos et al 2013; Calcagnoli et al 2015). In human males, intranasal administration of oxytocin inhibits the activity of the amygdala in response to social stress (Kirsch et al 2005) and facilitates trust behavior (Domes et al 2007). Further, intracerebroventricular (i.c.v.) administration of oxytocin lead to an increase in positive social contact and increased preference for a familiar social partner in both male and female prairie voles (Cho et al 1999).

However, chronic peripheral administration of this peptide produced many of the same responses that occur in the autonomic nervous system following natural release of oxytocin (Arletti et al 1992; Petersson et al 1996; Liberzon et al 1997). Petersson and colleagues (1996) reported that chronic administration of oxytocin decreased blood pressure by 10-20 mmHg. Holst and colleagues (2002) reported that exogenous oxytocin and postnatal stroking were able to separately decrease blood pressure and diastolic blood pressure respectively in prenatally stressed female rats. Further, intraperitoneal injections of oxytocin were capable of preventing the effects of isolation on cardiac function and also prevented depression-like behaviors (Grippo et al 2007e). Detillion and colleagues (2004) reported that exogenous oxytocin suppressed HPA reactivity to stress and improved wound healing by decreasing CORT concentrations during the inflammatory stage in hamsters. Previous studies showed an inhibitory effect of peripherally

administered oxytocin on basal ACTH and CORT secretion in men (Legros et al 1984;

Legros et al 1987). This suggests that peripheral oxytocin may be acting directly in the

autonomic nervous system and may cause downstream effects on HPA axis function.

However, the full extent of oxytocin's role in mitigating the effects of CORT on

oxidative stress and telomere shortening are not well elucidated.

Study System:

　　　Prairie voles (*Microtus ochrogaster*) are a socially monogamous rodent species

that are a useful model for studying social stressors such as isolation due to their

similarities with human social structure and behaviors. There are several species of

microtine, including pine voles, prairie voles, montane vole and meadow vole. These four

species are closely related genetically and have similar physical appearances, however

only the prairie vole and pine vole possess a socially monogamous mating systems

(Dewsbury 1988; Carter et al 1995). This mating system may be adapted to enhance

fitness values in their natural environment that consists of low densities with suitable

territories and food resources (Hall and Kelson 1959; Getz et al 1981; Getz and Carter

1996; Carter et al 1995). However, montane voles and meadow voles are adapted to

environments with plentiful resources that permit a polygamous system. Specifically,

these voles exhibit uniparental maternal care and dispersal of young after they are

weaned so they can acquire their own territories.

　　　This monogamous mating system in prairie voles is developed through pair bonds

with their partner. Pair bonding is defined as a strong affinity that develops between a

pair consisting of a male and female or as same-sex sibling pairing (Kleiman 1977; Carter

1995; Carter 1997; Young and Wang 2004). In prairie voles, it is formed through 24-48 hours of cohabitation with multiple copulation events during that time period (Williams et al 1992; Carter et al 1995). It is also formed by sibling pairs and possesses similar physiological and neuronal consequences as male-female bonds (Aragona and Wang 2004). After pair bonding, males develop selective aggression or mate guarding, developing aggressive responses toward unfamiliar conspecifics that enter the pair's territory (Carter et al 1995). After the pair bond is formed, both males and females develop a selective attraction to each other and aversion toward other unfamiliar conspecifics. This was shown by Williams and colleagues (1994) and Cho and colleagues (1999) demonstrating that male and female prairie voles demonstrated partner preference for a familiar conspecific versus an unfamiliar conspecific. However this preference can be broken by an oxytocin receptor antagonist or enhanced by oxytocin administration, showing that oxytocin plays a significant role in pair bond formation (Winslow et al., 1993; Williams et al., 1994; Insel and Hulihan, 1995; Cho et al., 1999; Liu et al., 2001).

In addition, prairie voles have higher densities of oxytocin receptors (OTR) in the medial prefrontal cortex compared to the other promiscuous vole species (Insel and Shapiro, 1992; Insel et al., 1994; Lim et al 2004) further proving this peptides role in pair bonding. It also suggests that this species is able to pair bond because of this high concentration, compared to other species that do not have high oxytocin receptor levels and are non-monogamous. Moreover, these studies demonstrate the forebrain receptors are involved in pair bond formation. Lim and colleagues (2004) demonstrated that both monogamous prairie voles and pine voles had high levels of oxytocin receptors in the

nucleus accumbens, a region associated with maternal behavior and reward system, whereas polygamous montane voles and meadow voles had small amounts of oxytocin binding receptors in this region. This further suggests that these oxytocin receptors may enhance pair bonding through the reward circuitry.

These elevated oxytocin levels and receptors may play a role in parental care as well. Both parents are involved in caring for offspring, which is not common in many rodent species. After offspring are born, they remain in the breeding pair's territory for several weeks after weaning. These offspring may engage in alloparental care to subsequent litters of their parents.

Moreover, prairie voles exhibit atypical CORT levels that are estimated to be ten times higher compared with many other rodent species (Taymans et al 1997). This may be caused by the heightened ACTH concentrations found in prairie voles (Taymans et al 1997). While this species has elevated CORT receptors, there is still a significant amount of circulating CORT (Taymans et al 1997), suggesting that CORT is not binding to receptors and causing heightened HPA regulation. Unstressed adult prairie voles normally have circulating CORT levels ranging from 800-1300ng/ml compared with laboratory rats that average around 100 ng/ml. This may be due to their greater adrenal to body weight ratio and HPA axis hyperactivity (Taymans et al 1997). Regardless of this species' higher regulation of the HPA axis set point, it still responds to circadian cues and stressors (DeVries 2002).

<u>Purpose/Hypothesis:</u>

From this study, we hope to further understand the physiological and behavioral consequences of social isolation. It is known that chronic isolation causes elevated CORT(prairie voles: reviewed in Carter et al 1995; Ruscio et al 2006, rats: Weiss et al 2004; Serra et al 2000; McCormick et al 1998; and primates: Mendoza and Mason 1986; reviewed in Gilmer and McKinney 2003) and anhedonia (Grippo et al 2007a,b,d; Grippo et al 2008; Grippo et al 2011). However, we do not know how isolation impacts oxidative stress and aging. Moreover, oxytocin can decrease HPA reactivity during chronic stress (Legros et al 1988; Heinrichs et al 2003; Nomura et al 2003; Detillion et al 2004) and reduce depression-like (Grippo et al 2007e) and anxiety-like behaviors (Uvas-Moberg et al 1994; McCarthy et al 1996; Klenerova et al 2009). However, we do not know how oxytocin may mitigate the negative physiological behavioral consequences of chronic isolation. This study will enable us to compare other findings to our own and expand previous knowledge on the detrimental effects of chronic social isolation and the potential mitigating factors of oxytocin.

Here we examined the hypothesis that chronic isolation causes increased aging, elevated CORT and oxidative stress along with depression-like behavior. We further hypothesize that with peripheral administration of oxytocin, these negative consequences will be mitigated.

:

The following measures described here were used to assess both the physiology
and behaviour aspects of this experiment. These methods and measures were researched
to determine exact procedures to ensure accurate and successful outcomes. Further, these
methods were validated and calibrated for our prairie vole model through piloting
conducted before the start of the experiment.

Anhedonia

Sucrose preference tests were used to measure anhedonia, which is the reduced
responsiveness to pleasurable stimuli. Anhedonia is a critical feature of depression in
humans (Willner et al 1987; American Psychiatric Association 2000). Hedonic behaviors
are associated with the reward circuit in the brain, but in depressed patients, this is often
disturbed resulting in the loss of the reward feeling associated with positive
behaviors.This test was previously used to measure depression-like behavior in prairie
voles and other rodents (Willner 1987; Neumann 2000; Grippo et al 2005; Grippo et al
2007a,b,c; Grippo et al 2011; Lieberwirth 2012; McNeal 2014) and is an accurate
measure of a negative behaviour associated with depression (Carter and Shieh
2015). While there are other methods of measuring depression-like behavior that were
discussed previously, we found that sucrose preference testing was highly used and
comparable to other prairie vole studies.

For measuring anhedonic behaviors, we applied methods that were similarly
conducted by Grippo and colleagues (2005; 2007a,b,c; 2011). Briefly, there was a
habituation period of 48 hours before the beginning of the experiment to acclimate prairie

voles to 1% sucrose solution. Two days after this habituation period, animals were exposed to two preference tests to determine baseline sucrose consumption levels once per day on subsequent days. These two measures were then averaged for analysis. This was to ensure that we accurately measured baseline values and wouldn't skew our results with first time preference testing. Preference tests were also conducted at 3 and 6 weeks during the experiment. Similar procedures from baseline measurements were applied at these time points. To ensure non-skewed results, animals that were isolated from their partners were continuously isolated during this part of the study. While consumption values are important values to consider, the preference value (sucrose consumption as a percentage of total fluid consumption) is a more meaningful method of determining anhedonia.

Blood sampling

There are several methods of blood collection in animal models and many require anesthesia. These include collecting blood from the tail vein, orbital sinus, jugular vein, and tail snips (Parasuraman et al 2010). However, it Puri and colleagues (1981) reported anesthesia such as ketamine can alter the release of hormones including CORT. Krystal and colleagues (1994) supported this earlier claim by reporting that ketamine injections caused elevated CORT levels shortly after administration. From this previous research, we determined that collecting blood while animals were awake would allow us to obtain the most accurate physiological measures.

Blood samples were collected via cheek bleeding at the start, 3 weeks, 6 weeks, and during acute stress tests. Blood samples were collected within 3 minutes of taking the

animals' cage from the study room to obtain baseline values. Approximately 100-200 µl of whole blood was collected to ensure enough was able to be used for assay measures.

Acute Stress Test

While chronic stress can result in elevated stress hormones, increased oxidative damage, and increased depression, acute stress responses may be amplified by these effects. Grippo and colleagues (2007b, 2009) found that while chronic isolation caused elevated ACTH and oxytocin levels in prairie voles, it did not impact CORT levels. However, an acute stressor that followed an isolation period resulted in a significant increase in CORT levels when compared to paired voles. This prompted us to conduct acute stress tests to determine if chronic stress can significantly impact the acute stress response.

In our study, the acute stressor occurred one day after the final blood collection day. For this acute stress test, animals participated in a resident intruder test (RIT), which is a stressor in rodents (Bosch et al 2004; Grippo et al 2007b, 2009; Koolhaas et al 2013).This test consists of placing the experimental animal (intruder) into the cage of an unrelated and unfamiliar prairie vole (resident) for 5 minutes. Blood samples were collected 20 minutes and 45 minutes after the start of the RIT. This allowed us to measure the stress induced reaction and the recovery period of the prairie voles.

Assay Measures

Once all samples from individuals were collected, several measures were conducted to determine physiological results. CORT levels (ng/mL) was determined

using Radioimmunoassay (RIA). Assessment of oxidative damage was determined using d-ROMs test. Antioxidant levels were analyzed using total antioxidant capacity (TAC) test. Finally, telomere lengths were analyzed using qPCR.

Corticosterone-Radioimmunoassay.CORT levels are interpreted as an index of stress (McEwen and Wingfield 2003; Korte et al 2005). Higher levels of baseline CORT are routinely assumed to indicate an individual in poor condition as compared to individuals with lower levels of CORT. Moreover, this can potentially correlate to other measures such as oxidative damage and telomere degradation (Epel et al 2006; Haussmann et al 2010; Costantini et al 2011; Haussmann et al 2012).

We quantified plasma levels of total corticosterone (the primary glucocorticoid in voles) in each sample using a radioimmunoassay with methods from Wingfield et al. (1991), Moore et al. (1986), and Breuner et al. (2006). This is a five day procedure that measures antigens through the use of isotope analysis and antibody binding capacity. Days 1 through 3 prepare samples for the actual radioimmunoassay. These days consist of filtering and removing other molecules such as lipids from samples and extracting the CORT steroids from the samples. Once this is completed, 3H is added to the sample along with rabbit CORT antiserum. The antiserum binds to both the 3H CORT and non-tritiated CORT until equilibrium is reached. During this time, competitive binding occurs to the antibody until total CORT is bound. Samples are then filtered with dextran coated charcoal to remove CORT that is unbound to the antibody from the solution. Standard curve ranges from 7.8 to 2000 pg to determine the correlation between radioactivity and

unlabeled CORT, which can then be calculated. Radioactivity in samples was counted in a scintillation counter and calculated into ng/mL of CORT in the sample.

Oxidative Stress

1. Oxidative Damage-Lipids and Proteins

Measuring ROS levels is ineffectual as these molecules have very short half lives resulting in inaccurate measures. Instead, measuring Reactive Oxygen Metabolites (ROMs) allows us to measure the damage that already occurred. These molecules are more stable and give accurate measures to depict the oxidative damage that occurred to proteins and lipids. Oxidative damage to lipids and proteins can be measured using the d-ROMs Test (Diacron). d-ROM stands for derivatives of Reactive Oxygen Metabolites and measures the oxidant ability of a plasma sample toward an oxidizer using an indicator (chromogen). For this test, plasma samples were combined with an oxidizing reagent and then a chromogen reagent. This combination allows us to measure the amount of ROMs within each sample. Specifically, since the combination of the reagents and plasma changes the color and fluorescence of each well, the plate reader measures the absorbance every minute for thirty minutes. This determines the oxidizing capacity of the sample, with more fluorescence over time resulting from more oxidative damage. Determining the total ROMs within a sample is calculated as:

$$\left(\frac{\left(Abs/min \right) sample}{\left(Abs/min \right) calibrator} \right) \times Calibrator$$

2. Antioxidants

While measuring ROMs assists us in understanding the damage being done , it does not give us the full picture of how the body is defending itself. Measuring total antioxidant capacity allows us to quantify antioxidant levels. OXY-Adsorbent Test measures how a sample reacts to oxidative damage. The use of hypochlorous acid and chromogen allows us to measure antioxidants. Hypochlorous acid is an oxidizer that is effective in fighting bacterial infections, however, in the absence of antioxidants, can cause many negative consequences on other biomolecules. Adding this to a sample and then adding chromogen as an indicator after mixing into the solution enables us to determine how many and how effective antioxidants are at neutralizing this reactive oxygen species. Once the solution is mixed, it is placed in the plate reader (Gen 5 2.00) and absorbance is measured every minute 25 times. The calculation for absorbance is:

$$\left(\frac{Abs\ blank - Abs\ sample}{Abs\ blank - Abs\ claibrator} \right) \times Concentration\ of\ calibrator$$

Telomeres

There are two main methods of measuring telomeres. The telomere restriction fragment (TRF) assay and quantitative polymerase chain reaction (qPCR). The former gives a more informative description of all telomere lengths in a sample while qPCR gives one measure. Specifically, the TRF assay measures total telomere lengths in all cells within the sample, allowing us to measure the average and median values (Montpetit et al 2014). The use of qPCR allows us to only measure the average telomere lengths of all cells within the sample (Montpetit et al 2014). However, TRF requires a larger

quantity of blood for mammals since most do not have nucleated red blood cells. This poses a problem for taking multiple longitudinal blood samples from prairie voles since these animals weigh approximately 28-40 grams. qPCR enables an accurate and effective measure of telomeres while requiring a smaller amount of blood. This method is used in a variety of species. Masi and colleagues (2011) used qPCR to measure leukocyte telomeres in humans and reported a negative correlation between telomere length and age along with oxidative stress. Debes and colleagues (2016) used qPCR to measure and determine relative telomere length was negatively correlated to altered stream temperature and size in brown trout. Heidinger and colleagues (2016) recently used qPCR to measure telomere lengths in European shags determining that chicks produced by older parents had greater telomere loss compared to those produced by younger parents.

