Investigating the behavioral effects of juvenile stress in the prairie vole model

Marigny C. Normann
marignycollins@gmail.com

Follow this and additional works at: https://huskiecommons.lib.niu.edu/allgraduate-thesesdissertations

Part of the Applied Behavior Analysis Commons, and the Neurosciences Commons

Recommended Citation
Normann, Marigny C., "Investigating the behavioral effects of juvenile stress in the prairie vole model" (2020). Graduate Research Theses & Dissertations. 7498.
https://huskiecommons.lib.niu.edu/allgraduate-thesesdissertations/7498

This Dissertation/Thesis is brought to you for free and open access by the Graduate Research & Artistry at Huskie Commons. It has been accepted for inclusion in Graduate Research Theses & Dissertations by an authorized administrator of Huskie Commons. For more information, please contact jschumacher@niu.edu.
ABSTRACT

INVESTIGATING THE BEHAVIORAL EFFECTS OF JUVENILE STRESS IN THE PRAIRIE VOLE MODEL

Marigny C. Normann, M.A.
Department of Psychology
Northern Illinois University, 2020
Dr. Angela J. Grippo, Director

Early life stress during the juvenile period, such as emotional neglect, interpersonal difficulties, or other forms of non-violent maltreatment, can have consequences into adulthood. Specifically, the negative effects include increased risk of psychiatric or physical illnesses, social deficits, and maladaptive behavioral responses to stress. Since these effects have far-reaching implications that can negatively alter later behavior and physiology, the present study assessed the effects of early life social stress on later social and affective behaviors in a social rodent species – the prairie vole. The prairie vole displays behavioral, cardiovascular, and neuroendocrine responses to social stress similar to those of humans and therefore is a valuable model for investigating the consequences of social stress. The present study was specifically designed to investigate the consequences of early life social stress in female prairie voles on adolescent and adulthood family interactions, measures related to depression and anxiety, novel social interactions, and pair-bond formation. The early life stress design implemented post-weaning social isolation during a targeted period of development and then re-socialized the animals with their siblings for a 2-week period until adulthood, relative to a control group that
remained paired with their respective siblings. During adolescence, paired and isolated prairie voles were exposed to a social interaction test. During adulthood, the groups underwent several additional behavioral tests focused on anxiety- and depression-related behaviors and social interactions.

It was hypothesized that early life social isolation would negatively influence later emotion-related and social behaviors. The socially isolated animals displayed increased play behaviors with a previous sibling during the juvenile period. In adulthood, socially isolated prairie voles displayed increased instances of mating behaviors with a novel male partner and spent less time huddling with a stranger male in a partner preference paradigm involving a choice between the previous familiar male partner or a novel male stranger. These behavioral differences were not initially hypothesized but may suggest that early life social isolation alters the development of adulthood social behaviors. No behavioral differences were observed between paired and isolated prairie voles in tests of anxiety- and depression-related behaviors during adulthood. The lack of these behavioral differences may indicate that 2 weeks of resocialization during the juvenile period, applied after social isolation, may serve a protective role against later maladaptive emotion-related behaviors in the isolated group. Exploratory correlations further supported the primary results and also suggest that early life social stress may influence the development of stress responses. Together, these results provide insight into consequences of early life social stress on later social behaviors and risk of anxiety- and depression-related behaviors in a social species, providing a translatable methodology to further explore these developmental processes.
INVESTIGATING THE BEHAVIORAL EFFECTS OF JUVENILE STRESS
IN THE PRAIRIE VOLE MODEL

BY

MARIGNY C. NORMANN, B.S.
©2020 Marigny C. Normann

A THESIS SUBMITTED TO THE GRADUATE SCHOOL
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE
MASTER OF ARTS

DEPARTMENT OF PSYCHOLOGY

Thesis Director:
Angela J. Grippo, PhD
DEDICATION

This document is dedicated to my family, who have supported me throughout this process.

Particularly to Kirk, who has always given me hope, propped me up when I could not stand on my own, and reminded me that nothing is impossible.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF FIGURES</th>
<th>vii</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>ABBREVIATIONS USED IN THIS DOCUMENT</td>
<td>x</td>
</tr>
</tbody>
</table>

Chapter

1. INTRODUCTION ................................................. 1
   General Introduction to the Present Study ........................ 1
   Stress – Definitions ........................................... 5
   Stress – Physiology ............................................ 8
      The Stress Response System: Adaptive Communication .......... 8
      The PVN .......................................................... 13
      The Stress Response System: Maladaptive Outcomes ............... 17
   Social Stress – Behavioral and Physiological Consequences on
   Development in Humans ........................................... 22
      Importance of Social Support in Adulthood ....................... 22
      Parental Influence on Development ................................ 24
      Importance of Peer Interactions on Development .................. 25
      Absence of Social Support in Development ......................... 27
      Effects of Early Life Stress on Adulthood Health ................ 29
   Animal Models and Consequences of Early Life Stress ............... 30
   Models of Maternal Influence/Separation .......................... 30
Models of Play and Social Interactions During Animal Development …… 32

Models of Post-Weaning Social Isolation ........................................... 36

The Prairie Vole Model .................................................................. 38

Parental Influence ........................................................................ 39

Play ................................................................................................ 41

Post-Weaning Manipulations .............................................................. 43

2. THE CURRENT EXPERIMENT .......................................................... 49

   Goals and Specific Aims of the Present Study ................................. 49

   Specific Aims and Hypotheses ....................................................... 53

   Methods ....................................................................................... 54

      Animals .................................................................................... 54

      Sample Size and Groups ............................................................ 55

      Power Analyses ......................................................................... 55

      Conditions ................................................................................ 57

   General Study Design .................................................................... 58

   Specific Methods ......................................................................... 60

      Social Isolation Paradigm ............................................................ 60

      Resocialization and Juvenile Social Interaction Test ...................... 61

      EPM Test of Anxiety and Locomotion ........................................ 65

      FST of Depression-Related Behavior ........................................... 66

      Male-Female Social Interaction Tests ........................................ 68

      Social Bonding Period ............................................................... 69
Partner Preference Test .................................................. 71
Adult Social Interaction Test ............................................. 73
Tissue Collection and Preparation ..................................... 75
Behavioral Analyses .......................................................... 75
Statistical Analyses ........................................................... 76
Results .................................................................................. 81
Body Weight ......................................................................... 81
Preliminary Correlations ..................................................... 83
Juvenile Social Interaction Test .......................................... 83
Elevated Plus Maze ............................................................ 88
Forced Swim Test ............................................................... 90
Male-Female Social Interaction Tests .................................. 91
  At Initial Pairing .............................................................. 91
  Following 24 Hours of Cohabitation .................................. 91
Partner Preference Test ....................................................... 100
Adult Social Interaction Test .............................................. 106
Exploratory Analyses ........................................................ 107
Discussion ............................................................................ 110
Juvenile Social Behaviors: Juvenile Social Interaction Test ...... 113
<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adulthood Emotion-Related Behaviors: EPM and FST</td>
<td>115</td>
</tr>
<tr>
<td>Adulthood Opposite-Sex Preferences: PPT</td>
<td>121</td>
</tr>
<tr>
<td>Adulthood Social Behaviors: Adult Social Interaction Test</td>
<td>122</td>
</tr>
<tr>
<td>Exploratory Analyses</td>
<td>124</td>
</tr>
<tr>
<td>Conclusions, Implications for Humans, and Recommendations for Future Research</td>
<td>127</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>134</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1.</td>
<td>The Stress Response</td>
</tr>
<tr>
<td>2.</td>
<td>The HPA Axis and Peripheral Nervous System</td>
</tr>
<tr>
<td>3.</td>
<td>Rodent Play Behaviors</td>
</tr>
<tr>
<td>4.</td>
<td>General Study Design</td>
</tr>
<tr>
<td>5.</td>
<td>Graph of Body Weights</td>
</tr>
<tr>
<td>6.</td>
<td>Graph of Investigative Behaviors in the Juvenile Social Interaction Test</td>
</tr>
<tr>
<td>7.</td>
<td>Graph of Play Behaviors in the Juvenile Social Interaction Test</td>
</tr>
<tr>
<td>8.</td>
<td>Graph of Huddling Behaviors in the Juvenile Social Interaction Test</td>
</tr>
<tr>
<td>9.</td>
<td>Graph of Time Spent Alone in the Juvenile Social Interaction Test</td>
</tr>
<tr>
<td>10.</td>
<td>Graph of Duration in the Open Arms of the EPM</td>
</tr>
<tr>
<td>11.</td>
<td>Graph of Center Crossings in the EPM</td>
</tr>
<tr>
<td>12.</td>
<td>Graph of Immobility in the FST</td>
</tr>
<tr>
<td>13.</td>
<td>Graph of Positive Responses to Male Approach in the Male-Female Social</td>
</tr>
<tr>
<td></td>
<td>Interaction Test at Initial Pairing</td>
</tr>
<tr>
<td>14.</td>
<td>Graph of Negative Responses to Male Approach in the Male-Female Social</td>
</tr>
<tr>
<td></td>
<td>Interaction Test at Initial Pairing</td>
</tr>
<tr>
<td>15.</td>
<td>Graph of Huddling Behaviors in the Male-Female Social Interaction Test</td>
</tr>
<tr>
<td></td>
<td>at Initial Pairing</td>
</tr>
<tr>
<td>16.</td>
<td>Graph of Aggressive Behaviors in the Male-Female Social Interaction Test</td>
</tr>
<tr>
<td></td>
<td>at Initial Pairing</td>
</tr>
</tbody>
</table>
17. Graph of Mating/Lordosis Behaviors in the Male-Female Social Interaction Test at Initial Pairing ........................................................................................................ 96

18. Graph of Positive Responses to Male Approach in the Male-Female Social Interaction Test Following 24 Hours of Cohabitation ........................................................................................................ 97

19. Graph of Negative Responses to Male Approach in the Male-Female Social Interaction Test Following 24 Hours of Cohabitation ........................................................................................................ 98

20. Graph of Huddling Behaviors in the Male-Female Social Interaction Test Following 24 Hours of Cohabitation ........................................................................................................ 99

21. Graph of Mating/Lordosis Behaviors in the Male-Female Social Interaction Test Following 24 Hours of Cohabitation ........................................................................................................ 100

22. Graph of Huddling Behaviors with Male Partner in the PPT ........................................................................................................ 101

23. Graph of Huddling Behaviors with Stranger Male in the PPT ........................................................................................................ 102

24. Graph Comparing Huddling Behaviors with Male Partner and Stranger Male in the PPT ........................................................................................................ 103

25. Graph of Aggressive Behaviors with Male Partner in the PPT ........................................................................................................ 104

26. Graph of Aggressive Behaviors with Stranger Male in the PPT ........................................................................................................ 105

27. Graph of Time Spent in the Neutral Arena in the PPT ........................................................................................................ 105

28. Graph of Huddling Behaviors in the Adult Social Interaction Test ........................................................................................................ 106

29. Graph of Aggressive Behaviors in the Adult Social Interaction Test ........................................................................................................ 107
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Comparison of Operational Definitions of Play</td>
<td>44</td>
</tr>
<tr>
<td>2. Definitions of Behaviors in the Juvenile Social Interaction Test</td>
<td>63</td>
</tr>
<tr>
<td>3. Definitions of Behaviors in the EPM</td>
<td>66</td>
</tr>
<tr>
<td>4. Definitions of Behaviors in the FST</td>
<td>67</td>
</tr>
<tr>
<td>5. Definitions of Behaviors in the Male-Female Social Interaction Tests</td>
<td>70</td>
</tr>
<tr>
<td>6. Definitions of Behaviors in the PPT</td>
<td>72</td>
</tr>
<tr>
<td>7. Definitions of Behaviors in the Adult Social Interaction Test</td>
<td>74</td>
</tr>
<tr>
<td>8. Correlations of Behaviors Theorized to be Related</td>
<td>78</td>
</tr>
<tr>
<td>9. Active Coping Behaviors in the FST</td>
<td>90</td>
</tr>
<tr>
<td>10. Exploratory Correlations</td>
<td>109</td>
</tr>
</tbody>
</table>
ABBREVIATIONS USED IN THIS DOCUMENT

ACTH - Adrenocorticopic Hormone
BLA – Basolateral Amygdala
BNST - Bed Nucleus of the Stria Terminalis
CeA – Central Amygdala
CRH – Corticotropin Releasing Hormone
EPM – Elevated Plus Maze
FST – Forced Swim Test
GC – Glucocorticoids
GR – Glucocorticoid Receptor
HPA – Hypothalamic-Pituitary-Adrenal
LC – Locus Coeruleus
MeA – Medial Amygdala
mPFC – Medial Prefrontal Cortex
NTS - Nucleus of the Solitary Tract
PND – Post-natal Day
PPT – Partner Preference Test
PVN – Paraventricular Nucleus
SEM – Standard Error of the Mean
CHAPTER ONE:
INTRODUCTION

General Introduction to the Present Study

Social relationships and emotional processing abilities are important for the health of humans and other social species. When these relationships are fostered during childhood and adulthood they promote psychological and physical health. Research in humans has observed several associations between appropriate socialization and health, such as decreased risk of psychological and emotional disorders, physical diseases, and overall mortality (for reviews, see Holt-Lunstad, Smith, & Layton, 2010; Uchino 2006). This research is supported by animal models, that have demonstrated deficits in physical health, emotional reactivity, and behavior following exposure to social stressors during multiple phases of development, including early life, the juvenile period, and adulthood (Amiri et al., 2015; Grippo, Gerena, et al., 2007; Hol, Van den Berg, Van Ree, & Spruijt, 1999; Hong et al., 2012; Lieberwirth, Liu, Jia, & Wang, 2012; Ruscio, Sweeny, Hazelton, Suppatkul, & Carter, 2007). The present study explores the influence of social stress in juveniles using a rodent model – the prairie vole – on behaviors related to social interactions, depression, and anxiety. Prairie voles (Microtus ochrogaster) provide a valuable model to investigate the effects of early life social stress which can negatively
influence pro-social behaviors in adulthood due to their intimate family structure and their susceptibility to the consequences of social stress in adulthood (Carter, Getz, & Cohen-Parsons, 1986; Getz, McGuire, Pizzuto, Hofmann, & Frase, 1993). Prairie voles have been used in previous studies to investigate the effects of social isolation on behaviors, neurological function, and stress reactivity in adult animals (Bosch, Nair, Ahern, Neumann, & Young, 2009; Grippo, Gerena, et al., 2007; Lieberwirth et al., 2012; Sun, Smith, Lei, Liu, & Wang, 2014), and to a lesser extent in young animals (Pan, Liu, Young, Zhang, & Wang, 2009; Perkeybile & Bales, 2015; Ruscio et al., 2007). Therefore, the present study utilized the highly social nature of the prairie vole to explore the effects of limited social experiences during development on behaviors in both adolescence and adulthood.

It is important to gain a detailed understanding of the developmental consequences of experiencing early life social stress. The presence of adaptive social stimulation and positive peer interactions are critical for developmental processes that promote psychological and physiological health throughout adolescence and into adulthood. Social stimulation promotes healthy cognitive development that facilitates social awareness and encourages cooperation (Cutting & Dunn, 2006). Specifically, socialization promotes adaptive behavioral, cognitive, and physiological regulation, such as impulse control, regulation of emotions and understanding of social norms and cues, as well as promotes healthy social interactions and stress-coping abilities (Cutting & Dunn, 2006; McAlister & Peterson, 2013). The absence of social stimulation in young individuals places them at risk for pronounced negative effects, which can perseverate throughout development into late adolescence or adulthood. For example, the absence of social stimulation or exposure to juvenile social ostracism has been associated with increased risk of
behavioral and mental health problems later in life (Cutting & Dunn, 2006; Dodge et al., 2003; Lansford et al., 2006; Wang, Chassin, Eisenberg, & Spinrad, 2015).

Animal models of development have also demonstrated consequences of early social stress. Specifically, rat studies suggest that the absence of social or physical stimulation during adolescence has negative effects on novelty processing in adulthood relative to those animals housed in an enriched environment (Brenes, Rodríguez, & Fornaguera, 2008). Additionally, social isolation during multiple age ranges is a stressor that significantly alters social behaviors, stress reactivity, and depression- and anxiety-related behaviors in rodents (Grippo, Gerena, et al., 2007; Grippo et al., 2014; Lukkes, Engelman, Zelin, Hale, & Lowry, 2012; Weiss, Pryce, Jongen-Rêlo, Nanz-Bahr, & Feldon, 2004). The results from these studies highlight the importance of investigating the processes through which early life social stress and experiences shape later behaviors.

Human and animal literature is consistent in the potential negative consequences of early life social stress and the importance of adequate socialization during development. To further investigate the importance of the social environment in an animal model, the unique social structure of the prairie vole provides valuable insight into the consequences of negative early life social experiences. The prairie vole was specifically chosen as the model for the present study given its translational social structure to humans. The prairie vole is a socially monogamous rodent species that forms opposite-sex pair bonds and lives in family groups. These family groups often consist of a male and female mating pair and some older female offspring (Carter et al., 1986; Getz et al., 1993). Together, the mating pair, and sometimes the older females, care for the younger offspring (Carter et al., 1986; Getz et al., 1993). Prairie voles experience several
negative behavioral, endocrine, and neurobiological consequences from being separated from their bonded partner or family members (Bosch et al., 2009; Lieberwirth et al., 2012; McNeal et al., 2014; Sun et al., 2014). These previous studies indicate that the prairie vole is a valid and useful model to explore consequences and benefits related to early life social experiences.

Given that the prairie vole is a useful translational model of development, the present study capitalizes on their highly social nature to investigate the effects of social isolation during a critical period in early life on the development of social and emotion-related behaviors during the juvenile period and into adulthood. The social stressor was 4 weeks of social isolation in female prairie voles immediately following weaning, representing children approximately 3 to 14 years of age (Semple et al., 2013). This design models social neglect that may be experienced by human children and has been used in several studies conducted in rats and mice (Chappell, Carter, McCool, & Weiner, 2013; Hol et al., 1999; Hole, 1991; Lukkes et al., 2012; Lukkes, Mokin, Scholl, & Forster, 2009; Lukkes, Watt, Lowry, & Forster, 2009). The study design also included a resocialization period with a female sibling following isolation to model the transition into late adolescence when children may experience more autonomous choices in their social interactions. Previous studies have demonstrated that female prairie voles are sensitive to the effects of early life stress (Ahern & Young, 2009) and can recognize their siblings following separation (Paz y Miñatno & Tang-Martínez, 1999), making this animal model valuable for the present study design. During the resocialization, the animals were assessed for measures of affiliative or agonistic behaviors upon resocialization, and then remained pair housed and undisturbed until they reached adulthood. Once in adulthood, the animals underwent a series of validated operational tests to assess anxiety- and depression-related behaviors and social
behaviors. The present study hypothesizes that social isolation during the juvenile period will have lasting negative effects on juvenile social interactions, adulthood social interactions, and emotion-related behaviors. This design contributes to our understanding of behavioral consequences of early life social stress.

**Stress – Definitions**

Stress is a universally discussed term that can describe almost any experience, such as transitions and encounters, work and home life, or interpersonal or personal struggles. More simply, stress is ubiquitous. Often stress is seen as a force that can either break people or make them stronger, a test to determine if they can succeed. This personal or psychological stress is analogous to another form of stress rooted in the field of engineering, described as the physical pressure placed on a material to determine how far it can stretch, how compressed it can become, or how much weight it can carry before breaking (“Stress|Physics,” 2019). This definition of stress measures an object’s or material’s strength and fluidity as an index of its ability to adapt.

In merging the physical sciences with the psychological, Hans Selye is credited with applying a slightly new meaning to the word “stress”. Selye postulated that while stress and stressors can be a danger to life, they also encourage the development of two response mechanisms: adaptation and resistance (Selye, 1950). Subsequently, the definition of stress has been expanded to include psychological and emotional traumas. In line with Selye’s concepts of adaptation or resistance, when an individual experiences a psychological trauma and is unable to adapt to or resist the effects of the stressor, the result can be serious and manifest as a mental
illness such as depression, anxiety, post-traumatic stress disorder, or a disrupted ability to cope with future stressors.

The theory of allostasis, as it relates to psychological stress, tries to account for why individuals may not be able to adapt to or resist an emotional or psychological stressor. Allostasis involves preparations that occur in response to or in anticipation of a change in environmental demands (McEwen, 2000, 2005; Robertson, Beveridge, & Bromley, 2017). The ability to psychologically and physiologically adapt to change is considered advantageous, but it can also be disadvantageous if the scenario requiring the change is prolonged or repeated. The concept of allostasis can explain how a person might alter their behavior to adapt to a change in the environment and, ideally, diminish additional stress that may result from that change. For example, traffic and road construction are problematic and agitating. If the main road a person takes to work is under construction and adds 20 minutes to their commute, they may experience stress about being late for work. An adaptive allostatic shift may encourage the person to arise a few minutes earlier and take an alternate route to work. By contrast, an example of a maladaptive response would be for an individual to resist changing their morning routine and sit through the traffic every day. The former response is considered to be more productive as it might lead to a less stressful commute to work, whereas the latter response results in repeated exposure to the stressor, potentially increasing frustration and additional stress levels. This adaptation is representative of the allostatic load, which describes the change in behavioral patterns or biological resources to adapt to a shift in the environment (McEwen, 2000, 2005; McEwen, Eiland, Hunter, & Miller, 2012; Robertson et al., 2017). When there is an unpredictable change and an individual overcorrects to address the change, allostatic overload may occur.
Social stressors, such as a lack of social support, feelings of loneliness, or experiencing social pressure, are valuable examples of how allostasis can become unbalanced. Constant shifting in the allostatic load to adjust for prolonged social stress, such as living in a crowded home environment, has been observed to have a negative influence on health via prolonged heightened arousal (Johnston-Brooks, Lewis, Evans, & Whalen, 1998). Prolonged heightened arousal uses a large amount of the body’s resources and can increase cardiovascular activity, alter glucose metabolism, change hormone synthesis, and alter central nervous system functioning, among other negative consequences (Herman et al., 2003; Jankord & Herman, 2008; Johnston-Brooks et al., 1998; Sapolsky, 1994; Uchino, 2006). These physiological changes are associated with persistent elevations in stress levels and reduced ability to appropriately respond to the stressor, which have been reported to increase the risk of illness (Johnston-Brooks et al., 1998).

Stress exposure can also elicit downregulation of some processes or a combination of activation and downregulation. For example, processes that promote survival during a short-term stressor increase heart rate and respiration by using energy that would have otherwise been diverted to maintenance processes (such as digestion) to allow for increased oxygen levels in the blood and redirect resources to the muscles to survive a threatening event (Sapolsky, 1994). These shifts in glucose and blood flow are allostatic responses to stress that can wear on the physiological systems involved over time (Sapolsky, 1994). Additionally, stress reactivity and integration of stress-related information involves a complex pattern of activation and downregulation in several brain regions. These interactions are critical for survival during short-term stressors but may have negative outcomes on emotionality and behavior in the context of
long-term stressors. The specific neurological processes involved in stress will be discussed in the following sections.

