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Behavioral and neuroendocrine consequences of disrupting a long-term monogamous social bond in aging prairie voles

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Abstract

Social support from a spouse, long-term partner, or someone who provides emotional or instrumental support may protect against consequences of aging, including mediating behavioral stress reactivity and altering neurobiological process that underlie short-term stress responses. Therefore, long-term social bonding may have behavioral and neurobiological benefits. The socially monogamous prairie vole provides a valuable experimental model for investigating the benefits of long-term social bonds on short-term stress reactivity in aging animals, given their unique social structure of forming enduring opposite-sex bonds, living in family groups, and bi-parental rearing strategies. Male-female pairs of long-term, cohabitating prairie voles were investigated for short-term behavioral and neuroendocrine stress reactivity following either long-term social pairing (control), or a period of social isolation. In Experiment 1, social isolation was associated with altered behavioral reactivity to an acute swim stressor, and greater neural activation in the hypothalamic paraventricular nucleus, as well as specifically the parvocellular region, following the swim stressor (vs. control). In Experiment 2, social isolation was associated with greater corticosterone reactivity following an acute restraint stressor (vs. pairing). No sex differences were observed. Exploratory correlation and subgroup analyses revealed systematic relationships among various demographic variables (such as age of the subjects, amount of time the pair cohabitated together, and number of litters the pair reared together) and the behavioral and neuroendocrine outcome measures. These findings may inform our understanding of the benefits of long-term social bonding on modulating short-term behavioral and neuroendocrine responses to stress.

LAY SUMMARY

Receiving social support from a long-term spouse or partner, or having a strong support network from friends, may have important health benefits, especially as people age. In aging monogamous prairie voles, social isolation from a long-term social partner disrupted behaviors and short-term stress responses, whereas living with a long-term partner protected against these disruptions. This research is important for our understanding of the benefits of social support on stress responses as we age.

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DECLARATION OF INTEREST

The authors declare no conflicts of interest.

Keywords

Acute stress; Aging; Behavior; Prairie vole; Social isolation; Social monogamy

INTRODUCTION

Social bonds are essential for supporting psychological and physiological health. Humans who report higher level of social engagement, larger social groups, or more meaningful social networks are at a lower risk of depression, heart disease, and other stress-related pathologies, relative to those with lower levels of social engagement, smaller, or less meaningful networks (Beutel et al., 2018; Cacioppo et al., 2010; Cacioppo & Cacioppo, 2018; Kaplan et al., 1988; Steptoe et al., 2004). Social bonds, such as lower rates of objective social isolation and lower levels of perceived loneliness, are also associated with an increased lifespan (Beller & Wagner, 2018; Steptoe et al., 2013).

Long-term and/or monogamous social bonds may be important for promoting short-term stress coping abilities in humans, especially during the aging years. Positive marital relationships and social interactions influence health in both men and women, including providing benefits for immune functioning, cardiovascular variables, stress reactivity, mood, and longevity (Grewen et al., 2003; Kiecolt-Glaser & Newton, 2001; Kiecolt-Glaser & Wilson, 2017; Robles et al., 2014; Sbarra, 2009; Uchino, 2006). Stress can transfer between spouses as well; for instance, salivary cortisol levels and negative mood states co-vary in married couples (Saxbe & Repetti, 2010). The influence of marital quality on health measures is estimated to be similar in effect size to the influence of diet and exercise (Robles et al., 2014). Although specific neurobiological mechanisms underlying these effects are not entirely elucidated, differences in marital satisfaction and relationship stability are associated with alterations in brain regions that mediate goal-directed behavior, motivation, empathy, stress reactivity, and reward (Acevedo et al., 2012a, 2012b).

Studies with rodent and non-human primate models provide support for protective effects of social bonds on responses to stress, behavior, and neurobiological processes (Carnevali et al., 2012; Fernandez-Duque et al., 1997; McCowan et al., 2016; Shively et al., 2009; Wood & Bhatnagar, 2015). In addition to these models, the socially monogamous prairie vole is a valuable rodent model for studying the influence of social bonding on behavioral, physiological, and neural stress reactivity. Prairie voles display characteristics of social monogamy, such as forming long-term opposite-sex partner bonds, living in extended family groups, and cooperatively caring for offspring (Carter & Getz, 1993; Young et al., 2011). As is the case for humans, adaptive social bonding in prairie voles promotes the health of this species. Appropriate social bonds in this species – such as living with an opposite-sex partner or family members, and engaging in positive social interactions with other animals – protect against behavioral disruptions, basal and reactive neuroendocrine dysfunction, and autonomic and cardiovascular responses to stress (Bosch et al., 2009; Grippo et al., 2011; McNeal et al., 2017; Smith et al., 2013; Sun et al., 2014). Several changes in stress-related brain circuitry have been suggested to play a role in the protective effects of social bonds, including peptide and hormone functions, and mechanisms involving the paraventricular

nucleus of the hypothalamus (PVN), nucleus accumbens, amygdala, and medial prefrontal cortex (Bosch et al., 2016; Bosch et al., 2009; Burkett et al., 2016; Carter et al., 2008; Lui et al., 2010; Stowe et al., 2005; Young et al., 2011).

The unique social structure of prairie voles allows for experimental investigations of the behavioral and neurobiological benefits of long-term social bonds. Our previous research has primarily focused on protective behavioral, physiological, and neural effects of social bonds among same-sex siblings (Grippo et al., 2007, 2012) or in sexually-inexperienced young adult prairie vole pairs (McNeal et al., 2014, 2017, 2019). However, the prairie vole model may provide valuable insight into the potential stress-buffering effects of social monogamy, such as engaging in long-term social interactions, cooperatively raising offspring, and living together as a family group. Therefore, the objective of the present study was to investigate the effects of disrupting a social bond in aging, long-term, monogamous pairs of prairie voles on short-term stress reactivity (compared to stress reactivity in control animals that remain paired with the long-term partner). Prairie voles were studied over a 6-year period at the end of their reproductive cycles, after living with and raising multiple offspring with a consistent partner of the opposite sex, to determine the influence of disrupted long-term social bonds on behavioral and neuroendocrine indicators of short-term stress reactivity. We hypothesized that disrupting the social bond between long-term, cohabitating pairs of prairie voles would increase acute behavioral and neuroendocrine stress responses. In addition to this primary hypothesis, we also conducted several exploratory analyses to determine whether demographic variables, such as age or sex of the subjects, amount of time cohabitated with the social partner, or number of litters raised with the social partner, were systematically related to the stress reactivity outcome measures.

METHODS

Animals

60 adult prairie voles of both sexes were used for the experimental procedures described here [Experiment 1: n=26 total (all male); Experiment 2: n=34 total (n=17 male and n=17 female)]. All animals were descendants of a wild stock originally captured near Champaign, IL, and bred in the laboratory at Northern Illinois University. All procedures were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, and approved by the Northern Illinois University Institutional Animal Care and Use Committee.

