THESIS

FEMALE- AND INTRUDER-INDUCED ULTRASONIC VOCALIZATIONS AS PROXY INDICATORS FOR ANIMAL WELL-BEING AND POSTOPERATIVE PAIN RECOGNITION IN C57BL/6J MICE

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In partial fulfillment of the requirements

For the Degree of Master of Science

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Spring 2019

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ABSTRACT

FEMALE- AND INTRUDER-INDUCED ULTRASONIC VOCALIZATIONS AS PROXY INDICATORS FOR ANIMAL WELL-BEING AND POSTOPERATIVE PAIN RECOGNITION IN C57BL/6J MICE

Mice are the most commonly used research animal and often undergo painful procedures. It is important to minimize pain and distress in research animals. However, recognizing pain and distress in mice is challenging. There is a need for new objective techniques to assess pain, distress, and animal well-being in laboratory mice. Female urine-induced male mice ultrasonic vocalizations (FiUSV) are ultrasonic vocalizations produced by adult male mice following presentation of adult female urine, while intruder-induced ultrasonic vocalizations (IiUSV) are produced by resident adult females when interacting with an intruder female mouse. These affiliative behaviors may be reduced with pain, distress, or decreased well-being.

Two studies were completed to determine if FiUSV and IiUSV can be used as proxy indicator assays to assess animal well-being and postoperative pain in mice. First, the role of FiUSV and IiUSV in identifying decreased animal well-being were assessed in mice using an acute inflammatory sickness model. Second, using a vasectomy pain model, the role of FiUSV in detecting postoperative pain was assessed in male mice. Findings from the first study showed mice injected with lipopolysaccharide (LPS) intraperitoneally produced significantly fewer FiUSV and IiUSV compared to saline-injected mice, and the decrease in the number of USV occurred prior to showing overt clinical signs of sickness. In the second study, vasectomized mice given no postoperative analgesics produced fewer FiUSV compared to baseline, while

vasectomized mice given postoperative analgesics had no change in the number of FiUSV compared to baseline. The findings from these studies provide evidence that FiUSV and IiUSV can be used as proxy indicator assays to assess animal well-being associated with acute inflammatory sickness, and FiUSV can be used to assess postoperative pain.

ACKNOWLEDGEMENTS

I would like to acknowledge and thank the following people who have supported me during the course of this project. I would like to thank my committee, Lon V. Kendall, Sue VandeWoude, and Khursheed Mama, for their guidance and insight. I would also like to acknowledge my fellow comparative medicine resident mates, Erin McWhorter, Katie Knapek, Whitney McGee, Alexa Personett, and Alyssa Strumpf, for their encouragement and support. Lastly, I would like to thank my wife, Jessy Prado-Smith, for her unwavering support while pursuing my academic goals.

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CHAPTER I.

LITERATURE REVIEW

1. Pain recognition in mice

Mice are the most widely used animal species in biomedical research, often undergoing potentially painful procedures. Implementation of analgesics can minimize the amount of pain mice experience. However, it is difficult to recognize pain in mice, and it is challenging to know if analgesics are warranted or if they are working effectively.

Pain recognition is difficult in all animal species due to an inability to directly verbalize the amount of pain they are perceiving. Additionally, mice are a prey species and hide their signs of pain or sickness until overtly painful. This means mice are most likely experiencing pain and distress for a prolonged period of time before displaying clinical signs of pain. There is also a lack of absolute objective methods to measure animal well-being or pain. A majority of the modalities rely on subjective scoring by a blinded or non-blinded individual, which can be problematic due to inter-individual variations in opinions creating significant confounding variables.

Researchers and animal care personnel must rely on indirect methods to measure an animal's state of well-being and potential level of pain. There are many reviews describing the various types of pain-recognition assays available in rodents. The majority of the modalities can be separated into four categories: reflexive pain or nociceptive tests, cage-side assessments of spontaneous behaviors, operant tests, and hedonic behavior assays [Carstens and Moberg, 2000; Flecknell, 2018; Graham, 2016; Gregory et al, 2013; Hawkins, 2002; Mogil, 2009].