**Stress – Physiology**

The primary objective of the stress response, like most innate physiological processes, is to promote survival. Evolutionarily, human hunter-gatherer ancestors experienced stress during life-threatening events, which played a major role in fight-or-flight reactions that would increase the likelihood of survival (Sapolsky, 1994). For example, the stress response that facilitates running from a lion is highly adaptive. Modern humans do not often encounter lions, but they may experience a similar stress response in the face of non-life-threatening stressors, such as presenting in public or meeting a significant other’s parents. The heightened arousal experienced during modern environmental changes has the potential to be harmful if the stress is chronic or extreme (Sapolsky, 1994). To parse apart the mechanisms of stress as they relate to social and emotional experiences, it is important to first understand how the stress response system operates in an adaptive way to promote health and longevity.

**The Stress Response System: Adaptive Communication**

The stress response as an adaptive survival instinct begins as soon as a threat is perceived, often prior to a person’s conscious acknowledgement of the threat. The majority of stress information integration occurs in the paraventricular nucleus of the hypothalamus (PVN), a brain region which receives information from various other brain regions, including the locus
coeruleus (LC), nucleus of the solitary tract (NTS), and bed nucleus of the stria terminalis (BNST; Jacobson & Marcus, 2011). Each of those brain regions specializes in processing vital information from other areas of the brain, including limbic regions such as the amygdala and hippocampus and cortical regions such as the medial prefrontal cortex (mPFC; Cunningham, Bohn, & Sawchenko, 1990). Together, these hindbrain, limbic, and cortical regions act to directly or indirectly influence the stress response system via communication with the PVN (Figure 1).

**Figure 1 - The Stress Response**: A depiction of the stress response and how various brain regions are collectively involved in the progression of chemical synthesis resulting from stress and the associated activation of the PVN. This figure also displays how glucocorticoids are involved in the downregulation of the stress response (from Arnett, Muglia, Laryea, & Muglia, 2016). Abbreviations: 5-HT = serotonin; ACTH = adrenocorticopic hormone; AVP = arginine vasopressin; BLA = basolateral amygdala; BnST = bed nucleus of the stria terminalis; CeA = central amygdala; CRH = corticotropin-releasing hormone; GABA = gamma-aminobutyric-acid; HC = hippocampus; NE = norepinephrine; NTS = nucleus of the solitary tract; PVN = paraventricular nucleus; Vsub = ventral subiculum.
Hindbrain structures, such as the NTS and LC, are critical for the survival instinct related to the stress response and have bidirectional projections to the PVN. The projections from the NTS transmit sensory information to the PVN, which promotes CRH release, as well as information supporting arousal and maintenance of hypotensive processes to relevant aspects of the central nervous system (Cunningham et al., 1990; Erdos, Cruickshank, Einwag, Schaich, & Wellman, 2017; Li et al., 2010). The LC has projections throughout the brain to regulate arousal and information processing related to basal neural functioning as well as survival-related vigilance (Jankord & Herman, 2008; Ziegler, Cass, & Herman, 1999). Both the NTS and LC have excitatory projections transmitting cardiovascular and autonomic information to the PVN (Luiten, ter Horst, Karst, & Steffens, 1985). The PVN then utilizes that information to regulate arousal via efferent connections to the spinal cord and dorsal vagal complex that are mediated by the hindbrain structures (Luiten et al., 1985). Together, this bidirectional communication plays a major role in the excitation and physiological arousal associated with stress. The hindbrain has critical connections to brain regions other than the PVN. Specifically, the LC has direct projections to the mPFC, which is another important component of the stress response neurocircuitry (Jankord & Herman, 2008; Marzo, Totah, Neves, Logothetis, & Eschenko, 2014). During times of threat, excitatory signals from the LC stimulate the mPFC (Jankord & Herman, 2008; Marzo et al., 2014) to encourage the mPFC process and convey important information regarding the cognitive and sensory components of stress to other brain regions.

The mPFC mediates executive functions, including higher cognitive processing and critical thinking, which is why it is an integral component of the stress response (Sapolsky, 1994). The mPFC is also a secondary sensory area because this region processes sensory
information related to the threat (Kollack-Walker, Day, & Akil, 2000). Additionally, the mPFC is involved in the stress response because it is responsible for the integration and dissemination of information related to survival mechanisms to the PVN via indirect projections (Figueiredo, Bruestle, Bodie, Dolgas, & Herman, 2003; McKlveen, Myers, & Herman, 2015). This information helps to regulate glucose metabolism requirements within the brain, effectively increasing and decreasing neural activity in other brain regions as needed (Figueiredo et al., 2003; McKlveen et al., 2015).

The projections connecting the mPFC to the PVN are complex because the mPFC promotes both excitation and inhibition. For example, the mPFC has excitatory projections to the NTS, which then communicates relevant information about arousal to the PVN (Cunningham et al., 1990; Jankord & Herman, 2008). The mPFC also has excitatory projections to the BNST, a limbic region that serves as a relay for sensory and emotion-related information to the PVN (via inhibitory signals; Figueiredo et al., 2003; McKlveen et al., 2015). This intricate communication from the mPFC to other brain structures helps to maintain adequate neural activation but also protects against hyperactivation in the stress system (Figueiredo et al., 2003; McKlveen et al., 2015).

As noted above, the mPFC has direct communication with the BNST. This limbic structure has various subregions that specialize in different actions and projections, including direct inhibitory projections to the PVN (Herman et al., 2003; Spencer, Buller, & Day, 2005). Subregions of the BNST include the anterior ventral, anterior medial, and posterior regions. These BNST subregions are interconnected. Specifically, the posterior BNST has inhibitory projections to the anterior ventral sub-region, which has excitatory projections to PVN (Choi et
al., 2007). These subregions integrate information originating from the mPFC and limbic regions, acting as a pathway to the PVN (Choi et al., 2007; Herman et al., 2003). The BNST then transmits this information via inhibitory projections to the medial parvocellular PVN, which is a neuronal cluster that is critical for the stress response (Watts, 2000).

An important function of the BNST is to mediate the indirect pathways connecting other brain regions to the PVN, making it a critical part of the stress response. The amygdala is one particular brain region that sends information to the PVN via the BNST. This limbic structure is, in part, focused on emotional regulation and fear responses. The amygdala has several subregions, that serve different functions and receive inputs about the stress response from hindbrain regions (such as the NTS) and cortical regions (Erdos et al., 2017; Li et al., 2010; Spencer, Buller, & Day, 2005; Ziegler et al., 1999). The central amygdala (CeA) receives sensory information from the NTS and cortical regions pertaining to pain and noxious stimuli (Fanselow & Gale, 2000). This subregion projects to the PVN, conveying information relevant to the endocrine system and fear conditioning responses (Fanselow & Gale, 2000). The CeA also has CRH-producing cells which respond to stress outside of the hypothalamic system but do not always lead to increased CRH in the PVN (Makino et al., 1999). Separately, the medial amygdala (MeA) has been observed to influence the stress response via norepinephrine, which is released from the LC during stress and binds to norepinephrine receptors in the MeA (Fanselow & Gale, 2000; Herman et al., 2003; Makino et al., 1999). This communication in turn influences PVN functions. Finally, the basolateral amygdala (BLA) is often associated with emotional memory and learned fear conditioning (Fanselow & Gale, 2000). While the BLA does not
communicate with the PVN via the BNST, it projects into the hippocampus, which is also important to the progression of the stress response.

In coordination with the amygdala, the hippocampus plays a critical role in the stress response and helps to promote survival. This limbic structure is responsible for a majority of memory, spatial processing, consolidation, and the integration of emotion and learning (Morris, Garrud, Rawlins, & O’Keefe, 1982). Specific to the stress response, the hippocampus receives information from the mPFC related to sensory perception and information from the BLA related to emotion or fear (Meaney & Lupien, 2000). The hippocampus integrates the information from those regions and relays specific information relevant for survival to the BNST, based on previous experiences with similar threats. The BNST then transmits the information, along with other relevant cognitive and emotion-related signals, to the PVN (Herman et al., 2003; Morris et al., 1982; Sapolsky, 1994). During stress, the hippocampus also works with the amygdala to indirectly send inhibitory signals to the PVN, via the BNST, to help to downregulate the stress response (Herman et al., 2003). Without any mechanisms to downregulate the stress response, the arousal related to the threat would not abate and cause damage to neurological structures and organs in the periphery. Therefore, the downregulation of the stress response allows the body to return various systems (immune, digestion, etc.) to basal levels of functioning (Herman et al., 2003; Sapolsky, 1994).

The PVN

The physiological chain of events that mediate the stress response promotes survival. The brain regions described above are integral components of the stress response system, by working
together to communicate with the PVN. The PVN is a part of the HPA axis and the larger hypothalamic system that integrates the incoming information directly from the LC, NTS, and BNST and begins the process of producing corticotropin-releasing hormone (CRH), adrenocorticopic hormone (ACTH), and glucocorticoids (GCs; Aguilera, 1994; Herman et al., 2003; Jacobson & Marcus, 2011; Li et al., 2010; Sapolsky, 1994). The synthesis and release of these chemicals are important for the maintenance of the stress response, including activation of peripheral organs, adaptive reallocation of resources (e.g., glucose), and downregulation processes after a stressor is experienced (Figure 2).

After receiving information from various brain regions regarding a stressor, the CRH produced from the PVN serves as a catalyst for the production of ACTH (Heimer & Van Hoesen, 2006). When CRH is released from the PVN, it then binds locally to CRH receptor 1 within the hypothalamus and throughout the limbic and hindbrain regions (Heimer & Van Hoesen, 2006; Sánchez, Young, Plotsky, & Insel, 1999). CRH receptor 1 is prominently expressed in the anterior pituitary, and a high concentration of CRH binds to those receptors (Heimer & Van Hoesen, 2006; Sánchez et al., 1999). This binding activates adenylate cyclase, which promotes the synthesis and release of ACTH into the vascular system (Aguilera, 1994; Heimer & Van Hoesen, 2006; Sapolsky, 1994).
Figure 2 – The HPA Axis and Peripheral Nervous System: A depiction of how the stress response system extends into the peripheral nervous system and how glucocorticoids are involved in the downregulation of the stress response (from Kulkarni, Gavrilidis, & Worsley, 2016). Abbreviations: ACTH = adrenocorticopic hormone; CRH = corticotropin-releasing hormone.

Once ACTH enters the circulatory system from the anterior pituitary, it binds to receptors to promote release of glucocorticoids. ACTH is a peptide hormone (Aguilera & Rabadan-Diehl, 2000) that binds to the melanocortin receptor 2 on the adrenal cortex situated above the kidneys (specifically the zona reticularis and zona fasciculata; Aguilera & Rabadan-Diehl, 2000; Gallo-Payet & Payet, 2003). The ACTH binding to melanocortin receptor 2 increases cyclic adenosine 3’, 5’-monophosphate concentration and promotes calcium influx (Aguilera & Rabadan-Diehl,
2000; Gallo-Payet & Payet, 2003). This action stimulates the production of the glucocorticoids (GCs), including corticosterone or cortisol (Aguilera & Rabadan-Diehl, 2000).

GCs are steroid hormones that are important to the function of the stress response. These products act on multiple organs to promote survival during stress and maintain daily physiological functioning. GCs bind to glucocorticoid receptors (GRs) throughout the body to promote general organ functions and survival processes (Garabedian, Harris, & Jeanneteau, 2017; Herman et al., 2003; Sapolsky, 1994). For instance, GRs are present in endothelial cells in the cardiovascular system, as well as in the liver, digestive system, and adipocytes (Garabedian et al., 2017). Due to the high presence of GRs throughout the body, the GCs alter the transcription factors in the cells responsible for maintenance of important functions such as bone strength, glucose metabolism, and cardiovascular tone (Garabedian et al., 2017). During stress, GC-GR interactions facilitate glucose utilization for critical organ functioning, promote autonomic processes, and also have cardioprotective and anti-inflammatory properties to aid in survival and recovery from a threat (Kadmiel & Cidlowski, 2013). Therefore, GCs are important to the stress response as they optimally regulate the distribution of glucose away from processes such as digestion and reproduction to the muscles and brain during stressful or threatening experiences (Chrousos, 2009; Garabedian et al., 2017; Herman et al., 2003; Sapolsky, 1994).

In addition to being critical to the active stress response in the peripheral nervous system, GCs are responsible for the downregulation of the stress response by engaging in negative feedback to the brain. After crossing the blood-brain barrier, GCs can bind to GRs or mineralcorticoid receptors, which are present in several brain regions. The GCs bind to receptors throughout the brain to downregulate chemical synthesis in those regions. Specifically, GC
binding in the pituitary inhibits ACTH production, and binding in the PVN reduces CRH production (Gjerstad, Lightman, & Spiga, 2018; Juruena, Cleare, & Pariante, 2004; displayed in Figures 1 & 2). GC binding in the hippocampus and mPFC also downregulates the stress response. This downregulation occurs through various mechanisms depending on the region, each culminating in a signal directing the PVN to decrease stress responding. For example, the GC binding within the hippocampus stimulates inhibitory signals to the PVN, via the BNST, to diminish the production of CRH (Herman et al., 2003; Sapolsky, 1994; Taves, Gomez-Sanchez, & Soma, 2011). Similarly, GC binding to GRs in the infralimbic mPFC reduce downstream GC production, via BNST connections, by inhibiting CRH production at the level of the PVN (McKlveen et al., 2015).

The Stress Response System: Maladaptive Outcomes

The previously described mechanisms underlying the stress response involve a delicate balance of neural activation and hormone regulation, with the short-term response to a threat serving a critical function of promoting survival. However, several negative consequences may occur as a result of prolonged exposure to stress or an inability to cope with stressors. Throughout the millennia, the main threats that animals were required to cope with and overcome revolved around survival. These stressors included goals such as having enough food, finding a mate, and escaping predation. By contrast, modern-day stressors may include paying off student loans or focusing on perfecting a presentation for work in the hopes of earning a promotion. These changes are recent in our evolutionary history. The stress response system has yet to adapt to the stress of traffic or work in a high-pressure field, which may explain why
social, emotional, or psychological stressors evoke the same physiological response in humans as a zebra encountering a lion.

The threat of being attacked by a lion elicits activation of the stress response. The stress response is commonly discussed in the context of fight-or-flight, which implies a physical threat. The shifts in glucose metabolism and cardiovascular tone specifically promote adaptive physical reactions to the physical threat. Unfortunately, modern-day individuals cannot fight or run from a demanding work environment without experiencing some professional consequences, even if the brain and body are preparing to run out of the conference room door. Enduring the physiological and neurological consequences of an otherwise adaptive stress response for a prolonged period of time (i.e., chronic stress) can have serious negative effects on neurobiological and psychoemotional health.

Consequences of stress on the health of neurobiological and psychological function are related to CRH, ACTH, and GCs, which are produced as a part of the stress response. As discussed above, the PVN integrates information related to stress from other brain regions and subsequently secretes CRH. While this production is critical during exposure to a life-threatening stressor, this process can become disrupted following excessive neural excitation in response to chronic stress, resulting in hyper-secretion of CRH from the PVN (Herman & Tasker, 2016; Makino et al., 1999; Pournajafi-Nazarloo et al., 2011; Sink et al., 2013). Research with animal models has consistently demonstrated that chronic stress paradigms upregulate CRH mRNA within the PVN, which consequently increases production of CRH (Herman & Tasker, 2016). Additionally, evidence suggests that chronic stress damages the inhibitory projections from the BNST to the PVN, which are important for decreasing CRH release from the PVN (Herman &
Tasker, 2016). These effects can have lasting negative consequences on proper functioning of inhibitory projections and the HPA axis as a whole (Makino et al., 1999; Pournajafi-Nazarloo et al., 2011; Sink et al., 2013). For example, the CeA and lateral BNST become more active during prolonged stress exposure, which increases production of CRH from the cells within those brain regions, increasing total levels of CRH throughout the brain (Makino et al., 1999; Sink et al., 2013).

Because CRH acts upon the anterior pituitary to produce ACTH (Heimer & Van Hoesen, 2006), CRH-ACTH interactions may also become dysfunctional in the context of chronic stress. Chronic stress increases POMC levels, which may be responsible for reports of increased pituitary sensitivity to CRH and the subsequent associated increase of ACTH (Franco et al., 2016). This sensitivity and increased ACTH production can damage and affect the size of the adrenal glands (Herman & Tasker, 2016) due to ACTH binding to the adrenals and stimulating the hyper-production of GCs during prolonged stress exposure (Herman et al., 2003; Sapolsky, 1994).

Although steroid hormones are important for a variety of physiological process and play a critical role in the short-term stress response, several consequences can occur if GC production or binding becomes dysregulated. Chronic production of GCs, resulting from high levels of CRH and the subsequently elevated levels of ACTH binding to the adrenal glands, can result in altered energy distribution in the periphery (Gallo-Payet & Payet, 2003; Garabedian et al., 2017; Kadmiel & Cidlowski, 2013). This can lead to overactivation of the survival mechanisms that prioritize cardiovascular and muscular systems over other important processes such as digestion and reproduction (Chrousos, 2009; Garabedian et al., 2017; Sapolsky, 1994). These systems are
critical for general physiological processes and long-term functions in the body. Therefore, chronic stress-induced downregulation of these systems can be damaging and have long-term consequences. Additionally, high levels of GCs encourage increased glucose production and fat storage, which would be useful if running from a lion but can cause weight gain and insulin imbalances if sitting at a desk (Chrousos, 2009; Garabedian et al., 2017; Sapolsky, 1994; Shpigel, Chen, Avidar, & Bogin, 1996).

The negative effects of chronic GC production are not limited to the periphery. GC binding to GRs and mineralcorticoid receptors throughout the brain are important for downregulating the stress response (Gjerstad et al., 2018; Uchoa et al., 2014). However, chronic stress and the hyper-production of GCs lead to a disproportionate number of GCs relative to receptors, resulting in an excess of unbound GCs (Brinks, de Kloet, & Oitzl, 2009). If the concentration of unbound GCs gets too high, then they can cause neural damage. For example, the receptors to which the GCs bind are located in the cytosol of the cell, but when there is a high concentration of GCs, they bind to inappropriate receptors, which can alter transcriptional factors within the nucleus of the cell (Godoy, Rossignoli, Delfino-Pereira, Garcia-Cairasco, & de Lima Umeoka, 2018).

The damaging effects of the GCs can have a lasting impact on the health and function of the stress response. GCs preferentially bind to the mineralcorticoid receptors in the hippocampus and the amygdala (Garabedian et al., 2017; Gjerstad et al., 2018; Herman & Tasker, 2016; Uchoa et al., 2014), and under high stress conditions the GCs bind to all of the available mineralcorticoid receptors (Garabedian et al., 2017; Herman & Tasker, 2016). When the mineralcorticoid receptors become full, the GCs begin binding to GRs, which usually triggers
inhibitory projections to the PVN. However, chronic stress can lead to an overproduction of GCs, which can dysregulate calcium channels on these receptors (Brinks et al., 2009; Fries, Hesse, Hellhammer, & Hellhammer, 2005). The calcium channels are critical for the maintenance of electrical signals from one cell to another, and when these channels become dysregulated, the neuron can experience dendritic arborization and possibly apoptosis - both of which can have lasting consequences on neural functions (Brinks et al., 2009; Fries et al., 2005).

Specifically, cells in the hippocampus are at high risk of injury due to the high levels of GRs in the region (Brinks et al., 2009; Fries et al., 2005). When the GRs become damaged or unreceptive to chemical signals, following damage to the calcium channels, the inhibitory projections from the hippocampus to the PVN (which downregulate the CRH - ACTH - GC process) are weakened (Fries et al., 2005; Herman et al., 2003; Jankord & Herman, 2008). This prolongs the stress response and promotes further damage to the hippocampus and related brain regions (Fries et al., 2005; Godoy et al., 2018).

The effects of chronic stress can also have lasting implications on the physiological processes involving GCs. For example, chronic stress exposure results in increased basal levels of GCs. The subsequent GC response to stress can become either sensitized or habituated to later exposures to the stressor depending on the context (Lowrance, Ionadi, McKay, Douglas, & Johnson, 2016; Weinberg, Johnson, Bhatt, & Spencer, 2010). Specifically, repeated exposure to the same stressor results in a habituated stress response (Lowrance et al., 2016), whereas unpredictable or a variety of different stressors results in a sensitized stress response (Weinberg et al., 2010). This may be due to chronic unpredictable stress reducing GR expression within the
PVN (Herman & Tasker, 2016), which would then result in increased levels of unbound GCs throughout the brain (Brinks et al., 2009).

**Social Stress – Behavioral and Physiological Consequences on Development in Humans**

The previous sections discussed the degree to which the stress response, while generally adaptive, can be maladaptive following extreme or prolonged stress exposure. These consequences are not limited to physiological processes, but also can influence mood and behavior when experienced in children. A large body of research suggests that exposure to chronic or intense stress during the childhood or juvenile period of development can have significant maladaptive effects into adulthood (Heim, Shugart, Craighead, & Nemeroff, 2010). During the early years of development, children are heavily dependent on others for survival and social stimulation. This dependency increases their vulnerability to abuse and neglect, beginning the vicious cycle of early life stress on adulthood health. Early life social stress increases the risk of behavioral and communication problems in childhood (Dodge et al., 2003; Lansford et al., 2006), as well as mental and physical health problems in adulthood (Johnson, Smailes, Cohen, Brown, & Bernstein, 2000).

**Importance of Social Support in Adulthood**

To better understand the influence of early life social stress on various health systems in adulthood, it is important to first understand the benefits of a positive social environment during adulthood. Social support has health benefits in people of all ages. Older individuals with a
social support system, such as a spouse, are less likely to develop diseases, have a better immune system, display more adaptive coping strategies, exhibit fewer symptoms related to mood disorders, and live generally happier and healthier lives (López-Cerdá, Carmona-Torres, & Rodríguez-Borrego, 2019; Moore et al., 2015; Sbarra, 2009; Sherman, Cheng, Fingerman, & Schnyer, 2016). Similarly, younger adults in college have reported lower levels of academic-related anxiety if they have a significant other in whom they can confide and receive social support (Renk & Smith, 2007).

The absence of social support or feelings of loneliness have been associated with measures of poor health. Loneliness negatively affects the immune system in adults (Uchino, 2006). The lack of social support in older single men increases levels of C-reactive protein, which is associated with higher levels of inflammation (Sbarra, 2009). Loneliness also alters basal and diurnal fluctuations in cortisol levels in adult men and women (Doane & Adam, 2010). Levels of loneliness are positively correlated with cardiovascular reactivity to a mental stressor, with lonely men and women displaying altered neuroendocrine responses to the stressor (Steptoe, Owen, Kunz-Ebrecht, & Brydon, 2004). Together, these data indicate that an absence of social support has consequences on physical health as well as exacerbates the physiological responses to stress.