These studies used similar techniques for using qPCR that we applied to our study. DNA was extracted from packed blood cells and concentrations were measured using Nanodrop techniques. Samples were combined with mastermix solution comprised of SYBR green and primers for prairie vole telomeres or Glyceraldehyde 3-phosphate dehydrogenase (GAPdh). Samples were analyzed and converted to a ratio of the telomere length relative to the standard reference DNA (T/S ratio).

Chapter 2-Paper

Introduction

  Social isolation and loneliness are the cause of increasing health problems for many in today's society. These negative social experiences are considered to be a chronic stressor. Chronic stressors can cause physiological damage leading to many negative downstream consequences, including increased aging (Epel et al 2004; Epel et al 2006; Damjanovic et al 2007; Houben et al 2008; Kiecolt-Glaser et al 2011). This is thought to be caused by elevated stress hormones and enhanced molecular damage from constant stimulation of the stress response (Kurz et al 2004 Costantini et al. 2011; Haussmann et al. 2012; Herborn et al. 2014; Haussmann and Heidinger 2015). Moreover, psychological risk factors such as increased depression (Cacioppo et al 2007; Cacioppo et al 2010) and heightened anxiety (Heinrichs et al 2003) are exacerbated by chronic stressors in the form of social isolation. Recently, symptoms of depression were correlated to increased aging (Simon et al 2006; Hartmann et al 2010; Verhoeven et al 2014), linking negative physiological consequences with psychological consequences. While the area of chronic social isolation and depression are well studied, there is limited knowledge on how chronic social isolation affects aging. Moreover, social support may mitigate and potentially prevent these consequences through the release of oxytocin, but this area is not well elucidated. In this study, we examined the effects of chronic social isolation on stress hormones, oxidative stress, symptoms of depression and aging along with the potential protective effects of oxytocin on these systems.

The eventual consequences from chronic stress may result from an altered stress response due to elevated cortisol or corticosterone (CORT). CORT is a hormone released during normal daily cyclic activities and stress events. During stress events, CORT is released through the hypothalamic pituitary adrenal (HPA) axis and enhances survival by mobilizing energy stores, inhibiting unnecessary physiological functions and regulating energy intake and expenditure (Wingfield et al 1998; Sapolsky et al 2000). After the stressor is removed, negative feedback of CORT to the rest of the HPA axis restores homeostasis and allows the body to effectively cope with the stressor. When CORT binds to these receptors after the stressor, it causes the hypothalamus and pituitary to diminish the release of their respective hormones to baseline levels. For acute stressors, which are sudden and short lived, individuals are able to cope relatively quickly and physiological functions are able to return to normal. However chronic stressors, such as chronic isolation or loneliness, are inescapable and persist for long periods of time. This leads to a weaker negative feedback system of the HPA axis, which remains active for longer periods, allowing more CORT to be continuously secreted (Sapolsky et al 1984; Eldridge et al 1989; Spencer et al 1991).

These chronically elevated CORT levels may lead to downstream effects including depression-like behaviors in a variety of species (prairie voles: reviewed in Carter et al 1995; Ruscio et al 2006; primates: Mendoza and Mason 1986; reviewed in Gilmer and McKinney 2003; humans: Cacioppo et al 2000; Steptoe et al 2004; Pressman et al 2005; Doane and Adam 2010). However, the mechanisms connecting CORT and depression are still not well understood. Yet individuals with Cushing's disease, which causes elevated

CORT secretion, show unusually high rates of depression in patients (Sonino and Fava 2002). Elevated CORT can also cause damage to the hippocampus and other brain regions during receptor binding which may result in neuronal changes in these regions leading to altered behaviors such as depression (Sapolsky et al 1984; Sapolsky and McEwen 1989; Vyas et al 2003). This may be correlated to chronically lonely individuals who are less likely to actively cope and more likely to feel depressed, anxious and threatened by their environment and the people around them (Cacioppo et al 2006). In animal models, symptoms of depression can be measured in several ways. Anhedonia, the reduced responsiveness to pleasurable stimuli, is measured in animal models and represents a symptom of depression (Willner et al 1987; American Psychiatric Association 2000). Chronic isolation causes an increase in anhedonia in sucrose preference testing in socially monogamous prairie voles (*Microtus ochrogaster*) (Grippo et al 2007a,b,d). Still, it is important to note that there are many symptoms of depression and that some are easily studied in animal models while others can only be studied in humans.

Besides its effects on behavior, chronic stress can impact oxidative stress potentially through elevated CORT levels (Epel et al 2004). Oxidative stress occurs from cells generating endogenous oxygen radicals during normal energy metabolism, leading to an increase in oxidative damage and a decrease in antioxidant capacity. Oxidative damage results from elevated Reactive Oxygen Species (ROS) and targets proteins, lipids and nucleic acids changing the molecular structure and making them ineffectual. Oxidative stress is implicated in a variety of diseases including cancer, cardiac disease,

diabetes, vascular disease and neurodegenerative diseases (Finkel et al 2000; Hulbert et al 2007; Monaghan et al 2009). There is currently limited knowledge on the effects of chronic isolation on oxidative damage. However, several studies demonstrated a positive correlation between chronic stress and increased oxidative damage, which we may be able to apply to chronic isolation situations. Exposure to chronic restraint stress, a model used to measure emotional stress, can result in elevated ROS production to many regions both centrally and peripherally (Sosnovsky and Kozlov 1992; Liu et al 1994; Oishi et al 1999; Manoli et al 2000; Fontella et al 2005). Chronic psychological stress, such as caring for a chronically ill child, can also cause increased oxidative damage to DNA (Epel et al 2004). Finally, major depressive disorder may cause elevated elevated ROS production possibly leading to increased neuronal degradation (Bilici et al 2001; Michel et al 2007). These studies and others note that different models of chronic stress can lead to different levels of ROS production.

Much of this molecular damage can be repaired by antioxidants and other repair mechanisms that are specific to each macromolecule (Monaghan et al 2009). Antioxidants neutralize the spread of free radicals without passing on their reactivity. Endogenous antioxidants are the primary defense against ROS attack while exogenous antioxidants are the secondary defense. von Zglinicki (2002) found that antioxidants not only reversed accelerated DNA shortening induced by increased oxidative damage, but some antioxidants even prolonged the replicative lifespan and impeded this shortening. Antioxidants can also neutralize the deleterious behavioral effects of chronic stress by preventing oxidative damage within the brain resulting in a decrease in depressive

symptoms (Eren et al 2007; Silva et al 2016). However, chronic stress may result in restricted antioxidant defense through elevated CORT levels (McIntosh et al 1998; Orzechowski et al 2002; Costantini et al 2011) and overwhelming amounts of oxidative damage (Djordjevc et al 2010). While the effects of chronic stress on antioxidants is under study, we do not know the impact of chronic social isolation on antioxidant defense.

Besides affecting proteins and lipids, ROS can also attack nucleic acids, specifically telomeres. Telomeres are structures at the end of chromosomes that protect against degradation (Hayflick 1965; Harley et al 1990; Blackburn 1991) and are implicated in aging (Aubert and Lansdorp 2008). While telomeres degrade from end-replication problem, oxidative damage is reported to cause a much more significant deleterious effect (von Zglinicki 2002). Epel and colleagues (2004) found that women with higher stress levels, had shorter telomeres and higher oxidative stress compared with women with lower levels of stress. Subsequent studies in both humans and animal models reported that exposure to a variety of stressors including depression (Wolkowitz et al 2010; Wikgren et al 2012), mood disorders (Simon et al 2006) and infectious disease (Vallejo et al 2004; Fitzpatrick et al 2011) are correlated with increased telomere degradation. However, some human studies found no effects of chronic stress or aging on telomere length or degradation (Martin-Ruiz et al 2005). While chronic isolation is a chronic stressor, there is limited knowledge on how social isolation may impact telomere degradation. Aydinonat and colleagues (2014) were the first to report that social isolation in monogamous African Grey Parrots resulted in significantly shorter telomeres over

time. However, this area needs to be further studied in hopes of applying it to human social isolation cases.

Numerous studies indicate that people with social support in the form of spouses, friends, and family members are healthier and happier than those with fewer supportive social contacts (Broadhead et al 1983; Leavy 1983; Mitchell et al 1982; Uchino et al 1999). Human and animal studies report that social support significantly alters wound healing, cardiovascular output, and CORT responses to lab stressors (Thorsteinsson and James 1999; Detillion et al 2003). However, the neuroendocrine mechanisms underlying social buffering are not well understood. It is thought that oxytocin, a neuropeptide released during positive social experiences, nursing and birth, is involved in the mechanisms of regulating the HPA axis, wound healing, pain response and depression-like behaviors during chronic stressors (Karelina et al 2011; Smith and Wang 2014). This is further supported by Neumann and colleagues (2000) reporting that oxytocin antagonist caused higher CORT levels following a chronic stressor compared to control animals. Previous studies reported an increase in oxytocin release during chronic stress (Grippo et al 2007b,d; Grippo et al 2009) possibly because it is part of the regulation of the HPA axis. Interestingly exogenous oxytocin decreases neuroendocrine responses during chronic stress events, showing that oxytocin may potentially regulate the HPA axis (Detillion et al 2003; Heinrichs et al 2003; Nomura et al 2003). However, there is limited knowledge of the effects of peripheral oxytocin's effects on the neuroendocrine system and its potential protective impacts against oxidative damage and increased aging.

Oxytocin is also involved in pair bonding in prairie voles (*Microtus ochrogaster*). Prairie voles are are a socially monogamous species that forms long-term pair bonds between partners, whether of the opposite sex or a sibling pair (Aragona and Wang 2004). These social bonds may protect against the negative effects of chronic stress by attenuating the HPA axis (Carter, 1998; DeVries et al 2007). However, this dependence on social bonds allows us to study the effects of social isolation and the potential mitigating effects of oxytocin. Previous studies reported that social isolation in prairie voles causes elevated CORT levels (reviewed in Carter et al 1995; Klein et al 1997; Ruscio et al 2006), anhedonia (Grippo et al 2007a,b,d; Grippo et al 2008; Grippo et al 2011), and autonomic dysfunction (Grippo et al 2007c, Grippo et al 2009; Grippo et al 2011; McNeal et al 2014). However the impacts of chronic isolation on oxidative damage and aging are not elucidated. Moreover, the potential beneficial and mitigating effects of oxytocin during this chronic isolation were not previously studied.

The objectives of this study were to determine how social isolation affects CORT, anhedonia and oxidative stress and if oxytocin administration can mitigate any of these negative consequences. Here we examined the hypothesis that chronic isolation causes elevated CORT and oxidative stress along with depression-like behaviors. We further hypothesize that peripheral administration of oxytocin mitigates all of the negative effects of social isolation.

Methods

Study System:

Reproductively naive female prairie voles (*Microtus ochrogaster*, n=80) were 7 to 8 weeks old and weighing 32-35g at the onset of the experiment. Voles were the F3 generation descended from wild stock originally caught in Champagne-Urbana, IL, USA. *Ad libitum* access to food (high fiber rabbit formula, PMI) and water was provided except during preference test experiments (see below). A 14-10 light-dark cycle was maintained through the experiment and prairie voles were housed in plastic containers (Ancare, 7.5" X 11.5" X 5"). All procedures were conducted in accordance with the Bucknell University Institutional Animal Care and Use Committee.

General Procedures

Animals were randomly assigned to one of six treatment groups: isolated (I) , paired (P) with no injections, isolated or paired with daily vehicle injections (Iv or Pv) (0.9% saline, i.p., 10 mL/kg), and isolated or paired with daily oxytocin injections (Io or Po) (0.05mg/kg, dose based on Grippo et al 2009; i.p. 10 mL/kg), n = 10 for each group. Isolated voles were separated from sibling pairs at 7 or 8 weeks of age at the start of the isolation experiment, while paired animals remained in those sibling pairs.

Five days before the start of the study, voles were given *ad libitum* access to 1% sucrose solution and water for a 48 hour habituation period. One day after this habituation period, two baseline sucrose preference tests were conducted on consecutive days using 1% sucrose solution and tap water. In previous studies, sucrose intake measures represented the reduced responsiveness to pleasurable stimuli (anhedonia)

which is often observed in human depression (Grippo et al 2006; Grippo et al 2007). In order to assess longitudinal physiological measures, blood was collected at the start of the experiment on day 0 and during the study period on days 21 and 42. Subsequent sucrose preference testing occurred during the experimental period on day 20 and 41. Finally, on day 43, an acute stress test was conducted using a resident-intruder paradigm.

<u>Blood Collection</u>:

Blood collection began at 1300hr. Samples (~100-250uL) were collected within 3 minutes of taking the cage from the study room to capture baseline CORT concentrations before stress induction (Vahl et al. 2005). Blood samples were spun at 3500 rpms for 5 minutes and plasma was collected. Packed red blood cells were mixed with a 500ul solution of 90% New Born Calf Serum (NBS) and 10% Dimethyl sulfoxide (DMSO) and both blood and plasma were stored at -80˚C until analysis.

<u>Sucrose Preference Testing</u>:

Sucrose preference measures began at 1300hr. Four hours prior to preference testing, food and water were removed from cages to ensure that excess food and water were not consumed prior to the start of preference testing (protocol adapted from Grippo et al 2007). At the start of the preference test, voles were moved into individual cages and allowed to habituate to the new environment for 30 minutes. During the preference test, voles had access to both a 1% sucrose solution and tap water for one hour. At the end of the preference test, sucrose and water consumption were measured and animals were returned to their home cages. This procedure was repeated on days 20 and 41 to measure changes in hedonic behavior during chronic stress. Sucrose preference was calculated as

the percentage of sucrose solution consumed relative the total amount of liquid consumed (Sucrose preference=[consumed sucrose solution/total liquid consumed (sucrose solution+water)] x 100).

<u>Acute Stress Test</u>:

All prairie voles were subjected to an acute stress test in the form of a resident intruder test (RIT) for five minutes (Grippo et al 2007; Koolhaas et al 2013). All RIT procedures started at 1300hr. During the test, the study animal (intruder) was placed into the cage of an unrelated and unfamiliar female prairie vole that was not on study (resident) for 5 minutes. This test was previously demonstrated as a stressor in female rodents (Bosch et al 2004; Grippo et al 2007a; Grippo et al 2007b; Grippo et al 2010).

Fifteen minutes after the completion of the stressor, a blood sample was collected (stress-induced) within 3 minutes to determine how the acute stress affected plasma CORT and oxidative stress levels. Twenty-five minutes after the stress-induced sample, animals were deeply anesthetized for 5-10 seconds in an isoflurane chamber. The animals were then euthanized by decapitation and trunk blood was collected to determine changes in CORT levels and oxidative stress levels during the recovery period.