1.1 Nociceptive tests

Nociceptive tests, also referred to as reflexive pain tests or evoked withdrawal responses to noxious stimuli, measure and quantify an animal's response to an aversive stimuli, which can be applied using cold or heat, electric shock, chemical compounds, or mechanical stimuli [Barrot, 2012]. For example, the Randall-Selitto testing method applies a mechanical pinch with linearly increasing force to an animal's body part (usually hind paw or tail) to elicit a reflex withdrawal response [Barrot, 2012; Smith et al, 2016]. The amount of force is recorded at the time the animal reaches its pain threshold, as demonstrated by the withdrawal reflex. Animals experiencing increased hypersensitivity (peripheral or central sensitization) due to injection of an aversive compound will have a lower pain threshold and reflex sooner. Threshold tests can assess increasing stimuli or assess duration of a constant stimuli depending on the assay used. Other examples of commonly used nociceptive tests include tail flick, hot plate, and Von Frey filament.

Reflexive pain tests measure spinal reflexes associated with hypersensitivity (hyperalgesia and allodynia) and not the presence of on-going, non-evoked pain. Nociceptive tests are advantageous for studying pathways of pain, but do not always translate well to clinical applications [Barrot, 2012; Rice et al, 2008]. There tends to be less individual variability when using nociceptive tests compared to cage-side assessments or behavioral scoring [Barrot, 2012]. Thus, fewer animals can be used to show significant differences between experimental groups. Reflexive pain tests have mixed results when using neuropathic pain models due to potential peripheral or central nerve pathologies secondary to the experimental model [Flecknell, 2018; Rice et al, 2008]. Other modalities, such as operant tests, spontaneous pain behaviors, and

hedonic behavior assays, are used if conscious experience of pain or the presence of on-going, non-evoked pain is to be evaluated.

1.2 Operant tests

Operant tests, also known as avoidance of evoked stimuli test, can be used to measure conscious experiences of pain by requiring complex central processing of the animal. Typically, these tests measure pain or nociception by using a reward-conflict paradigm by forcing the animal to decide between receiving a reward and escaping an aversive stimuli [Flecknell, 2018; Gregory et al, 2013; Neubert et al, 2008]. Examples of operant tests include the thermal escape test, conditioned place avoidance, and orofacial operant pain assay [Gregory et al, 2013; Neubert et al, 2008; Ramirez et al, 2015].

The orofacial operant pain assay has been used to measure pain and test the efficacy of analgesics in mice [Neubert et al, 2008; Ramirez et al, 2015]. This specific assay requires an animal to choose between receiving a reward (sweetened milk) paired with a noxious stimuli (thermal stimuli applied to the mouse's cheek) or avoid both the reward and noxious stimuli. Mice would choose to avoid both the reward and noxious stimuli more often if the thermal stimuli temperature is increased or if an inflammatory agent (capsaicin) is applied to the cheek prior to testing [Neubert et al, 2008]. The avoidance behavior towards the reward could be reversed if analgesics were given to the mice prior to testing. Thus, this method could be used to test the efficacy of various analgesic regimens [Ramirez et al, 2015].

Although operant tests can objectively measure pain, they require a large amount of time to train animals to complete the desired task. For example, seven 20-min testing sessions were required for a mouse to comfortably and consistently place their face through an opening to receive the sweetened milk reward and have their cheeks simultaneously sustain contact with the

thermodes [Ramirez et al, 2015]. This upfront time commitment and personnel costs associated with animal conditioning decreases the use of operant tests as a pain recognition tool. Additionally, these tests can be difficult to reproduce due to the specialized, sometimes hand-made, testing equipment that are often needed. For instance, the previously described study required a specialized acrylic testing chamber with an opening to allow access to the reward paired with an electrical circuit to apply the noxious stimuli [Ramirez et al, 2015].

1.3 Cage-side assessments

Cage-side assessments of spontaneous behaviors require minimal to no operant conditioning and do not typically require specialized equipment. Assessments usually involve an ethogram, which is a list of behaviors or activities exhibited by an animal that can be quantified by an observer. Ethograms can measure normal or abnormal behaviors, such as those only seen when an animal is in pain. Examples of behaviors associated with pain or decreased animal well-being include hunched posture, wound licking, orbital tightness, or decreased grooming and activity.