In addition to physiological consequences, loneliness and isolation are associated with mood disorders such as depression (Cacioppo, Cacioppo, Capitanio, & Cole, 2015). Specific investigations using subclinical populations observed a positive relationship between levels of self-reported depressive symptoms and feelings of social stress (Wang, Cai, Qian, & Peng, 2014). This relationship was statistically stronger in the group reporting lower levels of social
support, suggesting that the association between depressive symptoms and stress is moderated by social support (Wang et al., 2014). Similarly, a positive relationship exists between perceived stress and rates of depression and anxiety in adult clinical and subclinical populations (Bergdahl & Bergdahl, 2002; Galaif, Sussman, Chou, & Wills, 2003). Together, the results of these studies support the positive connection between social support and health as well as highlight the various consequences of social stress in adulthood.

Parental Influence on Development

Social connections are important in adulthood but are critical for infants and young children, and exposure to social stressors during development, such as difficult social bonds, may have serious implications. Parents serve an important role in a child’s development, and their influence on temperament can be seen in early infancy. One paradigm, the Strange Situation, investigated the degree to which infant reactions to a mild social stressor might relate to levels of parental engagement at home, as a measure of parent-infant attachment styles. This design focused on the infant’s reactions to their parents leaving them in a room and then returning to the room. These methods provided insight into parent-child attachments, and the development of social awareness in children (Ainsworth, 1979, 1985; Bretherton, 1992). Some children cried when their parents left and were consoled when they returned; while other children did not appear upset when their parents left but cried upon their return (Ainsworth, 1979). This flagship study suggested a level of emotional understanding that is not usually associated with such young children (Ainsworth, 1985). The behaviors of the children were, perhaps, the result of the inconsistent parenting styles causing disappointment and distress in the child, such that the
variation in attachment styles may represent a coping mechanism (Ainsworth, 1985). The researchers speculated that children as young as one year old were capable of understanding that a negative relationship with a parent causes pain and learned to protect themselves from that pain (Ainsworth, 1979; 1985). If infants experience social pain, then they are also suffering the effects of the stress of a tenuous parent-infant relationship.

Similar research has focused on the behavioral consequences of older children who experience the stress of being raised by a single mother. The experience of children raised by a single mother is different from children raised by two parents, possibly because these children receive less attention from their single parent than bi-parentally raised children (Waldfogel, Craigie, & Brooks-Gunn, 2010). Children raised by single mothers are more likely to score higher on aggression indexes, controlling for age, race, and socioeconomic status of the mother, relative to children raised by two parents (Osborne & McLanahan, 2007; Waldfogel et al., 2010). Interestingly, the single mothers themselves report higher levels of stress and depression relative to mothers in a two-parent household, suggesting that a single-parent household can have consequences on the caregiver as well as the children (Waldfogel et al., 2010). Collectively, these data highlight the consequences associated with early life social stress in the form of being raised by a single parent on the behavioral and emotional health of the child and on the psychological well-being of the parent.

Importance of Peer Interactions on Development

While parents have important roles in a child’s development, siblings and peers also provide critical early social experiences. These social connections help children to understand
that there are different perceptions, thought processes, and experiences from their own, which is important for later development of social understanding and emotional regulation. Children who have several sources of social interactions experience a greater diversity of social experiences at a young age and develop social skills and emotional regulation at an earlier age than children who are more socially restricted (Cutting & Dunn, 2006; McAlister & Peterson, 2013; Peterson et al., 2016). Overall, the ability to understand how other people interact with the world is critical to social and emotional cognition and influences how children behave in various situations. However, if these social skills are not fully developed due to poor socialization, the lack of emotional regulation can manifest as antisocial behaviors later in childhood (Cutting & Dunn, 2006; McAlister & Peterson, 2013; Peterson et al., 2016). These reports highlight the importance of beneficial social experiences during early childhood.

Given the importance of sibling and peer relationships for promoting behavioral development, an absence of positive social experiences during childhood or adolescence has been associated with negative outcomes. Childhood rejection decreases social information processing and increases risk of developing depression later in life (Dodge et al., 2003; Lansford et al., 2006; Wang et al., 2015). Socially rejected females display a higher level of negative affect and lower levels of social competence relative to socially accepted females (Lansford et al., 2006). These data suggest that diminished social experiences during early childhood can have lasting negative consequences on behaviors and social competency, including important skills such as cooperation, communication abilities, and problem solving (Cutting & Dunn, 2006; Dodge et al., 2003; McAlister & Peterson, 2013; Peterson et al., 2016).
The importance of positive social experiences and the consequences of their absence can be investigated by studying the experience of ostracism in children. Ostracism or intentional exclusion is considered to be a form of bullying in school-age children (Abrams, Weick, Thomas, Colbe, & Franklin, 2011). Ostracism has been associated with negative effects on the social and psychological needs of young individuals (Abrams et al., 2011). Such experiences have been reported to increase prevalence of negative emotions during childhood and adolescence (Wölfer, Bull, & Scheithauer, 2012). Additionally, the way in which children respond to the individuals inflicting the ostracism can have lasting effects on the child’s behavior. Children who respond by avoiding engagement with the bullies have been observed to be more withdrawn and display more physically aggressive behaviors compared to children who respond to the ostracism with pro-social behaviors (Wölfer et al., 2012). Collectively, the conclusions of these studies highlight the consequences of exclusion on emotional experiences and social behaviors.

**Absence of Social Support in Development**

The previous sections discussed in detail the importance of social experiences for development and possible consequences of an absence of socialization. Consequently, the experience of social deprivation can last for long periods of time and have permanent consequences. This is categorized as social neglect. According to the Child Welfare Information Gateway (2019), child “neglect” primarily focuses on physical and medical needs such as food, clothing, and shelter, with only some attention given to the importance of a child’s emotional needs. Emotional neglect has recently been included in the overall definition of “neglect” as
defined by the Child Welfare Information Gateway (2019) but varies by state. The social and emotional development of a child is, as described in the previous sections, critical for their physical, psychological, and behavioral health. Most research focusing on social or environmental deprivation has studied children in institutions or orphanages. Specifically, children exposed to these harsh environments have higher scores on indices of anxiety and depression, attentional issues, aggression, and social or emotional problems when compared to children who had been adopted from international agencies (Spratt et al., 2012).

These behavioral and emotional alterations are not only observed in children exposed to neglect in orphanage settings but can be experienced by children who encounter any level of deprivation. The effects of understimulation, lack of socialization, or other forms of environmental deprivation have been reported to stunt almost every measure of social-emotional development. These measures include the ability to cooperate with others, ability to conceptualize and understand that other people have different experiences, and the tools to process emotions appropriately instead of responding impulsively (Dodge et al., 2003; Lansford et al., 2006; Peterson, O’Reilly, & Wellman, 2016; Wang et al., 2015). Social neglect and associated outcomes increase the likelihood of children developing maladaptive stress responses and consequently multiple psychopathologies (Dodge et al., 2003; Lansford et al., 2006; Peterson et al., 2016; Spinhoven et al., 2010; Wang et al., 2015). These data provide strong evidence to support the hypothesis that early life stress has persistent and serious consequences on behaviors, stress responses, and psycho-emotional well-being.
Effects of Early Life Stress on Adulthood Health

Early life social stress can have consequences that perseverate into adulthood and influence behavior, health, and emotions. Early life maltreatment, such as emotional, physical and sexual abuse, maternal rejection, or harsh punishment, produce significant consequences in adulthood. These consequences include increased inflammation, increased life stress, and fewer health-related behaviors, compared to adults who did not report experiencing childhood maltreatment (Danese, Pariante, Caspi, Taylor, & Poulton, 2007). These data highlight the range of early life stressors that can have a lasting impact on health and behaviors in adulthood.

In addition to the influence of physical childhood maltreatment, emotional abuse and neglect are also associated with later negative psychological and physiological consequences. For example, childhood emotional neglect has been associated with increased risk of developing avoidant and paranoia-related personality disorder symptoms during adolescence and early adulthood (Johnson et al., 2000). Similarly, emotional maltreatment has also been associated with increased vulnerability to develop depression in adulthood (Gibb et al., 2001). Finally, a timed regimen of dexamethasone followed by an exogenous increase of CRH resulted in elevated cortisol levels in healthy control subjects, but the physiological stress response was blunted in the individuals who experienced childhood maltreatment (Carpenter et al., 2009). Interestingly, in a follow-up study conducted by the same lab with the same methods, the researchers reported a significant effect of age on cortisol reactivity, indicating that older adults (35-61) displayed a larger difference in cortisol responses from the controls than the younger (18-35) cohort of participants (Carpenter, Shattuck, Tyrka, Geracioti, & Price, 2011). This specific conclusion might represent evidence of early life stress beginning a cumulative
emotional process involving repeated exposures to various social stressors, possibly due to a diminished capacity to cope with the early life stressor. These studies provide evidence that supports the data presented in the previous sections, which highlight the impact of early life experiences on physical, emotional, and behavioral health in humans.

**Animal Models and Consequences of Early Life Stress**

Impoverished environments can be damaging to human development, as previously discussed; however, there are confounds and ethical considerations involved with human research. Due to such limitations, there is a wealth of research and background literature on animal models of environmental manipulations during early life and the juvenile period. The following sections will summarize the rodent research related to the present study and discuss the strengths and limitations of paradigms that manipulate the early social environment.

**Models of Maternal Influence/Separation**

Rodent models have been developed to investigate the behavioral and physiological effects of early life stressors. For example, some paradigms include early life pre-weaning manipulations such as maternal separation and maternal deprivation. These study designs focus on the importance of consistent maternal contact during rodent development. Deprivation is composed of one 24-hour period where the pups are separated from the dam, whereas separation involves multiple sessions of shorter (approximately 3 hours) separations of the pups from the
dam (Lehmann & Feldon, 2000). Some studies have observed that maternal separation or maternal deprivation increases measures of behaviors related to anxiety in the elevated plus maze (EPM); however, these effects have not been observed consistently across all studies (Aisa, Tordera, Lasheras, Del Río, & Ramírez, 2007; Daniels, Pietersen, Carstens, & Stein, 2004; Hulshof et al., 2011; Lajud, Roque, Cajero, Gutiérrez-Ospina, & Torner, 2012). Other studies have explored the effects of maternal separation in combination with social isolation at a later timepoint (post-weaning) on the stress response system. For instance, social isolation with and without maternal separation increased circulating corticosterone following a forced swim test (FST) stressor, compared to control conditions and maternal separation (without social isolation) (Vargas, Junco, Gomez, & Lajud, 2016). Maternal separation with and without social isolation was associated with increased levels of blood glucose compared to control conditions and isolation only (Vargas et al., 2016), which is likely also associated with GCs and the stress response. These paradigms highlight the importance of early life experiences on long-term behavioral and physiological consequences in the offspring.

Maternal care research focuses on variations in the amount of time that a dam spends attending to her offspring and has several implications for behavioral and physiological responses to stress. The amount of time that a dam spends licking and grooming her pups in conjunction with the time that she spends in the optimal nursing position (arched back) is a heavily researched area of maternal care (Caldji et al., 1998; Francis, Young, Meaney, & Insel, 2002; Meaney, 2001). High levels of maternal care reduce the HPA axis response to stress in adulthood, possibly due to increased sensitivity of the HPA axis to the downregulation signals (Fish et al., 2004; Kappeler & Meaney, 2010). Variations in maternal care, via a laboratory
manipulation or the dam’s natural behaviors, can have lasting consequences on the health of the offspring. Studies involving cross-fostering, in which the offspring of one dam are reared by a different dam, have demonstrated that offspring reared by dams with a naturally high level of maternal care display lower levels of adulthood anxiety- and depression-related behaviors (Weaver et al., 2004; Weaver, Meaney, & Szyf, 2006). Those maternal behaviors are then transmitted to the second generation of females regardless of the care level of the biological dam (Weaver et al., 2004, Weaver et al., 2006). These studies provide further support for the importance of adaptive early social experiences on the development of behavioral and physiological processes that promote health.

**Models of Play and Social Interactions During Animal Development**

In addition to animal models that focus on investigating the consequences of early life mother-offspring interactions, other models have focused on the influence of additional social interactions during early developmental periods. For example, models focused on peer and sibling relationships in rodents provide valuable information regarding the benefits of pro-social interactions, such as play behaviors. Similar to social interactions and play in developing human babies and children, littermates in rodents provide rewarding social stimulation in the form of play (Pellis & Pellis, 1987). In rodents and many other animal species, play behaviors can appear to be aggressive because the specific actions include lunging, biting, and other aggressive behaviors. However, these actions are playful. Play behaviors of rats have been described in detail by Pellis and Pellis (1987). These behavioral categories include lunges toward a conspecific; wrestling or tumbling, pinning with the conspecific underneath the animal usually in
the supine position with their ventral surface exposed; and boxing behaviors with the animals, on their hind legs swatting at each other (Pellis & Pellis, 1987; 1998; Figure 3). For the purpose of the present study, the term “play” will be used, although “play” and “play-fighting” are both used throughout the literature. These pro-social interactions are beneficial for developing the animal’s locomotor abilities and reflexes (Pellis & Pellis, 1987; 1998) and to help encourage natural and non-threatening exposure to competition (Pellis, 1988; Pellis, Pellis, & Bell, 2010; Vanderschuren, Achterberg, & Trezza, 2016). When adolescent male rodents engage in play behaviors, they also learn strategies to prepare for the unexpected, including possible attacks from predators and from conspecifics within the nest hierarchy, which is one reason that the play behaviors may appear to be aggressive (Pellis, 1988; Pellis et al., 1989).

Given the benefits of pro-social play behaviors, a lack of these interactions can have negative consequences on the brain and behavior. Pro-social play behaviors are associated with increased cellular coordination in various brain regions (van Kerkhof, Damsteegt, Trezza, Voorn, & Vanderschuren, 2013). Consequently, an absence of play opportunities via social isolation reduces biomarkers of plasticity in the prefrontal cortex (Leussis & Andersen, 2008), negatively influencing social/emotional regulation and other higher-order brain functions such as cognition, decision making, and the ability to manage stress.

Previous research has provided useful information about possible mechanisms by which variations in naturally occurring levels of play behaviors may influence development. Some studies have investigated the importance of access to play behaviors by housing animals together that have mismatched naturally occurring levels of play behavior (Bell, McCaffrey, Forgie, Kolb, & Pellis, 2009). When access to play is restricted in animals with naturally high levels of play
by housing them with animals displaying lower levels of play), animals show deficits in certain physical and cognitive tasks, including issues with movement and memory during the Morris water maze task (Bell et al., 2009; Pellis, Field, & Whishaw, 1999). Additional studies that have isolated animals to prevent play have reported consequences on anxiety- and depression-related behaviors (Leussis & Andersen, 2008). Considered together, these studies provide evidence that

**Figure 3 – Rodent Play Behaviors:** A depiction of rat play behavior (from Pellis & Pellis 1987). Animal 1 approaches from the rear (a) and pounces on Animal 2’s neck (b), Animal 2 then turns to face Animal 1 (c & d), but is then pushed on to their side by Animal 1 and Animal 2 responds by reaching for Animal 1’s neck (e-h). The animals continue to alternate among these play behaviors (i-o).
social interactions in the form of play behaviors are associated with the development of executive function and other cognitive skills, similar to previously discussed results in highly socialized children (McAlister & Peterson, 2013; Pellis, Pellis, & Himmler, 2014; Vanderschuren et al., 2016). Healthy development of these executive functions is related to emotional regulation and stress-coping abilities, which may in turn protect individuals from later development of anxiety- and depression-related behaviors.

The absence of pro-social interactions during development has been reported to affect later social interactions as well. Young rodents show an increase in play-related behaviors upon being reunited with a sibling following up to 20 hours of separation from the sibling (Hole, 1991; Holloway & Suter, 2004). Interestingly, increases in play behaviors occur after 20 hours of isolated housing, as well as after animals are denied physical contact but allowed other types of interactions via a porous divider in the cage, such as sight and smell of the other animal (Hole, 1991). Therefore, it may be the case that the increase in play observed following a period of imposed isolation or physical separation (via a barrier) represents a compensatory response to the lack of opportunities for play. The increases in play behaviors following a more prolonged period of play deprivation (2 weeks, via wire mesh separation) are not altered by access to a running wheel during separation, suggesting that the drive to engage in play is not associated with total amount of physical activity (Holloway & Suter, 2004). Moreover, separating rodents with a divider during peak times in play behaviors has negative consequences on intromission frequency in males during social interactions, similar to those seen in animals that were completely isolated (Spevak, Quadagno, Knoeppel, & Poggio, 1973). Together, these studies suggest that play behaviors are important for adequate development, not solely due to the
physical activity associated with play but also due to the social interactions that are inherent to physical play.

Models of Post-Weaning Social Isolation

Combined with studying the influence of early life (pre-weaning) social behaviors, investigation of several consequences in rodents following weaning can inform our understanding of behavioral and physiological responses to adulthood stress. Rodent models have been used in this context to study the consequences of social isolation during different periods of development. Several studies have reported increased locomotor activity in elevated maze tasks, increased immobility in the FST, and other behavioral deficits as a function of early life social isolation in rodent models (Amiri et al., 2015; Brenes et al., 2008; Butler, Ariwodola, & Weiner, 2014; Chappell et al., 2013; Hong et al., 2012; Lukkes, Mokin, et al., 2009; Schubert, Porkess, Dashdorj, Fone, & Auer, 2009; Weiss et al., 2004; Wilkinson et al., 1994). However, the time frame during which isolation is presented can influence the outcomes of the study. For example, a 3-week period between post-natal days (PNDs) 21 and 40 is considered to be critical for the development of neural and behavioral health in rodents (Bledsoe, Oliver, Scholl, & Forster, 2011; Lukkes et al., 2012; Lukkes, Mokin, et al., 2009; Ruscio et al., 2007; Semple et al., 2013). During this particular phase of rodent development, the neurological maturation is similar to what is seen in humans between the ages of 3 and 11 years old (Semple et al., 2013). Isolation and other stressful manipulations between PNDs 21 and 40 have significant consequences on measures of affective and social behaviors into adulthood (Lukkes, Watt, et al., 2009). During a social interaction task as a measure of social competence in rats, animals display
maladaptive social behaviors following isolation beginning at PND 22 (from PNDs 22-28 or from PNDs 22-35; Hol et al., 1999). These behaviors were not evident in animals following isolation beginning at PND 28 (PNDs 28-35; Hol et al., 1999). Following social isolation from PNDs 21-42 and pair housing through adulthood, animals display increased anxiety-related responses to a novel environment (Lukkes et al., 2012). These studies highlight the importance of considering the time period during which social isolation is applied, as well as the timing and method of resocialization, for understanding consequences on social and emotional behaviors.

Studies focused on the effects of post-weaning social isolation have also examined physiological consequences of early life stress. For example, social isolation housing between PND 23 through PND 60 has resulted in decreased volume of the frontal cortex, which is associated with higher-order cognition (Schubert et al., 2009), and reductions in norepinephrine in the ventral striatum, which is associated with mood disorder symptoms (Brenes et al., 2008). Early life social stress has been associated with altered neurophysiology and corticosterone levels, which have negative behavioral outcomes. These consequences include increased ethanol intake and blunted sensitivity to corticosterone in adulthood, which is associated with increased measures of anxiety (Butler et al., 2014; Weiss et al., 2004). Together, these data highlight adulthood behavioral and physiological consequences of juvenile social isolation.

The Prairie Vole Model

The previous sections provide evidence that rodent models of early life social experiences can have long-lasting behavioral and physiological consequences. In addition
to these models, the prairie vole model has been used previously in several contexts to explore
the effects of social stress, the disruption of social bonds, parental behaviors, and reproductive
strategies. Prairie voles provide a unique rodent model of social interactions due to their
cooperative living, social monogamy, and bi-parental nature – all of which contribute to the
translation of the early life rearing environment to that of humans (Carter, DeVries, & Getz,
1995; Getz et al., 1993). These animals provide an interesting perspective on possible processes
through which social structure and health are intertwined. Prairie voles in the wild live in
extended family groups and cohabitate together in male-female pairs (Getz et al., 1993). These
animals engage in bi-parental rearing of the offspring, a rare behavior for mammals but which is
observed in humans, making the prairie vole a representative model of human family structures
(Ahern et al., 2011; Carter et al., 1995; Getz et al., 1993). Due to their involved family structure,
in-breeding is rare; there have been few reported instances of siblings or parent-offspring dyads

Several previous studies have capitalized on the prairie vole’s natural behaviors to
explore various combinations of social stress and buffering effects of social bonds. This research
has discussed possible mechanisms through which social connections buffer against the
behavioral, endocrine, neurological, and cardiovascular consequences of social stress (Bosch et
al., 2009; Fowler, Liu, Ouimet, & Wang, 2002; Grippo et al., 2014; Grippo, Lamb, Carter, &
Porges, 2007; Grippo, Wu, Hassan, & Carter, 2008; Lieberwirth et al., 2012; McNeal et al.,
2014; Pournajafi-Nazarloo et al., 2011; Stowe, Liu, Curtis, Freeman, & Wang, 2005; Sun et al., 2014; Watanasriyakul et al., 2017). For instance, prairie voles provide a unique perspective on the degree to which social isolation or pair-bond disruption influences anxiety- and depression-related behaviors, social behaviors, and physiological functions (Grippo et al., 2008; Lieberwirth et al., 2012; McNeal et al., 2014; Sun et al., 2014). In addition to this previous research, other studies have focused on parental care and developmental manipulations using the prairie vole model, which will be discussed in the sections to follow.

Parental Influence

Studies conducted in juvenile prairie voles have investigated the influence of various pre-weaning manipulations on later pro-social behaviors. Because prairie voles engage in bi-parental rearing of the offspring, one valuable experimental manipulation involves removing the sire from the dam prior to the birth of the litter. Animals reared by the dam alone receive less overall attention than those reared by both parents, an effect exclusively driven by the absence of the sire and not due to the dam reducing her level of attention devoted to the pups (Ahern & Young, 2009). A follow-up experiment indicated that adult females reared by a single mother displayed altered pup-directed behaviors such as licking/grooming duration of a novel pup and an increased latency to form a pair bond with a stranger male, compared to bi-parentally reared females (Ahern & Young, 2009). A similar study in mandarin voles, which are also socially monogamous, investigated the influence of parental involvement on play behaviors of the offspring. The absence of the sire resulted in decreased overall play behaviors of the offspring, likely due to the observation that male vole pups mostly engage in play-related behaviors with
the sire (Chau et al., 2008; Wang, Tai, Yan, & Yu, 2012). The data from these studies address the importance of a bi-parental rearing environment in prairie voles, for whom intact family groups are a critical component of early life. In conjunction with the previously discussed importance of play behavior for the development of social behaviors and general survival, these conclusions suggest that a lack of paternal interaction may interfere with the development of the pups’ pro-social behavioral lexicon.