<u>Assays</u>:

Corticosterone

Plasma CORT levels were determined following a combination of the methods of Wingfield et al. (1991), Moore et al. (1986), and Breuner et al. (2006). The assay was validated and optimized in our lab on prairie vole plasma. Briefly, we diluted plasma samples to 1:250, followed by equilibrating overnight with 3H CORT measuring at 2,000

counts/min (cpm) (3H, 250uCi, Perkin Elmer, #NET399250UC) for determination of individual recoveries. Steroids were extracted via snap freezing using ether. Following this, the sample-ether solution was dried under nitrogen and resuspended in 90% ethyl alcohol. Once equilibrium was reached, samples were dried under nitrogen again and resuspended in phosphate-buffered saline with 1% gelatin. Samples were assayed in duplicate. Rabbit CORT antiserum (MP Biomedicals, #07-120016) was added, binding to both 3H CORT and CORT from the sample. Once equilibrium was reached, samples were filtered with dextran coated charcoal to remove labeled CORT that was not bound to the antiserum. Radioactivity in samples was counted in a scintillation counter and calculated into ng/mL of CORT in the sample. Standard curve range limit was 7.8 to 2000 pg. Interassay coefficients of variation was 5.38%.

Oxidative Stress

We assessed total antioxidant capacity (TAC) and reactive oxygen metabolites (ROMs) in plasma using the Oxy-adsorbent and d-ROMs test kits (Diacron International, Grosseto, Italy) respectively. Briefly, the Oxy-adsorbent test quantifies the antioxidant barrier's ability to cope with oxidation using hypochlorous acid (HClO). The assay was optimized for vole plasma in our lab with a dilution of 1:50. Samples were run in duplicates where plasma was combined with HClO for a ten minute period followed by chromogen to allow the sample to fluoresce. Absorbance was measured at 490nm (BioTek ELx800) and TAC was calculated in millimolars of HClO neutralized using the equation:

$$\left(\frac{\left(Abs/min \right) sample}{\left(Abs/min \right) calibrator} \right) \times Calibrator$$

The d-ROM test measures lipid and protein oxidative damage via hydroperoxides. The assay was optimized for prairie vole plasma in our lab with a final volume of 8ul of plasma. Samples were run in duplicates and a combination of an oxidizing agent and a fluorescent agent to measure reactive oxygen metabolites within each sample. Absorbance was measured at 490nm (BioTek ELx800) and ROM concentrations were calculated in millimoles of H_2O_2 with the equation:

$$\left(\frac{Abs\ blank - Abs\ sample}{Abs\ blank - Abs\ claibrator} \right) \times Concentration\ of\ calibrator$$

Telomeres

DNA was extracted using the Gentra Purgene Blood kit (Qiagen), following the manufacturer's protocol with some minor changes. Briefly, 100 ul of whole blood was added to red blood cell lysis solution and the remaining white blood cells were lysed at 56˚C. Proteins were precipitated out and the DNA was precipitated using isopropanol. The DNA pellet was subsequently washed with 70% ethanol and resuspended in DNA hydration solution. DNA concentrations and purity were measured spectophometerically using the Nanodrop 1000 (Thermo Scientific).

Telomeres were measured using the quantitative PCR (qPCR) method described by Cawthon (2002) on a Lightcycler 96 Exigon (Roche). Real time PCR calculates the cycle threshold within each sample. This is defined as the total number of cycles a sample

requires to accumulate fluorescence to cross the threshold (i.e. exceed background levels). The threshold cycle is inversely proportional to the initial concentration of the target. The Cawthon method gives a relative measurement of telomere length (RTL) and is calculated as a ratio (T/S) of telomere repeat copy number (T) to a control single copy gene (S). The Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was chosen as the single copy gene. The prairie vole (*microtus ochrogaster*) GAPDH sequence (NCBI accession number: NW_004949099.1, GENE ID: 101996351) was used to design primers. The GAPDH primers selected were vole GAP6 F 5'-GCGTCCAATACGGCCAAATC-3' and voleGAP6 R 5'-GAGCATTTGTTGACCCAGCC-3'. For amplification of telomeric sequences the universal primers designed by Cawthon (2002) were used:

Tel1b 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3' and Tel2b 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'. The telomere (T) and GAPDH (S) assays were carried out on separate 96 well plates. Each sample was measured in triplicate, with the average used to calculate RTL. In addition to the samples, each plate also contained a standard curve (a five-fold dilution series of a pool of DNA; ranging from 10 - 0.625ng/well), a reference sample and a no template control (NTC). Each reaction contained 10ul of SYBR (FastStart Essential DNA green master), forward and reverse primers, DNA (sample, standards or reference) or water (NTC) in a total volume of 20ml. Both T and S assays were performed using 2.5ng of DNA (equivalent to 5ml). Primer concentrations were 500/500nM for Tel1b/Tel2b and 200/200nm voleGAP6 F/voleGAP6 R. The thermal cycling profile for the T assay was a

preincubation of 95^0C for 5 minutes followed by 95^0C for 15sec, 58^0C for 30sec, 72^0C for 30sec for 30 cycles, and for the S assay was a preincubation of 95^0C for 5 minutes followed by 95^0C for 30sec, 55^0C for 30sec, 72^0C for 30sec for 40 cycles. Following amplification, a dissociation curve was performed in order to confirm the specificity of the reactions. The mean assay efficiencies for the T and S assays were 1.94 and 1.91.

To calculate the relative T/S ratios we used a modification of the delta-delta Ct method, to include efficiency for each assay as described by Pfaffl (2001):

$$\frac{1 + ET\Delta Cq^{T(control-sample)}}{1 + ES\Delta Cq^{S(control-sample)}} = RTL$$

Statistical Analysis

Statistical analyses were run using JMP software (version 10.0.0, SAS Institute Inc. 2012, Cary, NC, USA). For all analyses, we performed Generalized Linear Mixed Models of Restricted Maximum Likelihood (REML-GLMM). For every model, we checked for homogeneity of variances (Levene's test), and examined residuals to confirm that they approximated a normal distribution (Kolmogorov-Smirnov test). In each model, individual was introduced as a random factor to control for variance among individuals. Sucrose preference, CORT, ROMs, TAC, and telomere length were each modeled over the isolation period (three time points) or acute stress period (three time points). These models included the fixed effects of time, treatment, and the time*treatment interaction. Non-significant interactions were sequentially removed from the models and the analyses

were repeated until we obtained a model with only significant terms. Post-hoc

comparisons were carried out using Tukey HSD tests.

Results

Social Isolation Experiment

There was a significant treatment by time effect for plasma CORT levels (F=14.5;

p<0.0001; Figure 1). Specifically, I voles showed a significant increase in baseline

CORT across the 42 day social isolation period (Tukey HSD, $p < 0.05$), whereas the P,

Io, and Po voles all had stable baseline CORT levels over the experiment (Tukey HSD, p

> 0.05). In addition, while CORT levels were similar between all treatments at 0 days

(Tukey HSD, $p > 0.05$), I voles had higher plasma CORT levels compared to P voles at

21 days (Tukey HSD, $p < 0.05$), and had higher plasma CORT levels compared to all the

other groups at 42 days (Tukey HSD, $p < 0.05$).

There was a significant treatment by time effect for sucrose preference (F=29.2;

p<0.0001; Figure 2). The I voles' preference for sucrose significantly decreased across

the 42 d social isolation period (Tukey HSD, $p < 0.05$), whereas the P, Io, and Po voles

all had a high and stable preference for sucrose over the experiment (Tukey HSD, p >

0.05). In addition, while sucrose preference was similar between all treatments at 0 days

(Tukey HSD, $p > 0.05$), I voles had a lower preference for sucrose compared to all other

treatments at both the 21 d and 42 d sucrose preference test (Tukey HSD, $p < 0.05$).

There was a significant treatment by time effect for ROMs levels (F=16.9;

p<0.0001; Figure 3), though neither treatment nor time affected levels of TAC.

Specifically for ROMs, the I voles showed a significant increase in ROMs across the 42 d

social isolation period (Tukey HSD, p < 0.05), whereas the P, Io, and Po voles all had stable ROMs levels over the experiment (Tukey HSD, p > 0.05). In addition, while ROMs levels were similar between all treatments at 0 and 21 d (Tukey HSD, p > 0.05), I voles had higher ROMs levels compared to all the other groups at 42 days (Tukey HSD, p < 0.05).

There was a significant treatment by time effect for telomere length (F=5.1; p<0.003; Figure 5). Specifically, the I voles showed a significant decrease in telomere length during the 21d period of isolation in which telomere length was assessed (Tukey HSD, p < 0.05), whereas the P, Io, and Po voles telomere length did not change over this period (Tukey HSD, p > 0.05).

Acute Stress

There was a significant treatment by time effect for plasma CORT levels (F=7.9; p<0.0001; Figure 6). Prairie voles in all treatments responded to the acute stress by increasing CORT levels between the baseline and 20 min samples (Tukey HSD, p > 0.05). However, the I voles continued to increase to the 45 minute time point, whereas CORT levels in all other treatments returned to baseline levels (Tukey HSD, p > 0.05). In addition, I voles had higher levels of CORT at baseline compared to all other groups, which has similar baseline levels (Tukey HSD, p > 0.05).

There was a significant treatment by time effect for ROMs levels (F=3.8; p<0.002; Figure 7). I, Io, and P voles had significant increases in ROMs at 20 min, but, levels returned to baseline at the 45 minute time point. Po voles showed no change in ROMs levels over the 45 min stress test (Tukey HSD, p > 0.05). Interestingly, I voles had higher

levels of ROMs at every timepoint of the stress test in comparison to the other treatment group (Tukey HSD, p > 0.05).

While TAC levels did not vary by treatment group, they did change over the acute stress test (F=8.2; p<0.0005; Figure 8). TAC levels were similar between the baseline and the 20 min sample, but significantly decreased at the 45 min sample (Tukey HSD, p > 0.05).

Discussion

This is the first study to demonstrate that six weeks of social isolation caused increased aging, elevated CORT levels and increased oxidative damage in prairie voles while exogenous oxytocin prevented these negative consequences. Further, chronic isolation significantly altered prairie voles' acute stress response, while oxytocin prevented this abnormal stress response seen in isolated animals. Taken together, these results enhance our understanding of the consequences of social stress in both chronic and acute situations and the importance of social support. Moreover, this study shows the positive effects of oxytocin as a treatment to prevent these consequences when faced with chronic stress.

Chronic social isolation caused a significant decrease in telomere lengths during the study period. Specifically, telomere lengths decreased from the three week to the six week time periods for the isolated treatment group. However, prairie voles in isolation with daily oxytocin injections had no change in telomere lengths and were similar to paired treatment groups. This shows that social isolation caused significant biological aging compared to all other treatment groups. While telomere degradation is a natural

process that occurs over time and is associated with biological aging (Bakaysa et al 2007; Blasco 2007; Entringer et al 2011; Fyhrquist et al 2013), the rate of degradation over time due to chronic stress is correlated with mortality (Cawthron et al 2003; Joeng et al 2004; Monaghan 2010; Barrett et al 2013). Chronic stress may affect telomere lengths through increased oxidative damage, elevated CORT and decreased antioxidant efficiency (Epel 2009; Haussmann and Marchetto 2010; Correia-Melo 2014). Our results are novel for animal models but are similar to human studies. Aydinonat and colleagues (2014) reported that socially isolated African Grey Parrots had shorter telomeres compared to pair housed conspecifics. However the rate of telomere degradation was not significantly different between these two groups while we demonstrated a significant increase in the rate of degradation in the isolated treatment group. In comparison, loneliness during early childhood and adulthood are associated with shorter telomeres in later life (Drury et al 2011; Carroll et al 2013), suggesting that the rate of degradation is correlated to this type of isolation. Our results are the first to show that chronic social isolation does cause increased telomere degradation rates, causing elevated biological aging, which can lead to enhanced morbidity and possible early mortality.

Daily oxytocin administration during isolation was able to prevent significant telomere degradation during the study period. This is the first study to demonstrate that exogenous oxytocin is able to prevent this negative physiological impact of chronic stress. Elevated plasma oxytocin is associated with longer telomeres (Boeck et al 2016) and oxytocin receptor gene expression is correlated to lower telomere degradation rates during stress events (Smearman et al 2016). However, there is still limited knowledge on

how endogenous and exogenous oxytocin may reduce the effects of aging during chronic stress events. Our results suggest that through mitigating factors, exogenous oxytocin is able to prevent increased biological aging during chronic social isolation and may have implications for other chronic stressors.

Paired prairie voles, both with and without daily oxytocin injections, had no change in telomere degradation. This suggests that this six week time period was not long enough to degrade telomere lengths due to the natural aging process and without the influence of chronic stress. Human studies corroborate these findings showing that individuals with spousal support in daily life have a longer telomeres and decreased rates of changes compared to individuals lacking spousal support (Barger and Cribbet 2016). It is probable that telomeres were unaffected by these treatments because pair housing did not cause elevated CORT or increased oxidative damage. Epel and colleagues (2004) reported that oxidative stress and telomere degradation was enhanced in mothers caring long-term for a chronically ill child compared to those that had a healthy child. Moreover, increased oxidative stress is shown to increase the rate of telomere degradation (Xu et al 2000; Epel et al 2004; Houben et al 2008), however there is still a standard amount of telomere degradation leading to aging that occurs over time (Chang and Harley 1995; Haussmann et al 2003). While increased telomere damage from stressors is well studied, the area of standard telomere degradation needs to be further studied.

As previously mentioned, a potential mitigating factor of telomere degradation is elevated CORT. In our study, baseline CORT levels significantly increased when compared to other treatment groups during the six week study period. Isolation's effect

on baseline CORT levels suggests that six weeks of separation from a partner provides a significant chronic stressor to prairie voles. Other studies reported similar results demonstrating that chronic isolation causes elevated baseline CORT levels in prairie voles (reviewed in Carter et al 1995; Klein et al 1997; Ruscio et al 2006), rats (Weiss et al 2004; Serra et al 2000; McCormick et al 1998) and primates (Mendoza and Mason 1986; reviewed in Gilmer and McKinney 2003). Moreover, chronic loneliness in humans increases baseline CORT levels (Cacioppo et al 2000; Steptoe et al 2004; Pressman et al 2005; Doane and Adam 2010). However some studies do not report that social isolation result in increases in baseline CORT levels. For example, Grippo and colleagues (2007b) found that four weeks of chronic isolation had no effect on baseline CORT levels in prairie voles. This may be due to habituation of to the stressor, as suggested due to work in male Wistar rats who were able to effectively cope with their stressful environment (Djordjevic et al 2010; Zlatkovic et al 2014). While the mechanism underlying the rise in baseline CORT levels in our study is unknown, other studies demonstrated that chronically-elevated CORT levels reduces CORT receptor numbers leading to decreased negative feedback and increased CORT secretion (Sapolsky et al 1984; Eldridge et al 1989; Spencer et al 1991; Herman and Spencer 1998). This may suggest that chronic isolation causes elevated baseline CORT through a reduction in receptors and a decrease in negative feedback.

Interestingly, daily administration of oxytocin throughout the isolation period prevented baseline CORT elevations. Previous studies reported that centrally administered oxytocin down-regulates CRF expression in the PVN (Nomura et al 2003)

while intranasal oxytocin decreases circulating CORT in chronically stressed humans (Heinrichs et al 2003), showing that oxytocin may modify the HPA axis response during stress events. While it is unknown how peripheral oxytocin affects CORT levels, it is possible that a sufficient amount crosses the blood-brain-barrier to cause central effects or it produces effects in the periphery (Insel and Young 2000). Additionally, it is possible that exogenous oxytocin supplements endogenous oxytocin that is released during stressors (Insel 1992; Theodosis 2002; Bowen et al 2011) causing the eventual downregulation of CORT levels (Legros et al 1984; Coiro et al 1985; Legros et al 1987).