All animals were maintained on a 14/10h light/dark cycle (lights on at 6:30 am), with a temperature of 25±1°C and a relative humidity of 24±1 g/m^3, and allowed *ad libitum* access to food (Purina rabbit chow) and water. All animals were paired with a consistent partner of the opposite sex for the majority of their adult, reproductively-viable lives in the laboratory. Male-female pairs, and all litters produced by the pair (through weaning age at 21 days of age) were housed together as a family group in large, polycarbonate cage (25×45×60cm), with food, water, and cotton nesting material. All litters remained with the male-female pair until 21 days of age, at which time litters were weaned and moved into separate cages from the male-female pair. All male-female pairs were left undisturbed between and during rearing of the offspring, with the exception of standardized weekly cage changes.

Using a semi-naturalistic design, animals were studied at the end of their reproductive cycles over a multiple-year time period (between 2010 and 2016). A pair was considered to be at the end of its reproductive cycle, and therefore eligible for inclusion in this study, if it met one of the following criteria: (a) the pair completely stopped producing litters; (b) the pair showed a progressive reduction in the number of offspring per litter over several successive breeding cycles; and/or (c) the pair had significant delays between breeding cycles over several successive cycles. The animals that were included in this study had a mean ± standard error of the mean (SEM) age of 20.7±1.1 months (range 4.5–33.5 months); and a mean body weight of 45.1±0.9 g (range 27–68g). The animals were paired together for a mean ± SEM of 16.4±1.1 months (range 2.2–30 months); and produced (and raised through weaning age at post-natal day 21) multiple litters of offspring together (19±1 litters, range 2–36 litters).

General Experimental Design and Housing Conditions

Experiment 1—At the end of their reproductive cycles, male prairie voles were randomly assigned to one of two conditions: (a) paired (immediately tested; n=13); or (b) isolated (delayed tested; n=13). Animals in the paired group were removed from the respective female partners and immediately placed into a 5-minute forced swim test (FST; in a separate room, without visual, auditory, or olfactory cues from the female partner), followed by euthanasia and brain extraction 2 hours after the end of the FST. Animals in the isolated group were removed from the respective female partners and housed in isolation for 4 weeks (in a clean cage in a separate room, without visual, olfactory, or auditory cues from the previous female partner), and then placed into a 5-minute FST, followed by euthanasia and brain extraction 2 hours after the end of the FST.

Experiment 2—At the end of their reproductive cycles, prairie voles of each sex were randomly assigned to one of two conditions: (a) paired (immediately tested; n=9 male and 10 female); or (b) isolated (delayed tested; n=8 male and 7 female). The paired group was removed from its opposite-sex partner and immediately placed into a 30-minute restraint session (in a separate room, without visual, auditory, or olfactory cues from the opposite-sex partner), followed by blood collection and euthanasia 10 minutes after the end of restraint. The isolated group was removed from its opposite-sex partner and housed in isolation for 4 weeks (in a clean cage in separate room, without visual, olfactory, or auditory cues from its previous opposite-sex partner), and then placed into a 30-minute restraint session, followed by blood collection and euthanasia 10 minutes after the end of restraint.

Specific Methods, Experiment 1

FST—Prairie voles in each group were exposed to a 5-minute FST during the light period, using procedures described previously (Cryan et al., 2005b; McNeal et al., 2017). A clear, cylindrical Plexiglas tank (height 46cm; diameter 20cm) was filled to a depth of 18cm with room temperature tap water (25–26°C). Each vole was placed individually into the tank for 5 minutes. The tank was cleaned thoroughly and filled with clean water prior to testing each prairie vole. Immediately following the swim period, the prairie vole was placed into its previous cage (paired or isolated), allowed access to a heat lamp for 15 minutes, and

subsequently was left undisturbed for an additional 1 hour and 45 minutes (for a total of 2 hours).

During the FST, durations of behaviors were recorded using a digital video camera, and scored manually, off-line, by 2–3 trained coders that were blind to the experimental conditions using The Observer XT 8.0 (Noldus Information Technology, Leesburg, VA). Behaviors were defined as durations of: (a) swimming, involving coordinated movements of the forelimbs and hindlimbs without breaking the surface of the water; (b) struggling, involving forelimbs breaking the surface of water; (c) climbing, involving attempts to climb the walls of the tank, with the forelimbs breaking the surface of the water; and (d) immobility, involving no limb or body movements (passive floating) or using limbs solely to remain afloat without corresponding trunk movements; these are mutually-exclusive and exhaustive categories. Struggling, climbing and swimming were summed to provide one index of active coping behaviors, similar to previous studies involving a 5-minute FST exposure in prairie voles; immobility was defined as a passive behavioral response, consistent with the hypothesis that this behavior represents a maladaptive response to the short-term stress of swimming in previous prairie vole protocols (Bosch et al., 2009; Grippo et al., 2012; McNeal et al., 2014, 2017; Sun et al., 2014). Durations of each behavioral category were averaged among multiple coders, and coders were trained to a level of at least 90% inter-rater reliability.

Euthanasia and Brain Extraction—Two hours following the 5-minute FST, animals were removed from the home cage and euthanized under anesthesia (ketamine, 67mg/kg, subcutaneous (sc), NLS Animal Health, Owings Mills, MD; xylazine, 13.33mg/kg, sc, NLS Animal Health). Brains were immediately extracted from the skulls and processed with a spin immersion technique that has been validated previously for use in prairie voles (Cushing et al., 2001). Brains were immersed in a fixative solution consisting of 4% paraformaldehyde containing 5% acrolein (pH 8.6) for a total of 4 hours. Brains were postfixed for 24 hours in 4% paraformaldehyde, and sunk in 25% sucrose. Tissue was stored in 25% sucrose at 4°C until it was sectioned at 40 μm on a cryostat. Sliced serial sections were stored in wells in cryoprotectant antifreeze solution at −20°C until assayed for cFos using standard avidin:biotinylated enzyme complex (ABC) immunocytochemistry.

Immunohistochemistry—Serial brain slices (40 μm) from the PVN were assayed for the expression of cFos using standard ABC immunohistochemistry, as described previously (Grippo et al., 2007). Anti-cFos (Oncogene Science, Cambridge, MA; generated in rabbit) was used at a concentration of 1:100,000, and was visualized using nickel-diaminobenzadine (DAB) dissolved in 0.175 M sodium acetate.

Free-floating sections were rinsed 6 times during a 1 hour period with potassium phosphate buffered saline (KPBS). Sections were then incubated in 1% sodium borohydride for 20 minutes at room temperature. After multiple washes in KPBS, sections were incubated in 0.014% phenylhydrazine for 15 minutes at room temperature. Tissue was rinsed 6 times during a period of 1 hour in KPBS. Sections were then incubated in primary antibody for cFos diluted in KPBS + 0.4% Triton X-100 for 1 hour at room temperature, and then incubated for 42 hours at 4°C. Following this incubation period, sections were rinsed 10

times during a period of 1 hour with KPBS. Sections were then incubated in anti-rabbit IgG (BA-1000; Vector Laboratories, Burlingame, CA; 1:600) for 1 hour at room temperature. Sections were rinsed 5 times during a period of 50 minutes with KPBS, and then incubated in A/B solution (Vectastain Elite PK-6100; Vector Laboratories, Burlingame, CA; 45μl A, 45μl B per 10 ml KPBS + 0.4% Triton X-100) for 1 hour at room temperature. Sections were rinsed 3 times in KPBS and then 3 times in 0.175 M sodium acetate. cFos was visualized by incubation in DAB and nickel sulfate, dissolved in sodium acetate, for 9 minutes at room temperature, followed by rinsing 3 times with sodium acetate and 3 times with KPBS.