Additionally, disruptions in early life parental influence produce neurobiological changes in monogamous voles. The absence of the sire during post-weaning development in male mandarin voles increased plasma corticosterone in the PVN and anterior hypothalamus but decreased oxytocin immunoreactivity in the PVN in adolescence (Wang et al., 2012). Interestingly, female prairie voles reared in the absence of a sire displayed increased oxytocin immunoreactivity in the PVN in adulthood (Ahern & Young, 2009), suggesting a moderating effect of sex or age on the oxytocin system following pre-weaning social stress. Elevations of oxytocin in the brain are generally associated with pro-social and anxiolytic responding, suggesting an interesting potential influence of stress on the oxytocin system (Hammock, 2015). Together, these data suggest that there are lasting neural effects of early environmental manipulations, which may be sexually dimorphic, species specific, and time dependent. These studies can provide insight into neurobiological consequences of early life social interactions in a region of the brain that is associated with stress reactivity and social behavior.
Prairie voles have also been used to explore the influence of play behaviors on the development of social behaviors. As described previously, play behaviors are beneficial for development for several reasons. The interactions with a conspecific via play teach the animals how to prepare for the unexpected, defend against attack, adapt to changing social structures, and help them protect their place in the nest hierarchy (Pellis, 1988; Pellis et al., 1989). Those coordinated movements and developed social cognition help the animal to be more reproductively successful and increase survival by promoting adaptation to shifting social structures and potentially hostile environments (Spevak et al., 1973; Vanderschuren et al., 2016).

In the prairie vole, play usually begins around PND 14 (pre-weaning) and peaks between PND 20 and 30 (Chau et al., 2008). In the beginning of play, the prairie vole will usually engage with the sire and their littermates but will rarely try to engage in any play-related behaviors with the dam, which highlights a unique facet of the prairie vole rearing environment (Chau et al., 2008; Wang et al., 2012). The prairie vole’s familial social structure is different from rats and mice because they are reared with the sire, but it is reasonably similar to that of humans (Ahern et al., 2011; Carter et al., 1995; Getz et al., 1993; McGuire & Getz, 1991). Therefore, the prairie vole is a useful model for studying the effects of social isolation and play deprivation during the juvenile period.

Although there is some literature on the influence of play behaviors in other rodents (discussed above), the literature is less clear on the definitions and functions of play behaviors in prairie voles. Further, there are some disagreements about the distinction between specific behaviors that represent play versus aggression. For example, acts of play in prairie voles may include mutual upright (standing on hind limbs) boxing, lunging/attacking (almost never
resulting in a wound), tumbling/fighting, and submissive movements such as assuming the supine position (Tamarin & Sheridan, 1987; Turner & Iverson, 1973). However, while these behaviors might appear similar to aggression, but they have been interpreted as playful actions in several rodent species (Chau et al., 2008; Pellis & Pellis, 1987; 1998; Turner & Iverson, 1973; for comparison of play behaviors in vole literature, see Table 1). Differences in the definitions of play and aggressive fighting across studies may mediate some of the species differences observed in these variables. Because play behaviors are associated with the development of beneficial characteristics later in life, miscategorization of these behaviors as aggressive may lead to incorrect assumptions regarding the functions and consequences of these behaviors.

Pellis and colleagues (1989) previously attempted to clarify species differences in play behaviors among male prairie voles, montane voles, rats, and deer mice. Rats made overall contact with their conspecific on average twice as often as prairie voles (Pellis et al., 1989). Interestingly, while rats almost exclusively aim for the nape of the neck/back of the head region of their conspecific, prairie voles aim for that region almost as often as they aim for the rump area (Pellis et al., 1989). This comparison suggests that play between juvenile rats may differ from the prairie vole and thus may be interpreted in different ways. Studies directly comparing montane and prairie voles suggest that the play behaviors displayed by voles may be more closely associated with precopulatory behaviors (Pierce, Pellis, Dewsbury, & Pellis, 1991). This is different from traditional laboratory animals, such as rats, which may engage in play to prepare for possible agonistic encounters later in life (Pierce et al., 1991). Taken together, the target body region and the associated significance of the region of play behaviors (nape of neck) may be important in distinguishing between acts of aggression and acts of attention. However,
further research is necessary to better understand the initiation and response of play behaviors in the prairie vole model.

**Post-Weaning Manipulations**

The prairie vole has also been used as a model to understand the influence of post-weaning experiences on the development of stress reactivity, behavior, and neurobiological functions. In a previous study, male prairie voles displayed altered social behaviors and increased vasopressin immunoreactivity in the PVN following 6 weeks of post-weaning social isolation (Pan et al., 2009). Additionally, an increase in plasma corticosterone and CRH immunoreactivity in the PVN was observed following 4 or 21 days of post-weaning social isolation in male and female prairie voles (Ruscio et al., 2007). These previous data suggest that post-weaning social stress negatively alters HPA axis responses and associated behaviors.

Additional research has investigated the effects of combined environmental manipulations prior to weaning and post-weaning social isolation. Four weeks of post-weaning social isolation in combination with low-contact rearing from both parents has been reported to increase male-male aggression in adulthood (Perkeybile & Bales, 2015). Further, female prairie voles reared by high-contact parents and exposed to 4 weeks of post-weaning social isolation displayed increased vasopressin immunoreactivity in the PVN and elevated plasma corticosterone versus low-contact parents + post-weaning social isolation (Perkeybile & Bales, 2015). This suggests that the early rearing environment may differentially influence responses to post-weaning social isolation (Perkeybile & Bales, 2015). Together, these data indicate that post-
Table 1 – Comparison of Operational Definitions of Play: Definitions of various pro- and anti-social behaviors in previously published studies investigating the importance of juvenile social interactions.

<table>
<thead>
<tr>
<th>Author, Year &amp; General Study Design</th>
<th>Type of Behavior</th>
<th>Name of Behavior</th>
<th>Definition of Behavior</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wang et al., 2012</td>
<td>Play</td>
<td>Pinning</td>
<td>One of the animals lies with its dorsal surface on the floor with the other animal standing over it</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Boxing/Wrestling</td>
<td>A group of behaviors including boxing, wrestling, and pouncing</td>
</tr>
<tr>
<td><em>Effects of paternal deprivation on juvenile play behavior (males)</em></td>
<td></td>
<td>Chasing</td>
<td>Moving in the direction of or pursuing the test partner, who moves away</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Social Exploration</td>
<td>Sniffing or licking any part of the body of the test partner, including anogenital area</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Contact Behavior</td>
<td>Includes crawling over and under the test partner and social grooming</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Huddling</td>
<td>Gathering or lying together</td>
</tr>
<tr>
<td>Chou et al., 2008</td>
<td>Play</td>
<td>Wrestling</td>
<td>Facing another animal, often in a ventrum-to-ventrum embrace, and biting the other animal’s body without inflicting wounds</td>
</tr>
<tr>
<td><em>Sex differences in play behavior in two different species of monogamous animals</em></td>
<td></td>
<td>Tackling</td>
<td>Jumping or pouncing on conspecific</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Boxing</td>
<td>Standing on hind legs and batting at another animal with forepaws. Other animal usually reciprocates</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Other Play</td>
<td>Other forms of play, such as biting another animal’s ear, or chasing another animal when the chase leads to one of the behaviors described above</td>
</tr>
</tbody>
</table>

Continued on following page
Table 1 – Continued from previous page

<table>
<thead>
<tr>
<th>Paz y Miñatno &amp; Tang-Martínez, 1999</th>
<th>Amicable</th>
<th>Huddle</th>
<th>One animal lies or sits with its body in contact with that of the other</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amicable Groom</td>
<td>One animal gently grooms another without showing dominance over the animal being groomed</td>
<td></td>
</tr>
<tr>
<td>Agonistic</td>
<td>Teeth-Chatter</td>
<td>A rapid jaw movement prior to or just after a fight</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vocalization</td>
<td>An animal makes sounds prior to or just after a fight</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chase</td>
<td>One animal runs after another</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Up-One</td>
<td>An animal stands with one front paw on the ground and the other raised</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Upright</td>
<td>The long axis of the animal is raised perpendicular to the substrate, or the anterior part of the body is raised from the substrate with both front paws raised off the substrate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pin</td>
<td>A subordinate animal lies motionless on its side or back</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pounce</td>
<td>One animal jumps or lunges at another animal</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fight</td>
<td>Tumbling and biting</td>
<td></td>
</tr>
<tr>
<td>Tamarin &amp; Sheridan, 1987</td>
<td>N/A - Not specified</td>
<td>Latency to Approach</td>
<td>Time from start of encounter to the time mice are within 5-8 cm of each other</td>
</tr>
<tr>
<td></td>
<td>Approach</td>
<td>Movement of one animal to within 5-8 cm of the other</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Naso-nasal</td>
<td>Nose to nose contact</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Naso-anal</td>
<td>Nose to anal region contact</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vocalization</td>
<td>Animal makes sounds</td>
<td></td>
</tr>
</tbody>
</table>

Continued on following page
<table>
<thead>
<tr>
<th>Action</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upright</td>
<td>Long axis of the animal perpendicular to substrate</td>
</tr>
<tr>
<td>Pounce</td>
<td>Animal pounces from an upright position</td>
</tr>
<tr>
<td>Box</td>
<td>Paddle of forepaws in upright position</td>
</tr>
<tr>
<td>Wrestle</td>
<td>Tumbling and biting</td>
</tr>
<tr>
<td>Teeth Chatter</td>
<td>Rapid jaw movements</td>
</tr>
<tr>
<td>Side-Away</td>
<td>One animal turns away from the other</td>
</tr>
<tr>
<td>Submit</td>
<td>Animal lies motionless on back</td>
</tr>
<tr>
<td>Lean</td>
<td>One animal lies against the side of the other</td>
</tr>
<tr>
<td>Groom - Other</td>
<td>One animal grooms the other</td>
</tr>
<tr>
<td>Groom - Self</td>
<td>Animal grooms itself</td>
</tr>
<tr>
<td>Contact</td>
<td>Animals touching each other without moving</td>
</tr>
<tr>
<td>Avoid</td>
<td>One animal moves from within 5-8 cm of the other</td>
</tr>
<tr>
<td>Chase</td>
<td>One animal follows within 5-8 cm of the other</td>
</tr>
</tbody>
</table>
weaning social isolation can have lasting behavioral, hormonal, and neurological consequences on prairie voles.

In addition to understanding the influence of early life social interactions on behavior and physiology, it is also important to focus on the benefits of re-pairing animals after periods of social isolation. A previous experiment specifically tested various durations of post-weaning social isolation in both sexes on the pro-social and agonistic behaviors displayed by the siblings upon re-pairing (Paz y Miñatno & Tang-Martínez, 1999). Pro-social behaviors, such as huddling and grooming each other, did not differ between females that were exposed to 2 weeks of post-weaning social isolation versus unseparated females. By contrast, agonistic behaviors were higher in females following 3 weeks of post-weaning social isolation, versus unseparated females (Paz y Miñatno & Tang-Martínez, 1999). A similar pattern of behaviors was seen in males. However, the investigators reported some sex differences in agonistic behaviors at various timepoints, which may have been the result of testing during peak play timepoints yet defining typical play-related behaviors (such as chasing and pinning) as agonistic behaviors (Paz y Miñatno & Tang-Martínez, 1999). On PND 31 and PND 36, which is approximately peak play age (Chau et al., 2008), the unseparated males displayed more agonistic behaviors than females, and this pattern disappeared at the PND 41 timepoint, when play is usually diminished (Chau et al., 2008; Paz y Miñatno & Tang-Martínez, 1999). Sex differences in frequency of play behaviors have been reported, with males engaging in these behaviors more often than females (Chau et al., 2008). Together, these data suggest that excluding play behaviors from the measurements in previous research may have artificially inflated levels of agonistic behaviors.
seen in the unseparated animals, which has implications for interpreting the behaviors of isolated animals as well.
CHAPTER TWO:
THE CURRENT EXPERIMENT

Goals and Specific Aims of the Present Study

The present study builds on the foundational research detailed previously by using the unique and translatable prairie vole model to investigate behavioral consequences of early life stress. Previous research in humans has provided evidence that social stress early in development can have lasting negative effects on neurological, psychological, and behavioral health in adulthood (Carpenter et al., 2009; Danese et al., 2007; Heim et al., 2010; Johnson et al., 2000; Johnston-Brooks et al., 1998). To further explore whether early life social stress perpetuates behavioral consequences, the present study capitalized on the highly social nature of the prairie vole by investigating the effects of post-weaning social isolation on later social behaviors and behaviors related to mood disorders. This project expanded our knowledge on specific consequences of juvenile social isolation through several novel components: (a) the inclusion of a resocialization period following the social isolation phase, (b) the specific analysis of play behaviors in female prairie voles, (c) behavioral measures related to depression and anxiety following post-weaning social isolation, (d) assessment of male-female social interactions, and
(e) a design that is specifically focused on females. Therefore, the current study provided valuable novel information about the influence of social isolation during a targeted window of development on social and emotion-related behavioral measures, which can be directly translated to humans.

Specifically, following social isolation, the present timeline included a sibling reunification period, which was designed to expose the animals to social stress only during the juvenile period of development (Semple et al., 2013). This methodology is translatable to the human experience. While other species have been used to study the effects of early life stress on behavioral and endocrine measures of stress reactivity and neuropsychological development (Hol et al., 1999; Lukkes, Mokin, et al., 2009; Lukkes, Watt, et al., 2009; Weiss et al., 2004), the methodology has not been consistent. Most other animal models have used prolonged post-weaning social isolation, keeping the animals separated into adulthood or until the time of testing (Lukkes et al., 2012; Pan et al., 2009; Weiss et al., 2004). These previous designs may not adequately model social stress during the juvenile period because exposure to social stress during childhood is unlikely to continue through adulthood in humans as individuals gain independence with age. Therefore, the present study modeled the human experience by implementing a reunion between the experimental animal and their sibling following 4 weeks of post-weaning social isolation and allowing the sibling pairs to remain undisturbed for 10-14 days prior to testing.

The present study design included specific characterization of play behaviors between the female siblings, which has not been thoroughly researched. Play behaviors are associated with several social behaviors in adulthood that are relevant for survival, such as mating (Pellis et al., 2014; Spevak et al., 1973) and, specific to the prairie vole, pair-bond formation (Ahern &
Therefore, the present study design included a measure of play behavior to assess social interactions following the social isolation stressor in order to better characterize juvenile social behaviors in the female prairie vole. Additionally, assessing play allowed for the possibility to explore play behavior as a mediating factor between social stress during development and social behaviors in adulthood.

Following an analysis of social behaviors during the juvenile period, the experimental females were subsequently housed together with their respective female siblings until adulthood, modeling the transition to social independence during adolescence. When the animals reached adulthood, the experimental females were assessed for behaviors related to psychological disorders. Although the prairie vole model has previously been used to characterize the negative consequences of social isolation in adult animals, this was the first study to implement social isolation for a limited duration during juvenile development with follow-up behavioral tests in adulthood. More specifically, the behavioral tests in adulthood explored the effects of early life social stress on behaviors related to anxiety and depression (Amiri et al., 2015; Bledsoe et al., 2011; Hong et al., 2012; Lukkes et al., 2012). These behavioral measures informed our understanding of the extent to which exposure to juvenile social stress in the form of isolation might influence the development of abnormal emotion-related behaviors, providing insight into possible mechanisms that can increase an individual’s risk of developing emotional deficits.

In addition to measuring juvenile social behavior and behaviors related to mood disorders in adulthood, the present study design was also advantageous because of the behavioral measures related to adaptive social behaviors in adulthood. The absence of proper socialization during the juvenile period alters later social behaviors, including mating, sexual behavior, and pair bond
formation (Ahern & Young, 2009; Spevak et al., 1973). The present study has expanded our knowledge of developmental mechanisms that might influence male-female social interactions and pair bond formation by characterizing the behaviors of the female with a male partner at various timepoints, including during the initial male-female introduction, 24 hours following introduction, and during a specific social preference test. These social behavioral analyses provided the opportunity to conceptualize how early life social isolation alters juvenile and adult social behaviors, specifically adaptive behaviors related to procreation and pair bonding.

Finally, in addition to the improved knowledge of social consequences of early life social stress, the present study focused on social and emotion-related behaviors in a model of an at-risk population: females. A large portion of the background information on the effects of early life social stress on later behaviors is focused on male animals (Hole, 1991; Pan et al., 2009; Ruscio et al., 2007), which does not adequately represent the human population. Understanding how early life social stress influences later social and psychological development in females is important for several reasons. First, females are more likely than males to develop depression (American Psychiatric Association, 2013); therefore, research focused on possible mechanisms of mood disorder development is critical for this population. Second, females are more likely than males to report experiencing stress (American Psychological Association, 2012), which may be a factor that influences their increased risk of developing psychiatric disorders and supports focusing stress research on female subjects. Finally, young girls are more likely than boys to be victims of any type of maltreatment (Centers for Disease Control, 2019), which highlights the importance of understanding the consequences of early life social stress in females. The
knowledge gained by the results of the present study will increase awareness about the importance of positive social environments during development.

Specific Aims and Hypotheses

**Specific Aim 1:** To examine the effects of juvenile social isolation on affective behaviors.

*Prediction 1a – EPM*

Exposure to social isolation will increase anxiety responding in the EPM during adulthood, relative to the paired control condition.

*Prediction 1b – FST*

Exposure to social isolation will increase passive behaviors in the FST, representative of depression-related behaviors, relative to the paired control condition.

**Specific Aim 2:** To examine the effects of juvenile social isolation on social behaviors.

*Prediction 2a – Juvenile Social Interaction Test*

Exposure to social isolation will decrease affiliative behaviors toward a female sibling during a Juvenile Social Interaction Test at PND 48, relative to the paired control condition.

*Prediction 2b – Male-Female Interaction Tests*

Exposure to social isolation will decrease affiliative behaviors in the Male-Female Social Interaction Tests during adulthood, relative to the paired control condition.

*Prediction 2c – Partner Preference Test (PPT)*

Exposure to social isolation will decrease time spent huddling with the male partner in the PPT during adulthood, relative to the paired control condition.
Prediction 2d – Adult Social Interaction Test

Exposure to social isolation will decrease duration spent huddling with a same-sex sibling in the Adult Social Interaction Test, relative to the paired control condition.

Methods

Animals

The present study utilized a total of 64 prairie voles (20 experimental females, 20 female siblings, 12 male partners, and 12 male strangers). Prior to testing, all animals were sexually naïve. All animals were descendants of a wild stock originally caught in Illinois and bred at Northern Illinois University. Animal housing rooms were maintained on a 14/10 hours light/dark cycle (lights on at 6:30 am) in a temperature of 25 +/- 1°C and humidity of 40-70%. All animals were given ad libitum access to food (Purina rabbit chow) and water. Prior to weaning (at PND 21), animals were housed in family groups (dam, sire, and pups) in 24x45x60 cm polycarbonate cages. Following weaning, the animals were then placed into smaller polycarbonate cages (12x18x28 cm) with a small amount of pelleted food and a piece of cotton nesting material. All procedures were in compliance with the NIH Guide for the Care and Use of Laboratory Animals and the study design has been approved by the Animal Care and Use Committee of Northern Illinois University.
Sample Sizes and Groups

Power Analyses

Power analyses were conducted and revealed an appropriate sample size of 12 animals per group for the present study. The power analyses used previously published work to calculate the effect size. Using the previously published works, Cohen’s d was calculated, the probability level of p < 0.05 was used, and the desired power level of 0.80 was used to minimize the chances of a Type II error. Cohen’s d values were calculated based on previously published literature that is relevant to the present study based on the measures used, such as social interaction tests or post-weaning social isolation, or the use of isolation or early life manipulations in the prairie vole model.

Pellis, Pellis, and Dewsbury (1989) were the first to take an in-depth look at play-related behaviors in prairie voles and reported that the behaviors themselves were more complex than those of rats: Cohen’s d = 2.58 for a sample size of three per group. Hol and colleagues (1999) demonstrated that the time frame in development when an animal experiences social isolation has an impact on later social behavior: Cohen’s d = 1.49 for a sample size of seven per group. Pan et al., (2009) investigated anxiety-relevant behaviors in male prairie voles following six weeks of post-weaning social isolation: Cohen’s d = 2.04 for a sample size of four per group. Mosaferi, Babri, Ebrahimi, and Mohaddes (2015) investigated the effects of post-weaning social isolation in male rats followed by exposure to a common environment in adulthood on behaviors in the EPM: Cohen’s d = 7.09 for a sample size of two per group, and FST: Cohen’s d = 2.73 for a sample size of three per group. Grippo, Wu, Hassan, and Carter (2008) exposed adult female prairie voles to four weeks of social isolation, similar to the present study but at a different time
point, to investigate forced swim behaviors: Cohen’s $d = .78$ for a sample size of 22 per group. Ahern and Young (2009) explored the effects of being reared by a single mother vs. bi-parental rearing on the development of social preferences in adulthood and reported that after 48 hours of male-female cohabitation, single-mother-reared females did not display a preference for their male partner: Cohen’s $d = .59$ for a sample size of 37 per group. Ruscio and colleagues (2007) investigated the consequences of post-weaning social environment on basal plasma corticosterone levels in male and female juvenile prairie voles and observed that any exposure to post-weaning social isolation increased plasma corticosterone concentrations when sexes are collapsed: Cohen’s $d = .79$ for a sample size of 21 per group. By averaging the suggested sample size from each of the studies included in this power analysis (average = 12.4 animals per group), a sample size of 12 animals per group was deemed appropriate.

The power analysis indicated that 12 animals should have been tested in the present study, but complications impeded the use of the appropriate sample size. Testing for the present study began in January of 2020 and was only partially completed in March when the novel coronavirus (COVID-19) pandemic apexed in the US. The statewide lockdown hindered the timely completion of the study, as students were not allowed to work on campus. In consultation with the committee overseeing this project, the decision was made to complete the data analyses on the subset of animals that was tested prior to the lockdown, resulting in reduced and uneven sample sizes for certain behavioral measures. Therefore, the final number of animals tested in each component of this project were the following: (a) Juvenile Social Interaction Test – 8 control and 9 isolated; (b) EPM – 6 control and 6 isolated; (c) FST – 10 control and 10 isolated;
(d) Male-Female Social Interaction Tests – 6 control and 6 isolated; (e) PPT – 6 control and 6 isolated; (f) Adult Social Interaction Test – 6 control and 6 isolated.