However, the combined treatment of pairing and oxytocin injections produced no measurable advantages when compared to paired treatment groups. This suggests that in this experiment, the potential benefits of exogenous oxytocin are only effective during stress events. Others reported that the combination of social support and oxytocin administration did not result in different baseline CORT levels when compared with those that only had social support in the prairie vole model (Grippo et al 2009; Grippo et al 2012) and humans (Heinrichs et al 2003; Hennessy et al 2009). This suggests a ceiling effect potentially prohibited other effects from oxytocin administration. Because there was no difference in CORT levels between those that were paired with oxytocin and other paired treatment groups, we combined all paired treatment groups into one category when discussing these findings.

Besides CORT levels, isolation also caused an increase in ROM production at three weeks, which further increased at six weeks when compared to all other treatment groups. While the specific connection between CORT and oxidative damage isn't clear,

many studies show elevated CORT results in higher levels of oxidative damage (Sapolsky et al 2000; Agostinho et al 2010; Bjelkovic et al 2010; Haussmann and Marchetto, 2010; reviewed in Costantini et al 2011; Haussmann et al 2011; Fletcher et al 2015). Because CORT is generally involved in glucose mobilization and increased energy metabolism, it is likely elevated CORT levels increase reactive oxygen species production (Sapolsky 1985; Rebuffe-Scrives et al 1991; Sapolsky et al 2000; Lin et al 2004; Le et al 2005; Lin et al 2006; Karatsoreos et al 2011). Since CORT receptors are present within the mitochondria, it is possible that elevated CORT secretion leads to altered mitochondrial gene expression and formation causing elevated ROS production (Scheller et al 2000). You and colleagues (2009) demonstrated that blocking CORT receptors in the hippocampus decreased ROS production. Further, Yi and colleagues (2016) found that dexamethasone, a synthetic glucocorticoid, caused oxidative damage to lipids in cell cultures of thymocytes by increasing ROS production and inhibiting antioxidants. Fletcher and colleagues (2015) reported that isolation and lack of exercise results in elevated ROMs in prairie voles. However other studies report elevated CORT may up-regulate antioxidant defense and decrease free radical production for short periods (Long et al 2005; Yoshioka et al 1994; Atanasova et al 2009). In our study, we did not see a direct relationship between elevated CORT and ROMs in any treatment group, suggesting that CORT may cause an indirect effect on ROM production during chronic isolation.

ROM production was unchanged in prairie voles receiving oxytocin during isolation and was similar to the paired treatment groups. Currently, there is limited

knowledge on how oxytocin may impact oxidative stress. However recent research shows exogenous oxytocin can significantly decrease the gene expression of both NADPH oxidase and other oxidative damage markers (Rash et al 2011). Further, Stanic and colleagues (2016) found that chronic administration of oxytocin protected lymphocytes from high intensity damage from oxidative damage that leads to cellular death. These studies and our results show several possible explanations for this correlation. First, oxytocin could reduce CORT secretion causing downstream effects, such as regulating ROM production. Second, oxytocin could directly impact ROMs, and may be acting as a repair mechanism. Finally, both of these scenarios could occur concurrently.

While oxidative damage was significantly increased by chronic isolation, we did not observe any difference in TAC between treatment groups. Djordjevic and colleagues (2010) found isolated male wistar rats exhibited unaltered levels of antioxidants. Hau and colleagues (2015) observed that chronic immune stress in eurasian blackbirds showed no increase in antioxidant levels. Interestingly, Yoshioka and colleagues (1995) found superoxide dismutase and catalase activities to significantly increase during chronic stress events. However, other studies reported a negative correlation between elevated CORT and antioxidant capacity during chronic stress experiments (von Zglinicki et al 1995; von Zglinicki 2002; Monaghan et al 2009; Haussmann and Marchetto 2010; Zlatovick et al 2014; Fletcher et al 2015). This may suggest for our results that antioxidants are being depleted by oxidative damage and created at a similar rate and therefore are not measurably affected by chronic isolation.

Chronic isolation also affected the behavior of prairie voles. To measure depression-like behavior, we used sucrose preference testing, a measure of anhedonia, which is a symptom of depression in humans. Three weeks of isolation significantly decreased sucrose preference when compared to baseline values, and continued to decrease throughout the six week study period. While the connection between CORT and depression are not well elucidated, our study and many others demonstrated a strong correlation between elevated CORT and depression (Sheline et al 1996; Cacioppo et al 2000; Steptoe et al 2004; Pressman et al 2005; Adam et al 2006; Doane and Adam 2010). Previous studies reported that 4 weeks of isolation decreased sucrose preference, which declined further through 7 weeks of isolation in the prairie vole model (Grippo et al 2007a,b,d; Grippo et al 2008; Grippo et al 2011). While other models for measuring depression-like symptoms demonstrated negative behavioral impacts from chronic isolation (Djordjevic et al 2010; Carnevali et al 2012; Lieberwirth et al 2012; Zlatovic et al 2014), our results and those reported by other labs (Willner et al 1987; Bosch et al 2004; Grippo et al 2007a,b,d; Grippo et al 2008; Grippo et al 2011) using sucrose preference testing indicate that these depression-like behaviors potentially build up over time, similar to human depression.

Oxytocin was able to completely prevent depression-like behaviors in isolated prairie voles. Grippo and colleagues (2009) found similar results after four weeks of isolation and two weeks of oxytocin injections, suggesting that oxytocin not only prevents anhedonic behaviors, but it can potentially reverse them. Moreover, Norman and colleagues (2010) demonstrated an oxytocin receptor antagonist caused an increase in

floating time, or despair behavior, in the forced swim test paradigm in mice. This further supports oxytocin's direct impact on reducing depression-like behaviors. These studies and our results suggests that exogenous oxytocin may be protective against negative behavioral consequences of isolation and may be implicated in treatment for depression in humans.

While elevated CORT levels from chronic stress can cause many negative consequences both physiologically and behaviorally, elevated CORT is also essential for adapting to acute physical stressors by diverting energy to exercise muscles (reviewed in Sapolsky et al 2000), heightening vascular tone (Ullian, 1999), and suppressing unessential processes including digestion, growth, reproduction, and immune responses (Wingfield et al 1997; Boonstra et al 1998; Sapolsky et al 2000). What is even more interesting is the interaction of the responses to these two types of stress events. Our study also found that chronic stress had major physiological impacts on the acute stress response from the RIT and oxytocin was able to prevent an abnormal acute stress response.

Isolated voles not only had elevated CORT levels during the RIT, but they also did not show a standard acute stress response. These voles already had elevated baseline CORT levels, leading to even higher CORT levels compared to other treatment groups at the stress induced time point. Other studies on chronically isolated prairie voles showed similar rises in CORT levels following a five minute RIT (Grippo et al 2007b; Grippo et al 2009; McNeal et al 2013). Human studies also coincided with these findings reporting similar rises in baseline CORT concentrations following an array of acute stressors (Pike

et al 1997; Nephew et al 2003; Jackson et al 2006). What is most interesting is the results of the recovery time point, 45 minutes after the stressor. At this time point, CORT levels were still increasing, indicating that chronically isolated voles were not recovering in the the normal time period as all other treatment groups. Few studies measure the HPA axis response 45 minutes or longer after an acute stressor is lifted following chronic stress. Haussmann and colleagues (2012) found embryonic exposure to elevated CORT levels caused an increase in CORT levels 40 minutes after stress induction compared to individuals who weren't exposed to CORT during embryonic development. Pike and colleagues (1997) found that chronically stressed humans produced elevated peak CORT levels compared to unstressed participants. They further reported that chronically stressed individuals still had elevated CORT levels during the recovery time point. While we did not measure receptor densities, these studies may suggest for our study that CORT receptors diminish within the neuroendocrine system causing upregulation of CORT. However, there are several studies showing a suppression of the acute stress response after chronic stress exposure (Garcia et al 2000; Matthews et al 2001; Rich and Romero 2001), possibly due to habituation, adrenal exhaustion or downregulation of receptors. Since CORT levels continued to rise in isolated prairie voles, habituation and adrenal exhaustion were not apparent suggesting that the acute stress amplified CORT release and caused further damage.

Oxytocin administration during isolation caused a similar stress response to those prairie voles that were paired throughout the study. Specifically, these prairie voles had an increase in CORT levels at the stress induced time period, but levels returned to

baseline by the recovery time period. Moreover, these elevated CORT levels were significantly lower than chronically isolated prairie voles. This suggests that oxytocin administration regulates CORT release during acute stress events that are amplified from chronic stress. This additional finding confirms that prairie voles living in isolation and receiving daily oxytocin injections were not chronically stressed, allowing them to effectively cope with the acute stressor in isolation. Similar results were reported in other studies on isolated prairie voles (Grippo et al 2007b; Smith and Wang 2014) and chronically stressed humans (Heinrichs et al 2003) showing decreases in CORT levels following oxytocin administration during acute stressors. Detillion and colleagues (2003) found that isolated hamsters with oxytocin administration had similar CORT levels to paired animals during an acute stressor. These animals also had increased wound healing, further showing oxytocin's equivalence to social support. While these studies vary in timing and intensity, results still show a consistent trend of oxytocin parallelling the effects of social support, and mitigating the HPA axis function during acute stress events.

Prairie voles that were paired during isolation produced a standard acute stress response. Specifically, baseline CORT levels were similar to levels measured throughout the study, followed by a significant rise in CORT concentrations during the stress induced time period. A key factor is the return of CORT levels to baseline measures during the recovery time point, demonstrating an effective recovery from this acute stressor. This suggests that social support before and after the acute stressor enabled these prairie voles to effectively cope with the acute stressor compared to those in isolation. Social animals, spanning from rodents through non-human primates, recover faster from

negative experiences when with their partners. Social pairing during acute stressors results in decreased HPA reactivity (Detillion et al 2004; Grippo et al 2007b,d; McNeal et al 2014), improved wound healing (Detillion et al 2004), standard hedonic behaviors (Grippo et al 2007b,d; Zlatkovic et al 2014), protection from increased resting heart rate and exaggerated cardiac responses (Grippo et al 2009) and resistance to infectious agents (Balasubramaniam et al 2016). These animal models are correlated to human studies. Social support in the form of friends and loved ones buffers and dampens the acute stress response, enabling individuals to produce lower CORT concentrations compared to those who suffer from loneliness (Cacioppo et al 2000; Hennessy et al 2009; Barger and Cribbet 2016). Our findings and previous results are contributing to the understanding of emotional support along with its potential mitigating factors during acute stressors.

In addition to CORT concentrations, ROMs were also impacted during the acute stress test. Specifically, chronically stressed voles produced elevated ROMs during the stress induced time period, but these levels returned to baseline values by the 45 minute time point. However, these baseline levels were still significantly higher than all other treatment groups. Currently, there is limited knowledge on the impact of both chronic and acute stressors on ROMs. However, increased circulating CORT and increased oxidative stress in humans is correlated after acute exercise stress following chronic stress(Quadrilatero and Hoffman-Goetz 2005), suggesting that there is a potential amplification of ROMs during an acute stressor that follows a chronic stress. While our study showed a similar result between CORT and ROMs there was no primary correlation suggesting that CORT did not directly impact ROM production.

Interestingly, oxytocin administration during isolation caused no change in ROMs during the RIT, similar to the paired groups. While there are currently no studies on oxytocin's impacts on oxidative damage during an acute stress, previous studies reported that acute stressors do impact CORT levels and that oxytocin is able to diminish these rises in CORT levels (Hennessy et al 2009; Cacioppo et al 2015a). If these CORT concentrations cause direct or indirect effects on ROMs, then oxytocin administration might also regulate downstream effects from elevated CORT production such as ROMs. However, oxytocin did not entirely suppress the acute stress response suggesting that there may be other factors impacting oxidative damage.

Not only did the RIT affect ROMs, but it also impacted TAC levels. Antioxidant capacity stayed the same for all groups until the recovery sample when all treatment groups significantly declined in TAC. Zlatovic and Flipovic (2011) found that the combination of chronic and acute stress caused decreases in antioxidant capacity and acute stress alone did not change antioxidant levels. Other studies reported that antioxidants are exhausted during the acute stress response to regulate oxidative damage to lipids, proteins and nucleic acids (Monaghan et al 2009; Silva et al 2016). This suggests that antioxidants were depleted during the acute stress test to combat the elevated ROM production and effectively removed the oxidative damage molecules by the recovery time point.

Conclusion

Chronic stress in the form of chronic isolation resulted in both physiological and behavioral consequences in prairie voles. The most compelling result, isolation causing significant increases in telomere degradation while oxytocin prevents this effect, suggests that this chronic stress results in increased aging but oxytocin is protective against cellular senescence. This enhanced rate of degradation was very likely caused by several factors: elevated CORT, elevated oxidative damage or a combination of the two factors. In this study, we report that isolation caused an increase in both CORT concentrations and oxidative damage. Moreover, we demonstrated that oxytocin is able to prevent these rises during isolation.

Social isolation and perceived loneliness are associated with an increased risk of mortality in older men and women (Steptoe et al 2013), increased levels of depression (Cacioppo et al 2002, 2006), unwillingness to cope with new stressors (Caccioppo et al 2000), decreased immune function (Hawkley and Cacioppo 2010), increased likelihood of autonomic dysfunction (Thurston and Kubzanskey 2009) and impaired learning and memory (reviewed in Cacioppo et al 2009) just to name a few. Thankfully, social support mitigates many of these negative consequences. LaRocco and colleagues (1980) reviewed many types of social support including instrumental assistance, emotional empathy and understanding, and provision of information, however emotional empathy and understanding are the most important type of social support. The presence of a companion can moderate or fully prevent stressor-induced HPA activation along with the sympathetic nervous system through the natural release of oxytocin (Porges, 1998, 2007;

Hennesy et al 2009). This study was able to elucidate how oxytocin can mitigate the negative consequences of chronic social stress both physically and behaviorally without social support. Hopefully this and future studies can further annotate the mechanisms within this system to better understand and apply it to human cases.

Chapter 3-Thesis Conclusion

From this study, we determined that three and six weeks of isolation caused significant detriments on both physiology and behavior in our prairie vole model. However, oxytocin was able to protect animals from these negative consequences. The chronic elevation in CORT levels during isolation potentially aided in the rise of oxidative damage to lipids and proteins and may indirectly impact depression-like behavior. These two physiological responses may be the cause of increased telomere degradation leading to accelerated aging. Moreover, there was a significant impact of chronic isolation on the acute stress response. Chronically isolated prairie voles already had elevated baseline CORT levels and produced significantly higher CORT levels at the stress induced time point compared to other treatment groups. These individuals did not effectively cope with the stressor leading to higher CORT levels at the recovery time point compared as all other treatment groups. However, daily oxytocin injections during chronic isolation resulted in a standard stress response, similar to paired treatment groups. This further suggests that oxytocin is able to not only prevent the negative consequences of chronic isolation, but also regulate normal responses of CORT and oxidative damage levels during acute stressors. These results enhance the understanding for chronic isolation in animal models, but more importantly, demonstrate larger implications for human cases of isolation and loneliness and the potential additive effects of oxytocin as treatment.