Ethograms attempt to objectify behaviors that are subjectively measured. Thus, cage-side assessments are sensitive to bias, especially if observers are not blinded to experimental groups or treatments. The effectiveness of cage-side assessments is dependent on many factors such as the mouse strain and sex, degree and type of pain, type and dose of analgesics, and the methods used to score the spontaneous behaviors. For example, C3H/NeH mice show more pain-related behaviors after vasectomy compared to C57BL/6 mice [Wright-Williams et al, 2013].

The methods used to analyze cage-side assessments can have a large impact on results. For example, a study in rats found differences between behavior scores when analyzing videos, still photographs, or real-time scoring [Nunamaker et al, 2018]. Differences between these

modalities could occur for many reasons. Mice are a prey species and change their behaviors in the presence of predators [Hacquemand et al, 2013]. Studies have shown mice can hide their signs of pain depending on the presence or sex of the experimenter [Sorge et al, 2014]. Thus, the presence of an observer versus a remote-controlled video recording system during real-time scoring can change the behavior of mice. Other studies have shown behavior assessments of mice can differ between experimenters and research laboratories [Bohlen et al, 2014]. Thus, it is difficult to reproduce studies using cage-side assessments due to the lack of standardized methods, subjective bias, and individual experimenter effects in scoring.

1.4 Hedonic behavior assays

Hedonic behaviors are innate behaviors that are associated with positive emotional states, like nest building, wheel running, marble burying, and digging [Flecknell, 2018; Graham, 2016]. Assays measure the suppression of these behaviors after induction of pain or decreased well-being. For example, mice have decreased quality of nest building following surgery, and the decrease in quality can be prevented with postoperative analgesics [Arras et al, 2007; Jirkof et al, 2014]. These kind of assays use objective measurements and do not require an experimenter to be present, minimizing experimenter effects and subjective bias compared to cage-side assessments. For these reasons, hedonic behavior assays have increased in popularity.

Quantifying activity is a common modality to evaluate animal well-being and pain. Activity is an innate behavior that is suppressed with pain or decreased well-being and can be quantified using hedonic behavior assays. Various techniques can be used to measure activity such as wheel running, radiotelemetry, or cage-side assessments. Each technique has advantages and disadvantages. For example, both radiotelemetry and wheel running are absolute objective measurements but typically require animals to be singly housed. This adds confounding variables

to a study as individual housing of social species can increase stress levels, which has previously been shown to alter nociception [Marcinkiewcz et al, 2009]. Additional equipment and invasive procedures may be needed for these measurements. Radiotelemetry requires expensive equipment and surgical implantation of the devices into the mice, and wheel running requires numerous rodent wheels and specialized caging. Cage-side assessments of activity require minimal equipment, but animals are subjectively scored based on number of activity bouts or number of vertical rises. Cage-side assessments are highly dependent on the methods used to quantify the behavior, and thus can be poor indicators of pain and animal well-being [Nunamaker et al, 2018].

Hedonic behavior assays are advantageous because they can be objectively measured without observer bias and can estimate the overall well-being of the animal in real-time. One disadvantage to this method is some of the assays require animals to be individually housed, such as radiotelemetry, marble burying, and nest building. Singly housing social animals can alter results due to increased animal stress. Some assays can minimize this by using shorter assays to assess the behavior, allowing animals to stay socially housed for a majority of the time. Another pitfall of hedonic behavior assays is that multiple causes, other than pain or decreased well-being, can alter these innate behaviors. For example, various psychiatric disorders such as obsessive-compulsive disorder or autism spectrum disorder can alter both nest building and marble burying [Angoa-Perez et al, 2013].

1.5 Ultrasonic Vocalizations (USV)

Rodents produce ultrasonic vocalizations (USV) above the human range of hearing (>20 kHz) in addition to audible vocalizations. There have been attempts to use USV to measure pain with mixed results. Studies assessing USV as proxy indicators of pain have had more success

with rats than mice. This is because rats produce alarm cries between 22-28 kHz in response to aversive stimuli, which can be induced using nociceptive tests [Knapp and Pohorecky, 1995; Martino and Perkins, 2008]. Evidence suggests mice do not produce equivalent USV to rat alarm cries, thus less experiments have assessed USV for pain recognition in mice [Williams et al, 2008; Wallace et al, 2005].