Conditions

Experimental females were removed from the family group at PND 21 and assigned to either post-weaning social isolation or paired control conditions. The present study used 20 experimental female voles, 10 in the post-weaning social isolation condition and 10 in the paired control condition. An additional 20 female siblings were also used (one animal per experimental female), which were not specifically studied, except during the Juvenile Social Interaction Test with the experimental female (described below in section titled “Re-socialization and Juvenile Social Interaction Test”). This study also included a pair-bonding component and a PPT when the experimental female animals reached adulthood, which required one male per experimental female for cohabitation and bonding (male partner) and an additional stranger male for the PPT itself (stranger male), which totals 24 male prairie voles. The following group names and used herein: (a) experimental animals: female animals to be studied, housed in either the social isolation or paired control conditions; (b) female siblings: respective siblings of each experimental female (not studied); (c) male partners: unrelated adult males that are paired with each experimental female during adulthood; and (d) stranger males: unrelated adult males that are used as an unfamiliar stranger during the PPT.
General Study Design

The general study design is described here and in Figure 4, with specific methodological details in the following sections. The present study design used female prairie voles beginning on the date of weaning (PND 21). On this date, experimental females were assigned into one of two possible groups: social isolation or paired-control. The paired-control animals were housed together and remained pair housed with a female sibling for the duration of the study until adulthood, while the social isolation females were separated from their female sibling at weaning and housed in isolation for 4 weeks. After the 4-week social isolation period, socially isolated animals were reunited with their previous female siblings, and their behaviors immediately following resocialization were recorded and analyzed for social behaviors. The paired-control animals also experienced a brief separation from their sibling at that time point, followed by reunion with their respective siblings, which increased the likelihood of exhibiting social behaviors and other measures of conspecific interactions (Wang et al., 2012). Following the Juvenile Social Interaction Test, the experimental females in both groups (isolation and control) remained undisturbed, pair housed with their respective female siblings, until adulthood (approximately PND 65).
Figure 4 – General Study Design: The general timeline of the present study beginning when the animals are placed into conditions, through the sample collection at the end of the study.

In adulthood, all experimental females underwent the EPM to measure exploratory behavior and an index of anxiety-related behaviors. Forty-eight hours following the EPM, all animals were exposed to the FST to measure behavioral despair, as an index of depression-related behavior (Slattery & Cryan, 2012). Forty-eight hours after the FST, all experimental females were separated from their respective female siblings and then paired with an unrelated male partner. Following the introduction of the experimental female with her male partner, the male-female pair was evaluated for social behaviors (Peirce et al., 1991) in the Male-Female Social Interaction Test at Initial Pairing. The male-female pairs were then housed together for 24 hours and then observed for social behaviors a second time in the Male-Female Social Interaction Test Following 24 Hours of Cohabitation. The 48 hours of male-female housing with the social behavior observations acted as a period of time for the animals to develop a pair bond.
Following the pair bonding period, all experimental females were exposed to a PPT to measure the social preference of the female for her familiar male partner compared to a stranger male (Williams, Catania, & Carter, 1992). Following the PPT, the experimental females were separated from their familiar males and housed in isolation overnight. The following day, the experimental females were re-introduced to their same-sex sibling in an Adult Social Interaction Test to observe how a second period of separation might alter social behaviors between the siblings. Two hours after the end of this final Adult Social Interaction Test, the experimental females and their female siblings were euthanized and brain tissue was collected for future analyses (outside the scope of the present study).

**Specific Methods**

**Social Isolation Paradigm**

Beginning on PND 21, experimental females were semi-randomly assigned to either the social isolation condition (n = 10) or the paired control condition (n = 10). Animals were assigned to conditions in a semi-random fashion, ensuring that each cohort of animals contained sibling pairs for both paired and isolated conditions and that multiple sibling pairs or litters from the same breeder pair were assigned to different conditions to ensure an appropriate level of genetic diversity within each condition. Animals in the social isolation condition were removed from their family group and housed in individual cages for 4 weeks, without auditory, visual, or olfactory cues from the respective female siblings. The paired-control animals were removed from the family group and housed in pairs with a female sibling during that phase of the study. Cage changes and measurement of body weight were standardized between the groups.
Resocialization and Juvenile Social Interaction Test

Following the 4-week social isolation phase of the study, the socially isolated animals were re-paired with their respective female siblings in new, clean cages with half the standard amount of bedding. The behaviors of each animal in the pair were digitally video recorded for the first 20 minutes of resocialization. Because the animals in the paired control condition did not experience any social isolation, these animals were first separated for a brief 6-hour period before being re-paired in a new, clean cage with half the standard amount of bedding. The behaviors of the sibling pairs were digitally video recorded for the first 20 minutes of resocialization, in the same manner as the isolated animals (Vanderschuren et al., 2016; Wang et al., 2012).

Following the Juvenile Social Interaction Test, all animal pairs were given additional bedding, a new clean piece of cotton nesting material, a clean water bottle, clean cage top, and food. The animals were then returned to the housing room and left undisturbed, in pairs (experimental females housed with their respective female siblings), until adulthood (approximately PND 65), with the exception of standardized room inspections and weekly cage changes.

Behaviors during the sibling interaction test included the following: play behaviors, general investigation, aggression, huddling, and time spent alone engaging in individual behaviors. The play behaviors included: (a) animals rear/sit on their hind paws and mutually swat at each other (often categorized in the literature as “boxing”); (b) one animal pushes the other to the ground and they roll around, with each animal taking turns pinning the other animal (wrestling); (c) sometimes the result of wrestling, one animal places its forepaws on top of the
other animal (pinning); and (d) one animal is pinned on its back, ventral surface exposed, and the animal doing the pinning is usually licking or grooming the pinned animal’s ventral surface (pinning + supine). All play behaviors were combined into a single category based on previous published protocols (Chau et al., 2008; Paz y Miñatno & Tang-Martínez, 1999; Wang et al., 2012).

Investigative behaviors included: (a) sniffing of the face, neck, rump, ano-genital region; (b) other grooming of the conspecific; and (c) one animal following another with their nose within ~1 cm of the other’s rump (sometimes categorized in the literature as “chasing”).

Huddling was defined, simply, as side-by-side contact that does not include any aspects of play or investigation. Time spent alone was defined as the duration of time that the animals spent not engaging with each other, sometimes engaging in individual behaviors such as self-grooming.

Aggression included any lunges, bites, or attacks that caused harm to the conspecific, demonstrated by the attacking animal having a tuft of the other animal’s fur in their mouth, the attacked animal vocalizing, or any evidence of blood. To specifically distinguish aggression from play, the definitions of each category included the attacked animal’s reaction to those behaviors. Specifically, aggression is usually a fast sequence of events that does not stop until one animal has escaped.

In play behaviors, the pinning interactions (with or without the pinned animal in the supine position) are common sequences of behaviors, and in the prairie vole, the pinned animal in the supine position often remains in that position for several seconds without struggling to escape (perhaps due to the fact the conspecific is not causing them pain). This is one example of the complex play behaviors of prairie voles (Pellis et al., 1989; Pierce et al., 1991). The animal
who is pinning their conspecific often grooms the pinned animal during this encounter, further supporting the categorization of a play interaction as amicable or pro-social. That lack of response on behalf of the pinned animal during play is an important part of the operational distinction between the play and aggressive behaviors. Additionally, as this study focused on female prairie voles, it may be important to note that aggression is not a common behavior in sexually naïve female prairie voles during interactions with a family member (Bowler, Cushing, & Carter, 2002). For more details of the social behaviors, see the comparisons of behaviors in Table 1; and the specific behaviors are defined in Table 2 (see Figure 3; adapted from Pellis & Pellis, 1998).

Table 2 – Definitions of Behaviors in the Juvenile Social Interaction Test: Operational definitions of behavioral categories in the Juvenile Social Interaction Test used in the present study.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Investigative Behaviors</td>
<td>Ano-genital sniffing</td>
</tr>
<tr>
<td></td>
<td>Grooming conspecific (sometimes while sitting in side-by-side contact, but grooming is mutually exclusive from huddling)</td>
</tr>
<tr>
<td></td>
<td>Facial sniffing</td>
</tr>
<tr>
<td></td>
<td>One animal follows the other animal with their nose within 1cm other the other animal's rump (chasing)</td>
</tr>
<tr>
<td></td>
<td>Other general active interactions while all four paws remain on the ground (walking side-by-side, grooming conspecific in supine position)</td>
</tr>
</tbody>
</table>

(Continued on following page)
| Play Behaviors | One animal engages the other in a manner to subdue the other without kicking up much/any bedding (usually because one animal submits to the other; pinning)  

Pinning often results in one animal in the supine position (on their back) with the other animal on top of them (pinning + supine)  

The animal in the supine animal might try to push the other animal into the supine position, both animals switching positions (wrestling)  

The animal in the supine position is not fighting back/ trying to escape/ other behaviors indicative of aggression  

Animals will rear on their hind legs to pounce on the other animal or swat lightly at them (boxing)  

All behaviors are directed toward the rump or the back of the head/facial area (but not the throat) |
| --- | --- |
| Aggressive Behaviors | Lunges, bites, or other attacks that cause the other animal harm (tufts of fur in the attacking animal's mouth). These behaviors usually occur in a very fast sequence, much quicker than seen in play  

Interaction results in wrestling-like behaviors with bedding being kicked up and one animal actively trying to escape  

Attacks are typically toward the face/throat  

One animal vocalizes |
| Huddling | Side-by-side contact  

Not interacting or grooming each other while in side-by-side contact |
| Time Alone | Animals not engaging or interacting with each other  

Animals engaging in individual behaviors away from sibling |
EPM Test of Anxiety and Locomotion

When the animals reached adulthood (~ PND 65), the socially isolated and paired-control animals were exposed to the EPM for 5 minutes, which is a measure of anxiety-related behaviors (Walf & Frye, 2007). This test has been repeatedly used in adult prairie voles as well as in other rodent models of adulthood and post-weaning social isolation (Grippo et al., 2014; Lukkes, Mokin, et al., 2009; Lukkes, Watt, et al., 2009). The EPM apparatus consisted of two opposing open arms, without walls, made of clear Plexiglas (49.5x10 cm) elevated 30 cm from the floor and two opposing closed arms that have tall black walls to enclose the arms (49.5x10x30.5 cm). The experimental animal was placed in the center square of the apparatus (10x10 cm) at the start of the test and the animal’s movements were digitally video recorded from above for later analysis. Following the 5 minutes of testing, the experimental animals were then removed from the apparatus and returned to their home cage with their siblings. The apparatus was cleaned with a Neutrad cleaning solution and dried prior to being used to test the next animal.

The behaviors scored during this test included (a) time spent in the closed arms, (b) time spent in the open arms, (c) time spent in the center, and (d) number of center crossings. A reduced amount of time spent in the open arms of the apparatus is indicative of an anxiety-like response and the total number of crossings is a measure of locomotor activity (Walf & Frye, 2007; Table 3).
Table 3 – Definitions of Behaviors in the EPM: Operational definitions of behavioral categories in the EPM test used in the present study.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time Spent in Closed Arms</td>
<td>All 4 of the animal's paws have crossed into the closed arm of the apparatus</td>
</tr>
<tr>
<td>Time Spent in Open Arms</td>
<td>All 4 of the animal's paws have crossed into the open arm of the apparatus</td>
</tr>
<tr>
<td>Time Spent in Center</td>
<td>All 4 of the animal's paws are within the center square of the apparatus</td>
</tr>
<tr>
<td>Number of Center Crossings</td>
<td>Total number of times the animal entered the center square of the apparatus</td>
</tr>
</tbody>
</table>

FST of Depression-Related Behavior

Forty-eight hours following the EPM, social isolation and paired-control animals were exposed to the FST for 5 minutes, as a measure of helpless behavior in an inescapable task and representative of depression-related behaviors in an animal model (Slattery & Cryan, 2012). The test apparatus was a clear Plexiglas cylinder (height 46 cm, diameter 20 cm) filled to a height of 18 cm with room-temperature water (approximately 21-25°C). The animal was then placed in the tank for 5 minutes with its behaviors digitally recorded for later analysis. Following the end of the 5 minutes, the animal was removed from the water and replaced in the home cage. A corner of the cage, approximately < 25% of the total area of the cage, was then placed under a heat lamp to help the wet animal thermoregulate for 10-15 minutes. The water in the tanks used for testing was changed between each animal.

The behaviors in the FST included measures of active coping versus a passive (maladaptive) response, with the passive response as a demonstration of learned helplessness or
behavioral despair by the animal during an inescapable experience. Passive behaviors included immobility, defined as floating without any limb movement or with just enough movement to keep afloat (Grippo et al., 2008). The behaviors characterized as active coping represent the animal actively trying to escape the water, including (a) swimming, defined as the animal moving its fore and hind limbs in a coordinated manner without breaking the surface of the water; (b) struggling, defined as the forelimbs moving and breaking the water, in the middle of the tank; and (c) climbing, movements similar to struggling except that the actions are directed at the wall (Grippo et al., 2008). For more details of the behaviors in the FST, please see Table 4.

**Table 4 – Definitions of Behaviors in the FST:** Operational definitions of behavioral categories in the FST used in the present study.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Active Behaviors</strong></td>
<td>Swimming: Moving its fore and hind limbs in a coordinated manner without breaking the surface of the water</td>
</tr>
<tr>
<td></td>
<td>Struggling: Forelimbs moving and breaking the water in the middle of the tank</td>
</tr>
<tr>
<td></td>
<td>Climbing: Similar to struggling except that the actions are directed at the wall</td>
</tr>
<tr>
<td><strong>Passive Behaviors</strong></td>
<td>Immobility: Floating without any limb movement or with just enough movement to keep afloat</td>
</tr>
</tbody>
</table>
Male-Female Social Interaction Tests

Forty-eight hours after the FST, each social isolation and paired-control females was paired with an unfamiliar male partner. The male partners were age and weight matched and unrelated to the females. Each experimental female was then removed from its home cage and subsequently placed into a new, clean cage with half the standard amount of bedding with the male partner. The behaviors of the male-female interaction were digitally video recorded for the first hour of their cohabitation for later analysis. Following this Male-Female Social Interaction Test at Initial Pairing, the male-female pairs were given a new clean piece of cotton nesting material, new cage top, new water, the additional amount of bedding, and fresh food. Twenty-four hours after the first male-female social interaction test, the animals were brought back to the testing room and exposed to a second identical observation referred to as the Male-Female Social Interaction Test Following 24 Hours of Cohabitation.

The social behaviors during the 1-hour recorded cohabitation period included: (a) huddling, which is defined as side-by-side contact that does not include any aspects of mounting or any behaviors categorized as approach/contact; (b) approach/contact by the male toward the female; (c) positive reaction from the female to the approach/contact; (d) negative reactions from the female to the approach contact; (e) any indicators of aggression by one animal towards the other, defined as any lunges, bites, or attacks that cause harm to the other animal; (f) investigative behaviors; and (g) time spent alone, defined as the duration of time the animals spent not interacting with each other. The approach/contact behaviors were counted each time that the male, defined as the larger animal of the pair, approached the female. The reactions of the experimental female to the advances by the male were adapted from Pierce et al., (1991) and
included a positive reaction defined as mating/copulation (the female allows the male to mount her from behind to copulate with her), mutual sniffing, or grooming or negative reactions, specifically defined as (a) the female turns to face the male and stands on all four paws or rears on her two hind paws (postural defense), which is sometimes followed by (b) mutual slapping, animals usually reared on hind legs, batting at each other (boxing); (c) the female lunges toward the male, pushes, swats or bites him (agonistic defense); (d) female moves away from the male when he approaches (withdrawal) – these animals were included in one category as negative female responses to male approach. Additionally, the duration of huddling behaviors (side-by-side contact) and instances of aggression (lunges, bites, or other attacks that cause the other animal harm: the attacking animal might have a tuft of the other’s fur in their mouth, the attacked animal may vocalize) were also counted. The male-female pairs were monitored for aggression in a manner identical to the sibling interaction test, with experimenters encouraged to intervene if the aggressive behaviors persisted. The male and female animals were differentiated by body size, as the males are consistently larger than the females. For a more detailed description and explanation of these behaviors, please see Table 5.

The second male-female observation occurred approximately 24 hours after the initial test using the same methods and behavioral observations as described above.

Social Bonding Period

Following the initial male-female pairing and behavioral observation, the animals were left undisturbed except for the second male-female observation. The 48-hour pair housing period provided sufficient time for the animals to form a social bond, as suggested in previous research (Ahern & Young, 2009; Bosch et al., 2009; McNeal et al., 2014).
Table 5 – Definitions of Behaviors in the Male-Female Social Interaction Test: Operational definitions of behavioral categories in the Male-Female Social Interaction Tests used in the present study (adapted from Pierce, Pellis, Dewsbury, & Pellis, 1991).

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Approach/Contact</strong></td>
<td></td>
</tr>
<tr>
<td>By male towards the female</td>
<td>Ano-genital sniffing</td>
</tr>
<tr>
<td></td>
<td>Grooming / Licking conspecific</td>
</tr>
<tr>
<td></td>
<td>Facial sniffing</td>
</tr>
<tr>
<td></td>
<td>Any attempts to mount/copulate with female</td>
</tr>
<tr>
<td><strong>Positive Reaction to Male</strong></td>
<td>If the male attempts to mount/copulate with the female and she does NOT respond with any of the defensive or withdrawal behaviors described below, and the animals appear to engage in mating behaviors (successful mounting/copulation)</td>
</tr>
<tr>
<td><strong>Approach/Contact</strong></td>
<td></td>
</tr>
<tr>
<td>By female</td>
<td></td>
</tr>
<tr>
<td><strong>Negative Reactions to</strong></td>
<td>Turning to face the male standing on all four paws or in a reared position on their two hind paws (postural defense)</td>
</tr>
<tr>
<td><strong>Approach/Contact</strong></td>
<td>Mutual slapping, animals usually reared on hind legs, batting at each other, usually oriented towards head/face as point of contact - note: this category may follow postural defense (boxing)</td>
</tr>
<tr>
<td>By female</td>
<td>Lunges, pushing, ‘slaps’, or biting (agonistic defense), mutually exclusive from aggression (tufts of fur, vocalizing, blood, etc)</td>
</tr>
<tr>
<td></td>
<td>Female moves away from male when he approaches or attempts contact (withdrawal)</td>
</tr>
<tr>
<td><strong>Huddling</strong></td>
<td>Side-by-side contact</td>
</tr>
<tr>
<td></td>
<td>Not interacting or grooming each other while in side-by-side contact</td>
</tr>
<tr>
<td><strong>Aggressive Behaviors</strong></td>
<td>Lunges, bites, or other attacks that cause the other animal harm (tufts of fur in the attacking animal's mouth). These behaviors usually occur in a very fast sequence, much quicker than seen in play</td>
</tr>
<tr>
<td></td>
<td>Interaction results in wrestling-like behaviors with bedding being kicked up and one animal actively trying to escape</td>
</tr>
<tr>
<td></td>
<td>Attacks are typically toward the face/throat</td>
</tr>
<tr>
<td></td>
<td>One animal vocalizes</td>
</tr>
<tr>
<td><strong>Mating/Lordosis Behaviors</strong></td>
<td>Successful mounting and copulation attempts</td>
</tr>
<tr>
<td></td>
<td>Female engages in the lordosis posture with rump elevated</td>
</tr>
</tbody>
</table>
Following the 48-hour social bonding period, each experimental female was exposed to a 3-hour PPT. This test measured the preference of the experimental female for its previously bonded male partner versus a stranger male. The apparatus consisted of three clear Plexiglas arenas (30x20 cm each). The male partner was tethered to one of the arenas while the stranger male was tethered to a separate arena, such that each male was painlessly confined to only one arena. These two arenas were connected by a neutral, middle arena, which had two connecting tunnels (one tunnel to each of the arenas housing a male; 7.5cm in diameter, 8cm long). Each arena had bedding, food, and water. The males were tethered and left to acclimate to the tether for 45 minutes prior to the beginning of the test. The experimental female was placed into the neutral middle chamber at the beginning of the test and was allowed to move freely throughout all three chambers. After the end of the PPT, the female animals were removed from the apparatus and housed in a standard cage alone with access to food and water for approximately 20-24 hours. After each PPT, the boxes were disassembled, cleaned of bedding and wiped out with a Neutrad solution before being wiped dry and re-assembled with fresh food, bedding, and water prior to testing the next animal.