Chronic Isolation

      In humans, loneliness is the feeling that emerges when social relationships are perceived as deficient and may arise from a lack of intimacy or a lack of companionship (Steptoe et al 2004). However, in other species, isolation signals danger. Examples include fish evolved to swim in schools while traveling to avoid predation attacks (Ioannou et al 2012), mice altering sleep patterns when housed in isolation (Kaushal et al 2012), and isolated monkeys showing elevated CORT (Levine 1993), elevated ACTH levels (Parker et al 2005) and elevated antagonistic behaviors (Watson et al 1998). These behaviors demonstrate the emphasis of self preservation, which increases the chance for survival. Moreover human and animal research suggest that multiple brain regions involved in social threat, surveillance, aversion, and self-preservation are activated during social isolation (Cacioppo et al. 2009; Bickart et al. 2012; Eisenberger & Cole 2012; Klumpp et al. 2012). Research also indicates that loneliness increases the attention to negative social stimuli (Jones et al 1981), eventually leading to severe depression (Cacioppo et al 2006). While social isolation is linked with depression it also predicts mortality (House et al 1988). The direct mechanisms by which isolation and loneliness cause increased rates of mortality and decreased health are still being studied. However, there is considerable research conducted on the impact of social isolation on neuroendocrine, immune, sympathetic nervous system and cardiovascular responses which directly impact health and mortality.

Isolation and CORT

In both chronic and acute stress testing, CORT concentrations are used as a biomarker of an individual's response to stress. Our study aligns with previous research, reporting that isolation does cause elevated baseline and stress-induced CORT levels. The extent of human research suggests that loneliness or perceived social isolation increase CORT levels (Steptoe et al. 2004; Hawkley et al. 2006; Edwards et al. 2010; Dickerson et al 2011; Hawkley et al 2012), but the reaction may depend on the severity of the isolation and the time of day of the measurement (Pressman et al 2005; Cacioppo et al 2015). While social interaction and isolation were discussed, the social structure and dynamics of the species and the encounters may aid in our understanding of the consequences of both situations (Cacioppo et al 2015). This refers to the brain's interpretation of the social environment. For instance, the response in animals that are chronically isolated in monogamous species, such as nonhuman primates, is dependant on how connected animals are to their partners (Mendoza and Mason 1986). Specifically monkeys have higher CORT levels when isolated from a mate or an offspring as compared to those isolated from a sibling or unmated conspecific (Mendoza and Mason 1986; Smith and French 1997). These connections need to be further studied to understand the link to human loneliness and isolation.

CORT and Neurodegeneration. Loneliness is associated with poor emotional well-being and with depression (Prince et al 1997; Nolen-Hoeksema and Ahrens 2002). Many individuals with depression or major depressive disorder secrete abnormally high levels of CORT (Sheline et al 1996). These elevated CORT levels from chronic social

stress may cause neurodegenerative effects throughout the brain including the

hippocampus and amygdala (McEwen 1992; Sapolsky 1999; McNeal et al 2014) and

activating the prefrontal cortex (Hennessy et al 2009). All of these regions impact

cognitive behavior, decision making and overall executive functioning (Cacioppo et al

2009). The hippocampus is vital to learning and memory and possesses a high

concentration of CORT receptors, particularly MR. Chronic stress, leading to chronically

elevated CORT levels, results in higher binding in the hippocampus, which can

compromise neurons and their ability to survive more stressful events such as seizures

(Pavlides et al 1993; Ekstrand et al 2008). If stressors persist for weeks or months, these

elevated CORT levels can further cause irreversible degradation of the hippocampus and

its neurons through decreased dendritic arborization (Sheline et al 1996; Duman et al

2001; Henn and Vollmayr 2004). This degradation of the hippocampus from elevated

CORT levels impairs learning and memory and causes greater cognitive decline

(Sapolsky 1999; Cacioppo et al 2009; Yau 2016). Further, elevated CORT levels altering

neurogenesis or neurodegeneration within the hippocampus causes significant impacts on

psychiatric disorders specifically depression (Ho and Wang 2010; Campbell and

Macqueen 2004; Kempermann et al 2008; Yang et al 2016). These studies may imply for

our results that CORT is potentially directly or indirectly impacting behavior through

compromising the hippocampus. However, we were unable to examine hippocampal

function or structure during our study.

Isolation and immune

Besides neurodegeneration and behavioral consequences, the immune response is another biomarker of chronic social isolation. Many cases of social isolation both in animal models and human clinical experiments reported that chronic social isolation is correlated with immune response and healing. Loneliness was also associated with impaired lymphocyte proliferative responses to bacteria in psychiatric patients (Kiecolt-Glaser et al 1984; Scanlan et al 2001). Detillion and colleagues (2003) found that wounded hamsters who were chronically isolated and experienced acute stressors had larger wounds with longer healing time spans relative to unstressed and paired hamsters. The human literature on the impacts of chronic social isolation on overall health and well being is extensive (Perkins, 1991; Gupta and Korte, 1994; Ernst and Cacioppo, 1999). Many of these consequences were attributed to elevated CORT levels, which can inhibit proinflammatory signal pathways (Raison and Miller 2003; Rhen and Cidlowski 2005; Collado-Hidalgo et al 2006). Specifically, chronically elevated CORT from social isolation is linked with decreased leukocyte response to mitogens (Manuck et al 1991), reduced cytokine response and increased natural killer (NK) cell lysis, causing a decrease in the regulation of tumor growth (Cacioppo et al 1995; Hawkley and Cacioppo 2003; Kunz-Ebretch et al 2003; Cole et al 2007). Interestingly, the literature is mixed on how CORT affects the immune response and proinflammatory cell proliferation. While CORT can cause a reduction in these cell numbers, other studies found the opposite effect with elevated pro-inflammatory response during social isolation (Manuck et al 1991 and Bachen et al 1992). Cacioppo and colleagues (1995) found that socially isolated

individuals had a significant under-expression of the GR target genes in leukocytes, meaning that even though CORT was circulating, it was unable to bind to leukocytes to regulate proliferation. With these potential negative impacts on the body from social isolation, it is imperative that we further understand the downstream effects of these negative consequences and how to potentially counteract them.

Isolation on Sympatho-Adrenal System

Epinephrine and Norepinephrine. Another potential biomarker for stress is elevated release of catecholamines, specifically epinephrine and norepinephrine (Schmidt and Kraft 1996). These hormones are released within seconds to minutes after the initiation of the stressor, proving difficult to distinguish between baseline and stress-induced values. Moreover, there is evidence that these hormones and those secreted by the HPA axis are connected and correlated during the stress responses (Van de Kar and Blair 1999; Sapolsky 2000; Carrasco and de Kar 2003 Schommer et al 2003; Palme et al 2005). However, there are mixed views on how chronic isolation impacts these hormones. Many studies report that extended periods of social isolation do not produce any significant changes in these catecholamines (Pajovic et al 2006; Sladana et al 2007; Jovanovic et al 2016) while others report a significant increase in plasma concentrations (Adzic et al 2009; Gavrilovic 2010; Qin et al 2015). Since they are released so quickly, it is difficult to distinguish between a significant baseline elevation and a stress induced rise in animal models. Interestingly, the combination of a chronic stressor and acute stressor cause the most profound effect on epinephrine and norepinephrine (Pajovic et al 2006; Sladana et al 2007; Gavrilovic et al 2013). It is important to note that while measuring

CORT levels are more feasible, measures of epinephrine and norepinephrine may give insight into the sympathetic nervous system during chronic and acute stressors and a combination of these areas.

  Cardiac Function. Part of the Sympatho-Adrenal system includes the autonomic system. Measures of the autonomic system, specifically the circulatory system can be important biomarkers of chronic stress effects. Individuals with low levels of social engagement experience an increased risk of cardiovascular disease (CVD) mortality (Ramsay et al 2008). Further, social isolation in humans is associated with several cardiovascular risk factors including coronary artery calcification, increased blood glucose levels, and hypertension (Hemingway et al 2001; Strike and Steptoe 2004; Kop et al 2005; Rutledge et al 2008). Since those who are socially isolated tend to exhibit symptoms of depression, it is likely that these depressed individuals have altered cardiovascular outputs (Grippo et al 2001). For example, Lahmeyer and colleagues (1987) reported that individuals who are physically well, but psychologically depressed often exhibit elevated resting heart rates. Steptoe and colleagues (2004) supported this by reporting significant increases in diastolic blood pressure in lonely women who also exhibit symptoms depression. Further, raised fibrinogen concentrations were reported in individuals who were socially isolated or who lack social support (Davis and Swan 1999; Wamala et al 1999) and in people with lower socioeconomic status (Brunner et al 1996). These elevated levels of fibrinogen are associated with coronary heart disease (Danesh et al 1998). These and other symptoms of CVD must be further studied in both humans and animals to understand the impacts and possible preventative measures. Unden and

colleagues (1991) reported that individuals with higher social support tended to have lower heart rates and blood pressure in high stress work environments than those who had limited social support.

Animal models of social isolation produced similar results from human literature. Social isolation in prairie voles resulted in elevated resting and stressor-induced heart rate, which correlated with elevated stress-induced CORT levels and depression-like behaviors (Grippo et al 2007c, Grippo et al 2011; McNeal et al 2014). Two to four weeks of isolation also caused reduced heart rate variability (Grippo et al 2007c), suggesting an inadequate functioning of the autonomic system leading to decreased resilience to other stress events (McCarty and Shaffer 2015). Specifically, this may be mediated by autonomic dysregulation, by increased sympathetic and decreased parasympathetic innervation to the heart (McNeal et al 2014). Gorban and colleagues (2014) further supported this reporting elevated heart rates and increased cardiac dysfunction within isolated and depressed cynomolgus monkeys compared to pair housed individuals.

However, social support can reverse these cardiac dysfunctions associated with isolation. Grippo and colleagues (2012) reported a significant increase in resting heart rate in prairie voles when isolated from their pair-bonded partner, but then a return to basal levels when pairs were reunited. This study demonstrates that while social isolation does cause increased cardiac dysfunction, social support can diminish this response after the stressor has occurred. This is one of the first studies to show the importance of social support on cardiac function. While we were unable to study the autonomic system within

this study, it is possible that prairie voles in isolation had elevated heart rate throughout the study period.

<u>Salivary alpha-amylase</u>

Recently, alpha-amylase was described as a potential indicator of norepinephrine activity (Chatterton et al 1996; Rohleder et al 2004; Nater et al 2005). Salivary alpha-amylase is a protein enzyme that breaks starch into maltose and dextrin. It is also produced under sympathetic innervations (Tobin et al 1995; Leicht et al 2011). It is thought that alpha-amylase increases with physiological (Walsh et al 1999; Li and Gleeson 2004; Leicht et al 2011) and psychosocial stressors (Nater et al 2004; Nater et al 2006; Rohleder et al 2004). Specifically, there is a significant correlations between alpha-amylase and catecholamine levels (Chatterton et al 1996). This suggests that it may be a potential biomarker for gauging the level of activity of the sympathetic nervous system (Rohleder et al 2004; Nater et al 2005; Nater et al 2006).

<u>Oxytocin</u>

Interestingly, our study and previous studies found that oxytocin has protective mechanisms from chronic stress situations. While the physiological function and regulation of oxytocin on the stress response are not well understood, the examination of its regional release (Rhodes et al 1981; Carrasco and de Kar 2003; Donaldson and Young 2008), receptor expression (Insel 1992; Insel and Shapiro 1992; Champagne et al 2001) and binding (Klein et al 1995; Carter 1996; Purba et al 1996; Gimpl and Fahrenholz 2001) are being studied.

Oxt on HPA and Hippocampus/Neuroendocrine functions

Oxytocin is released from the posterior pituitary for peripheral circulation (Higuchi et al 1986) and through dendrites into the extracellular space moving by diffusion through the brain (Moos et al 1989; Neumann et al 1993). It is thought that oxytocin affects the HPA axis directly in the central region inhibiting CRH and ACTH release (Chiodera et al 1991; Legros 2001; Erkut et al 2004). This inhibition could potentially cause a cascading effect, leading to decreased CORT secretion. Oxytocin also binds within the hippocampus during both positive and negative social encounters. Specifically, during chronic and acute stress situations, oxytocin increases its binding within the hippocampus causing downstream effects and signalling to the PVN in the hypothalamus (Legros 2001). This is supported by Nomura and colleagues (2003) who reported that administration of oxytocin into the PVN downregulated CRH gene expression, suggesting another potential cause for decreased CRH. Recently, Jurek and colleagues (2015) demonstrated that i.c.v. injections of oxytocin delayed the stress-induced rise in CRH transcription in the PVN following a chronic stressor. However, during chronic stress, oxytocin receptors transcription is diminished within the hypothalamus (Schoenemann et al. 1995; Zheng et al 2010), potentially limiting the amount of oxytocin able to bind and decrease CRH release. It is possible that the combination of oxytocin binding within the hippocampus and the limited binding within the PVN may be enough to decrease hormone secretion to the rest of the HPA axis.

Interestingly, endogenous and exogenous oxytocin may be unable to eliminate the HPA response to acute stressors. Yee and colleagues (2016) found that endogenous

oxytocin levels increased 2.5 times higher than baseline during an acute stressor. Further, even with the combination of exogenous oxytocin administration and these elevated endogenous oxytocin levels, prairie voles still had elevated CORT levels following an acute stressor (Yee et al 2016). Grippo and colleagues (2007b) found that even with elevated endogenous oxytocin, there was still a significant increase in ACTH and CORT levels during an acute stressor. Minas and colleagues (2015) found male and female Sprague-Dawley rats still produced elevated ACTH and CORT levels along with elevated oxytocin levels following an acute stressor. Neumann and colleagues (2000) further proved this theory, reporting that endogenous levels of oxytocin did not prevent an increase in ACTH or CORT following an acute stressor. However, an oxytocin antagonist injected into the PVN or an i.c.v. injection caused higher and more significant rises in these hormones following an acute stressor (Neumann et al 2000). This may suggest that oxytocin cannot completely eliminate the stress response in acute stress situations, which is beneficial to ensure that both animals and humans can physiologically cope with this type of stressor.

Oxytocin and GABA

While endogenous oxytocin may directly impact the PVN through receptor binding, there is still limited data on how it actually diminishes CRH expressing neurons (Dabrowska et al 2011). It is thought that a combination of oxytocin and gamma-aminobutyric acid (GABA) signaling may play a crucial role in inhibiting CRH expression during a variety of stress events (Theodis et al 1986; Englemann et al 2001; Leng et al 2001; Englemann et al 2004; Smith et al 2016). GABA is a dominant

inhibitory neurotransmitter in the mammalian brain (Decavel and Van den Pol 1990). It's principle role is reducing neuronal excitability throughout the nervous system. It also has neurons in the PVN and surrounding area (Roland and Sawchenko 1993; Herbison 1994; Herman et al 2002). Furthermore, over half of the CRH-expressing neurons express GABAa receptors (Cullinan 2000). These receptors within the PVN inhibit stress-induced CRH binding (Bali and Kovacs 2003; Bartanusz et al 2004). Bulbul and colleagues (2011) reported that exogenous oxytocin's effects on the PVN are blocked by the GABAa receptor antagonist, suggesting a significant link between these two neuronal systems. Smith and colleagues (2016) further proved this by demonstrating that oxytocin administered in the PVN inhibited CRH release by increasing GABAergic neuron activity. This area needs to be further studied to elucidate this and other inhibitory mechanisms within this stress response system.