Assays to measure nociception have been developed in rats utilizing USV. For example, rats produce alarm cries in the ultrasonic range when puffs of air are applied to the face [Knapp and Pohorecky, 1995; Martino and Perkins, 2008]. The number of USV in response to the air jet intensities are amplified if the rat had LPS injected into their intracerebroventricular space (lateral ventricle). The increase in USV was avoided if analgesics (morphine) were given [Martino and Perkins, 2008]. Similar assays have not been successful in mice. For instance, C57BL/6J mice do not produce any vocalizations when using mechanical nociceptive tests in limbs injected with formalin [Wallace et al, 2005]. Similarly, mice inconsistently produce USV and audible vocalizations during acute painful procedures, such as tail snip or ear punch [Williams et al, 2008]. This suggests mice do not appear to produce spontaneous vocalizations in response to aversive procedures. To successfully use mouse USV for pain recognition, one must understand when and why mice produce USV.

2. Review of mouse USV

Mice produce USV mainly during non-aggressive interactions with conspecifics [Portfors, 2007]. USV emitted by adult mice differ between sexes, which are also different from USV emitted by pups. Although mice emit USV during many different situations, there are three scenarios that are well described to consistently obtain high amounts of USV in a short period of

time. The three types of USV can be termed pup isolation-induced USV, female urine-induced male mice USV (FiUSV), and intruder-induced female USV (IiUSV).

2.1 Pup isolation-induced USV

Neonatal pups of both sexes produce isolation-induced USV. While USV from pups can be elicited by a variety of techniques, the most common method is to simply remove the pups from the nest [Portfors, 2007]. Mouse pups produce isolation-induced USV between 35-100 kHz when removed from the nest. The production of these USV elicits a search and retrieval behavior of the dam to come collect the pup and return it to the nest. There is debate on why pups produce these vocalizations. Initially, it was thought that pups produced these vocalizations as a cry for help, meaning pups were motivated to communicate with the dam. However, there are arguments that the USV produced by neonatal mice are a by-product of increased abdominal compressions to maintain cardiac blood flow in response to cold stress [Blumberg and Sokoloff, 2001].

Pup isolation-induced USV are naturally induced by many aversive stimuli [Portfors, 2007]. Thus, assessing the production of these vocalizations in response to pain/stress would be challenging due to the possibility of numerous confounding variables. Additionally, pup isolation-induced USV drastically change between 3 d to 14 d post parturition [Rieger and Dougherty, 2016]. Furthermore, pain pathways are still developing in neonates, and the degree and nature of responses can change with age [Hatfield, 2014]. Due to these variabilities, creating an assay using pup isolation-induced USV to detect pain or well-being would be challenging.

2.2 Female urine-induced male mice USV (FiUSV)

Adult male mice produce USV between 30 to 120 kHz in the presence of a female or her urine, and this has been referred to as a song [Portfors, 2007]. Assays have been used to reliably quantify the number of syllables produced by male mice in response to female urine [Chabout et

al, 2017]. These vocalizations have been termed female urine-induced male mice USV (FiUSV) [Roullet et al, 2011]. These songs contain different syllable types organized in patterns [Hanson and Hurley, 2012]. A syllable consists of a unit of sound surrounded by a period of silence. Syllables can be short and simple with few fluctuations in frequency or they can be long and complex, containing jumps in frequency or multiple frequencies produced at the same time, as seen in Figure 1.1.

