

Column E Explanation (Amendment)

This form is intended as an aid to complete the Column E explanation. It is not an official form and its use is voluntary. Annual Reports and explanations should NOT include PI information such as names (principal investigators and research staff), addresses, protocols, meeting notes (either in part or in full), the animals room numbers, grant information, veterinary care programs, and the like. A Column E explanation must be written so as to be understood by lay person as well as scientists.

1. Registration Number 48-R-0002, Customer ID Number: 1459
2. Number 760 of animals categorized as column E used in this study.
3. Species (common name) Prairie Voles of animals used in this study.
4. Explain the procedure producing pain and/or distress. Explanations should include a brief description of the procedure, but also explain what the animal's experience, examples of which may include, but are not limited to: Neurological signs, seizures, tremors, paralysis, lethargy, inappetence, respiratory signs, GI distress, vomiting and diarrhea.

The overall scientific objectives of the research project are to evaluate the role that stress has on physiology, neurochemistry, and behavior associated with social behavior to determine the influence that stress has on sociality. In order to reach these objectives, prairie voles are utilized because of their unique social systems including mating induced formation of pair bond-related behaviors including partner preferences and selective aggression in prairie voles with male prairie voles displaying paternal behavior toward their own offspring or conspecific pups. These behaviors are modulated by stressful life events tested through various methodologies described in further detail below.

Animals involved in these two specific experimental test methods are considered Category E due to the induced stress as part of the research objectives. They are unable to escape a known stressful situation that occurs for a duration longer than what is considered "momentary." Analgesics, topical antiseptics, and antibiotics are utilized for wounds incurred during these tests whenever possible as long as it does not interfere with study objectives. To minimize pain and distress from wounding, there is an established rubric to evaluate wound size and severity with the humane endpoint of euthanasia for significant wounding.

Resident intruder test (RIT): This is a commonly used test to examine aggressive behavior. The subject is placed in an arena (20x25x45cm) to establish "residence." After 10 min, an intruder (a same or different sex individual from the same species) is introduced into the arena and aggressive behavior on the part of the resident is quantified for a further 10 min. Aggression typically is manifested by threat displays and chases, although biting does occasionally occur. The use of large cages gives the animals sufficient room for separation. Animals are closely monitored throughout the test to ensure that neither animal is

seriously harmed. The test will be terminated if either animal appears to be unable to defend themselves. Frequency could include a single exposure only or once per day for up to seven days. Bite wounds are common. Animals with wound(s) exceeding 1 cm, wound(s) that involve the face or genitals, or full thickness wounds that expose bone or viscera will be euthanized. Mild wounds will be treated with a topical antiseptic (i.e., chlorhexidine).

Social defeat (SD): This is a commonly used test to examine the consequences of an agonistic interaction. The subject is placed in an arena (20x25x45cm), an established "residence" home cage. Aggressive behavior on the part of the resident is quantified for 30 min with an additional 30 min of non-physical contact using a divider between resident and intruder. Aggression typically is manifested by threat displays and chases, although biting does occasionally occur. The use of large cages gives the animals sufficient room for separation. Animals are closely monitored throughout the test to ensure that neither animal is seriously harmed. The test will be terminated if either animal appears to be unable to defend themselves. Frequency could include a single exposure only or once per day for up to seven days. Bite wounds are common. Animals with wound(s) exceeding 1 cm, wound(s) that involve the face or genitals, or full thickness wounds that expose bone or viscera will be euthanized. Mild wounds will be treated with a topical antiseptic (i.e., chlorhexidine).

5. Attach or include with the reason(s) for why anesthetics, analgesics and tranquilizers could not be used. (For federally mandated testing, see Item 6 below).

There may be times when medical treatment of wounds inflicted during these tests is not possible due to interference with data collected related to stress response. When performing this type of stress modeling, behavior is a component measure that is evaluated; however, neurotransmitter levels in the brain are the most critical variable for analysis. Use of systemic drugs including analgesics or antibiotics can alter the production and thus measurement of various neurochemicals of interest, which would negatively impact research outcomes. To minimize pain and distress from wounding, there is an established rubric to evaluate wound size and severity with the humane endpoint of euthanasia for significant wounding.

6. What, if any, federal regulation require this procedure? Cite the agency, the code of Federal Regulations (CFR) title number and the specific section number (e.g. APHIS, 9 CFR 113.102): If the requirement is per a guidance document, such as an Agency notice or harmonization guideline, please provide specific sufficient information to identify the cited document.