Stained sections were mounted on electrostatically-charged slides, air-dried, dehydrated in a series of ethanol dilutions, cleared with Histoclear (National Diagnostics, Atlanta, GA), and protected with coverslips using Histomount mounting medium (National Diagnostics).

Images were captured using a Nikon Eclipse E 800 microscope, Sensi-cam camera and IPLab software (Scanalytics, Inc., Fairfax, VA). The density of cFos-immunoreactive cell bodies was determined in the PVN with a 10x objective, using a standardized sampling area, according to procedures described previously (Pan et al., 2009; Ruscio et al., 2007). Measurements within the PVN were taken in a caudal section of the nucleus where the stained cells take a characteristic shape demonstrated in previous studies (Ruscio et al., 2007; Wang et al., 1996). This section is further characterized by the medial-lateral position of the fornix (relative to the third ventricle) and medial and dorsal location of the optic tract (relative to more central and ventral position in more rostral sections), approximate to Figure 49 in Paxinos and Watson (Paxinos & Watson, 2005).

Density measurements in the PVN were manually determined from sections matched in rostral-caudal orientation to minimize variability, and are expressed as the number of cells per standardized area. Density measurements of the parvocellular region of the PVN were characterized as an index of activation in corticotropin-releasing factor (CRF) perikarya (McCann et al., 2000; Saper & Lowell, 2014). Using ImageJ (National Institutes of Health, Bethesda, MD), images were first converted into 8-bit images to increase the contrast between the cells and the image background. A region of interest (ROI) of 51×152 pixels was placed on each side of the third ventricle, specific to the parvocellular region (Chen et al., 2013). An additional background ROI of 51×51 pixels for each image was placed in an area of the tissue devoid of cells, structural landmarks, or artifact, and was subtracted from the parvocellular density measurement. These values are expressed as an arbitrary density unit.

For all subjects, density measures were conducted by 2–3 trained, experimentally-blind raters. Two to three brain sections were analyzed from each subject, and measurements were performed separately for each hemisphere. Density measures for each subject were then averaged across brain slices, hemispheres, and raters to provide an accurate estimation of cell density in the full PVN and the parvocellular region. Damaged sections were excluded from analysis.

Specific Methods, Experiment 2

Restraint—Prairie voles in each group were exposed to 30 minutes of restraint during the light period in a custom-designed apparatus (Pournajafi-Nazarloo et al., 2009). Each animal was placed individually into a clean wire mesh screen that wrapped around the animal and was closed at each end with binder clips, which restricted all limb movement (Dayas et al., 2001). Care was taken to ensure that the binder clips did not pinch the animal's nose or tail. Following the restraint session, the screen material was removed, and the animal was replaced into its previous cage (paired or isolated), and was left undisturbed, for 10 minutes.

Blood Sampling and Plasma Extraction—10 minutes following the end of the 30-minute restraint session, animals were removed from the home cage and anesthetized for blood collection using procedures described previously (Grippo et al., 2007). This time point was specifically chosen based on previous evidence of increased circulating hormone reactivity following a 5-minute behavioral stressor in prairie voles (McNeal et al., 2017; Wardwell et al, 2020). Animals were deeply anesthetized with a mixture of ketamine (67 mg/kg, subcutaneous (sc); NLS Animal Health) and xylazine (13.33mg/kg, sc; NLS Animal Health). Blood was sampled within two minutes of the anesthetic injection, from the periorbital sinus via a heparinized capillary tube, and was collected during a period of 1.5 minutes. Therefore, blood was sampled at 13.5 minutes following the end of the restraint session. The blood was placed immediately on ice, and subsequently centrifuged at 4°C, at 3500 rpm, for 15 minutes, to obtain plasma. Plasma aliquots were stored at −80°C until assayed for circulating corticosterone.

Analysis of Circulating Corticosterone—Plasma concentrations of corticosterone were assayed using a commercially-available enzyme-linked immunosorbent assay kit, according to the kit instructions (Enzo Life Sciences, ADI-900–097, Farmingdale, NY). Plasma was diluted in assay buffer at 1:500 to yield results reliably within the linear portion of the standard curve, as described previously (McNeal et al., 2017). The minimum detection limit is 0.027ng/ml. Inter- and intra-assay coefficients of variation are <5%, and cross-reactivity with other steroids or peptides is <1.7%.

Data Analyses

General—The data are presented as means and SEM for all analyses, tables, and figures. A probability value of $P<0.05$ was considered to be statistically significant, using a 2-tailed distribution.

Evaluation of Demographic Variables—Given the semi-naturalistic study design, the following demographic variables were evaluated for statistical outliers: (a) body weight at the time of testing; (b) number of litters at the time of testing; (c) age at the time of testing; and (d) number of months spent together. Demographic data points that exceeded 2 standard deviations above or below the overall mean were considered to be statistical outliers. The means of each demographic variable including all data were compared to the means after the statistical outliers were removed, using Student's t-tests. Year of testing was not evaluated for outliers.

Primary Analyses—Primary, hypothesis-driven analyses were conducted with single-factor analyses of variance (ANOVA) and/or Student's t-tests. In Experiment 1, behaviors in the FST and immunoreactivity in each the full PVN and the parvocellular region were compared as a function of housing condition (paired vs. isolated). A Pearson's r correlation coefficient was used to compare density measurements in the full PVN vs. the parvocellular region. In Experiment 2, corticosterone reactivity following restraint was compared as a function of housing condition (paired vs. isolated).

Exploratory Analyses—Pearson's r correlation coefficients were conducted to determine whether any dependent measures were linearly related to the demographic variables. Correlations are reported for illustrative purposes only; statistical significance is not included. For the purpose of discussing the relationships, advice from Cohen (Cohen, 1992) was used to describe a correlation of approximately 0.1 as small, approximately 0.3 as moderate, and approximately 0.5 as large.

Given the large range of data in the demographic variables, the dependent measures in Experiments 1 and 2 were further evaluated as a function of the following demographic variables: (a) body weight at the time of testing; (b) age at the time of testing; (c) number of litters produced; (d) number of months spent together; and (e) test year (years 2010–2016, based on the time point that the pair was included in the study). For each of these demographic variables, a median split was conducted to separate paired and isolated groups into a *low* subgroup (below the median value) and a *high* subgroup (above the median value). This median split was then applied to the associated dependent measures: (a) immobility in the FST; (b) immunoreactivity in the PVN; and (c) corticosterone levels. Student's t-tests were used to compare the low and high subgroup for each demographic variable, and for each dependent measure. A Bonferroni correction was applied to each set of Student's t-tests conducted on the dependent variables.

In Experiment 2, additional analyses of the demographic and dependent variables were conducted as a function of sex. Further exploratory analyses were conducted as a function of escaping from the restraint chamber prior to the end of the 30-minute session; 6 animals in the paired group escaped from the chamber, whereas 0 animals from the isolated group escaped. This outcome was not anticipated. These animals were left undisturbed until the end of the 30-minute period, and were roaming freely in the cage which surrounded the restraint chamber. Following the 30-minute period, these animals were treated in the same manner as the non-escapees, and were subsequently replaced into their previous home cage (paired or isolated) to be left undisturbed for 10 minutes. Blood was collected from these escapees in the same manner as the non-escapees, for evaluation with exploratory post-hoc analyses.