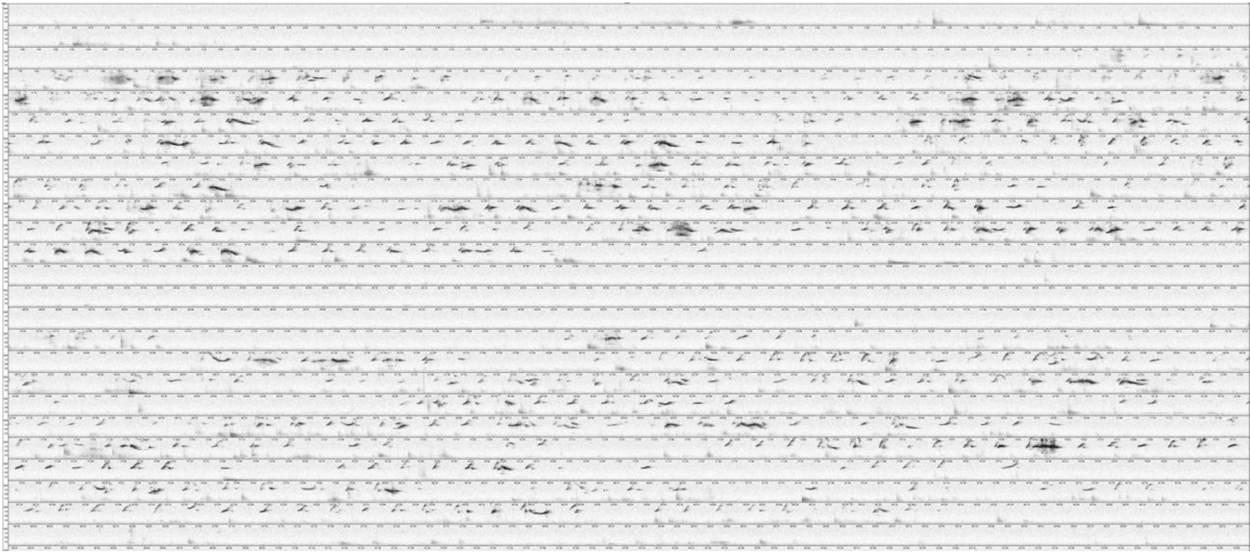
Methods to adequately collect FiUSV are well described [Chabout et al, 2017]. For example, a testing chamber (Figure 1.2) is commonly used to decrease background noise and provide a visual barrier between the test subjects and experimenter [Kurajova et al, 2010]. This is advantageous, because background noise could potentially conceal USV, leading to falsely low quantification of vocalizations. Additionally, it conceals the presence of an observer which can alter mouse behavior.

Methods for maximizing FiUSV production are also well described in the literature. Courtship vocalizations are dependent on strain, social status, previous heterosexual experience, and freshness of urine stimulus. For example, some strains, such as C57BL/6, BALB/c, and FVB mice, produce FiUSV almost 100% of the time compared to other strains (HMI, PGN2, and CAST/Ei) producing FiUSV far less reliably (< 40%) [Sugimoto et al, 2011]. An additional study showed fresh urine (less than 5-min old) induces males to emit more FiUSV than if old urine (1 to 2 h old) was used [Roullet et al, 2011]. Furthermore, male mice with previous female experience emit more FiUSV than males with no previous female experience [Dizinno et al, 1978; Nyby et al, 1983; Roullet et al, 2011]. There are a variety of methods described to adequately provide prior heterosexual experience to male mice. These range from multiday

male-female dyad exposures to a single overnight exposure of one female per cage of males [Chabout et al, 2017; Nyby et al, 1983].

As FiUSV are an innate behavior dependent on the animals' affective state, they can potentially be used as a proxy indicator for pain recognition or animal well-being. Courtship USV have been shown to be suppressed in sick, wild, male mice when housed with a female mouse overnight [Lopes and König, 2016]. Using FiUSV as a hedonic behavior assay would be valuable compared to cage-side behavior assessments because FiUSV are objectively measured and can be collected without an experimenter present. Additionally, quantification of the vocalizations can be beneficial over other hedonic behavior assays, such as marble burying and nest building, because male mice can remain socially housed for a majority of the study. Male mice are only singly housed while in the testing chamber, which lasts around 20 min.