Agency _____ Not applicable _____ CFR _____ Not applicable _____

Explanation of procedures for Column E

Removal from study

If necessary, what criteria will be used to remove an animal from use prior to the planned conclusion of the study? For each criterion, define a specified duration or endpoint. For example, "animals will be removed from the study if diarrhea is observed for greater than 24 hours".

Any animal from any protocol with a body condition score of 1 will be removed from the breeding colony. For social defeat specifically, there is an associated risk of wounding with this procedure. Animals with wound(s) exceeding 1 cm, wound(s) that involve the face or genitals, or full thickness wounds that expose bone or viscera will be euthanized. Animals will be evaluated at least once per daily for the first 48 hrs after surgery. Animals that fail to eat, groom and assume normal body postures or show obvious signs of impairment will be euthanized. In addition, during tethering protocols, if animals continue to display distressed behavior or posturing (biting or pulling tether, repeated jumping, arched back) beyond a 10 min habituation period, the tether will be removed, and animals removed from study and returned to their home cage. Abnormal repetitive behavior, either impulsive/compulsive behavior or non-purposeful repetitive behaviors called stereotypies, may manifest as a result of experimental conditions. These behaviors will be categorized as expected experimental outcomes and used as behavioral markers of distress within the study. However, in standard housing conditions, animals may develop similar abnormal repetitive behavior. Under these conditions, these behaviors will be categorized as aberrant. Enrichment and social housing will be employed to discourage such aberrant behaviors. Barbering will be the only abnormal repetitive behavior that justifies study endpoint, as this can be a social learned behavior and difficult to deter.

Partner preference test: The apparatus consists of a central cage joined by hollow tubes with two parallel identical cages each of which houses a stimulus animal. The subjects being tested are free to move throughout the whole apparatus, while stimulus animals are loosely tethered (3 hr maximum) within their separate test cages and have no direct contact with each other. Tethering is accomplished by using a lightweight chain attached to a plastic wire-tie loosely placed around an individual animal's neck. Tethered animals have full access to food and water and are able to move around and to have normal behavioral interactions with the subject (such as side-by-side contact, grooming, and mating). These animals are checked regularly (every 30 mins) to ensure they do not get tangled. For the partner preference test, the familiar mate (one that had previous experience with the subject) and an unfamiliar conspecific (one that did not previously encounter the subject) are used as stimulus animals. The subject is put into the central cage and its behavior is recorded for 3hrs using a time-lapse video recording system. Food and water are provided ad libitum throughout the test. Stimulus animals will only be tethers twice per week with a 48 hr minimum interval. Before use by other animals, the apparatus will be cleaned by 70% ethanol, rinsed by water, and dried.

- b. Intensity and duration of stimulus: Not applicable
- c. Frequency of tests: up to twice in a 12-month period
- d. Length of time in test apparatus: 3 hours
- e. Potential adverse events: Aggression is extremely rare and has never been observed to be prolonged or result in severe injury. Any mild wounds from aggressive encounters will be treated with a topical antiseptic (i.e., chlorhexidine). During tethering protocols, if animals continue to display distressed behavior or posturing (biting or pulling tether, repeated

jumping, arched back) beyond a 10 min habituation period, the tether will be removed, and animals removed from study and returned to their home cage.

a. Describe animal methodology/test(s) to be used: ***Social affiliation test***: The apparatus consists of two identical cages (20x25x45cm) connected by a hollow tube (7.5x16cm) or the same three-chamber apparatus used in the *Partner preference test*. Motion sensors mounted on the tube will automatically record the time subjects spend in each cage as well as the frequency of cage entries. A stimulus animal will be loosely tethered in one cage, as described for the partner preference test (above). The subject will be placed into the empty cage (without the stimulus animal) and its behavior will be recorded for 30mins using a time-lapse video recording system. Before use by other animals, the apparatus will be cleaned by 70% ethanol, rinsed by water, and dried.

- b. Intensity and duration of stimulus: Not applicable
- c. Frequency of tests: up to twice in a 12-month period
- d. Length of time in test apparatus: up to 30 min
- e. Potential adverse events: During tethering protocols, if animals continue to display distressed behavior or posturing (biting or pulling tether, repeated jumping, arched back) beyond a 10 min habituation period, the tether will be removed, and animals removed from study and returned to their home cage.

a. Describe animal methodology/test(s) to be used: ***Open field test***: The subject's locomotor activity will be tested in an open-field apparatus. The apparatus (60x60cm) is surrounded by a 20cm-high wall and consists of 16 quadrants (15x15cm). Subjects will be placed into the open-field apparatus for 30mins of locomotion testing. Passage from one quadrant to the next is recorded as a grid cross. Before use by other animals, the apparatus will be cleaned by 70% ethanol, rinsed by water, and dried.