Behaviors were digitally recorded from above to be analyzed later for (a) time spent in side-by-side contact with the male partner or the stranger male, (b) aggressive behaviors toward the male partner or male stranger, (c) investigative/pro-social behaviors directed toward the male partner or the male stranger, and (d) time spent in each of the three chambers in total and (e) time spent in each of the arenas housing a male without interacting with the conspecific. For more details, please see Table 6.
Table 6 – Definitions of Behaviors in the PPT: Operational definitions of behavioral categories in the PPT used in the present study. All behaviors were coded separately for behaviors directed towards the male partner vs. the stranger male.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Investigative/Pro-Social Behaviors</strong></td>
<td>Ano-genital sniffing</td>
</tr>
<tr>
<td></td>
<td>Grooming / Licking conspecific</td>
</tr>
<tr>
<td></td>
<td>Facial sniffing</td>
</tr>
<tr>
<td></td>
<td>One animal engages the other in a manner to subdue the other without kicking up much/any bedding (usually because one animal submits to the other; pinning)</td>
</tr>
<tr>
<td></td>
<td>Pinning often results in one animal in the supine position (on their back) with the other animal on top of them (pinning + supine)</td>
</tr>
<tr>
<td></td>
<td>The animal in the supine animal might try to push the other animal into the supine position, both animals switching positions (wrestling)</td>
</tr>
<tr>
<td></td>
<td>The animal in the supine position is not fighting back/ trying to escape/ other behaviors indicative of aggression</td>
</tr>
<tr>
<td></td>
<td>Animals will rear on their hind legs to pounce on the other animal or swat lightly at them (boxing)</td>
</tr>
<tr>
<td></td>
<td>All behaviors are directed toward the rump or the back of the head/facial area (but not the throat)</td>
</tr>
<tr>
<td><strong>Aggressive Behaviors</strong></td>
<td>Lunges, bites, or other attacks that cause the other animal harm (tufts of fur in the attacking animal's mouth). These behaviors usually occur in a very fast sequence, much quicker than seen in play</td>
</tr>
<tr>
<td></td>
<td>Interaction results in wrestling-like behaviors with bedding being kicked up and one animal actively trying to escape</td>
</tr>
<tr>
<td></td>
<td>Attacks are typically toward the face/throat</td>
</tr>
<tr>
<td></td>
<td>One animal vocalizes</td>
</tr>
<tr>
<td><strong>Huddling</strong></td>
<td>Side-by-side contact</td>
</tr>
</tbody>
</table>

(Continued on following page)
Not interacting or grooming each other while in side-by-side contact

<table>
<thead>
<tr>
<th>Time Spent in Each Arena</th>
<th>Total time spent in neutral arena (without either male)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of times the female crosses from the neutral arena into either arena housing a male (locomotion)</td>
</tr>
</tbody>
</table>

**Adult Social Interaction Test**

Twenty-four hours following the PPT, the experimental females were re-introduced to their female sibling in a new clean cage containing half the standard amount of bedding, and their behaviors were digitally recorded. The behaviors included (a) investigative behaviors – defined as mutual sniffing or grooming; (b) aggressive behaviors – defined as lunging, swatting, biting, or fighting the conspecific where one animal had a tuft of the other’s fur in their mouth, one animal struggled to get away from the other, or the attacked animal vocalized; (c) huddling – defined as the duration of side-by-side contact without any behaviors previously defined as investigative; and (d) time spent alone – defined as the duration of time the animals spent not engaging with each other (Table 7). Following the conclusion of the test, the siblings remained pair housed in the cage where the test was conducted and given food and water until tissue collection.
<table>
<thead>
<tr>
<th>Behavior</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Investigative Behaviors</strong></td>
<td>Ano-genital sniffing</td>
</tr>
<tr>
<td></td>
<td>Grooming conspecific (sometimes while sitting in side-by-side contact, but grooming is mutually exclusive from huddling)</td>
</tr>
<tr>
<td></td>
<td>Facial sniffing</td>
</tr>
<tr>
<td></td>
<td>One animal follows the other animal with their nose within 1cm of the other animal's rump (chasing)</td>
</tr>
<tr>
<td></td>
<td>Other general active interactions while all four paws remain on the ground (walking side-by-side, grooming conspecific in supine position)</td>
</tr>
<tr>
<td><strong>Aggressive Behaviors</strong></td>
<td>Lunges, bites, or other attacks that cause the other animal harm (tufts of fur in the attacking animal's mouth). These behaviors usually occur in a very fast sequence, much quicker than seen in play</td>
</tr>
<tr>
<td></td>
<td>Interaction results in wrestling-like behaviors with bedding being kicked up and one animal actively trying to escape</td>
</tr>
<tr>
<td></td>
<td>Attacks are typically toward the face/throat</td>
</tr>
<tr>
<td></td>
<td>One animal vocalizes</td>
</tr>
<tr>
<td><strong>Huddling</strong></td>
<td>Side-by-side contact</td>
</tr>
<tr>
<td></td>
<td>Not interacting or grooming each other while in side-by-side contact</td>
</tr>
<tr>
<td><strong>Time Alone</strong></td>
<td>Animals not engaging or interacting with each other</td>
</tr>
<tr>
<td></td>
<td>Animals engaging in individual behaviors away from sibling</td>
</tr>
</tbody>
</table>
Tissue Collection and Preparation

One hundred ten minutes following the end of the Adult Social Interaction Test, the females were removed from their temporary housing cage and euthanized under anesthesia for tissue collection. The heavy anesthetic was administered via a subcutaneous injection of ketamine (67 mg/kg, sc; NLS Animal Health, Owings Mills, MD) and xylazine (13.33 mg/kg, sc; NLS Animal Health, Owings Mills, MD) in a 5:1 ratio. The brain was then carefully removed, the forebrain sliced 3mm posterior from the olfactory bulbs to expose the lateral ventricles, submerged in FormaldeFresh (Fisher Scientific, Waltham, MA) and lightly agitated for 4 hours, similar to previously described methods (Cushing, Yamamoto, Hoffmann, & Carter 2003). The brains were then stored in fresh FormaldeFresh at room temperature for 24 hours before being sunk in 25% sucrose at 4ºC. The tissue was preserved in the sucrose until it was sliced.

Using a cryostat at -20ºC, the tissue was sliced at 40um and stored in a cryoprotectant solution at -20ºC for future analyses that are outside the scope of the present project.

Behavioral Analyses

Following procedures described previously, all behavioral tests were digitally recorded for manual analyses of behavior (Grippo, Cushing et al., 2007; Grippo et al., 2014; Grippo et al., 2008). The behavior scoring was conducted using the Observer XT software version 8 (Noldus Information Technology, Leesburg, VA), which allowed for the videos to be viewed and behaviors to be observed, scored, and reviewed. Using the Observer program, specific project files were made for each of the behavioral tests using the behavioral criteria described in the
previous sections. According to previous protocols used by our laboratory to ensure valid and reliable behavior scoring, the following procedures were used. All videos were scored by a condition-blind experimenter. To ensure that the operational definitions of social behaviors were appropriately interpreted and applied, approximately 25% of the videos from the social behavior tests were subsequently rescored by the same experimenter. The behavioral scores were then compared to ensure that the experimenter was consistent in their interpretation of the operational definitions for each behavioral test. To ensure that the operational definitions of anxiety- and depression-related behaviors were appropriately interpreted and applied, all videos from the EPM and FST were reviewed by the same experimenter, and corrections to the initial behavior scores were made as necessary.

Statistical Analyses

Primary analyses. A probability value of $p < .05$ was considered statistically significant for all analyses described here. A probability value of $p < .1$ was also used for purposes of discussing potentially meaningful differences in outcome measures in the present study.

Prior to conducting specific statistical analyses, all datasets were inspected for statistical outliers and logistical issues that interfered with data collection. Individual animals were considered to be outliers and were removed from the dataset on a test-by-test basis, based on the criterion of data falling 2 or more standard deviations above or below the mean. If a test was not completed due to an issue (such as an animal falling off of the EPM or camera error during a test), the data point for that specific test was not used, and behaviors and resulting data for all
subsequent tests were carefully assessed for abnormalities. Specifically, for the EPM, one animal fell off of the apparatus multiple times. This same animal displayed significantly higher levels of diving to the bottom of the tank, swimming on their back, or other behaviors not included in the predetermined categories of the FST. Therefore, the data from this animal were excluded from both the EPM and FST analyses. Another animal spent an amount of time in the open arms of the EPM exceeding 2 SDs above the mean and was excluded from this specific analysis. A third animal displayed an increased number of aggressive behaviors, 2 SD above the mean in the Male-Female Social Interaction Test at Initial Pairing, and therefore was also excluded from the final dataset for this test. Finally, two animals experienced camera difficulties, one in the Male-Female Social Interaction Test at Initial Pairing and another in the PPT. Their videos ended too early; therefore, their behaviors were not included in the analyses of those tests. Final analyzed sample sizes for each behavioral test are listed in the Results section.

Given that some behavioral tests included several variables - some of which have been hypothesized previously to be inter-related (Field, 2013) - several correlations were conducted to determine the most appropriate statistical comparison for each behavioral test. The results of the correlations are displayed in Table 8. These analyses indicated weak correlations between most variables and therefore indicated that either independent samples or paired student’s t tests were the most appropriate statistical test for each behavioral outcome measure. A Levene’s test was conducted to investigate homogeneity of variance for each set of means. If the Levene’s test revealed a significant result, unequal variances were assumed for the specific test. Comparisons with significant Levene’s tests, which violated the homogeneity of variance assumption, are noted in the Results and figures.
Table 8 – Correlations of Behaviors Theorized to be Related: Table of correlations displaying the Pearson’s r correlation values and effect size (Field, 2013; Taylor, 1990) for the behaviors initially hypothesized to be related, based on preliminary (unpublished) experiments.

<table>
<thead>
<tr>
<th>Test</th>
<th>Correlated Variables</th>
<th>Pearson’s r and Effect Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Juvenile Social Interaction Test</td>
<td>Investigate with Play</td>
<td>r = 0.25, Small Effect</td>
</tr>
<tr>
<td></td>
<td>Huddling with Play</td>
<td>r = -0.51, Moderate Effect</td>
</tr>
<tr>
<td>Male-Female Social Interaction Test at Initial Pairing</td>
<td>Positive Response with Huddling</td>
<td>r = -0.01, Small Effect</td>
</tr>
<tr>
<td></td>
<td>Negative Response with Aggressions</td>
<td>r = 0.44, Moderate Effect</td>
</tr>
<tr>
<td>Male-Female Social Interaction Test Following 24 Hours of Cohabitation</td>
<td>Positive Response with Huddling</td>
<td>r = -0.03, Small Effect</td>
</tr>
<tr>
<td></td>
<td>Negative Response with Aggressions</td>
<td>N/A - Aggressions = 0</td>
</tr>
<tr>
<td>Partner Preference Test</td>
<td>Stranger Huddle with Partner Aggressions</td>
<td>r = -0.22, Small Effect</td>
</tr>
</tbody>
</table>

Using independent samples $t$ tests, several variables were compared between the socially isolated and paired control conditions. Independent samples $t$ tests produced $p$ values for a two-tailed comparison. The following $t$ tests were conducted for the specific behaviors in the
following tests: (a) body weight at specific timepoints; (b) EPM – duration of time spent in the open arms and number of center crossings; (c) FST – duration of time spent immobile; (d) Juvenile Social Interaction Test - durations that the animals spent engaging in investigative, play, and huddling behaviors, as well as the amount of time the animals spent alone engaging in individual behaviors; (e) Male-Female Social Interaction Tests at Initial Pairing and Following 24 Hours Cohabitation - the number of times the female engaged in a negative response to the male’s approach, number of times the female engaged in a positive response to the male’s approach, number of aggressive behaviors, duration spent huddling, and number of instances of mating/lordosis between animals; (f) Adult Social Interaction Test – duration the animals spent huddling and the number of aggressive behaviors; (g) PPT – duration that the animal spent huddling with her male partner, duration that the animal spent huddling with the stranger male, duration of time the animals spent in the neutral cage, instances of aggression with the male partner, and instances of aggression with the male stranger. To further explore the partner preference behaviors, paired t tests were conducted to compare the duration each animal spent huddling with the male partner vs. the stranger male in each condition (paired and isolated) separately.

Exploratory analyses. Correlations were conducted to explore potential relatedness between several pairs of variables, as listed here. The duration spent immobile in the FST was correlated with (a) duration spent in the open arms of the EPM, (b) duration spent alone in the Juvenile Social Interaction Test, (c) number of aggressions in the Juvenile Social Interaction Test, (d) duration of play behaviors in the Juvenile Social Interaction Test, (e) duration spent alone in the Adult Social Interaction Test, (f) duration spent huddling with the male partner in
the PPT, (g) number of aggressions towards the stranger male in the PPT, and (h) duration spent huddling with the stranger male in the PPT. The duration spent in the open arms of the EPM was correlated with (a) duration spent alone in the Juvenile Social Interaction Test, (b) number of aggressions in the Juvenile Social Interaction Test, (c) duration of play behaviors in the Juvenile Social Interaction Test, (d) duration spent alone in the Adult Social Interaction Test, (e) duration spent huddling with male partner in the PPT, (f) number of aggressions towards the stranger male in the PPT, and (g) duration spent huddling with the stranger male in the PPT. Duration spent huddling in the Juvenile Social Isolation Test was correlated with the duration spent huddling in the Adult Social Interaction Test. The duration spent huddling with the male partner in the PPT was correlated with (a) the number of positive responses to male approach and (b) duration spent huddling in the Male-Female Social Interaction Test at Initial Pairing. The duration spent huddling with the stranger male in the PPT was correlated with (a) play behaviors in the Juvenile Social Interaction Test and (b) aggressive behaviors in the Juvenile Social Interaction Test. These analyses were conducted to explore the potential relatedness between behaviors related to depression and anxiety with social behaviors and the relatedness between social behaviors in different tests. Only correlations yielding a moderate or large effect size were reported. These correlations were subsequently conducted for each housing condition separately to assess potential differences by condition on the relationships between these variables.
Results

Body Weight

The animals were weighed at three timepoints throughout the study design: prior to the Juvenile Social Interaction Test (n=9 socially isolated; n=8 paired control), prior to the EPM (n=6 socially isolated; n=6 paired control), and after the Adult Social Interaction Test (n=6 socially isolated; n=6 paired control). Animals in the socially isolated group and animals in the paired control group were assessed for differences in body weight at each of the three timepoints: prior to the Juvenile Social Interaction Test (Time 1) \([t(15)=.08, p=.94]\); prior to the EPM (Time 2; unequal variances assumed based on Levene’s test) \([t(6)=1.30, p=.24]\); and after the Adult Social Interaction Test (Time 3; unequal variances assumed based on Levene’s test) \([t(6)=.76, p=.48]\). The body weights did not differ between groups at any timepoint (Figure 5).
Figure 5 – Graph of Body Weights: Mean (+SEM) body weight of animals in the socially isolated and paired control conditions at three timepoints during the study design. Data were compared using an independent samples *t* test for Timepoint 1, and independent samples *t* tests assuming unequal variances for Timepoints 2 and 3.
Preliminary Correlations

Pearson’s r correlation coefficients were conducted on variables in the Juvenile Social Interaction Test, Male Female Interaction Tests, and the PPT to determine whether these variables were statistically inter-related. These comparisons were conducted based on data from preliminary unpublished experiments, demonstrating a relationship between investigative and play behaviors in the Juvenile Social Interaction Test. This previously observed relationship informed a hypothesis that some positive or negative social behaviors may be inter-related in various behavioral tests. The Pearson’s r and effect size (Taylor, 1990) for the measures are reported in Table 8. These results suggest that only the durations of play behaviors and huddling behaviors in the Juvenile Social Interaction Test were related (r=-0.51, p=0.04; moderate effect size). Based on the results of these correlations, independent samples t tests were conducted to compare the paired and isolated groups in both positive and negative social behavioral outcome measures, for each social behavioral test.

Juvenile Social Interaction Test

Using independent samples t tests, the total durations of investigative, play, and huddling behaviors, and duration the animals spent alone were compared between the socially isolated (n=9) and paired control (n=8) conditions. For investigative behaviors, the isolated animals spent significantly more time investigating their same-sex sibling compared to paired-control animals [t(15)=2.48, p=0.03] (Figure 6). Similarly, the isolated animals spent slightly more time engaging in play behaviors with their sibling compared to paired control [t(15)=2.92, p=0.11]
(Figure 7). The isolated animals spent less time huddling with their sibling compared to paired-control animals \([t(15)=2.64, \ p=0.03]\) (Figure 8). Additionally, there was a significant difference in the duration that the animals spent alone and not engaging with their siblings (unequal variances assumed based on Levene’s test) \([t(15)=2.34, \ p=0.03]\), with socially isolated animals spending less time alone during the test compared to the paired-control animals (Figure 9).

![Graph of Investigative Behaviors in the Juvenile Social Interaction Test](image)

**Figure 6 – Graph of Investigative Behaviors in the Juvenile Social Interaction Test:** Mean (+SEM) duration spent engaging in investigative behaviors with a female sibling in socially isolated and paired-control prairie voles during the Juvenile Social Interaction Test, conducted at the beginning of resocialization. Data were compared using an independent samples \(t\) test (*\(P < 0.05\) vs. paired control condition).
Figure 7 – Graph of Play Behaviors in the Juvenile Social Interaction Test: Mean (+SEM) duration spent engaging in play behaviors with a female sibling in socially isolated and paired-control prairie voles during the Juvenile Social Interaction Test, conducted at the beginning of resocialization. Data were compared using an independent samples t test (#P = 0.11 vs. paired control condition).
Figure 8 – Graph of Huddling Behaviors in the Juvenile Social Interaction Test: Mean (+SEM) duration spent engaging in huddling behaviors with a female sibling in socially isolated and paired-control prairie voles during the Juvenile Social Interaction Test, conducted at the beginning of resocialization. Data were compared using an independent samples t test (*P < 0.05 vs. paired control condition).
Figure 9 – Graph of Time Spent Alone in the Juvenile Social Interaction Test: Mean (+SEM) duration spent alone engaging in individual behaviors (separately from the female sibling) in socially isolated and paired-control prairie voles during the Juvenile Social Interaction Test, conducted at the beginning of resocialization. Data were compared using an independent samples t test assuming unequal variances (*P < 0.05 vs. paired control condition).

EPM

The duration spent in the open arms for one animal exceeded two standard deviations from the mean of the socially isolated condition, and the animal’s data point was therefore excluded. One animal in the control group also fell off the apparatus multiple times and was excluded from analysis. Animals in the socially isolated group (n=5) and the paired control group (n=5) were compared for time spent in the open arms of the EPM using an independent samples t test. There were no significant differences between the socially isolated (n=5) and the paired control (n=5) conditions for duration spent in the open arms of the EPM [t(8)=1.41, p=0.20] (Figure 10).
The socially isolated animals (n=5) were also compared to the paired control animals (n=5) in a measure of locomotion in the EPM using an independent samples \( t \) test. There was no difference between the two groups in number of center crossings \([t(8)=0.33, p=0.75]\) (Figure 11).

**Figure 10 – Graph of Duration Spent in the Open Arms of the EPM**: Mean (+SEM) duration spent in the open arms of the EPM for the socially isolated and paired control animals in adulthood. The remainder of the 300 seconds was comprised of duration spent in the center of the apparatus and duration spent in the closed arms. Data were compared using an independent samples \( t \) test.
Figure 11 – Graph of Center Crossings in the EPM: Mean (+SEM) number of times the animals crossed the center square of the EPM for the socially isolated and paired-control animals in adulthood. Data were compared using an independent samples t test.

FST

At the time of the FST, one animal had fallen off of the EPM apparatus 48 hours earlier and engaged in several atypical behaviors during the FST (diving to the bottom of the tank, abnormal swimming behaviors). The duration of these behaviors exceeded two standard deviations from the mean of the control group, and therefore this animal was excluded from the analysis. The socially isolated animals (n=10) and the paired control animals (n=9) were assessed for duration of time spent immobile in the FST using an independent samples t test. The active coping behaviors (duration struggling, climbing, swimming) in the test were pooled together, as they did not differ between groups (Table 9). There was no significant difference in duration spent immobile in the FST [t(17)=0.29, p=0.78] (Figure 12).
Table 9 – Active Coping Behaviors in the FST: Table of mean (+/- SEM) duration of active coping behaviors in socially isolated and paired-control from the FST.

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Isolated</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Struggling</td>
<td>5.0 +/- 1.3</td>
<td>5.4 +/- 1.3</td>
</tr>
<tr>
<td>Climbing</td>
<td>179.5 +/- 31.4</td>
<td>185.4 +/- 21.3</td>
</tr>
<tr>
<td>Swimming</td>
<td>8.0 +/- 2.4</td>
<td>15.6 +/- 5.1</td>
</tr>
</tbody>
</table>

Figure 12 – Graph of Immobility in the FST: Mean (+SEM) duration spent immobile in the FST for the socially isolated and paired-control animals in adulthood. The remainder of the 300 seconds was comprised of climbing, swimming, and struggling behaviors. Data were compared using an independent samples t test.
Male-Female Social Interaction Tests

At Initial Pairing

One animal in the socially isolated group displayed high levels of aggressive behaviors and low levels of huddling behaviors that were equal to two standard deviations from the mean; therefore, this animal’s data were excluded from all analyses for this test. Using independent samples $t$ tests, there were no significant differences between the socially isolated ($n=4$) and paired control ($n=5$) conditions in any of the variables: positive response to male approach [$t(7)=0.10$, $p=0.93$] (Figure 13), negative response to female approach [$t(7)=0.25$, $p=0.25$] (Figure 14), duration spent huddling (unequal variances assumed based on Levene’s test) [$t(3)=1.72$, $p=0.18$] (Figure 15), number of aggressive behaviors (unequal variances assumed based on Levene’s test) [$t(5)=1.86$, $p=0.13$] (Figure 16), or instances of mating/lordosis (unequal variances assumed based on Levene’s test) [$t(3)=1.11$, $p=0.34$] (Figure 17).

Following 24 Hours of Cohabitation

Using independent samples $t$ tests, the socially isolated ($n=5$) were compared to the paired control animals ($n=3$) for: number of positive responses to male approach (unequal variances assumed based on Levene’s test) [$t(2)=0.45$, $p=0.70$], Figure 18; number of negative responses to male approach (unequal variances assumed based on Levene’s test) [$t(2)=0.69$, $p=0.56$], Figure 19; duration spent huddling with the male [$t(6)=1.66$, $p=0.15$], Figure 20; instances of mating/lordosis (unequal variances assumed based on Levene’s test) [$t(5)=1.44$, $p=0.02$], Figure 21. The number of aggressive behaviors was not analyzed because there were no aggressive behaviors displayed by either condition.
Figure 13 – Graph of Positive Responses to Male Approach in the Male-Female Social Interaction Test at Initial Pairing: Mean (+SEM) number of positive responses to male approach in socially isolated and paired-control prairie voles during the Male-Female Social Interaction Test At Initial Pairing, conducted in adulthood at the beginning of the male-female pair housing period. Data were compared using an independent samples $t$ test.
**Figure 14 - Graph of Negative Responses to Male Approach in the Male-Female Social Interaction Test at Initial Pairing:** Mean (+SEM) number of negative responses to male approach in socially isolated and paired-control prairie voles during the Male-Female Social Interaction Test at Initial Pairing, conducted in adulthood at the beginning of the male-female pair housing period. Data were compared using an independent samples $t$ test.
Figure 15 - Graph of Huddling Behaviors in the Male-Female Social Interaction Test at Initial Pairing: Mean (+SEM) duration spent huddling with the male partner in socially isolated and paired-control prairie voles during the Male-Female Social Interaction Test at Initial Pairing, conducted in adulthood at the beginning of the male-female pair housing period. Data were compared using an independent samples t test assuming unequal variances.
Figure 16 - Graph of Aggressive Behaviors in the Male-Female Social Interaction Test at Initial Pairing: Mean (+SEM) number of aggressive behaviors in socially isolated and paired-control prairie voles during the Male-Female Social Interaction Test at Initial Pairing, conducted in adulthood at the beginning of the male-female pair housing period. Data were compared using an independent samples t test assuming unequal variances.
Figure 17 – Graph of Mating/Lordosis Behaviors in the Male-Female Social Interaction Test at Initial Pairing: Mean (+SEM) number of mating or lordosis behaviors in socially isolated and paired-control prairie voles during the Male-Female Social Interaction Test at Initial Pairing, conducted in adulthood at the beginning of the male-female pair housing period. Data were compared using an independent samples t test assuming unequal variances.
Figure 18 – Graph of Positive Responses to Male Approach in the Male-Female Social Interaction Test Following 24 Hours of Cohabitation: Mean (+SEM) number of positive responses to male approach in socially isolated and paired-control prairie voles during the Male-Female Social Interaction Test Following 24 Hours of Cohabitation, conducted in adulthood 24 hours into the male-female pair housing period. Data were compared using an independent samples t test assuming unequal variances.
Figure 19 – Graph of Negative Responses to Male Approach in the Male-Female Social Interaction Test Following 24 Hours of Cohabitation: Mean (+SEM) number of negative responses to male approach in paired and isolated prairie voles during the Male-Female Social Interaction Test Following 24 Hours of Cohabitation, conducted in adulthood 24 hours into the male-female pair housing period. Data were compared using an independent samples t test assuming unequal variances.
**Figure 20 – Graph of Huddling Behaviors in the Male-Female Social Interaction Test Following 24 Hours of Cohabitation:** Mean (+SEM) duration of huddling with the male partner in socially isolated and paired-control prairie voles during the Male-Female Social Interaction Test Following 24 Hours of Cohabitation, conducted in adulthood 24 hours into the male-female pair housing period. Data were compared using an independent samples $t$ test assuming unequal variances.
Partner Preference Test

Using independent samples t tests, the socially isolated (n=5) and paired-control animals (n=6) were compared for several behaviors in the PPT. The most commonly used measure of partner preference is the duration spent huddling with the male partner (Williams et al., 1992). This analysis revealed a slight non-significant trend [t(9)=1.45, p=0.18], suggesting that the socially isolated animals displayed a slightly greater preference for the familiar male compared to the paired-control animals as measured by duration spent huddling with the male partner (Figure 22). The socially isolated animals spent significantly less time huddling with the stranger male than the paired control animals [t(9)=2.30, p=0.05] (Figure 23). Using a paired t test, the socially isolated animals displayed a significant preference for their male partners, displayed by

Figure 21 – Graph of Mating/Lordosis Behaviors in the Male-Female Social Interaction Test Following 24 Hours of Cohabitation: Mean (+SEM) number of mating/lordosis behaviors in socially isolated and paired-control prairie voles during the Male-Female Social Interaction Test Following 24 Hours of Cohabitation, conducted in adulthood 24 hours into the male-female pair housing period. Data were compared using an independent samples t test assuming unequal variances (*P < 0.05 vs. paired control condition).
increased duration huddling with the partner compared to the stranger male \(t(4)=3.81, p=0.02\) (Figure 24). The paired-control animals did not display such a preference, as there was no difference between the duration of time they spent huddling with their partners compared to the strangers \(t(5)=0.50, p=0.66\), Figure 24.