A



B

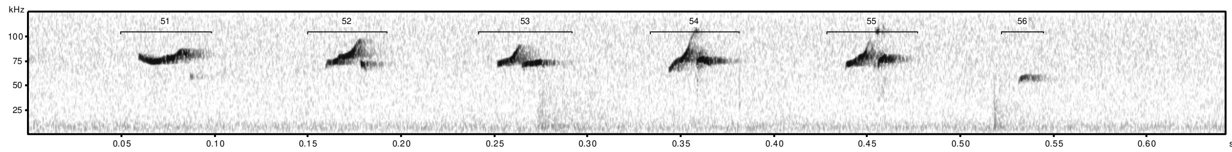


Figure 1.1. FiUSV spectrogram example. (A) A representative 90-s spectrogram (FFT-length = 256 points, overlap of 50%, and FlatTop window) of a male mouse song induced by fresh female urine. Each row is represented as a function of time (3.5 s per row) and frequency. (B) A 0.65 s spectrogram segment expanded from the 90-s sample showing FiUSV in greater detail. Time (s) is on the x-axis and frequency on the y axis. Individual syllables identified by black section labels.

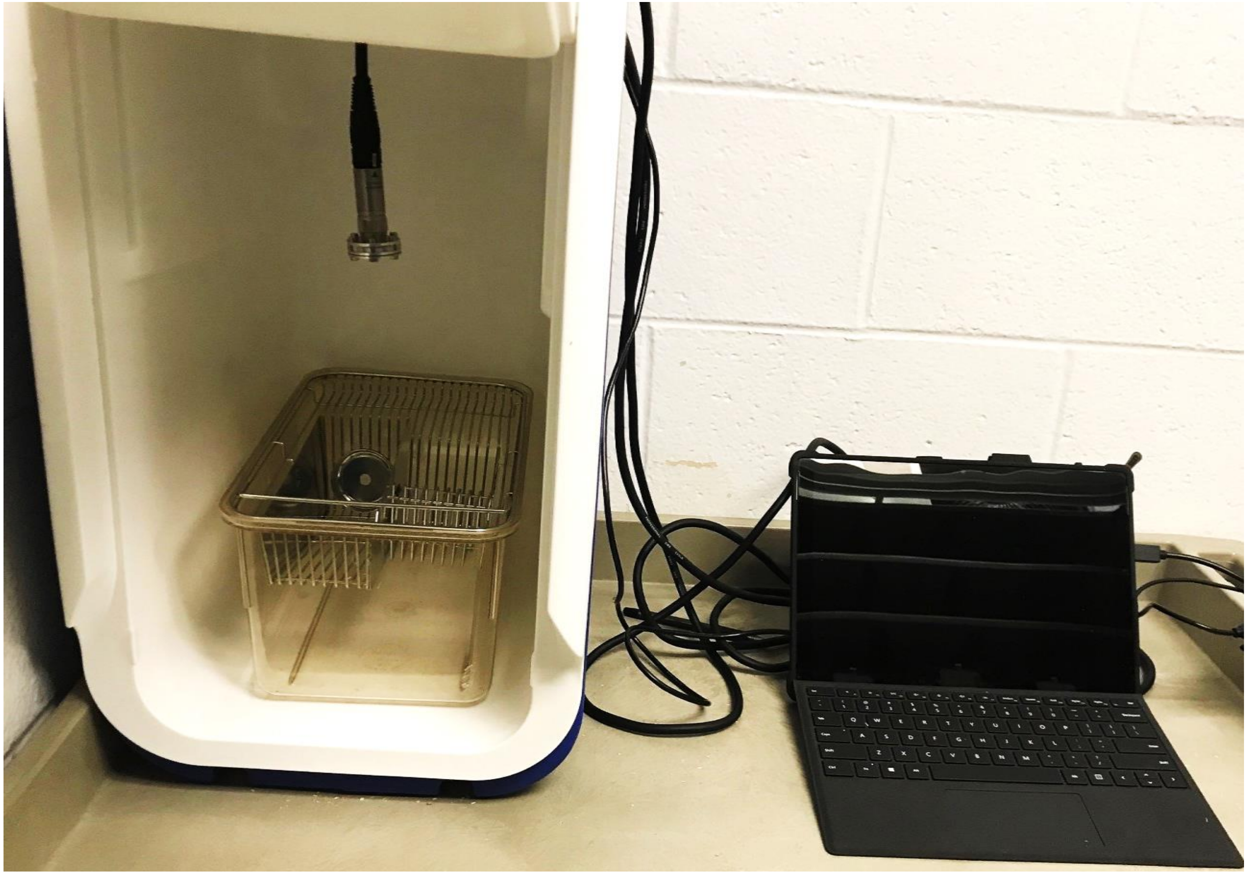
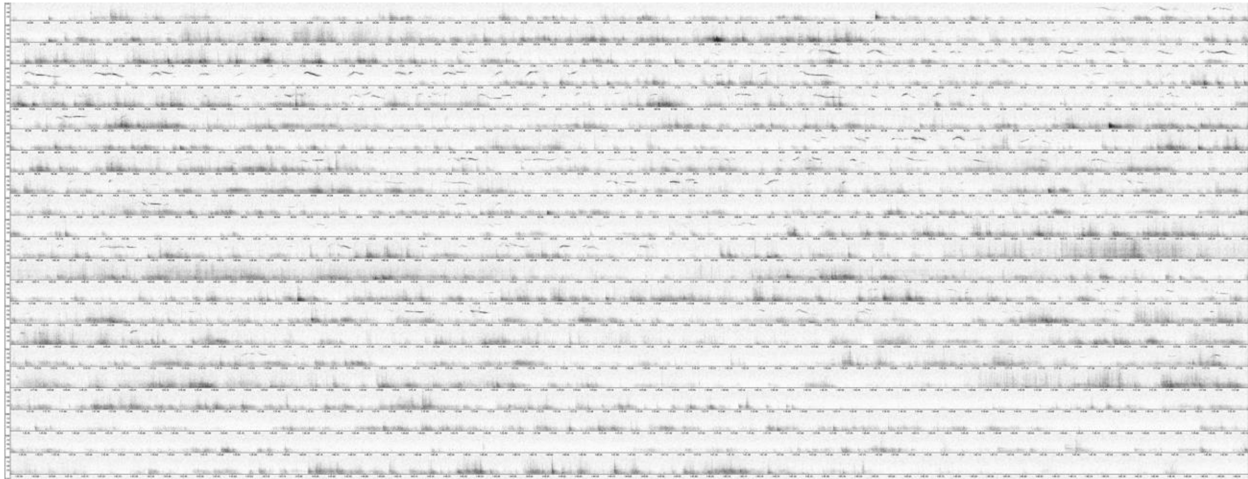