- b. Intensity and duration of stimulus: Not applicable
- c. Frequency of tests: up to twice in a 12-month period
- d. Length of time in test apparatus: up to 30 min
- e. Potential adverse events: Mild stress response

a. Describe animal methodology/test(s) to be used: ***Elevated plus maze (EPM) test***: This apparatus consists of a four-armed cross in which two arms have high walls (closed arms) and two arms lack walls (open arms). This cross is elevated approximately 40cm of the ground. Subjects are placed in the center of the maze and the amount of time spent in the open or closed arms is used as an index of anxiety. Several inches of bedding or a pad are placed in a box that runs the length under the open arms of the maze to provide a soft landing for the occasional animal that jumps from the maze. Before use by other animals, the apparatus will be cleaned by 70% ethanol, rinsed by water, and dried.

- b. Intensity and duration of stimulus: Not applicable
- c. Frequency of tests: up to twice in a 12-month period
- d. Length of time in test apparatus: up to 10 min
- e. Potential adverse events: Moderate stress response

a. Describe animal methodology/test(s) to be used: ***Resident intruder test (RIT)***: This is a commonly used test to examine aggressive behavior. The subject is placed in an arena

(20x25x45cm) to establish "residence." After 10 min, an intruder (a same or different sex individual from the same species) is introduced into the arena and aggressive behavior on the part of the resident is quantified for a further 10 min. Aggression typically is manifested by threat displays and chases, although biting does occasionally occur. The use of large cages gives the animals sufficient room for separation. Animals are closely monitored throughout the test to ensure that neither animal is seriously harmed. The test will be terminated if either animal appears to be unable to defend themselves.

- b. Intensity and duration of stimulus: Overt aggressive encounter
- c. Frequency of tests: Single exposure or once per day up to seven days
- d. Length of time in test apparatus: 20 min
- e. Potential adverse events: Bite wounds are common. Animals with wound(s) exceeding 1 cm, wound(s) that involve the face or genitals, or full thickness wounds that expose bone or viscera will be euthanized. Mild wounds will be treated with a topical antiseptic (i.e., chlorhexidine).

a. Describe animal methodology/test(s) to be used: ***Social defeat (SD)***: This is a commonly used test to examine the consequences of an agonistic interaction. The subject is placed in an arena (20x25x45cm), an established "residence" home cage. Aggressive behavior on the part of the resident is quantified for 30 min with an additional 30 min of non-physical contact using a divider between resident and intruder. Aggression typically is manifested by threat displays and chases, although biting does occasionally occur. The use of large cages gives the animals sufficient room for separation. Animals are closely monitored throughout the test to ensure that neither animal is seriously harmed. The test will be terminated if either animal appears to be unable to defend themselves.

- b. Intensity and duration of stimulus: Overt aggressive encounter
- c. Frequency of tests: Single exposure or once per day up to seven days
- d. Length of time in test apparatus: 1 hr
- e. Potential adverse events: Bite wounds are common. Animals with wound(s) exceeding 1 cm, wound(s) that involve the face or genitals, or full thickness wounds that expose bone or viscera will be euthanized. Mild wounds will be treated with a topical antiseptic (i.e., chlorhexidine).

a. Describe animal methodology/test(s) to be used: ***Social preference/recognition***: Voles will be habituated for five minutes in a standard small rodent three-chamber apparatus (subject in center chamber and stimulus animals in outside chambers, all separate). A stimulus vole (either a littermate of the subject or a stranger) will be tethered in the two adjoining chambers. Following habituation, subjects will be allowed to freely explore the three-chamber apparatus and interact with the two stimulus voles for five minutes. All voles will be returned to a neutral cage for 30 minutes. Subjects will then return to the three-chamber apparatus and be habituated and tested as in the first trial; however, the stimuli voles will be the original novel vole and a new unfamiliar vole. This test will provide information regarding social novelty preferences during trial 1, as previously literature demonstrated adult prairie voles demonstrate a social preference for novel social stimuli over littermates. In addition, we will be able to determine if our vole recognizes the original novel vole from trial 1 to 2 and if their interaction is distinct for a second unfamiliar vole. Before use by other animals, the apparatus will be cleaned by 70% ethanol, rinsed by water, and dried.