RESULTS

Demographic Variables

Demographic variables across Experiments 1 and 2 are show in Table 1. Single-factor ANOVAs indicated that paired and isolated groups did not significantly differ on any of the following variables in either experiment: (a) body weight at the time of testing; (b) age at the

time of testing; (c) number of months spent together; (d) number of litters produced; or (e) year of testing (P>0.05 for all comparisons).

Given the large range of demographic data in the present semi-naturalistic design, these variables were evaluated for statistical outliers using the threshold of 2 standard deviations above or below the mean. The following demographic variables contained statistical outliers: (a) body weight at the time of testing (4 total outliers, 2 from the paired condition and 2 from the isolated condition); and (b) number of litters at the time of testing (5 total outliers; 3 from the paired condition and 2 from the isolated condition). Removal of the outliers from these two variables did not significantly alter the means for either condition (P>0.05 for all comparisons; data not shown). No statistical outliers were detected in the following demographic variables: (a) age at the time of testing; or (b) number of months spent together at the time of testing. Therefore, the primary, hypothesis-driven analyses were conducted with all animals included (animals whose body weight or number of litters at the time of testing were considered outliers were not removed from these analyses).

Demographic variables are further discussed in the sections describing exploratory analyses for each experiment. Exploratory analyses were also conducted with all animals included, without removing the statistical outliers.

Experiment 1, FST

Behaviors in the FST—Social isolation significantly influenced behavior in the FST (Figure 1), such that the duration of immobility during the FST was significantly higher in the isolated group vs. the paired group [t(24)=5.59, P<0.0001]. The specific behaviors of swimming, struggling, and climbing did not differ between the groups (specific categories not shown); these behaviors were summed to provide one index of active behaviors for each group. This category is mutually-exclusive from immobility, and (because these are exhaustive behavioral categories) comprises the remainder of 300 seconds during the FST for each animal.

Immunoreactivity in the PVN—Social isolation (relative to pairing) significantly increased immunoreactivity in the full PVN [t(14)=4.06, P<0.0006] and in the parvocellular region [t(9)=3.3, P<0.009] (Figure 2). The density measurements in the full PVN and the parvocellular region were positively correlated (r =.80; large effect).

Exploratory Analyses—Exploratory comparisons were conducted as a function of the following 5 demographic variables: (a) body weight at the time of testing; (b) age at the time of testing; (c) number of months spent together; (d) number of litters produced; and (e) year of testing. First, several correlational analyses were conducted to determine whether any demographic variable was systematically associated with immobility levels in the FST. All correlations were larger in the paired group vs. the isolated group. Body weight was negatively correlated with immobility duration in both housing groups (r= −0.47 vs. −0.28 in paired and isolated groups, respectively). Age was positively correlated with immobility duration in both housing groups (r= 0.58 vs. 0.32 in paired and isolated groups, respectively). Amount of time spent together was positively correlated with immobility duration in both housing groups (r= 0.58 vs. 0.32 in paired and isolated groups,

respectively). Number of litters produced was positively correlated with immobility duration in both housing groups (r= 0.58 vs. 0.36 in paired and isolated groups, respectively). Test year was positively correlated with immobility duration in both paired and isolated groups (r= 0.54 vs. 0.31 in paired and isolated groups, respectively).

Several correlational analyses were conducted to determine whether any demographic variable was systematically associated with levels of immunoreactivity in the PVN. Body weight was positively correlated with PVN immunoreactivity in the paired group, with a smaller correlation in the isolated group (r= 0.66 vs. 0.23 in paired and isolated groups, respectively). Age showed a small association with PVN immunoreactivity in both groups (r= −0.18 vs. 0.05 in paired and isolated groups, respectively). Amount of time spent together was positively correlated with PVN immunoreactivity in the isolated group only (r= 0.00 vs. 0.42 in paired and isolated groups, respectively). Number of litters produced was positively correlated with PVN immunoreactivity in the isolated group only (r= −0.04 vs. 0.45 in paired and isolated groups, respectively). Test year showed a small correlation with PVN immunoreactivity in both groups (r= −0.08 vs. 0.11 in paired and isolated groups, respectively).

Additional exploratory analyses were conducted on the 5 demographic variables and 2 dependent measures (immobility in the FST and immunoreactivity in the PVN) using a median split of each demographic variable, for the paired and isolated conditions separately, therefore forming 2 subgroups: *low* and *high* for each variable. Table 2 displays the median value for each demographic variable, the mean of each the low and high subgroup for the demographic variable and associated dependent measure, and notations of statistically significant P values for each comparison (after applying the Bonferroni correction). These comparisons indicated that immobility in the FST varied as a function of number of litters the pair reared together, age at the time of testing, and amount of time the pair had cohabitated together at the time of testing, in both the paired and isolated conditions. The low subgroup on each demographic variable (i.e., fewer litters reared together, younger age, and fewer months cohabitating together) exhibited lower immobility values than the high subgroup on the demographic variable (i.e., more litters reared together, older age, and greater number of months cohabitating together), in each the paired and isolated groups ($P<0.05$ for all comparisons of low vs. high subgroups). By contrast, immunoreactivity in the PVN did not significantly vary in the low vs. high subgroups as a function of these same demographic variables in either group ($P>0.05$ for all comparisons of low vs. high subgroups). Neither immobility in the FST nor immunoreactivity in the PVN varied as a function of body weight or year of testing, in either the paired or isolated groups ($P > 0.05$ for all comparisons of low vs. high subgroups).

Experiment 2, Restraint

Circulating Corticosterone Levels—Isolation significantly influenced restraint-induced corticosterone levels (Figure 3), such that corticosterone levels were significantly higher in the isolated group vs. the paired group [$t(24)=3.68$, $P<0.001$]. This analysis excluded 6 animals in the paired group that escaped from the restraint chamber prior to the end of the

30-minute testing session (n=4 males; n=2 females; see the section describing exploratory analyses below for further analysis of these animals).

Exploratory Analyses—Exploratory comparisons were conducted as a function of the following 5 demographic variables: (a) body weight at the time of testing; (b) age at the time of testing; (c) number of months spent together; (d) number of litters produced; and (e) year of testing. Several correlational analyses were conducted to determine whether any demographic variables were systematically associated with circulating corticosterone levels. All correlations were 0.2 or lower for both housing groups, with the exception of test year, which showed a discrepant pattern of association with corticosterone values between the housing groups. A moderate-to-large negative correlation between testing year and corticosterone levels was observed in the isolated group ($r = -0.42$); however a (smaller) positive correlation between testing year and corticosterone levels was observed in the paired group ($r = 0.25$).