Oxytocin on Sympathoadrenal System

Epinephrine and Norepinephrine. Besides impacting the neuroendocrine system, oxytocin may impact epinephrine and norepinephrine release during stress events. There are few studies on the impacts of oxytocin on these catecholamines. Chan (1965) reported that administration of epinephrine resulted in decreased oxytocin levels and inhibited milk ejection, suggesting a negative correlation between these two systems. Moreover, it is possible that oxytocin is regulating the neuroendocrine and autonomic systems by upregulating the parasympathetic and reducing the sympathoadrenal response (Porges 1998; Higa et al 2002). Since oxytocin may impact CRH release during stress events (Nishioka et al 1998) and CRH also plays a role in activating the sympathetic

nervous system (Penalva et al 2002), it is possible that oxytocin may indirectly affect the release of epinephrine and norepinephrine. Moreover, exogenous oxytocin may be beneficial during chronic stress events to the sympathoadrenal response system through increasing the number of oxytocin-containing neurons and decreasing the number of CRH-containing neurons in the PVN (Grippo et al 2012). Vasopressin, a peptide similar to oxytocin and released from the PVN, has inhibitory effects on peripheral epinephrine and norepinephrine release, acting directly at nerve endings (Unger et al 1986) which may suggest another possible connection. Jovanovic and colleagues (2016) reported that peripheral oxytocin treatment reduced the significant elevation of epinephrine and norepinephrine following chronic social isolation. This suggests that while endogenous oxytocin may not significantly impact these catecholamines, exogenous doses may diminish their release in addition to other neuroendocrine hormones.

Cardiac Function. In addition to its central effects, oxytocin and its receptors are expressed in various peripheral tissues, including the heart and blood vessels (Jankowski et al 2000; Gimple and Fahrenholz 2001; Kiss and Mikkelsen 2005). It is thought that oxytocin present within the cardiovascular system is involved in maintaining homeostatic functions (Gutkowska et al 1997; Jankowski et al 2000; Carter et al 2003) and delaying cardiovascular diseases (Paredes et al 2006; Szeto et al 2008; Nation et al 2010). For example, Szeto and colleagues (2008) reported that oxytocin may prevent atherogenesis, thickening of the artery wall, by inhibiting inflammation in vascular endothelial and smooth muscle cells. Morris and colleagues (1995) found that oxytocin mediates stress-induced tachycardia while Petersson and colleagues (1996) reported that oxytocin

administration resulted in long term decreases in blood pressure in female and male rats.

Finally, Grippo and colleagues (2009) observed no change in heart rate or heart rate

variability in prairie voles that were chronically isolated with oxytocin injections,

suggesting that oxytocin protects against more than endocrine dysfunction.

Oxytocin on Behavior and Depression

Oxytocin administration decreases depression-like behavior in a variety of species

(rats: Windle et al 1997; Blume et al 2008; Bosch et al 2008; Slattery and Neumann

2010; Haoy et al 2016; Sanchez-Vidana et al 2016, mice: Ring et al 2008, prairie voles:

Cho et al 1999; Bales and Carter 2003; Bales et al 2007; Grippo et al 2009, monkeys:

Argiolas and Gessa 1999; Argiolas and Melis 2004). Interestingly, Darragh and

colleagues (2016) reported that intranasal oxytocin administration was able to

significantly decrease stress and anxiety in patients with depression. Further, Heinrichs

and colleagues (2009) reported that oxytocin assists in lowering CORT levels and

depressive behaviors in chronically stressed humans. However, we still don't know

exactly where oxytocin is acting within the brain to cause these changes. Acevedo and

colleagues (2012) showed when men and women were presented with a picture of

someone they love, there is a significantly greater blood-oxygen-level-dependent

(BOLD) response of oxytocin in the hypothalamus, ventral tegmental area, nucleus

accumbens, globus pallidus and the amygdala. These are regions that are expected to be

active during pair bond formation (Numan 2015) and this suggests that these regions are

also involved in long term bonds.

Besides its effects on depression, oxytocin also regulates the perceptions of relationships. Schneiderman and colleagues (2012) reported significantly higher oxytocin levels in both men and women who are in a relationship compared to those individuals that were single. Further, oxytocin levels can be predictive on the success of the relationship, showing that higher concentrations within the brain results in longer relationships than individuals with lower levels (Maner et al 2008). Scheele and colleagues (2012) found that men in monogamous relationships stayed farther away from attractive women after intranasal oxytocin treatment compared to a placebo, suggesting that oxytocin released within the brain promotes the maintenance of monogamy. Ditzen and colleagues (2009) found that intranasal oxytocin significantly increased the duration of positive communication behaviors in couples in relation to negative behaviors during conflict discussion. This study supports the connection that oxytocin promotes the maintenance of a stable pair bond in men and/or women through anxiolytic effects. However, it is important to note that intranasal oxytocin is likely affecting multiple neural sites and may impact more than one function. These are just a few examples of oxytocin's effects on different behaviors and more research must be conducted to understand the mechanisms, pathways and neuronal actions it affects.

Future directions:

Comparing pair bonds between male-female and female-female

While the impact of isolation is well studied in same-sex sibling pairs, the impacts of isolation on a bonded male-female pair needs to be further studied. Previous studies demonstrated that after a male and female are bonded, they consistently prefer their

partner when faced with a choice between them and an unfamiliar conspecific (Williams et al 1994; Carter et al 1995; Cho et al 1999; Carter et al 2008). However, studying both physiological mechanisms and alterations in behavior would be advantageous to further understand the impact of isolation. Specifically, while having positive social support is crucial, spousal support may be more impactful. Bower (1989) reported that widowers tend to be socially isolated and have a low happiness rating compared to men in the same age class that were still married. Moreover, Brown and colleagues (1975) reported that women who experienced a severe life event and had social support from their significant other had a lower disease prevalence rate compared to women who did not have the social support of a significant other. Lindstrom and Rossvall (2012) further proved this by reporting that individuals who were unmarried, widowed or divorced had poorer mental health compared to those that were married or cohabitating. This suggests that social support in the form of a significant other is in fact important for mental and physical health and that separation through isolation or death does have detrimental consequences. However, the mechanisms through which these negative consequences transpire are still unknown. Using an animal model such as the prairie vole that pair bonds and shows preference for their familiar partner may enable us to study these deleterious effects and determine if and how isolation may differ between pair-bonded mates and same sex sibling pairs.

Maternal stress effects and environmental mismatching

Besides its impact on the individuals affected, chronic isolation may influence future generations, specifically through maternal stress effects. Maternal stress effects are

physiological changes in the mothers and offspring after the mother experiences periods of chronic stress during gestation. Previous studies reported that elevated basal CORT levels from maternal stress can cause several negative behavioral outcomes including increased anxiety, depression and fearfulness (Seckl and Meaney 2004; Meaney et al. 2007; Love & Williams 2008). However, from an evolutionary perspective, altered phenotypes can achieve adaptive fitness values depending upon the environment in which they occur for both the mother and offspring (Gross 1996). While in some cases these maternal effects prepare offspring for stressful environments, many times offspring are born into environments that they are not physiologically or phenotypically prepared for, termed environmental mismatching. Embryos in several species showed developmental sensitivity following the exposure of elevated CORT levels from their mother (Love and William 2008), suggesting these hormones could result in predictive adaptive responses (Love et al 2009). For example, in the snowshoe hare model, during periods of high predation, maternal stress is high, producing offspring with higher basal CORT levels and an adapted stress response for this high predation environment compared to offspring born during low predation periods exposed to high predation environments in later life (Sheriff et al 2010). Although these characteristics are seemingly negative, they are actually highly advantageous when both the maternal and offspring environment are matched under high stress situations and enhance both survival and fitness for the mother and offspring. On the other hand, those offspring who are in mismatched environments are not prepared for the chronic stressful environments in which they are born into and may cause elevated CORT in later life leading to increased rates of cellular aging and

decreases in survival and fitness. Moreover, Marchetto and colleagues (2016) reported that mothers who were stressed during pregnancy gave birth to offspring with shorter telomeres, producing maladaptive traits and may shorten the life of those future children. While there are many studies on exogenous stressor effects on both mother and offspring, chronic social isolation as a possible stressor in maternal stress and environmental mismatching in offspring are not well elucidated.

<u>Final Remarks</u>

This is the first study to analyze not only how social isolation impacts oxidative damage and aging, but also how oxytocin is able to prevent all of these negative consequences. Moreover, this study has vast implications for human isolation and loneliness. Isolation does not have to be a completely physical experience. Cacioppo and colleagues (2000) stated that in many cases individuals are surrounded by people, but they still feel lonely and isolated perhaps because of differences or other psychological reasons relating to depression. Moreover, with today's technology, many people are not interacting with the outside world, potentially causing self-imposed isolation. This may lead to both physiological and psychological consequences. Our study is one of few that are currently being conducted to determine if oxytocin is able to treat these symptoms to alleviate and potentially eliminate disease and depression that are amplified from chronic stress in the form of chronic isolation. It is imperative that we continue to study this developing problem in hopes that we can someday cure the negative consequences of isolation both physiologically and psychologically.

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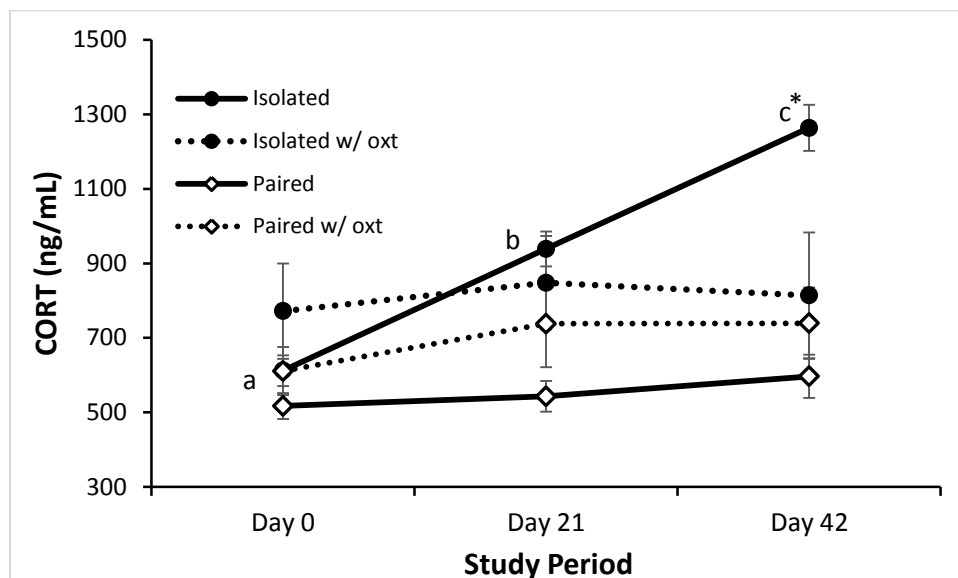
Figures