Figure 1.2. Testing chamber for USV collection. A beach cooler configured with a small hole to allow the ultrasonic-microphone wires to exit the chamber allows USV to be collected in a visually isolated environment with minimal background noise interference. Animals are placed in the empty shoe-box cage within the testing chamber. The ultrasonic microphone hangs from the top of the chamber, directly above the cage.

2.3 Intruder-induced female USV (IiUSV)

Female mice emit USV in various social interactions and are less well-described than FiUSV [Portfors, 2007]. There is a method to elicit a large amount of female mouse USV, called the resident-intruder paradigm. Using this paradigm, a resident female mouse will produce large amounts of USV in response to an intruder female mouse. The USV produced by the resident female mouse can be termed intruder-induced female mice USV (IiUSV). The number of syllables produced using this assay are similar in number and appearance to FiUSV (Figure 1.3) [Scattoni et al, 2008].

A



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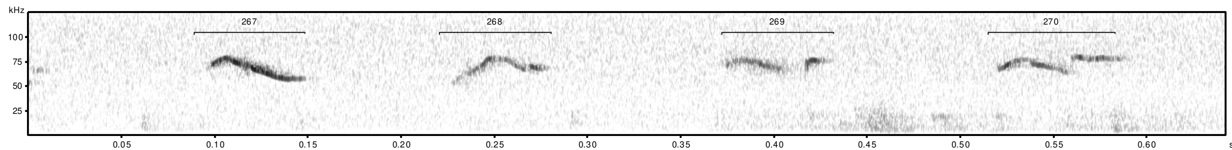

Figure 1.3. IiUSV spectrogram example. (A) A representative 90-s spectrogram (FFT-length = 256 points, overlap of 50%, and FlatTop window) of a resident female mouse response to an intruder female mouse. Each row is represented as a function of time (3.5 s per row) and frequency. Note the increased background noise (coloration arising from bottom of each row) compared to Figure 1.2. To elicit IiUSV there is bedding and 2 mice in the testing chamber, while to elicit FiUSV there is no bedding and 1 mouse in the testing chamber. (B) A 0.65 s spectrogram segment expanded from the 90-s sample showing FiUSV in greater detail. Time (s) is on the x-axis and frequency on the y axis. Individual syllables identified by black section labels.