Additional exploratory analyses were conducted on the 5 demographic variables and the dependent measure (corticosterone levels) using a median split of each demographic variable, for the paired and isolated conditions separately, therefore forming 2 subgroups: *low* and *high* for each variable. Table 3 displays the median value for each demographic variable, the mean of each the low and high subgroup for the demographic variable and associated dependent measure, and notations of statistically significant P values for each comparison (after applying the Bonferroni correction). These comparisons excluded the 6 animals from the paired group that escaped from the restraint chamber. Corticosterone levels in the paired group varied as a function of number of litters reared together, such that those animals who reared fewer litters together displayed lower corticosterone levels than those animals who reared a greater number of litters together ($P<0.05$). Corticosterone levels in the paired group did not differ as a function of low vs. high body weight, age, number of months cohabitating together, or test year ($P>0.05$ for all comparisons). In contrast, corticosterone levels in the isolated group varied as a function of both age and amount of time the pair cohabitated together, such that animals who were a younger age and had spent fewer months cohabitating together with the partner displayed lower corticosterone levels than those animals that were older and those that spent a great number of months cohabitating together with the partner ($P<0.05$ for all comparisons). Corticosterone levels in the isolated group did not differ as a function of low vs. high body weight, number of litters reared together, or test year ($P>0.05$ for all comparisons).

Exploratory sex comparisons were conducted in the paired and isolated groups, using Student's tests. No significant sex differences were observed in any variable, including: (a) body weight at the time of testing; (b) age at the time of testing; (c) number of litters produced; (d) number of months spent together; or (e) circulating corticosterone levels ($P>0.05$ for all comparisons; data not shown).

Additionally, because all animals that escaped from the restraint chamber were in the paired group (n=6 escapees in the paired group; n=0 escapees in the isolated group), post-hoc t-tests were conducted to compare this subgroup with the two housing groups (with a Bonferroni correction). The paired escapee group exhibited corticosterone levels that were

slightly (but non-significantly) higher than the paired non-escapee group (paired escapees: 2689.9ng/ml; paired non-escapees: 2196.2ng/ml; P>0.05); and corticosterone levels that were slightly (but non-significantly) lower than the isolated group (paired escapees: 2689.9ng/ml; isolated: 3113.3ng/ml; P>0.05). A z-test for a difference between two proportions indicated a lack of a sex difference in the escapees (2/6 escapees were female and 4/6 escapees were male; z=1.15, P>0.05). The following demographic variables were evenly distributed among the escapees: (a) body weight at the time of testing; (b) age at the time of testing; (c) number of litters reared together; and (d) number of months spent together. However, year of testing was not evenly distributed; all animals that escaped were tested during the latter year of the design (in 2016) relative to animals tested during the earlier years of the design (between 2013–2015).

DISCUSSION

The presence of social support – for instance in the form of a spouse, long-term social partner, or someone who can provide emotional and/or instrumental support – may buffer against various consequences of aging, including mediating the effects of stress on health and altering central functions (López-Cerdá et al., 2019; Moore et al., 2015; Sbarra, 2009; Sherman et al., 2016). Given similarities in social structure between humans and prairie voles (Carter & Getz, 1993; Sun et al., 2014; Young et al., 2011), the current study investigated behavioral and neuroendocrine responses to a short-term stressor as a function of disrupting long-term monogamous social bonds in the prairie vole model. The results shed light on the critical influence of adaptive social bonding on behavioral and neuroendocrine indicators of stress reactivity in long-term cohabitating, aging animals.

Disrupting a long-term social bond between a cohabitating pair of aging male and female prairie voles produces behavioral disruptions following a short-term swim stressor. Aging male prairie voles that were isolated for 4 weeks from a long-term female partner displayed increased duration of immobility in the FST, relative to paired males. An increase in immobility levels during the FST is hypothesized to represent helpless behavior in response to a short-term stressor in prairie voles (Bosch et al., 2009; McNeal et al., 2014), and has been used previously to assess the efficacy of pharmacological antidepressant treatments (Cryan et al., 2002; Cryan et al., 2005a). Some research, however, has debated whether immobility in the FST represents a maladaptive response or an adaptive behavior after an animal learns that there is no possibility of escape from the swim chamber (Molendijk & de Kloet, 2015, 2016). Although it is reasonable to hypothesize that immobility may serve an adaptive role to help the animal conserve energy in some rodents, previous research in prairie voles is not consistent with this hypothesis (Grippo et al., 2012). Online physiological data collected from socially isolated prairie voles during the FST indicated that, despite being less active than socially paired prairie voles in the FST, socially isolated animals displayed elevated heart rate, reduced heart rate variability, and increased incidence of both atrial and ventricular arrhythmias (Grippo et al., 2012). These physiological data support studies demonstrating the predictive and construct validity of the FST in the context of helpless behavior (Bielajew et al., 2003; Cryan et al., 2005b), and are consistent with observations of increased physiological reactivity during the FST in other rodents (Pintér et al., 2011). The combination of increased immobility and increased sympathetic reactivity

provides evidence that the observable behavior is a maladaptive response to this acutely stressful situation, rather than an energy conservation strategy, in socially isolated prairie voles (Grippo et al., 2012). Additional research from younger prairie voles also supports the value of the FST as an operational index of helpless behavior in the context of social stress. For instance, young adult prairie voles separated from a short-term social partner (relative to social pairing) exhibit a similar pattern of immobility during the FST, and to a similar magnitude, as observed in the present study (Bosch et al., 2009; Grippo et al., 2012; McNeal et al., 2014).

To gain a more comprehensive understanding of the consequences of disrupting long-term, monogamous social bonds in aging prairie voles, the present study also investigated neurobiological reactivity to short-term stressors as a function of social isolation from an opposite-sex partner. In Experiment 1, social isolation from an opposite-sex partner (vs. social pairing) was associated with greater neural activation in the PVN following the FST, both in the full PVN and specifically in the parvocellular region, which may suggest not only general increased reactivity of the PVN, but also neural activation in specific cell bodies that promote HPA axis functions. Similarly, in Experiment 2, social isolation from an opposite-sex partner (vs. paired) was associated with greater corticosterone reactivity following restraint. These observations are consistent with previous research demonstrating increased short-term physiological stress reactivity in both adolescent and young adult prairie voles exposed to social isolation paradigms (Bosch et al., 2009; McNeal et al., 2014; Ruscio et al., 2007; Sun et al., 2014). For example, young adult prairie voles separated from a same-sex partner display an increase in oxytocin- vasopressin- and CRF-immunoreactivity in the PVN (Sun et al., 2014), as well as increases in short-term circulating corticosterone and adrenocorticotropic hormone following a 5-minute swim stressor (McNeal et al., 2014). It has been hypothesized that the central CRF system plays a mediating role in a maladaptive passive response to stress in prairie voles (Bosch et al., 2009).

Although the present behavioral and physiological data support studies that have previously focused on younger prairie voles, both age and aspects of social monogamy appear to be important factors in the short-term stress responses to social isolation. Some of these demographic factors differentially influence behavioral and physiological responses to stress. The duration of immobility displayed in the FST was higher in older animals, animals that spent greater amounts of time in a cohabitating relationship, and animals that reared greater number of litters with a partner (vs. those lower on these variables); these correlations are further supported by the results of the median split subgroup analyses. These 3 demographic variables are inter-related; indeed, older animals have had increased time to spend cohabitating together and increased opportunities to produce litters, relative to younger animals. However, the patterns of correlations and subgroup observations in both paired and isolated groups suggest age, amount of social cohabitation, and number of litters reared together may influence behavioral responses to the FST. These data from prairie voles also support those from other rodent models focused on aging animals. For example, aging rats display altered behavior in the elevated plus maze relative to younger animals, and behavioral responses vary as a function of both age and previous experience to long-term stress (Shoji and Mizoguchi, 2010). Consistent with conclusions from rodent studies of stress and aging, declines in both psychological and physiological health with age may lead

to greater levels of perceived stress, which in turn relate to lower perceptions of successful aging in humans; these relationships are moderated in part by the presence of social support (Moore et al., 2015).