The other behaviors from the PPT that were analyzed included, aggression with partner (unequal variances assumed based on Levene’s test) \(t(5)=1.0, p=0.36\) (Figure 25), aggression with stranger (unequal variances assumed based on Levene’s test) \(t(4)=1.52, p=0.20\) (Figure 26), and duration spent in the neutral chamber \(t(9)=1.0, p=0.35\) (Figure 27).

**Figure 22 – Graph of Huddling Behaviors with Male Partner in the PPT:** Mean (+SEM) duration spent huddling with the male partner in socially isolated and paired-control prairie voles during the PPT, conducted in adulthood 48 hours into the male-female pair housing period. Data were compared using an independent samples \(t\) test.
**Figure 23 – Graph of Huddling Behaviors with Stranger Male in the PPT**: Mean (+SEM) duration spent huddling with the stranger male in socially isolated and paired-control prairie voles during the PPT, conducted in adulthood 48 hours into the male-female pair housing period. Data were compared using an independent samples *t* test (*P* < 0.05 vs. paired control condition).
Figure 24 – Graph Comparing Huddling Behaviors with Male Partner and Stranger Male in the PPT: Mean duration spent huddling with the stranger male compared to the duration spent huddling with the male partner in socially isolated and paired-control prairie voles during the PPT, conducted in adulthood 48 hours into the male-female pair housing period. Data were compared using a Paired Samples t test (*P < 0.05 vs. paired control condition).
Figure 25 – Graph of Aggressive Behaviors with Male Partner in the PPT: Mean (+SEM) number of aggressive behaviors with the male partner in socially isolated and paired-control prairie voles during the PPT, conducted in adulthood 48 hours into the male-female pair housing period. Data were compared using an independent samples t test assuming unequal variances.
Figure 26 – Graph of Aggressive Behaviors with Stranger Male in the PPT: Mean (+SEM) number of aggressive behaviors with the stranger male in socially isolated and paired-control prairie voles during the PPT, conducted in adulthood 48 hours into the male-female pair housing period. Data were compared using an independent samples t test assuming unequal variances.

Figure 27 – Graph of Time Spent in the Neutral Arena in the PPT: Mean (+SEM) duration spent in the neutral arena in socially isolated and paired-control prairie voles during the PPT, conducted in adulthood 48 hours into the male-female pair housing period. Data were compared using an independent samples t test.
Using independent samples *t* tests, the social behaviors in the Adult Social Interaction Test were compared between the socially isolated (n=6) and paired-control animals (n=6). The behaviors analyzed included the duration the animals spent huddling with their sibling (unequal variances assumed based on Levene’s test) \[t(7)=0.73, p=0.49\] (Figure 28), and the number of aggressive behaviors (unequal variances assumed based on Levene’s test) \[t(5)=2.70, p=0.04\] (Figure 29).

**Figure 28 – Graph of Huddling Behaviors in the Adult Social Interaction Test:** Mean (+SEM) duration spent huddling with the same-sex sibling in socially isolated and paired-control prairie voles during the Adult Social Interaction Test, conducted 72 hours after females were separated from their siblings and housed with male partners. Data were compared using an independent samples *t* test assuming unequal variances.
**Figure 29 – Graph of Aggressive Behaviors in the Adult Social Interaction Test:** Mean (+SEM) number of aggressive behaviors with the same-sex sibling in socially isolated and paired-control prairie voles during the Adult Social Interaction Test, conducted 72 hours after females were separated from their siblings and housed with male partners. Data were compared using an independent samples *t* test assuming unequal variances (*P < 0.05 vs. paired control condition).

**Exploratory Analyses**

To further investigate the relatedness of specific variables, several correlations were conducted with paired and isolated groups combined. These correlations are reported in Table 10. The duration spent immobile in the FST was significantly and negatively correlated with duration spent in the open arms of the EPM, which was expected given that anxiety and depression are often related (Bergdahl & Bergdahl, 2002; Galaif et al., 2003; Grippo et al., 2008). The duration spent immobile in the FST was also significantly and positively correlated with huddling with the male partner in the PPT. The duration spent in the open arms of the EPM was correlated with huddling behaviors in the PPT. Increased duration in the open arms was associated with decreased duration spent huddling with the male partner (negative correlation).
and increased duration spent huddling with the stranger male (positive correlation). The duration spent huddling with the sibling in the Juvenile Social Interaction Test was positively correlated with huddling behaviors with the sibling in the Adult Social Interaction Test. Similarly, the duration the animals spent alone in the Juvenile Social Interaction Test was positively correlated with the duration spent alone in the Adult Social Interaction Test.

For additional insight, the correlations were conducted again in the paired and isolated conditions separately. The correlations previous described generally differed between the groups, with the following exceptions, which were similar in both paired and isolated groups: (a) duration spent in the open arms of the EPM and immobility duration in the FST and (b) duration spent in the open arms of the EPM and duration spent huddling the male partner in the PPT.

The following correlations were stronger in the isolated group relative to the paired group: (a) immobility in the FST and huddling the male partner in the PPT and (b) duration spent in the open arms of the EPM and aggression in the Juvenile Social Interaction Test. The following correlations were stronger in the paired group, relative to the isolated group: (a) duration spent in the open arms of the EPM and duration huddling with the stranger male in the PPT, (b) duration spent huddling in the Juvenile Social Interaction Test and duration spent huddling in the Adult Social Interaction Test, and (c) duration spent alone in the Juvenile Social Interaction Test and duration spent alone in the Adult Social Interaction Test.
Table 10 – Exploratory Correlations: Exploratory correlations conducted with Pearson’s r value and effect size (Field, 2013; Taylor, 1990) for the averaged variables with socially isolated and paired-control values combined and for each housing condition on its own.

<table>
<thead>
<tr>
<th>Variable 1</th>
<th>Variable 2</th>
<th>Pearson's r &amp; Effect Size - Conditions Combined</th>
<th>Pearson's r &amp; Effect Size - Isolated</th>
<th>Pearson's r &amp; Effect Size - Paired</th>
</tr>
</thead>
<tbody>
<tr>
<td>FST - Immobility Duration</td>
<td>EPM - Open Arm Duration</td>
<td>$r = -0.69$, Large Effect</td>
<td>$r = -0.80$, Large Effect</td>
<td>$r = -0.51$, Moderate Effect</td>
</tr>
<tr>
<td></td>
<td>PPT - Huddling Duration with Partner</td>
<td>$r = 0.56$, Moderate Effect</td>
<td>$r = 0.91$, Large Effect</td>
<td>$r = -0.28$, Small Effect</td>
</tr>
<tr>
<td>EPM - Open Arm Duration</td>
<td>PPT - Huddling Duration with Partner</td>
<td>$r = -0.73$, Large Effect</td>
<td>$r = -0.78$, Large Effect</td>
<td>$r = -0.63$, Large Effect</td>
</tr>
<tr>
<td></td>
<td>PPT - Huddling Duration with Stranger</td>
<td>$r = 0.96$, Large Effect</td>
<td>$r = -0.05$, Small Effect</td>
<td>$r = 0.97$, Large Effect</td>
</tr>
<tr>
<td></td>
<td>Juvenile Social Interaction Test - Aggression Instances</td>
<td>$r = 0.40$, Moderate Effect</td>
<td>$r = 0.95$, Large Effect</td>
<td>N/A - No Aggression</td>
</tr>
<tr>
<td>Juvenile Social Interaction Test - Huddling Duration</td>
<td>Adult Social Interaction Test - Huddling Duration</td>
<td>$r = 0.66$, Large Effect</td>
<td>$r = -0.34$, Small Effect</td>
<td>$r = 0.72$, Large Effect</td>
</tr>
<tr>
<td>Juvenile Social Interaction Test - Duration Alone</td>
<td>Adult Social Interaction Test - Duration Alone</td>
<td>$r = 0.47$, Moderate Effect</td>
<td>$r = 0.09$, Small Effect</td>
<td>$r = 0.94$, Large Effect</td>
</tr>
</tbody>
</table>
Discussion

The present study employed novel methodology in the prairie vole model, to assess potential lasting behavioral consequences of early life social stress. Prairie voles provide unique insight into how social stress during different periods of life can influence social behaviors, cardiovascular function, neuroendocrine health, and behaviors related to affective disorders (Bosch et al., 2009; Fowler et al., 2002; Grippo et al., 2014; Grippo, Lamb, et al., 2007; Grippo et al., 2008; Lieberwirth et al., 2012; McNeal et al., 2014; Pournajafi-Nazarloo et al., 2011; Stowe et al., 2005; Sun et al., 2014; Watanasriyakul et al., 2017). This species provides a valuable model for understanding the interactions of social stress with behavior and physiology due to the unique social structure of prairie voles, including the ability to form lasting social bonds, bi-parental rearing of offspring, and cohabitation in large family groups (Carter et al., 1986; McGuire & Getz, 1991). Only a few previous prairie vole studies have investigated the developmental trajectories of females from early life through adulthood (Ahern et al., 2011; Ahern & Young, 2009; Perkeybile & Bales, 2015). This research has demonstrated the importance of early life experiences on adulthood social behaviors and appropriate neurological function.

Based on research demonstrating the value of the prairie vole model for investigating social interactions and the developmental consequences of early life stress (Ahern & Young, 2009; Pan et al., 2009; Perkeybile & Bales, 2015; Ruscio et al., 2007), the primary goal of the present study was to explore the influence of social stress during a targeted window of development on juvenile and adulthood social behaviors as well as behaviors related to mood.
and anxiety disorders. Given previous evidence supporting the interactions of social stress, behavior, and physiology (Bosch et al., 2009; Grippo, Gerena, et al., 2007; Grippo, Lamb, et al., 2007; McNeal et al., 2014), it was expected that animals exposed to 4 weeks of post-weaning social isolation would display decreased positive social behaviors (play, huddling, mating/lordosis, and partner preference behaviors), increased negative social behaviors (aggression and time spent alone during social interaction tests), and increased anxiety- and depression-related behaviors (decreased duration in the open arms of the EPM, increased duration spent immobile in the FST; Slattery & Cryan, 2012; Walf & Frye, 2007). The results generally indicate that post-weaning social isolation influenced important social behaviors such as play during the juvenile period, male-female interactions, partner preference, and responses to an adult sibling. However, social isolation did not have lasting negative consequences on anxiety- or depression-related behaviors or on certain adulthood social behaviors. The specific behavioral responses to social isolation will be discussed in the sections below.

The results from the present study provide insight into emotion-related and social behaviors as a function of early life social stress and resocialization after stress, which will inform our understanding of these constructs in humans. As previously described, prairie voles are a useful model for human experiences because of their social structure (Carter et al., 1995; Getz et al., 1993) and sensitivity to stress (Grippo, Lamb, et al., 2007; Lieberwirth et al., 2012; Normann et al., 2018). For these reasons, the present study was specifically designed to include several measures of behaviors that are important for understanding developmental consequences of stress in humans. These measures included behaviors related to mood disorders and social behaviors that contribute to the formation of family interactions and opposite-sex relationships. This design also focused the presentation of social stress during a developmental window that
represents approximately 3 years to 14 years of age in humans (Semple et al., 2013). Following
this period of social isolation, a phase of resocialization with a sibling was included to model the
transition from childhood, when humans are reliant upon parents and other family for social
stimulation, to young adulthood, when humans become more autonomous, expand their social
relationships, and engage in more activities outside of the home. This developmental transition is
critical for human socialization. Early life social stress has been associated with increased risk of
developing mood disorders and altered socialization in humans (Cutting & Dunn, 2006; Dodge et
al., 2003; Heim et al., 2010; Lansford et al., 2006; McAlister & Peterson, 2013; Peterson et al.,
2016; Wang et al., 2014). This transition has not been specifically studied in rodents prior to the
present study. The present data suggest that early life social stress has some negative influence
on social behaviors during both the juvenile and adulthood periods but also may positively
influence active social behaviors during the juvenile period. However, the presentation of early
life stress during the targeted window of development may not have a lasting negative impact on
adulthood anxiety- and depression-related behaviors or some adulthood social behaviors. The
lack of these behavioral changes may be due to the resocialization period (designed to model
increased social experiences in the later adolescent period in humans) or neurophysiological
changes related to stress reactivity, oxytocin, or the estrus cycle. These mechanisms may have
collectively or individually buffered against anxiety, depression, and some social behavioral
disruptions and will be specifically addressed in the following sections. The present results
indicate that adequate or elevated levels of socialization after early life social stress may protect
against the risk of developing anxiety- or depression-related behaviors and social deficits in
adulthood. This research can inform future treatment and prevention strategies in the context of
early life social stress.
Juvenile Social Behaviors: Juvenile Social Interaction Test

Given the importance of play and socialization during development and several consequences of inadequate social experiences, the present study included a specific social behavioral test following post-weaning isolation. The Juvenile Social Interaction Test was conducted to investigate whether post-weaning social isolation resulted in decreased positive social behaviors when animals were resocialized with a same-sex sibling. The absence of social experiences during the first 4 weeks of post-weaning development, when peak play behaviors usually occur in rodents (Chau et al., 2008; Wang et al., 2012), was hypothesized to impair social behaviors. The results of the present study partially supported this hypothesis. As expected, the socially isolated animals displayed decreased huddling behaviors, indicating that these animals were not engaging in adaptive passive social behaviors with their same-sex sibling. In contrast, the paired-control animals spent a majority of their time either huddling with their respective siblings or alone, but these animals did not engage in active social behaviors with their siblings. This pattern may represent a sense of familiarity or sibling recognition in the paired-control animals, that was absent in the socially isolated animals.

In addition to huddling behavior, another important behavior for juvenile prairie voles is play behavior. It was hypothesized that play behaviors would be reduced in socially isolated prairie voles. This hypothesis was based on previous research demonstrating that the absence of adequate social interactions during peak play periods of development leads to social impairments, increased anxiety, and altered reward-processing abilities in animals (Bell et al., 2009; Bledsoe et al., 2011; Hol et al., 1999; Lukkes, Mokin, et al., 2009; Lukkes, Watt et al., 2009; Vanderschuren et al., 2016). However, the pattern of results did not support this
hypothesis. Socially isolated animals engaged in increased levels of play behaviors, compared to the paired-control animals. This observation, coupled with reduced huddling behavior, suggests that the socially isolated animals chose to engage in active social behaviors rather than passive social behaviors. In contrast to the isolated group, play behaviors in the paired group were low. The paired-control animals may have already experienced an appropriate level of play behaviors during the paired housing period, which might be one reason to explain why this group displayed lower levels of play than the isolated group.

High levels of play behavior exhibited by the isolated group during the specific Juvenile Social Interaction Test may imply that these animals continued to play together after the test was concluded. Some previous evidence suggests that even minimal socialization during development can be enough to protect against negative consequences of early social stress (Colonnello, Petrocchi, Farinelli, & Ottaviani, 2017). Therefore, increased levels of play behaviors displayed by the socially isolated group might indicate that access to resocialization after social isolation is a useful protective strategy. This hypothesis supports data from human studies. For example, peer social support in humans influences emotions differently based on age (Weinstein et al., 2006), and therefore the period of development during which social support is provided may have an important influence on emotions and stress reactivity.

The opportunity for sibling prairie voles to play together during the Juvenile Social Interaction Test is a potential social mechanism that may protect against stress. Previous studies of juvenile socialization and play behavior in rodents have been primarily limited to males, but these indicate that play behavior has enriching qualities critical to the development of appropriate behaviors. Play provides tactile stimulation but also readies male rodents for social interactions in adulthood related to the nest hierarchy and mating (Pellis, 1988; Pellis et al.,
1989; van Kerkhof et al., 2013; Vanderschuren et al., 2016). The social component of play is important for development, over and above general activity, because depriving an animal the opportunity to play with a conspecific cannot be replaced with exercise alone (Holloway & Suter, 2004). The present results, demonstrating increased play behavior in prairie voles after social isolation, extend previous research on the importance of play to female rodents. Future research investigating sex differences in play behavior of prairie voles will contribute increased knowledge about the importance of play for appropriate developmental processes.

The neural mechanisms that underlie the benefits of play behaviors require further investigation. Play affects the brain in a sexually dimorphic manner because play behaviors can increase activation of specific estrogen receptors that have a masculinizing effect, increasing sexual maturity, physical agility, and neural plasticity (Auger & Olesen, 2009; van Kerkhof et al., 2013; Vanderschuren et al., 2016; Veenema, Bredewold, & De Vries, 2013). Play behaviors in female rodents have been less thoroughly examined than play in males but have been related to neural plasticity in brain regions associated with the stress response system such as the mPFC, BNST, and amygdala (Auger & Olesen, 2009; Veenema et al., 2013). Future research will benefit from a specific investigation of stress-related brain circuitry following resocialization and opportunities for play in the prairie vole model.

**Adulthood Emotion-Related Behaviors: EPM and FST**

In addition to investigating behaviors during the juvenile period, the present study also included a focus on behaviors during adulthood as a function of early life social isolation. Given previous research demonstrating that social stress contributes to anxiety and depression in humans and disrupted anxiety- and depression-related behaviors in animal models (Slattery & Cryan, 2012; Walf & Frye, 2007), the present study hypothesized that animals who had
experienced early life social isolation would display increased levels of anxiety- and depression-related behaviors in the EPM and FST, respectively. The EPM has been consistently used in voles (Grippo, Gerena, et al., 2007; Grippo et al., 2008) and other rodent species to measure anxiety-related behaviors, as defined by decreased duration in the open arms of the maze (Chappell et al., 2013; Lukkes, Mokin, et al., 2009; Walf & Frye, 2007). Additionally, the FST has been used to measure behavioral despair and depression-related constructs in voles (Grippo, Gerena, et al., 2007) and other rodent species (D’Andrea, Gracci, Alleva, & Branchi, 2010), by assessing the duration of immobility in the apparatus (Slattery & Cryan, 2012). The results of the present study did not support the hypothesis, with no group differences in duration spent immobile in the FST or duration spent in the open arms of the EPM. This outcome was unexpected, given that previous work using adult female prairie voles indicates that 4 weeks of social isolation consistently increases anxiety- and depression-related behaviors (Grippo, Gerena, et al., 2007; Grippo et al., 2014, Grippo et al., 2008). The lack of differences in the EPM and FST may be a function of theoretical or methodological reasons. The following considerations will be discussed below. First, the specific operational measures associated with anxiety and depression in the current study design may not have been adequate to detect significant differences in these behavioral constructs. Additionally, it is possible that the specific period of social isolation used here was not effective at producing adulthood anxiety- and depression-related changes. Finally, the introduction of a 2-week resocialization period following social isolation, which allowed for specific play behaviors, may have protected against long-term emotion-related consequences of the isolation.

As previously discussed, the data do not support the hypothesis that 4 weeks of social isolation would disrupt behaviors in the EPM and FST during adulthood. The EPM and FST
were chosen for use in the present study based on previous evidence that social stress in adult prairie voles disrupts behaviors in these measures (Grippo, Gerena, et al., 2007; Grippo et al., 2014; Grippo et al., 2008). However, previous human research has demonstrated mixed behavioral consequences of early life social stress. Human literature consistently indicates that early life stress increases the risk of developing mood disorder symptoms; however, the specific behaviors have been characterized as paranoid and avoidant behaviors (Johnson et al., 2000). It was not possible to measure these behaviors using the present study design, given the difficulty in operationally defining paranoid and avoidant behaviors in rodent models. Additional research from humans indicates that anxiety and depression may result from severe physical early life stressors (Danese et al., 2007; Gibb et al., 2001; Heim et al., 2010; Spratt et al., 2012). In contrast, the present study design did not expose prairie voles to early life physical stress or trauma, rather the social stress paradigm may be less severe than early life traumatic experiences in humans. Considering the present results in the context of this previous literature, the EPM and FST may not have been adequate operational tests for investigating anxiety- and depression-related constructs following a period of early life social stress in prairie voles.

Separate from the possibility that the EPM and FST were not effective operational indices of anxiety- and depression-related behaviors, the targeted window of development when the voles were exposed to social isolation may have occurred too late in their developmental trajectory to induce behavioral changes related to emotion in adulthood. Some previous studies have suggested that pre-weaning manipulations are more effective than post-weaning manipulations at producing deficits in anxiety- and depression-related behaviors, social behaviors, and neurological functioning (Caldji et al., 1998; Francis et al., 2002; Weaver et al., 2004; Weaver et al., 2006). Previous research has indicated that the first year of human
development and pre-weaning manipulations are part of the critical period of development. For example, maternal separation, maternal deprivation, or paternal deprivation in voles and other rodents induce maladaptive behaviors in social contexts such as increased anxiety in a novel social environment and increased latency to form a pair bond (Ahern & Young, 2009; Aisa et al., 2007; Daniels et al., 2004; Hulshof et al., 2011; Lajud et al., 2012; Perkeybile & Bales, 2015; Wang et al., 2012). Some of these previous studies limited their stress exposures to the pre-weaning period of development and observed behavioral effects of the manipulations in adulthood, which suggests that post-weaning social isolation may not be as impactful as pre-weaning manipulations. Given this hypothesis, it is possible that the present post-weaning isolation manipulation was not significant enough to produce anxiety- and depression-related changes. However, this possibility is less likely when considered in the context of evidence from other studies indicating that the period of development that is especially sensitive to stress occurs later in life (Alberini & Travaglia, 2017; Callaghan, Graham, Li, & Richardson, 2013).