- b. Intensity and duration of stimulus: Not applicable
- c. Frequency of tests: 3 times a 2 week period
- d. Length of time in test apparatus: 5 min sessions
- e. Potential adverse events: During tethering protocols, if animals continue to display distressed behavior or posturing (biting or pulling tether, repeated jumping, arched back) beyond a 10 min habituation period, the tether will be removed, and animals removed from study and returned to their home cage.

a. Describe animal methodology/test(s) to be used: *Social and object recognition*. Social recognition will be assessed by observing whether subject voles investigate a "novel" conspecific more than a "familiar" conspecific. *Two-trial test*. A stimulus vole will be placed into the test cage, and subject will be allowed to investigate it for 5 minutes. After a delay, the subject will either be presented with the same "familiar" conspecific or a new, "novel" conspecific. After several days, testing will again occur with presentation of the other group. For example, if on week 1, a male was given the "same" female on trial 2, then on week 2, the male will be given a "novel" female on trial 2. *Five-trial test*. A stimulus vole will be placed into test cage with the subject for one minute, for 5 trials, with 10 minutes between each trial. Social stimuli may be substituted with object stimuli (e.g., rubber duck, plastic block, glass beaker).

- b. Intensity and duration of stimulus: Not applicable
- c. Frequency of tests: up to 3 times a week for 2 weeks
- d. Length of time in test apparatus: 5 to 10 min accumulated over a multi-trial session
- e. Potential adverse events: None

a. Describe animal methodology/test(s) to be used: *Marble burying test*. When rodents are put in a cage with marbles they will bury the marbles. This behavior is seen as anxiety related. Voles will be placed for thirty minutes in a standard cage filled with 5 cm depth of bedding with 20 marbles evenly spaced. After thirty minutes the amount of marbles buried will be measured. In this procedure a marble is considered buried if 2/3 of the marble is covered with bedding.

- b. Intensity and duration of stimulus: Not applicable
- c. Frequency of tests: up to 3 times a week for 2 weeks
- d. Length of time in test apparatus: 30 min
- e. Potential adverse events: None

a. Describe animal methodology/test(s) to be used: *Forced swim test*. This test is designed to measure passive coping behavior to a stressful, uncontrollable event. Voles will be forced to remain for 5 min in a 4 l glass beaker (15 cm in diameter) filled to a height of 20 cm with tap water ($23 \pm 1^\circ\text{C}$). Behavior will be scored for swimming, struggling, and floating. After each test, voles will be removed and dried thoroughly before being returned to its home cage. A heating pad will be placed under $\frac{1}{2}$ of the cage until the animal is completely dry. Before use by other animals, the apparatus will be drained, cleaned by 70% ethanol, and rinsed by water.

- b. Intensity and duration of stimulus: immersion in tap water ($23 \pm 1^\circ\text{C}$) in a 4 l glass beaker for 5 min.
- c. Frequency of tests: up to 3 times a week for 2 weeks
- d. Length of time in test apparatus: 5 min

- e. Potential adverse events: Animals will be observed, and animals with agonal respiration will be removed from the test. Drowning has never been observed.

a. Describe animal methodology/test(s) to be used: ***Tail suspension test***. Voles will be suspended by the tail using adhesive tape to an aluminum stick (diameter 1 cm) and hung in the middle of a black covered box (40 x 40 x 40 cm³) approximately 80 cm above the ground. The duration of immobilization during a 5 min test will be recorded. Before use by other animals, the apparatus will be cleaned by 70% ethanol, rinsed by water, and dried.

- b. Intensity and duration of stimulus: Suspended by tail
- c. Frequency of tests: up to 3 times a week for 2 weeks
- d. Length of time in test apparatus: 5 min
- e. Potential adverse events: Potential limb damage from a fall if the tape is torn, but this has never been observed

a. Describe animal methodology/test(s) to be used: ***Light/dark box test***. This test is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behavior of rodents in response to mild stressors, that is, novel environment and light. The LDB apparatus consists of two plastic cages (29 cm L x 18 cm W x 13 cm H) that are visually distinct (white vs black) and connected to one another by a hollow tube (16 cm L x 7.5 cm radius). Voles will be placed in the dark box facing away from the opening. The amount of time spent in each cage and frequency of cage crosses will be quantified. Before use by other animals, the apparatus will be cleaned by 70% ethanol, rinsed by water, and dried.