In contrast to the systematic relationship between behavioral stress reactivity and some demographic variables in the present study, the same patterns were not observed in the physiological stress indicators. PVN activation was not systematically correlated with age, and activation did not vary as a function of high vs. low subgroups on any demographic variable for the paired and isolated conditions. Corticosterone levels following restraint varied as a function of high litter numbers (vs. low litter numbers) in the paired group, but varied as a function of both older age and greater amount of time the pair cohabitated together (vs. lower on these 2 variables) in the isolated group. These differential relationships may be a function of experimental artifact (e.g., a floor effect in the PVN levels of the paired group), or differential interactions among physiological stress reactivity, age, and factors related to social monogamy.

Some limitations of the present study design may limit the translational value of these findings. First, the semi-naturalistic design involved the study of animals over a 6-year period. Although all environmental conditions were standardized across the study period to the best of our ability, some shifts in physiological responses were observed over time for reasons that we cannot discern. Animals from paired and isolated conditions tested in later years (vs. earlier years) exhibited greater activation in the PVN; whereas the corticosterone levels in paired and isolated groups correlated in opposite directions with test years. By contrast, behavioral responses in the FST did not differ across the 6-year period in either group. The unsystematic shifts in physiological measures may be representative of confounding (and uncontrolled) variables, or potentially unknown (and uncontrollable) shifts in chemical assay materials, across time.

Additionally, the differential response pattern to restraint between paired and isolated groups was unanticipated in the present study. Six paired prairie voles escaped from the restraint chamber (by chewing through the mesh screen material) during the 30-minute period; by contrast, zero isolated prairie voles escaped from the chamber. The paired escapees displayed slightly higher corticosterone levels than paired non-escapees, but slightly lower corticosterone levels than isolated animals. This pattern of results might be expected; for instance, greater plasma corticosterone may reflect greater movement in the paired escapees, relative to paired non-escapees. However, it is interesting to note that only paired animals were successful in escaping the restraint chamber (despite all methods of restraining the animals being standardized). It might be adaptive for an animal to devise a strategy to escape the restraint chamber, or to be motivated enough to attempt an escape. Hence, the fact that isolated animals did not perform this putatively adaptive behavior may be an interesting research question to explore in future studies focused on stress-coping or problem-solving strategies as a function of social isolation.

In conclusion, the present data provide support for the benefits of long-term, monogamous social bonds in aging prairie voles on acute stress responses. Specifically, the present results indicate that short-term behavioral and physiological responses to acute stressors are

impaired following the disruption of a long-term social bond in aging prairie voles. Stress reactivity may in part increase with age as well as long-term monogamous behaviors, such as cohabitating and rearing offspring together, in the prairie vole model. The translational value of the prairie vole social structure can inform our understanding of potential stress-buffering benefits of social bonds, and this species may serve as an important model for continued studies of the interactions of social monogamy and aging. Elucidating the neurobiological mechanisms underlying social interactions, health, and behavior will promote treatment and prevention strategies for social stress-related consequences, particularly for at-risk older populations.

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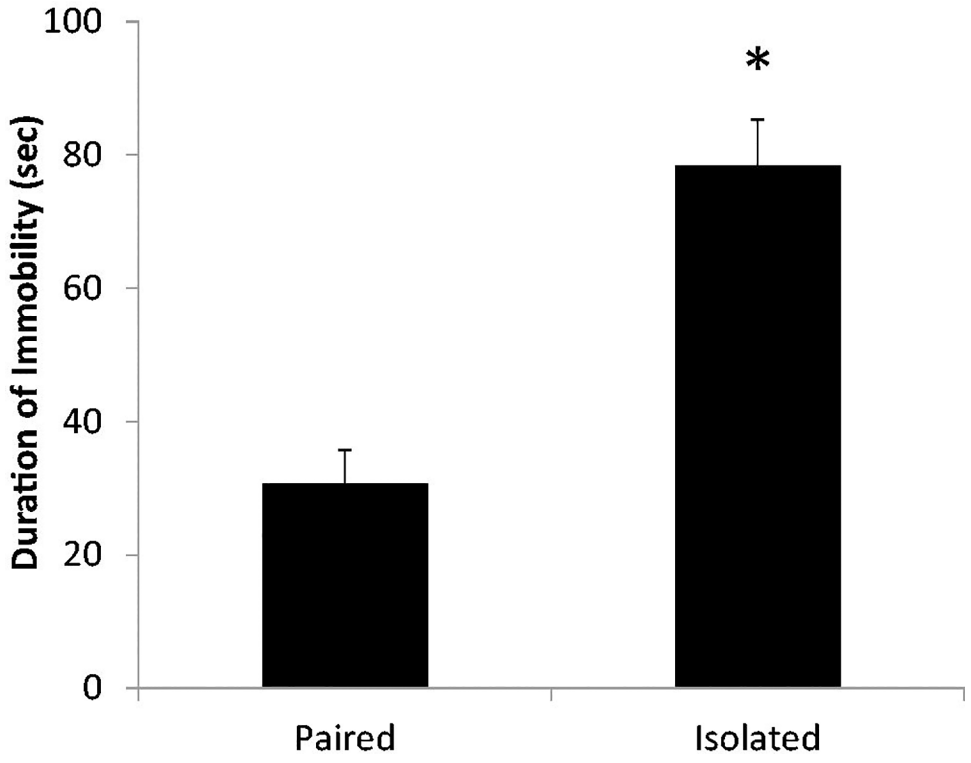

Figure 1.

Mean (+ SEM) duration of immobility during a 5-minute FST in paired (immediately tested in the FST) and isolated (delayed tested, following 4 weeks of isolation) in aging, socially-bonded male prairie voles. Note that the remainder of 300 seconds is comprised of combined active behaviors (swimming, struggling, and climbing), which are mutually exclusive from immobility. *P < 0.05 vs. paired group.