Figure 1. CORT levels following 42 days of study. Isolated prairie voles (solid line with filled in circle) had steadily increasing CORT concentrations from Day 0 (a), Day 21 (b) and day 42(c). The isolated group's CORT levels were significantly different from all treatment groups at day 42 (*). Prairie voles isolated with daily oxytocin injections (dotted line with filled in circle) showed no significant changes in CORT levels during the 42 day study period and were similar to those that were in paired treatment groups. Prairie voles that were paired both with (dotted line with diamond) and without (solid line with diamond) oxytocin injections had no significant changes in CORT concentrations during the study period and were not statistically different among each other.
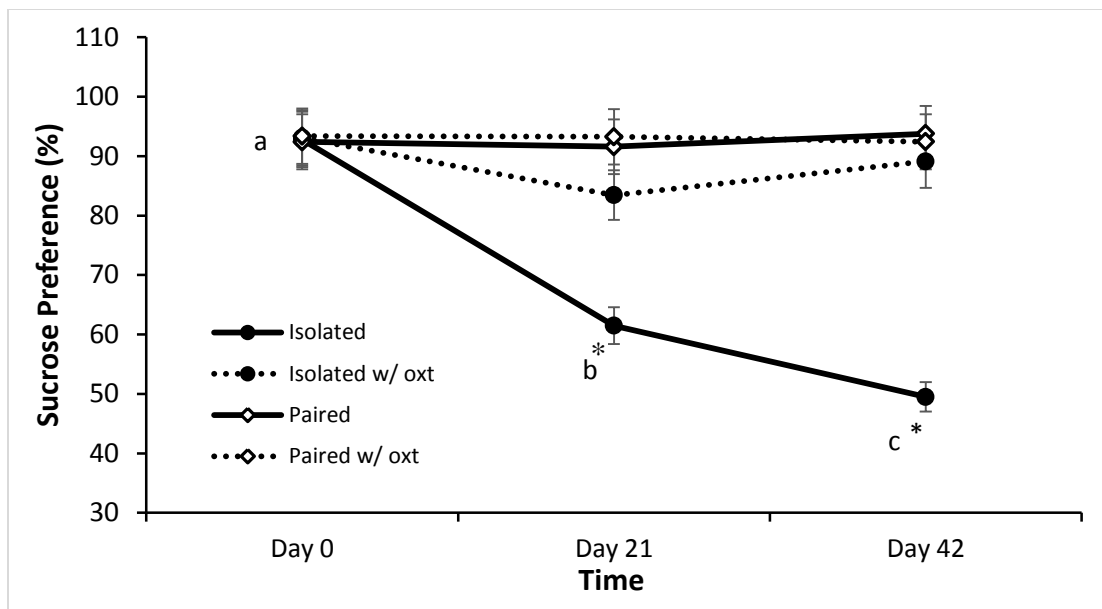
Figure 2. Sucrose preference tests during the 42 day study period. Isolated prairie voles (solid line with filled in circle) showed a significant reduction in preference for sucrose at 21 days of isolation (b) compared to their day 0 preference values (a). This preference further decreased by the day 42 time point (c) compared to previous time points.This isolated group also had significantly lower (*) preference for sucrose at day 21 and 42 than all other treatment groups. Prairie voles isolated with daily oxytocin injections (dotted line with filled in circle) had similar preference for sucrose over tap water throughout the study period, which was consistent with paired treatment groups. Prairie voles that were paired both with (dotted line with diamond) and without (solid line with diamond) oxytocin injections had no significant changes in sucrose preference during the 42 day study period.
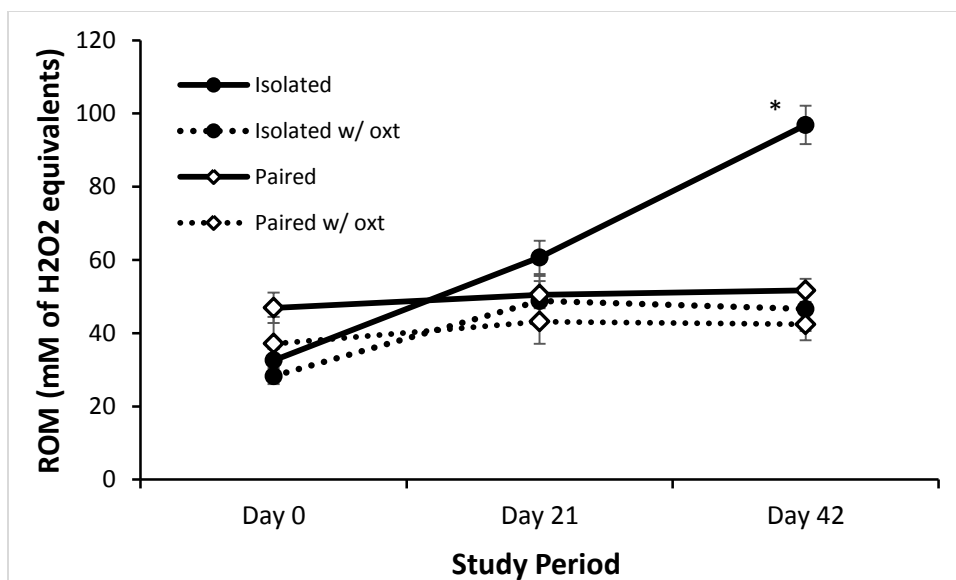
Figure 3. Effects of isolation on reactive oxygen metabolites (ROMs). ROMs levels were similar between all treatments at day 0 and day 21, but isolated prairie voles (solid line with circle) had higher ROMs levels compared to all the other groups at 42 days (*). ROMs in all other treatment groups were not significantly different from each other or within the time period of the study.
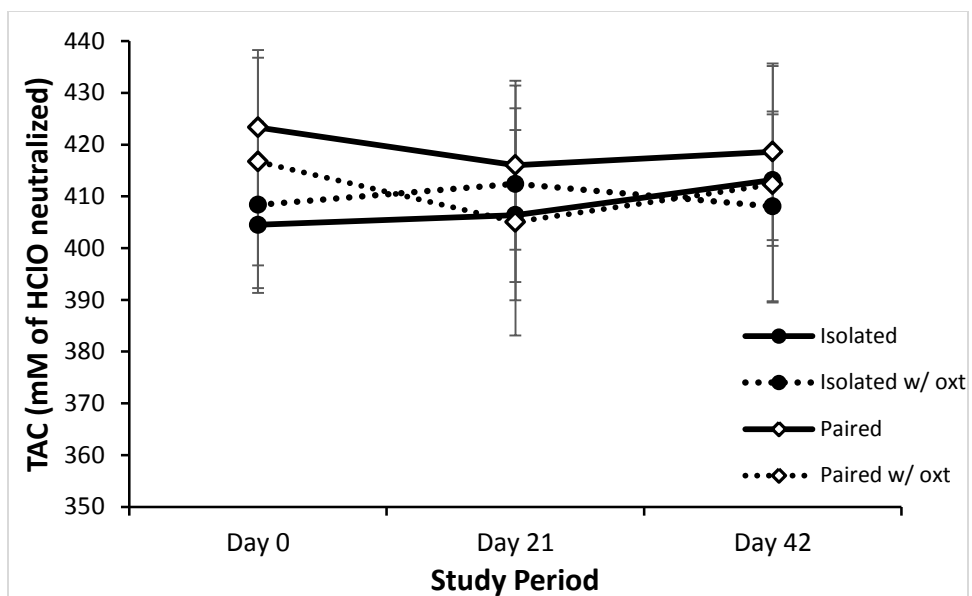
Figure 4. Effects of isolation on total antioxidant capacity (TAC). There was no significant changes in TAC concentrations in any treatment group during the 42 day study period.
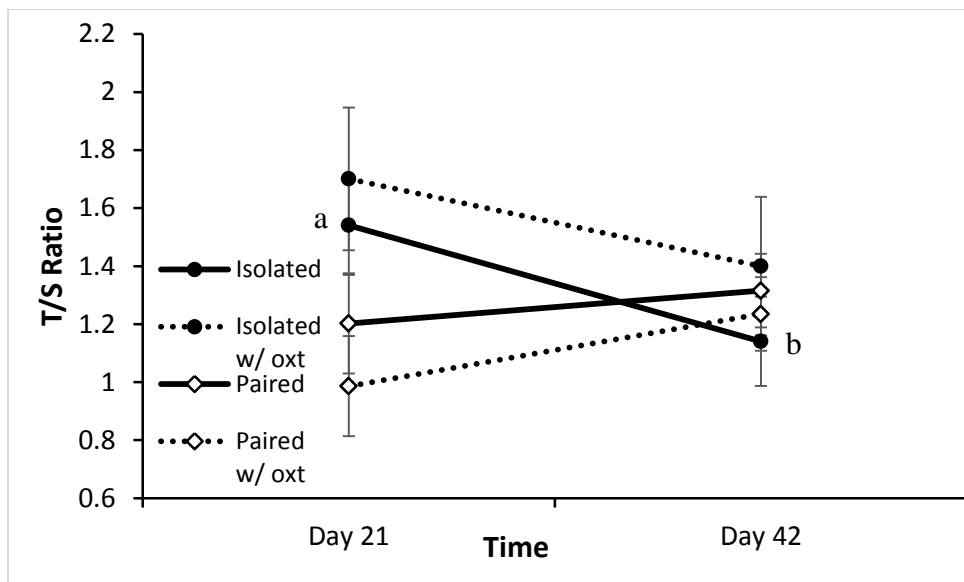
Figure 5. Impact of isolation on telomere length. All prairie voles had similar telomere lengths at day 21 of the study period. There was a significant decrease in telomere length from day 21 (a) to day 42 (b) in isolated prairie voles (solid line with circle). All other treatment groups showed no sign of significant degradation.
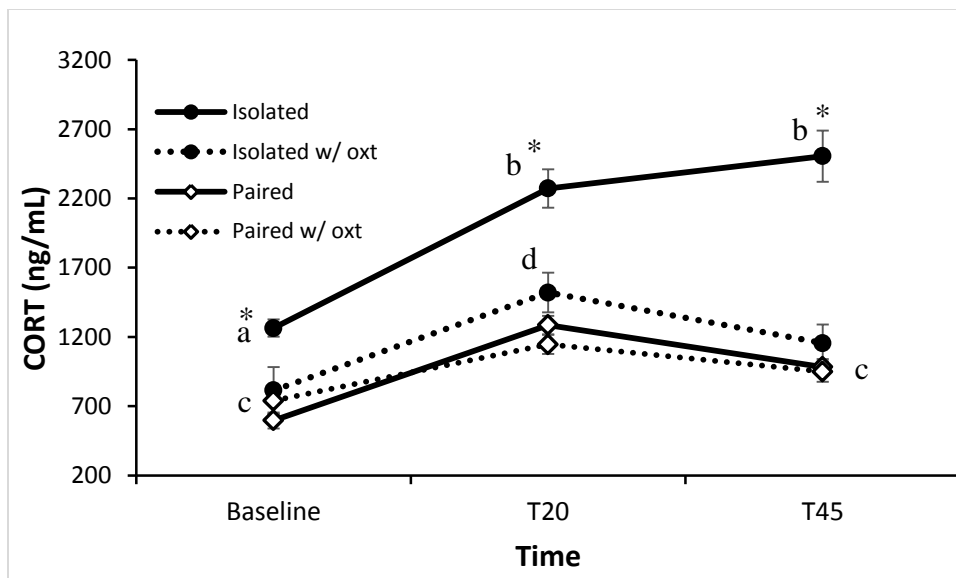
Figure 6. Acute stress test on CORT levels. Prairie voles in all treatments responded to the acute stress with significantly increased CORT levels between the baseline (a,c) and 20 minute samples (b, d). Isolated prairie voles (solid line with circle) had similar CORT levesl at the 45 minute time point (b), whereas CORT levels in all other treatments returned to baseline levels (c). Isolated prairie voles also had significnalty higher CORT levels at all time points during the acute stress test (*) compared to all other treatment groups.
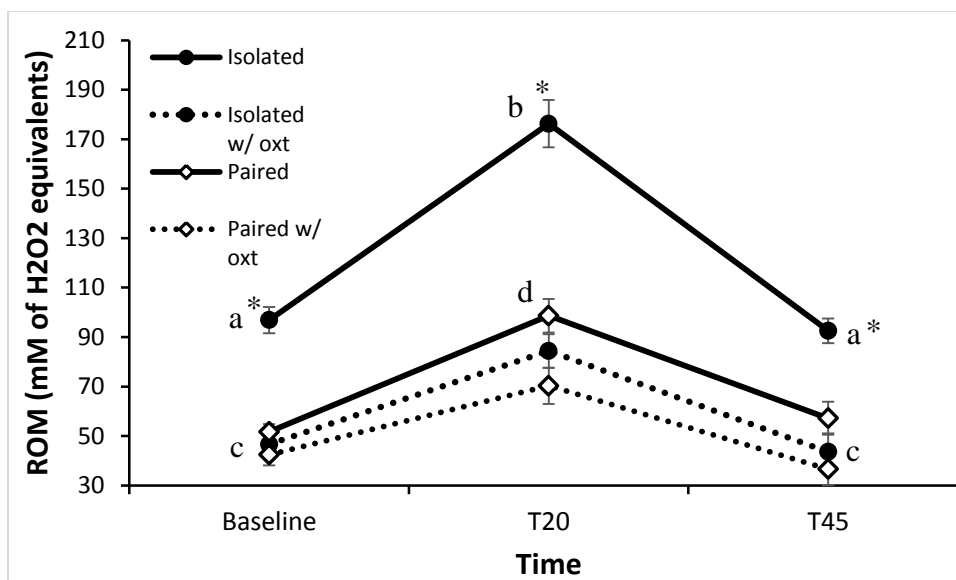
Figure 7. ROM effects from acute stress testing. Isolated (solid line circle) isolated with oxytocin (dotted line with circle) and paired (solid line with diamond) had significant increases in ROMs from baseline (a, c) to 20 minutes (b, d), but all levels returned to baseline by the 45 minute time point (a, c). Isolated prairie voles had significantly higher ROMs at all time points compared to all other treatment groups (*). Prairie voles that were paired and received daily oxytocin injections (dotted line with diamond) showed no change in ROMS during the acute stress test.
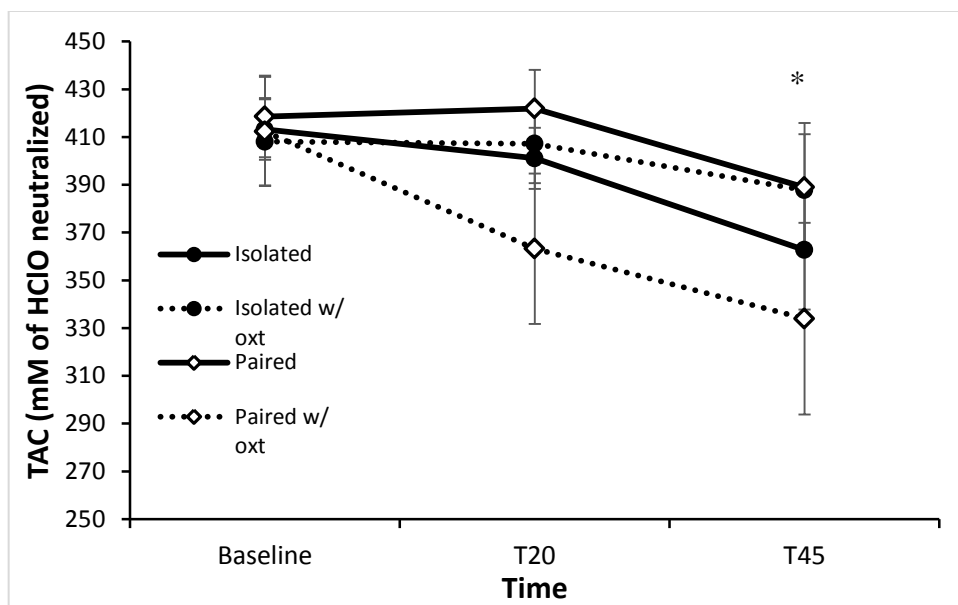
Figure 8. TAC levels from acute stress testing. TAC levels were similar between all treatment groups at the baseline and 20 minute time points, but significantly decreased by the 45 minute time point (*).

Appendix

A1. Protocol for CORT Radioimmunoassay

Day 1

1. Preparation
   a. **Samples**, **controls (CTL #)**, and **blanks (BLK #)** will be set up in **glass centrifuge tubes** (13x100).
   b. The **first** and **last** tubes are always **blanks** and **contain only dH$_2$O**, these tubes provide a negative control for background radiation.
   c. **Three controls** should be set up (run **2 at front, middle, and end on day 5**), these tubes allow measurement of intra-assay variation as well as inter-assay variation.
   d. Create a data sheet for plasma volumes, numbering all Samples, Blanks, and Controls.
2. Plasma Samples
   a. Vortex plasma and withdraw 50-150 ul (**100 ul** is ideal) with a pipette and place in a 13x100 glass tube.
   b. Add dH$_2$O to all samples with ER for a **total volume of 400 ul** (plasma + dH$_2$O).
3. Blanks
   a. Add **400 ul** of dH$_2$O to each [do NOT add hot steroid to blanks].
4. Controls
   a. Add **50 ul** of cold (non-radioactive) steroid to each with pipette.
   b. Add **350 ul** dH$_2$O to each tube for a **total volume of 400 ul**.
5. Recovery Set Up
   a. Add **20 ul (2000 cpm)** of labeled steroid to all samples and standard tubes [do NOT add hot steroid to blanks].
      i. Use ER set on 2 with 0.5 ml tip.
   b. Add **20 ul** of labeled steroid to each of two scintillation vials.
      i. Add **2.5 ml** scintillation fluid to each scintillation vial.
      ii. Vortex, cap, label, and store in a dark place for later.
6. Storage
   a. Vortex all tubes thoroughly.
   b. Cover with parafilm.
   c. Refrigerate overnight to allow equilibration.

Day 2

1. Preparation of Snap Freeze Apparatus
   a. Place dry ice (the denser the better) and methanol in a small ice bucket/two walled plastic container.
2. Extraction of steroids from samples (part 1)
   a. Add **3ml** of anhydrous ethyl ether to each sample.
   b. Allow samples to sit for 10 minutes after addition of ether to last tube.
   c. During the 10 minutes, vortex each sample at a low speed for several seconds.
      i. Grip the tube confidently about an inch from the top while vortexing to keep solution from rising out of tube (Be careful! Set the vortex at about 7. If the vortex is set at 10 it will overflow the tube).
   d. Place each tube in the super-cold liquid (day 2 step 1) until the aqueous phase (bottom portion) freezes.
      i. This will take approximately **5-10** seconds per tube.
      ii. **The perfect amount of times is when a little knob or spike forms on the frozen aqueous layer.**
   e. Pour off the ether phase (top portion) into a new 12x75mm test tube.
      i. If there are watery droplets in the bottom of this new tube after pouring off, you did not allow long enough for the tube to freeze
      ii. If there are white, frozen, shards present after pouring off (that do not result in watery droplets after thawing) you are freezing for too long.
   f. Allow tubes to thaw.
   g. Repeat extraction of the aqueous phase with another 3mls of ether.
3. Drying
   a. Prepare drying apparatus in Morgan BF's lab.
      i. The drying rack is below the hood, and consists of two parts: the base in which tubes are placed and the matching cover containing many metal needles.
      ii. Place the base in a water bath set at **37** Degrees Celsius containing ~1.5" of water (do not allow the needles to touch the water).
   b. Place samples in drying apparatus base.
      i. If your samples can fit on one half of the apparatus, place them all on one side, and use the clamp provided to close off the tube to the other side of the apparatus. This will reduce wasted nitrogen

c. Fit cover ensuring that each sample has a working needle inserted in its center.

d. Attach nitrogen gas to the drying rack and begin with a gentle flow of nitrogen.

      i. To turn on the nitrogen, open large valve on nitrogen canister the entire way (CCW), and open the small regulator valve just a small amount (CW). Regulator flow gauge should not register any flow.

e. Lower apparatus cover slowly so that the flow of nitrogen gas creates a dimple in the surface of the liquid, but does not pierce the surface.

f. Run until all ether has been evaporated.

g. Remove and dry the outsides of all test tubes.