Consistent with this alternative hypothesis, most human children do not have any memory of events that occurred prior to 3 years of age (Alberini & Travaglia, 2017), and rats do not retain fear memory at PND 16, but do at PND 23 (Callaghan et al., 2013). Therefore, events occurring after these periods of development may have more lasting effects, possibly because neurological processes related to stress and memory during infancy are not developed enough to alter behaviors during the earlier ages of development. Further research is necessary to resolve the inconsistencies in behavioral consequences of pre- vs. post-weaning social manipulations.

Although it is possible that the lack of anxiety- and depression-related changes were a function of the specific operational tests or the specific period of social isolation, it is more likely that this outcome is due to the stress-buffering effects of a resocialization period in the present
study design. This design used 4 weeks of post-weaning social isolation, followed by a period of resocialization for 2 weeks. This novel 2-week resocialization period allowed the isolated animals to resocialize with their respective female sibling and may have mediated the potential behavioral consequences of isolation related to mood disorders. This effect mirrors previous observations in prairie vole studies using various forms of protective stimulation such as exercise, enrichment, and/or re-pairing. For example, 4 weeks of isolation with an enrichment paradigm, as well as isolation followed by 4 weeks of enrichment or access to a running wheel, protected against anxiety- and depression-related consequences of social isolation in the EPM and FST respectively (Grippo et al., 2014). Similarly, male-female pairing for 5 days, followed by 5 days of isolation, and then 10 days of re-pairing or access to a non-social enrichment paradigm protected against the consequences of social isolation in the FST, compared to animals who were left isolated during the 10-day treatment period (Normann et al., 2018). These data suggest that environmental enrichment and/or exercise can protect against anxiety- and depression-related behaviors following isolation, similar to re-pairing with a familiar animal, even when the pairing and treatment times are short (Normann et al., 2018). These previous treatment paradigms in adult prairie voles may be similar to the social engagement and stimulation provided by the resocialization period with the siblings in the present study. In particular, it is possible that specific play behaviors displayed by the juvenile prairie voles during this resocialization period were important for the development of healthy neurological function, emotion-related behaviors, and social behaviors in adulthood.

**Adulthood Opposite-Sex Behaviors: Male-Female Social Interaction Tests**

Not only are behaviors related to depression and anxiety important in the present context, but adulthood social behaviors also provide insight into the long-term consequences of early life
social isolation. Previous research demonstrates the importance of early life social experiences on adulthood social behaviors (Bell et al., 2009; Hole, 1991; Holloway & Suter, 2004; Pellis, 1988; Spevak et al., 1973; Vanderschuren et al., 2016); therefore, the present study explored the effects of social isolation on pair-bond formation, specifically including observations of male-female social interactions prior to testing for a partner preference. Based on previous research highlighting the importance of adequate socialization and biological processes on sexual function and pair-bond formation (Ahern & Young, 2009; Holloway & Suter, 2004; Pierce et al., 1991; Spevak et al., 1973), it was hypothesized that social isolation would elicit fewer positive responses to male approach in the Male-Female Social Interaction Test at Initial Pairing compared to the paired controls. In contrast, the socially isolated animals did not display fewer positive responses to the male approach versus the control group. It is possible that social isolation altered sexual behaviors in an adaptive manner to facilitate later reproductive success. Some previous research from prairie voles is consistent with this hypothesis. For instance, 4 weeks of social isolation in adulthood accelerated reproductive function in prairie voles of both sexes, measured by copulatory behaviors and latency to male-induced estrus (Perry, Carter, & Cushing, 2016).

Related to social isolation potentially altering later sexual behavior, it is also possible that the isolation period influenced specific adulthood biological reproductive functions. Following the Male-Female Social Interaction Test at Initial Pairing, the pairs were allowed to cohabitate for 24 hours. This was an important component of the study design to allow for female prairie voles to enter estrus. Estrus in prairie voles is induced following olfactory stimuli and physical contact from a male (Carter, Witt, Schneider, Harris, & Volkening, 1987) and is hypothesized to be important for the formation of a pair-bond (Williams et al., 1992). It was hypothesized that
social isolation would impair pair-bond formation during this cohabitation period. Interestingly, and opposite to this hypothesis, after 24 hours of cohabitation with the male partner, the socially isolated females displayed increased levels of mating/lordosis behaviors compared to the paired-control animals. This pattern is not consistent with previous evidence that early life social stress increases the latency to form a pair bond in a specific PPT (Ahern & Young, 2009). However, this previous study differed from the present design by employing pre-weaning social stress via paternal deprivation, without any social isolation during pre- or post-weaning development. The current behaviors demonstrated by isolated females in the present study are more consistent with the hypothesis that social isolation accelerated reproductive function, similar to the previous observation in adult socially isolated prairie voles (Perry et al., 2016).

Adulthood Opposite-Sex Preferences: PPT

Prairie voles are unique rodents in that they form lasting opposite-sex pair bonds, and these bonds and preferences are sensitive to stress (Ahern & Young, 2009; DeVries, DeVries, Taymans, & Carter, 1995; Perry et al., 2016). Therefore, it was hypothesized that the male-female social bond would be weaker in the socially isolated animals due to their early life stress experience. Partner preference, which can be measured in the PPT, has previously been defined as increased duration spent huddling with a familiar male partner (relative to a stranger male) during the PPT (Williams et al., 1992). In addition to this measure, the present study also explored measures of aggression towards the partner, aggression towards the stranger, duration spent huddling with the stranger, and time spent in the neutral chamber of the PPT to assess female behaviors relevant to a preference for her male partner. The socially isolated females did not display the hypothesized lack of a preference for their male partners; instead, nearly all of the
time that the socially isolated animals spent huddling occurred with their male partners. This group also displayed slightly increased aggression toward the stranger male, relative to the control group. By contrast, the paired-control animals engaged in huddling with both their partners and the stranger males. This pattern of results suggests that socially isolated animals clearly preferred physical contact with the male partner over a stranger male, whereas that preference was not evident in the paired-control females. These behaviors mirror the behavioral patterns displayed in the Male-Female Social Interaction Tests and may have occurred for similar reasons, including altered sexual function combined with potential changes in biological reproductive functions of isolated prairie voles (Carter et al., 1987; DeVries et al., 1995; Perry et al., 2016; Williams et al., 1992). Future research will benefit from a more detailed investigation of the development of sexual and reproductive processes following early life social stress.

**Adulthood Social Behaviors: Adult Social Interaction Test**

As a final measure of how post-weaning social isolation impacts adult social behaviors, the animals of the present study were re-paired with same-sex siblings approximately 20 hours after the PPT. This specific social behavioral test was presented to assess sibling recognition and to investigate same-sex social behaviors following several adulthood social and behavioral manipulations. It was expected that isolated animals would display decreased affiliative behaviors during this behavioral test, including decreased huddling with the respective sibling. The results did not fully support that hypothesis. There were no differences observed in huddling behaviors between paired and isolated groups; however, the socially isolated group displayed increased instances of aggression toward their sibling, compared to the paired control group. Aggression was not expected during this social behavioral task because reports of aggressive behaviors toward familiar animals in prairie voles are rare (Lee, Goodwin, Freitas, & Beery,
2019; Simmons et al., 2017) and have generally been described in social tasks involving interactions with strangers (Grippo, Cushing, et al., 2007; Grippo, McNeal, Watanasriyakul, Cacioppo, Scotti, & Dagner 2019). Previous research indicates that female prairie voles can form same-sex bonds with unfamiliar animals even after re-pairing with a novel female (Lee et al., 2019). The differences in aggression observed here may be the result of the juvenile social isolation manipulation altering the long-term stress response. For example, increased stress reactivity may manifest as increased aggression in sexually experienced prairie voles. Although aggression is more commonly observed in males or in the presence of an unfamiliar animal (Bowler et al., 2002; Lee et al., 2019), the isolated females in the present study may have responded negatively to being removed from a previous male partner and subsequently re-introduced to a sibling.

One mechanism of altered stress reactivity in isolated prairie voles may have involved altered oxytocin. Oxytocin plays an important role in the stress response, male-female social behaviors, and social memory (Bales et al., 2013; Insel & Shapiro, 1992; Ross et al., 2009). Further, post-weaning social isolation decreases sociality and Fos-positive oxytocin cells within the PVN of female rats (Tanaka et al., 2019). This research highlights the connection between early life stress and the oxytocin system. Early life social stress may alter oxytocin functionality within the stress responses system, thereby influencing social behaviors in adulthood. Future research focused on the central oxytocin system (which will be conducted outside the scope of the present project) will provide valuable insight into a potential mechanism that underlies adulthood social behavior.
Exploratory Analyses

Given that the present study investigated the effects of early life stress on behaviors during the juvenile period and into adulthood, several correlations were conducted to further explore the relationships among variables related to juvenile social behaviors, anxiety- and depression-related behaviors, and adulthood social behaviors. Of the many correlations conducted, seven yielded a moderate or large effect, but only two of these relationships were equally driven by the socially isolated and paired control conditions. The results of the correlations with the conditions combined and the correlations for each condition separately provide insight into how the behaviors representing anxiety and depression relate to the social behaviors in the juvenile period and adulthood, as well as how juvenile social behaviors are related to adulthood social behaviors. In order to understand how post-weaning social isolation impacts later behaviors, these relationships and their directionality are important to discuss.

An unexpected pattern of results from the primary analyses was the absence of an effect of social isolation on behaviors in the EPM and FST. Therefore, exploratory analyses correlating the EPM and FST behaviors with each other, as well as with adulthood social behaviors, may be informative. To begin, the duration in the open arms of the EPM was strongly and negatively correlated with the duration spent immobile in the FST. This suggests that these measures of anxiety- and depression-related behaviors are related to each other, supporting previously published literature focusing on the inter-relatedness of these constructs in isolated female prairie voles (Grippo et al., 2008).

Second, the duration spent immobile in the FST was moderately positively correlated with the duration spent huddling with the male partner in the PPT, an effect largely driven by the socially isolated group. This indicates that the relationship between depression-related behaviors
and huddling with the male partner in the PPT was much stronger in the isolated animals. This pattern may relate to increases in corticosterone during different behavioral states following social isolation. For example, FST exposure increases corticosterone production (Drugan et al., 2005), which is hypothesized to represent a physiological stress response. Corticosterone is also increased during male-female social interactions in female prairie voles, as it facilitates the formation of a pair bond (DeVries et al., 1995). It is possible, therefore, that larger increases in corticosterone in the isolated condition may have mediated the stronger correlation between behaviors in the FST and the PPT in this group. Further research focused on corticosterone reactivity following different behavioral tests will help to confirm this hypothesis.

The duration spent in the open arms of the EPM was strongly and negatively correlated with the duration of huddling with the male partner in the PPT; this relationship was similar in paired and isolated conditions. These results support the previously discussed hypothesis that 2 weeks of resocialization might buffer against the consequences of post-weaning social isolation on the stress response. Interestingly, the duration spent in the open arms of the EPM was strongly positively correlated with the duration spent huddling with the stranger male in the PPT in the paired group only. This pattern of results indicates that paired-control animals who displayed lower levels of anxiety-like behaviors during the EPM spent more time with the stranger male during the PPT. In contrast, socially isolated animals spent almost no time huddling with the stranger males during the PPT, and therefore the correlation between EPM open arm duration and stranger huddling was very low. It is possible that lower levels of anxiety-related behaviors in the EPM also mediated lower levels of anxiety toward a novel stranger in the paired group. Collectively, these correlations further inform our understanding of the relationships between
emotion-related and social behaviors in prairie voles and provide a foundation for future studies to investigate these specific relationships.

Exploratory correlations were also conducted to assess the relationship between juvenile and adulthood social behaviors. These correlations inform our understanding of the potential stability of social behaviors throughout social development, based on the presence or absence of early life social stress. The huddling durations in the Juvenile and the Adult Social Interaction Tests were strongly and positively correlated only in the paired group. By contrast, the socially isolated group displayed a small to moderate negative correlation between the huddling behaviors of the Juvenile and Adult Social Interaction Tests. This pattern of results may suggest that appropriate social support during early life (as was the case for the paired control group) is related to appropriate social interactions with a sibling throughout development. Early life social isolation, on the other hand, may impair the development of appropriate social behaviors.

In addition to differential correlations between huddling behaviors in the paired and isolated groups, the duration the animals spent alone in the Juvenile and Adult Social Interaction Tests was moderately and positively correlated only in the paired group (but not the isolated group). These results may suggest that previous early life social experiences influence the amount of time an animal chooses to spend with a family member versus alone. Appropriate early life social support (in the paired control group) may influence this social behavioral process more consistently throughout development, relative to early life social stress. Additional research questions using the prairie vole model will provide valuable insight into the influence of early life stress on social developmental processes.
Conclusions, Implications for Humans, and Recommendations for Future Research

The present study used a novel animal model and a unique series of tests to explore the influence of early life social stress during a specific period of development on social behaviors in the juvenile and adult periods as well as behaviors related to depression and anxiety in adulthood. To accomplish these goals, the present study utilized female prairie voles and several assessments of social behaviors including a Juvenile Social Interaction Test and Male-Female Social Interaction Tests. The Juvenile Social Interaction Test was specifically used to investigate whether female prairie voles’ play and other social behaviors were altered as a result of early life social isolation, as well as whether post-weaning resocialization might affect behaviors in adulthood. Play behaviors in females have not been thoroughly examined in rodents, with very few studies focusing on female prairie voles (Chau et al., 2008; Paz y Miñatno & Tang-Martínez, 1999). Similarly, the behaviors between a female and novel male have not been extensively investigated outside of social preference behaviors and parental behaviors (Bales & Carter, 2003; McGuire & Getz, 1991). These unique social tests were included to provide an understanding of the importance of juvenile social interactions on later social and emotional behaviors.

The results of the present study indicate that social stress during the juvenile period alters juvenile social behaviors, including increasing play behavior with a sibling. Some specific social behaviors were also altered during adulthood in the isolated group, including increased mating/lordosis in the Male-Female Social Interaction Test Following 24 Hours of Cohabitation and increased behaviors related to partner preference in the PPT. These specific changes indicate that early life social isolation may influence some juvenile and adulthood social behaviors in an adaptive manner, in contrast to the maladaptive social behavioral changes that were initially hypothesized. However, following several behavioral tests in adulthood, isolated prairie voles
displayed a maladaptive response to being re-introduced to their previous siblings in the form of aggression toward them. This altered behavior suggests that social isolation may influence adulthood stress reactivity and associated social behaviors in a negative manner.

In contrast to some social behavioral changes in socially isolated prairie voles, other adulthood social behaviors and anxiety- and depression-related behaviors were not altered as a function of juvenile social isolation. These results suggest that 2 weeks of resocialization with a female sibling prior to adulthood may protect against some negative behavioral consequences of social isolation. This hypothesis is supported by the results of the Juvenile Social Interaction Test, suggesting increased levels of active positive social behaviors in the socially isolated animals compared to the paired control animals. These positive social interactions may have served as a form of enrichment, which has been associated with several behavioral and physiological benefits in prairie voles (Grippo et al., 2014; Normann et al., 2018; Watanasriyakul et al., 2017), other rodent species (D’Andrea et al., 2010), and even humans (Hamer, Endrighi, & Poole, 2012).

Given previous evidence from prairie vole social isolation studies in adults, the results of the adulthood behavioral tests in the present study suggest that resocialization may serve as a form of protective social enrichment. As detailed earlier in this discussion section, the absence of paired vs. isolated differences in the EPM and FST is similar to what has been reported by previous prairie vole studies using enrichment, physical exercise, or re-pairing with a previous social partner to prevent or reverse the consequences of social isolation in adulthood (Grippo et al., 2014; Normann et al., 2018; Watanasriyakul et al., 2017). Similar to these previous paradigms demonstrating enrichment’s ability to buffer the effects of social isolation, the
resocialization period in the present study may have provided a buffering effect on adulthood emotion-related behaviors.

The resocialization period as a protective mechanism may also help to explain some adulthood social behavioral observations in the present study. The absence of behavioral differences in the Male-Female Social Interaction Test at Initial Pairing suggests that the resocialization period mediated these opposite-sex interaction effects of juvenile social stress. Further, 24 hours of cohabitation with a male partner was associated with increased mating/lordosis instances in the socially isolated group, which might indicate that the resocialization period increased the level of adaptive social behaviors in the socially isolated animals. This outcome was not initially hypothesized; rather, isolated females were expected to show deficits in both juvenile and adulthood social behaviors. The importance of female play during the juvenile period on adulthood male-female social interactions has not been investigated in prairie voles prior to the present study. The unexpected increase in play behavior displayed in the isolated group during the Juvenile Social Interaction Test may have persisted throughout the resocialization period and subsequently may have mediated the unexpected increase in mating/lordosis behaviors during adulthood.

This unexpected pattern of increased positive social behaviors in the socially isolated group extends to partner preference behaviors. Partner preference has been defined as increased duration spent huddling with a familiar male partner, relative to a stranger male (Williams et al., 1992). However, as there were no differences in duration spent huddling with the male partner between the housing conditions, the duration of time spent huddling with the stranger male provided some useful information regarding the formation of a pair bond. The socially isolated animals spent almost no time huddling with the male stranger, whereas the paired-control
animals spent similar durations huddling with the male stranger and male partner. These behaviors indicate that the socially isolated animals preferred the male partner over the stranger male, which was not evident in the paired control group. This outcome was not hypothesized based on some previous research suggesting early life stress increases the latency of cohabitation necessary for females to form a pair bond (Ahern & Young, 2009). However, that previous study differed from the current study in that it employed paired housing during post-weaning development, rather than a social isolation component. The present results may indicate that the transition from an impoverished social environment to paired housing (resocialization) increased active social behaviors beyond the previously reported low levels of play in females (Chau et al., 2008) to improve their ability to form a pair bond in adulthood. This pattern of results is similar to the effects observed in pre-weaning prairie voles following short separation from their parents. Upon reuniting with the offspring, the parents engage in elevated levels of care behaviors (licking, grooming, huddling), which has been suggested to increase social behaviors in juvenile prairie voles (Perkeybile et al., 2019). Further research focused on the specific behaviors that the animals engaged in during the 2-week resocialization period will help to confirm this theory.

After the PPT, the animals were re-introduced to their female siblings from the resocialization period (or the previous paired female in the paired group) to assess social behaviors and sibling recognition. The Adult Social Interaction Test was the only behavioral test that supported the hypothesis that social isolation would impair social behaviors compared to paired control conditions. This prediction was supported by increased aggressive behaviors toward the previous sibling in the isolated group, compared to the control group. Interestingly, the resocialization buffering theory does not explain these results. A possible explanation for this increased aggression toward a sibling is an alteration in the long-term stress response system, as
described above, which may have been a function of the combination of early life social stress with additional social and behavioral manipulations prior to the final Adult Social Interaction Test.

The present study is an important investigation of behavioral consequences of early life social stress that was confined to a specified period of development in prairie voles. When integrated together, the results from both the juvenile and adulthood behavioral measures provide insight into the importance of socialization during development and the critical role of sibling interactions on behavioral measures in adulthood. Some limitations in the present study design, such as the sample size and the need to remove some statistical outliers from the data analyses, may limit the conclusions from this study. Future studies will provide an important extension to the current study by attempting to remedy uncontrolled variables and focusing on improvements in the study design.

First, conducting an EPM and FST test conducted during the juvenile period, prior to the resocialization period, will provide information about the consequences of post-weaning social isolation on anxiety- and depression-related behaviors during development. This testing strategy will also provide an interesting comparison between EPM and FST behaviors before and after resocialization to assess whether resocialization can reverse juvenile anxiety- and depression-related behaviors. Second, following the Juvenile Social Interaction Test, it would be informative to record additional observations throughout 2 weeks of resocialization, allowing for a better understanding of specific behaviors displayed by the animals during the resocialization period. For example, if isolated animals continued to engage in play behaviors after the conclusion of the test, this observation would provide support for the social enrichment theory. Third, the addition of a group of animals who experienced 5 weeks of social isolation and another who experienced
6 weeks of isolation (into adulthood) would also act to clarify whether the resocialization period was as enriching as it appeared to be in the present study. These comparisons will provide important information about the specific influence of resocialization on later behaviors, relative to both continued isolation and continued pairing (control conditions) into adulthood. Finally, the inclusion of physiological and neural measures, such as plasma oxytocin, oxytocin receptor binding in the nucleus accumbens, and PVN activation (immediate early gene c-Fos with or without oxytocin co-labeling, time linked to a social test or stressor), will inform questions about mechanisms underlying positive social behaviors and allow for further exploration of the effects of post-weaning social isolation on stress responses (Grippo, Gerena, et al., 2007). Collectively, these changes to the study design will provide critical additional information about the effects of post-weaning social isolation, with and without resocialization, on developmental trajectories of stress reactivity, emotion-related behaviors, and social behaviors.

The present study capitalized on the similarities in social structure between prairie voles and humans, including the importance of socialization during development and the prairie vole’s ability to be re-paired with their same-sex siblings to mirror human experiences (Lee et al., 2019). The present study was designed to investigate early life social stress on both juvenile and adulthood behaviors, thus modeling human social experiences from childhood, to adolescence, and into adulthood. Four weeks of post-weaning social isolation modeled social stress or neglect during the developmental equivalent of 3 to 14 years old in humans. This period was followed by 2 weeks of resocialization, which represented the transition from childhood to adolescence when human children gain autonomy and can engage in social experiences outside of the family unit (Semple et al., 2013). The adulthood behavioral tests were designed to assess the influence of early life social stress on behaviors related to depression and anxiety, as well as social behaviors
associated with male-female bonds and partner preference formation, to provide insight into these constructs in humans.

In summary, the present results have important implications for understanding human social and emotional development. Social stress early in life has been associated with communication problems, behavioral disorders, and increased risk of developing psychoemotional difficulties in adulthood (Dodge et al., 2003; Heim et al., 2010; Lansford et al., 2006). These outcomes are related to the presence or absence of social experiences during childhood. The absence of social interactions with peers inhibits the development of social skills and emotional regulation (Cutting & Dunn, 2006; McAlister & Peterson, 2013; Peterson et al., 2016). The results of the present study therefore inform our understanding of potential benefits of juvenile social interactions on later social and emotional responses. This research has valuable implications for designing prevention and treatment strategies for human children and adolescents who are exposed to social stress. Additional research using valid and reliable animal models, such as the prairie vole model described here, will continue to inform our understanding of social and emotional development in humans.
REFERENCES