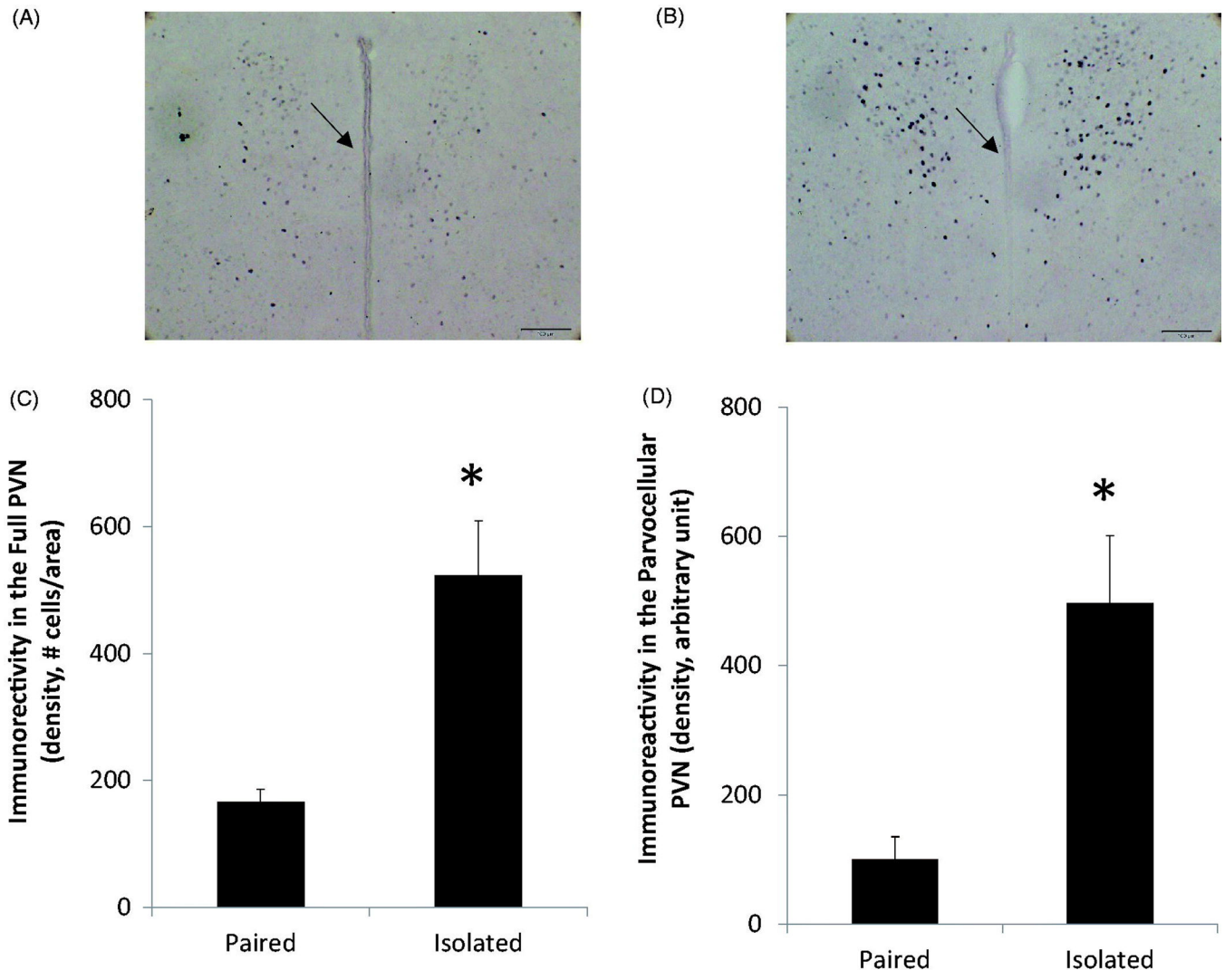

Figure 2.

Representative images of PVN activation (Panels A and B), and mean (+ SEM) level of immunoreactivity in the full PVN (Panel C) and the parvocellular region (Panel D) 2 hours following the FST in paired (immediately tested in the FST) and isolated (delayed tested, following 4 weeks of isolation) in aging, socially-bonded male prairie voles. Arrow is denoting the 3rd ventricle in Panels A and B. *P < 0.05 vs. paired group.

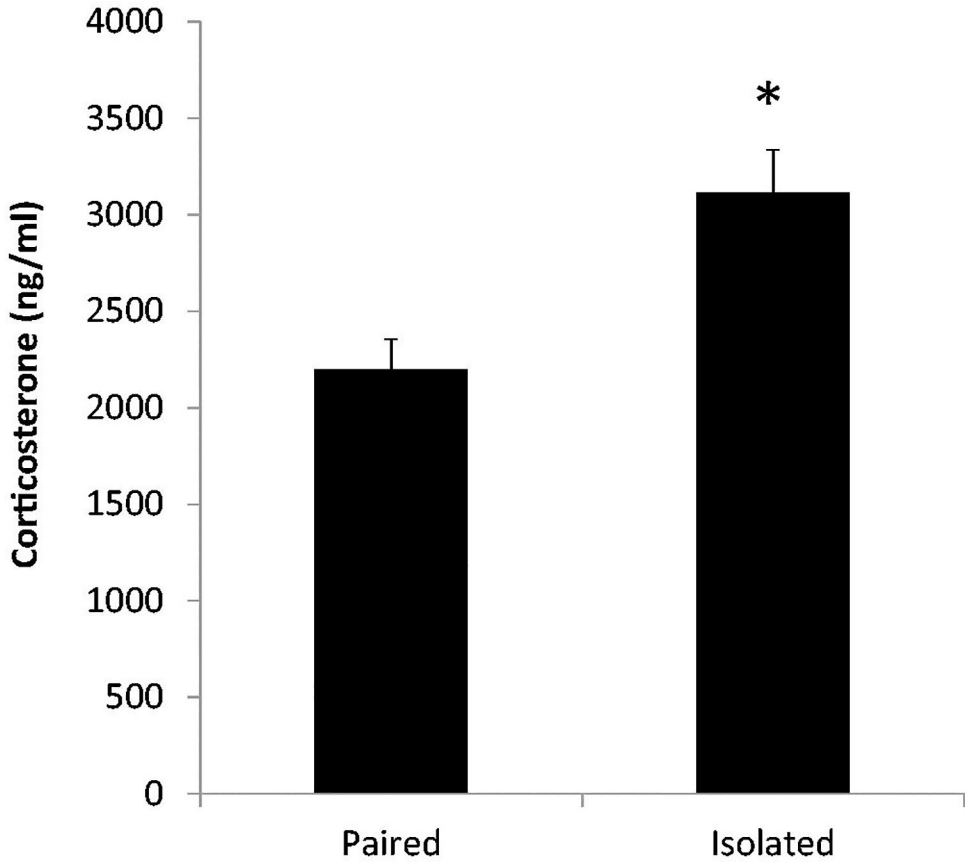

Figure 3.

Mean (+ SEM) circulating corticosterone levels 10 minutes following the end of a 30-minute restraint session in paired (immediately tested in restraint) and isolated (delayed tested, following 4 weeks of isolation) in aging, socially-bonded male and female prairie voles. *P < 0.05 vs. paired group.

Table 1.

Demographic variables across both experiments in paired and isolated groups.

Experiment 1

	Body Weight (g)	Age (mo)	Cohabitation (mo)	Litters (#)	Test Yr (yr)
Paired	42 ± 4	48 ± 5	38 ± 5	15 ± 3	2011 ± 0.4 years
Isolated	48 ± 3	55 ± 6	45 ± 6	16 ± 3	2011 ± 0.3 years

Experiment 2

	Body Weight (g)	Age (mo)	Cohabitation (mo)	Litters (#)	Test Yr (yr)
Paired	45 ± 2	52 ± 6	39 ± 6	18 ± 3	2015 ± 0.2 years
Isolated	46 ± 3	54 ± 5	42 ± 5	19 ± 3	2015 ± 0.1 years

Notes: All categories are shown as mean ± SEM. Test Yr column is displaying the actual mean test year (between 2010–2016), and the SEM in partial years.