- b. Intensity and duration of stimulus: Not applicable
- c. Frequency of tests: up to 3 times a week for 2 weeks
- d. Length of time in test apparatus: 15 min
- e. Potential adverse events: Mild stress response

a. Describe animal methodology/test(s) to be used: ***Rotarod performance test***. Voles will be placed on a horizontally oriented, rotating cylinder (3 cm diameter rod) suspended approximately 40 cm above a cage floor. Voles naturally try to stay on the rotating cylinder, and motor behavior will be measured. The speed of the rotarod is mechanically driven, and may either be held constant, or accelerated. The start speed is adjusted to 4 rpm, the acceleration rate to 20 rpm/min. Maximum speed is 40 rpm. Voles will be measured 5 times in a given session with up to 5 minutes between measures. Before use by other animals, the apparatus will be cleaned by 70% ethanol, rinsed by water, and dried.

- b. Intensity and duration of stimulus: a ramping increase from 2-40 rpm over 5 minutes
- c. Frequency of tests: up to every week for 3 weeks
- d. Length of time in test apparatus: 30-60 min
- e. Potential adverse events: Potential limb damage from the fall, but this has never been observed

a. Describe animal methodology/test(s) to be used: ***Startle response test***. The acoustic startle response can be used to assess anxiety levels and sensory-motor gating and is measured by placing an vole on a platform device that records motion in an enclosed chamber. The enclosure allows the vole to turn and freely move its limbs and tail, but does not permit it to rear or ambulate. During testing, a number of different acoustic stimuli are presented to the vole, ranging from background

white noise (65dB) to a loud startling stimulus (100-120 dB) for 30-40ms. Acoustic stimuli ≥ 100 dB has been shown to elicit a reliable whole-body startle response in most rodents. The process is repeated for up to approximately 80 stimuli, which can last a total of approximately 25 minutes. To assess differences in hearing sensitivity, voles can also be tested for hearing threshold in the acoustic startle response. In this test, 8 different intensities of acoustic stimuli are used (40, 50, 60, 70, 80, 90, 110, and 120 dB). Voles will be given two ascending and two descending series of acoustic stimuli. The amplitude of the acoustic startle response will be recorded. Before use by other animals, the apparatus will be cleaned by 70% ethanol, rinsed by water, and dried.

- b. Intensity and duration of stimulus: up to 80 40-120 dB tones (30-40 ms each) over 25 min
- c. Frequency of tests: up to 3 times a week for 2 weeks
- d. Length of time in test apparatus: 25 min
- e. Potential adverse events: Temporary or partial hearing loss may occur from > 120 dB tone.

a. Describe animal methodology/test(s) to be used: *Social fear conditioning (SFC)*. Voles will be placed in the conditioning chamber and, after a 30-s adaptation period, an empty wire mesh cage will be placed as a non-social stimulus near one of the short walls. Voles will be allowed to investigate the non-social stimulus for 3 min, before it will be replaced by an identical cage containing a conspecific. Unconditioned voles will be allowed to investigate the social stimulus for 3 min. Conditioned voles will be given a 1-s electric foot shock (0.7mA, pulsed current) each time they investigated the social stimulus, defined by direct contact with the conspecific. Foot shocks will be administered in a variable inter-shock interval, depending on when direct social contact is made. Conditioned voles will be returned to their home cage when no further social contact is made for 2 min. Before use by other animals, the apparatus will be cleaned by 70% ethanol, rinsed by water, and dried.

- b. Intensity and duration of stimulus: Approximately 2-10 1-s electric foot shock (0.7mA, pulsed current)
- c. Frequency of tests: Single exposure or once per day up to seven days
- d. Length of time in test apparatus: 6-9 min sessions
- e. Potential adverse events: Moderate transient discomfort

The following modification was approved by the IACUC on November 27, 2017:

The modifications to the current protocol is targeting to include specific language regarding removal from study and endpoint criteria based on wounding resulting from conspecific aggression during experimentation, particularly during, but not limited to, resident-intruder encounters and social defeat conditioning.

1. Other Study Procedures

- a. Describe animal methodology/manipulation/test(s) to be performed:

For conspecific aggression (e.g., resident-intruder encounters and social defeat conditioning), there is an associated risk of wounding with this procedure. Animals with wound(s) exceeding 1 cm, wound(s) that involve the face or genitals exceeding 3 mm in length or 0.5 mm in depth, or full thickness wounds that expose bone or viscera will be euthanized. Animals will be evaluated at least once per daily for the first 48 hrs after surgery. Animals that fail to eat, groom and assume normal body postures or show obvious signs of impairment will be euthanized.

- b. Duration: N/A
- c. Frequency of tests: Assessments will be completed after each conspecific aggression session
- d. Potential adverse events: None associated with wound assessment.