4. Storage

a. Add **1 ml** of 90% ethanol (in H2O) to each tube.

b. Vortex thoroughly (use the multi-tube vortex in Morgan's lab).

c. Cover with parafilm.

d. Freeze overnight at -20 Degrees Celsius.

Day 3

1. Extraction of steroids from plasma samples (part 2)

a. Pre-spin the Beckman refrigerated centrifuge on second floor for 10 minutes to cool it to 0 Degrees Celsius (Must first set temperature to 0 degrees Celsius).

      i. Use tan colored inserts stored in cabinet above plate reader in Haussmann lab

b. Spin the samples at 1000g for 5 minutes (set timer to 7 minutes, as the centrifuge takes about 2 minutes to get up to speed).

      i. Use J 5.3 rotor

      ii. Set acceleration to SLOW

      iii. Set deceleration to SLOW

c. Pour the supernatant into a 12x75 test tube.

d. Dry under nitrogen gas as described in Day 2 step 3 (Drying).

e. Resuspend the samples in **550 ul** assay buffer (PBSg).

f. Vortex thoroughly (use the multi-tube vortex in Morgan's lab).

g. Refrigerate samples overnight.

Day 4

1. Preparation

a. Place the appropriate number of 12x75 test tubes in racks.

      i.   Arrange 27 tubes for standard curve (3xS1, 3xS2, 3xS3, 3xS4, 3xS5, 3xS6, 3xS7, 3xS8, 3x29)

     ii.   Arrange 9 tubes for B's (3xB1, 3xB2, 3xB3)

   iii.   Arrange a pair of tubes for each sample.

2. Setting up the Samples
   a. Vortex each tube (from day 3) thoroughly.
   b. Using EP200, pipette **200 ul** of each sample into duplicate assay tubes from preparation step.
   c. Using EP200, pipette **100 ul** of each sample into a scintillation vial to count recoveries.
      i. Add **2.5 mls** scintillation fluid.
      ii. Cap, vortex, label, and set aside for counting on Day 5.

3. Setting up the Standard Curve
   a. The three sets of three tubes should be labeled 3x(B1-B3).
      i. B1 measures the total cpm in 100 ul of the labeled steroid solution.
      ii. B2 measures non-specific binding (background).
      iii. B3 measures the maximum binding of the labeled steroid with the antibody.
   b. The remaining 27 tubes should be labeled 3x(S1-S9) and are used to generate the curve.
   c. Add **200 ul** assay buffer (PBSg) to the B1 and B2 tubes.
   d. Add **100 ul** assay buffer (PBSg) to the B3 and S2-S9 tubes [do NOT add anything to the S1 tubes yet].
      i. Use the ER with a 5 ml tip set on 1.
   e. Add **100 ul** of cold (unlabeled) steroid to each of the S1 and S2 tubes using EP200.
      i. This will be **2000 pg** for Corticosterone.
   f. Thoroughly vortex one of the S2 tubes, Remove **100 ul** and add this to an S3 tube.
   g. Repeat above step to continue serial dilution.
      i. Discard the last 100 ul that is removed from the S9 tube.
   h. Repeat above two steps to create serial dilution for the second and third sets of standards.
   i. Add **100 ul** assay buffer (PBSg) to all B and S tubes to bring them up to the same volume as the samples.

4. Setting up the Radioimmunoassay
   a. Whirl mix [do NOT shake][do NOT vortex] the labeled (Hot) steroid and antibody solutions prior to use.

b. Using the ER add **100 ul** of labeled (Hot) steroid to all of the tubes (standard curve, B tubes, and samples).
c. Add **100 ul** antiserum to all tubes starting with B3 tubes (do NOT add antiserum to the B1 or B2 tubes).
d. Cover all tubes with parafilm.
e. Whirl mix.
f. Refrigerate overnight
    i. Curves may be left in refrigerator longer, but overnight is the minimum time required.

Day 5

1. <u>Separation of Bound and Free Counts</u>
    a. Pre-spin the Beckman refrigerated centrifuge on second floor for 10 minutes to cool it to 4 Degrees Celsius.
        i. Use tan colored inserts stored in cabinet above plate reader in Haussmann lab
    b. Add **0.5 mls** dH$_2$O to the B1 tubes.
        i. Use an ER set on 2 with a 12.5 ml tip.
    c. Place dextran coated charcoal in a beaker, add a magnetic stir bar, place on stirrer.
        i. For Corticosterone use half strength charcoal solution.
    d. The charcoal mix should be stirred continuously while adding **0.5 mls** to all remaining tubes.
        i. This step should be done as quickly as possible to avoid heating and to ensure all samples are exposed to charcoal for approximately the same amount of time.
    e. Whirl mix the racks of tubes and let stand for 12 minutes.
        i. Start the timer as soon as the charcoal mix is added to the last sample.
        ii. Load the centrifuge while waiting, be sure everything is balanced.
    f. Centrifuge at 1000 g for 10 minutes (set timer on centrifuge for 12 min, it takes approximately 2 minutes for the centrifuge to get up to speed).
        i. Use J 5.3 rotor
        ii. Set acceleration to SLOW
        iii. Set deceleration to OFF
    g. Remove at least 800 ul of supernatant from each tube with a Pasteur Pipette, place into a new 12x75 glass tube
    h. Remove **800 ul** of supernatant from each tube, and place into a scintillation vial.

      i.   Add **3.5 mls** scintillation fluid.
      ii.   Cap, vortex, and count on the scintillation counter.
      iii.   Be sure to run calibrator through Scint counter ahead of samples.
   i.   Discard centrifuge tubes with the charcoal pellets as radioactive waste.

A2. d-ROMs Protocol

Setting up the assay

1. Remove kit from refrigerator; all reagents must be at room temperature prior to use in the assay.
2. Prepare plate reader.
   a. Turn on power supply to plate reader (silver "box" on right of machine).
   b. Turn on power switch on front of machine (machine is powered up and ready when the green light stops blinking)
   c. Open Gen 5 2.00 software
   d. Create a new experiment from existing protocol "**dROMSKINETIC**."
      i. **Confirm the reader is heating to 37C.**
      ii. Once temperature is reached, and plate is inserted, this protocol will read the plate at **505 nm** thirty times. Reads take approx. 38 seconds apiece followed by a 22 second wait period. This allows reads to be taken once per minute.
3. Using the 96-Well Plate Template (found at the bottom of this sheet), set up your plate by writing which samples are going in each well.
   a. Samples run in duplicate should be in adjacent wells.
   b. Calibrator should go in A1, A2; Blank should go in B1, B2; Control sample (to be run on all plates for a sample set spanning more than one plate) should go in C1, C2.
4. If calibrator still in powder form (unopened), add **2mL** distilled H20 (included in kit) to powder. Invert carefully and let sit for **~15 minutes** to dissolve. Separate into **200uL** aliquots and freeze for later use.

Running the Assay

1. Add **8uL of calibrator** and **8uL of blank (water)** to appropriate wells.
2. Add **8uL of plasma sample** (not diluted) to appropriate wells.
   a. **2ul** of plasma per well for HOSP, TRES, GRCA, BRTH samples.
   b. **2ul** of plasma per well for Feral Quail samples (this should be confirmed).
   c. **8ul** of plasma per well for vole samples.
3. Prepare R1:R2 mixture directly before adding.
   a. **2uL R1: 200uL R2** (per well); make extra so don't run out when using the multichannel pipette.
4. Add **202uL of R1:R2 mixture** to each well using the multichannel pipette.
   a. R1:R2 mixture can be placed in a clean large reservoir and pipetted from there.

5. Mix gently by slowly tapping plate.
   a. Pop any large bubbles with a needle
6. Wipe bottom of plate with Kimwipe in order to remove fingerprints, dust, etc.
7. Read plate immediately.
   a. Record time of first read (following reads will begin at **[initial read time + (1.00 minutes * read number)].**
   b. d-ROMs calls for a one-minute incubation; discount first read from plate reader to account for incubation period when analysing data.
   c. Multiple reads in the program will allow for comparison between plates.

Data analysis

1. Subtract blank value from calibrator and samples
2. Results are expressed in Carratelli Units, see formula
   a. (Abs/min) are the mean differences of the absorbances recorded at 1,2,3,…n min;
   b. [Calibrator] is the concentration of calibrator**.**

$$\text{CARR U} = \frac{\text{(Abs/min) sample}}{\text{(Abs/min) calibrator}} \times \text{[calibrator]}$$

A3. OXY-Absorbent Protocol

1. Prepare plate reader.
    a. Turn on power supply to plate reader (silver "box" on right of machine).
    b. Turn on power switch on front of machine (machine is powered up and ready when the green light stops blinking)
    c. Open Gen 5 2.00 software
    d. Create a new experiment from existing protocol "**OXY 25 Read**."
        i. Executing this protocol will read the plate at 505 nm twenty five times. Reads take approx. 37 seconds apiece followed by a 23 second wait period. This allows reads to be taken once per minute.
2. Using a 96-Well Plate Template (in Protocols and Worksheets drawer), set up your plate by writing which samples are going in each well.
    a. Samples run in duplicate should be in adjacent wells.
    b. Calibrator should go in A1, A2; Blank should go in B1, B2
3. Dilute calibrator **1:100** (**10μL calibrator** into **990μL dH$_2$0**).
4. Dilute plasma, if necessary.
    a. **Do not dilute** samples for domestic quail
    b. **1:50** dilution for HOSP, TRES, GRCA, BRTH (**2μL sample into 98μL dH20**)
    c. **1:50** dilution used for voles
    d. **1:10** dilution for feral quail
5. Add **5μL calibrator** and **5μL blank (water)** to the appropriate wells.
6. Add **5 μL of diluted plasma** sample to each well.
7. Using a multichannel pipette, add **200 μL R1** reagent to each well.
    a. Do not blow out all the way in order to prevent bubbles.
8. Cover plate with clear, sticky cover to prevent evaporation.
    a. Mix by slowing tapping plate sideways. Be gentle so as to prevent liquid from sticking to the cover sheet.
9. Incubate plate **10 minutes** at **room temperature**.
10. Add **2μL R2** (chromagen) to each well and mix using the pipette.
    a. Blow out all of sample and mix with pipette tip.
    b. After R2 is added, go back with a larger pipette that can pick up ~200 μL and **mix wells**.
11. Wipe bottom of plate with Kimwipe in order to remove fingerprints, dust, etc.
12. Read plate immediately as described in step 2.
    a. Record time of first read (following reads will begin at **[initial read time + (1.00 minutes * read number)].**

b. Multiple reads in the program will allow for comparison between plates.

Calculation:

(Abs blank – Abs sample)   x   Concentration of calibrator = OXY concentration

(Abs blank – Abs calibrator)

Concentration of calibrator = **350** μmol HClO/mL (**check calibrator vial for each kit**)

OXY concentration expressed as μmol of HClO/mL of sample

A4. Puregene Extraction of Whole Blood

1. Thaw frozen blood sample.

2. Spin to pellet cells (3500rpm,5mins).

3. Remove supernatant and discard.

4. Resuspend cells in 3xcell volume with RBC lysis buffer. Leave at RT for 1 min.

5. Spin at 11.5k for 2mins.

6. Remove supernatant and discard.

7. Add 600ul cell lysis solution and 10ul of Prot K.

8. Incubate at 56oC while shaking until fully digested (between 1 & 3hrs)

9. Place tubes on ice for 10mins.

10. Add 230ul of protein precipitation solution and vortex to ensure solutions are mixed well.

11. Spin at 11.5k for 5min.

12. Remove supernatant to a fresh tube and add 1.5ul glycogen.

13. Add 700ul isopropanol and mix well by inversion.

14. Leave at -20oC for ~30min

15. Spin at 13.5k for 2min.

16. Remove supernatant and discard.

17. Add 600ul of 70% ethanol (made freshly each week) and spin at 13.5k for 1min.

18. Remove supernatant and respin if necessary to remove residual ethanol.

19. Air dry

20. Resuspend in 25ul DNA rehydration solution.

A5. Protocol for qPCR

Telomeres were measured using the quantitative PCR (qPCR) method described

by Cawthon (2002) on a Lightcycler 96 Exigon (Roche). The Cawthon method gives a

relative measurement of telomere length (RTL) and is calculated as a ratio (T/S) of

telomere repeat copy number (T) to a control single copy gene (S). The Glyceraldehyde-

3-phosphate dehydrogenase (GAPDH) gene was chosen as the single copy gene. The

prairie vole (*microtus ochrogaster*) GAPDH sequence (NCBI accession number:

NW_004949099.1, GENE ID: 101996351) was used to design primers. The GAPDH

primers selected were vole GAP6 F 5'-GCGTCCAATACGGCCAAATC-3' and

voleGAP6 R 5'- GAGCATTTGTTGACCCAGCC-3'. For amplification of telomeric

sequences the universal primers designed by Cawthon (2002) were used:

Tel1b 5'-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3' and Tel2b

5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'. The telomere (T)

and GAPDH (S) assays were carried out on separate 96 well plates. Each sample was

measured in triplicate, with the average used to calculate RTL. In addition to the samples,

each plate also contained a standard curve (a five-fold dilution series of a pool of DNA;

ranging from 10 - 0.625ng/well), a reference sample and a no template control (NTC).

Each reaction contained:

- 10ul of SYBR (FastStart Essential DNA green master)
- Forward and reverse primers,
- DNA (sample, standards or reference) or water (NTC)

- Total volume of 20ml. Both T and S assays were performed using 2.5ng of DNA (equivalent to 5ml).

Primer concentrations were 500/500nM for Tel1b/Tel2b and 200/200nm voleGAP6 F/voleGAP6 R. The thermal cycling profile for the T assay was a preincubation of 95^0C for 5 minutes followed by 95^0C for 15sec, 58^0C for 30sec, 72^0C for 30sec for 30 cycles, and for the S assay was a preincubation of 95^0C for 5 minutes followed by 95^0C for 30sec, 55^0C for 30sec, 72^0C for 30sec for 40 cycles. Following amplification, a dissociation curve was performed in order to confirm the specificity of the reactions.

To calculate the relative T/S ratios we used a modification of the delta-delta Ct method, to include efficiency for each assay as described by Pfaffl (2001),

$$\frac{1 + ET\Delta Cq^{T(control-sample)}}{1 + ES\Delta Cq^{S(control-sample)}} = RTL$$