Table 2.

Evaluation of demographic and dependent variables in Experiment 1 as a function of median splits in demographic variables, in paired and isolated groups separately.

Paired, FST Immobility Duration

	Body Weight (g)	Age (mo)	Cohabitation (mo)	Litters (#)	Test Yrs (yrs)
Median Value	43.0	19.2	13.8	16.5	2010–13
Subgroup Means of Demographic Variable	35.0 ± 2.0 (Low) 47.0 ± 0.9 (High) *	9.3 ± 0.8 (Low) 25.6 ± 0.9 (High) *	4.2 ± 1.3 (Low) 20.9 ± 1.4 (High) *	5.3 ± 2.4 (Low) 23.0 ± 1.6 (High) *	2010–11 (Low) 2012–13 (High)
Subgroup Means of Immobility Duration (s)	56.5 ± 3.5 (Low) 26.1 ± 4.5 (High)	20.8 ± 10.0 (Low) 35.2 ± 5.4 (High) *	20.8 ± 10.0 (Low) 35.2 ± 5.4 (High) *	20.8 ± 10.0 (Low) 35.2 ± 5.4 (High) *	24.8 ± 6.7 40.4 ± 5.3

Paired, PVN Immunoreactivity

	Body Weight (g)	Age (mo)	Cohabitation (mo)	Litters (#)	Test Yrs (yrs)
Median value	43.0	19.2	13.8	16.5	2010–13
Subgroup Means of Demographic Variable	35.0 ± 2.0 (Low) 47.0 ± 0.9 (High) *	9.3 ± 0.8 (Low) 25.6 ± 0.9 (High) *	4.2 ± 1.3 (Low) 20.9 ± 1.4 (High) *	5.3 ± 2.4 (Low) 23 ± 1.6 (High) *	2010–11 (Low) 2012–13 (High)
Subgroup Means of Immunoreactivity (density)	104.2 ± 29.4 (Low) 176.7 ± 10.4 (High)	159.7 ± 5.3 (Low) 168 ± 17.5 (High)	159.7 ± 5.3 (Low) 168 ± 17.5 (High)	159.7 ± 5.3 (Low) 168 ± 17.5 (High)	164 ± 17.8 (Low) 168 ± 15.2 (High)

Isolated, FST Immobility Duration

	Body Weight (g)	Age (mo)	Cohabitation (mo)	Litters (#)	Test Yrs (yrs)
Median Value	47.0	17.0	13.8	15.0	2010–2013
Subgroup Means of Demographic Variable	45.0 ± 0.4 (Low) 50.0 ± 0.9 (High)	6.8 ± 0.9 (Low) 24.4 ± 0.9 (High) *	2.7 ± 0.1 (Low) 19.2 ± 1.1 (High) *	7.8 ± 3.6 (Low) 23.4 ± 1.4 (High) *	2010–11 (Low) 2012–13 (High)
Subgroup Means of Immobility Duration (s)	80.9 ± 8.8 (Low) 70.0 ± 4.7 (High)	51 ± 2.5 (Low) 82.2 ± 7.6 (High) *	51 ± 2.5 (Low) 82.2 ± 7.6 (High) *	52.0 ± 7.6 (Low) 83.6 ± 9.1 (High) *	68.7 ± 13.0 (Low) 89.7 ± 8.1 (High)

Isolated, PVN Immunoreactivity

	Body Weight (g)	Age (mo)	Cohabitation (mo)	Litters (#)	Test Yrs (yrs)
Median value	47.0	17.0	13.8	15.0	2010–2013
Subgroup Means of Demographic Variable	45.0 ± 0.4 (Low) 50.0 ± 0.9 (High)	6.8 ± 0.9 (Low) 24.4 ± 0.9 (High) *	2.7 ± 0.1 (Low) 19.2 ± 1.1 (High) *	7.8 ± 3.6 (Low) 23.4 ± 1.4 (High) *	2010–11 (Low) 2012–13 (High)
Subgroup Means of Immunoreactivity (density)	514.3 ± 50.9 (Low) 555.3 ± 170.7 (High)	524.0 ± 60.2 (Low) 523.6 ± 75.6 (High)	524.0 ± 20.7 (Low) 523.7 ± 61.2 (High)	524.0 ± 20.7 (Low) 523.7 ± 61.2 (High)	590.9 ± 43.2 (Low) 445.4 ± 94.0 (High)

Notes: Mean values are shown as mean ± SEM. Test Yr column is displaying the actual test years in the Median Value cell, and actual mean test year(s) in the Means cells.

* P < 0.05 vs. respective *Low* subgroup (after Bonferroni correction applied to multiple comparisons).

Table 3.

Evaluation of demographic and dependent variables in Experiment 2 as a function of median splits in demographic variables, in paired and isolated groups separately.

Paired, Corticosterone

	Body Weight (g)	Age (mo)	Cohabitation (mo)	Litters (#)	Test Yrs (yrs)
Median Value	42.0	19.0	16.1	19.0	2013–2016
Subgroup Means of Demographic Variable	34.9 ± 1.4 (Low) 47.9 ± 1.9 (High)	11.3 ± 1.7 (Low) 26.1 ± 1.5 (High) *	7.9 ± 2.0 (Low) 22.1 ± 1.7 (High) *	6.2 ± 2.1 (Low) 24.4 ± 2.1 (High) *	2013–2015 (Low) 2016 (High)
Subgroup Means of Corticosterone (ng/ml)	2240.7 ± 153.3 (Low) 2168.3 ± 246.0 (High)	2182.2 ± 268.7 (Low) 2208.1 ± 201.6 (High)	2182.2 ± 268.7 (Low) 2208.1 ± 201.6 (High)	1516.0 ± 261.3 (Low) 2407.1 ± 202.3 (High) *	2164.0 ± 223.1 (Low) 2268.6 ± 146.9 (High)

Isolated, Corticosterone

	Body Weight (g)	Age (mo)	Cohabitation (mo)	Litters (#)	Test Yrs (yrs)
Median value	53.5	21.7	17.4	20.5	2014–2015
Subgroup Means of Demographic Variable	43.6 ± 0.9 (Low) 61.3 ± 3.0 (High)	11.5 ± 1.1 (Low) 28.1 ± 1.3 (High) *	5.8 ± 0,3 (Low) 24.0 ± 1.4 (High) *	16.3 ± 1.3 (Low) 27.8 ± 1.9 (High) *	2014 (Low) 2015 (High)
Subgroup Means of Corticosterone (ng/ml)	3153.2 ± 245.2 (Low) 2992.6 ± 288.9 (High)	2508.6 ± 276.7 (Low) 3207.1 ± 255.6 (High) *	2508.6 ± 276.7 (Low) 3207.1 ± 255.6 (High) *	3159.0 ± 289.8 (Low) 3037.5.1 ± 230.5 (High)	3606.5 ± 428.5 (Low) 2591.7 ± 192.9 (High)

Notes: Mean values are shown as mean ± SEM. Test Yr column is displaying the actual test years in the Median Value cell, and actual mean test year(s) in the Means cells.

* $P < 0.05$ vs. respective *Low* subgroup (after Bonferroni correction applied to multiple comparisons).