USV produced during the resident-intruder paradigm are emitted predominantly by the resident female mouse and not the intruder mouse [Gourbal et al, 2004]. This was demonstrated by anesthetizing either the resident mouse or intruder mouse and measuring the amount of USV produced when placed together. The study found that IiUSV were produced when both the resident and intruder were awake and when the resident was awake but the intruder was anesthetized. IiUSV were not measured when the resident was anesthetized and the intruder was awake. Thus, the resident mouse is the one that produces the IiUSV. However, it doesn’t prove

that the intruder also produces USV in response to the resident. A previous study developed a system to measure USV produced by individual mice when housed together. They used a special microphone array system to show both male and female mice produce courtship vocalizations while interacting [Neunuebel et al, 2015]. This special microphone array system has not been used to assess USV produced during the resident-intruder paradigm, but could potentially be used to show whether both the resident and intruder mouse produce vocalizations.

Since IiUSV are studied less often than FiUSV, there is less information known about how to maximize IiUSV production. The number of IiUSV produced by resident females are dependent on the resident's estrous cycle, age, and whether they have been exposed to the intruder previously [Gourbal et al, 2004]. For example, female resident mice produce fewer IiUSV when in proestrus or estrus compared to when sexually non-receptive (metestrus or diestrus), and 12-mo old resident female mice produce fewer IiUSV than 3- to 5-mo old resident females. Additionally, the study showed resident female mice produce fewer IiUSV if exposed to the same intruder compared to a different intruder if tested 30 min apart, but there was no change if tested 60 min apart [Moles et al, 2007].

IiUSV are produced by the resident female for the first few minutes after being introduced to the intruder mouse [Gourbal et al, 2004], which is similar to FiUSV in male mice [Roullet et al, 2011]. Thus, both of these paradigms can be completed in a short amount of time. Studies using the intruder-resident paradigm singly house the female mice for 3 days prior to testing [Gourbal et al, 2004; Moles et al, 2007]. Although this is not ideal, IiUSV would still be advantageous as a proxy-indicator assay due to being objective, quantifiable, and consistently inducible. Since evidence suggests mice do not produce spontaneous USV in response to noxious

stimuli, the suppression of these innate behaviors (FiUSV and IiUSV) can potentially be used as proxy indicator assays for animal well-being and pain.

3. Mouse pain models

Pain is a complex perceptual phenomenon associated with actual or potential tissue damage. There are many types of pain that have different clinical and physiological processes. Examples of various types of pain include inflammatory pain, neuropathic pain, postoperative pain, and cancer pain. There are many models described in the literature to study each of the above types of pain. This review will focus on inflammatory and postoperative pain as these are of specific interest to our research.

3.1 Inflammatory pain models

Inflammatory pain is commonly induced by injecting compounds that cause inflammation (inflammogens) into various tissues, such as the skin, paw, muscle, joint, or body cavity. The introduction of these inflammogens stimulates central and peripheral sensitization which can then be detected using nociceptive tests or quantification of spontaneous pain behaviors. Common inflammogens used in mouse inflammatory pain models include formalin, lipopolysaccharide (LPS), carrageenan, capsaicin, and Complete Freund's Adjuvant. The inflammatory pathway and thus the degree of inflammatory pain is dependent on the route of administration, dose, and type of inflammogen used.

LPS is a historically used inflammogen that mimics infection and sepsis caused by bacteria. LPS is a component of the cell wall of bacteria and can be derived from various gram-negative bacteria such as *E. coli* and *Salmonella*. LPS is typically injected into the plantar surface of the hindpaw to test peripheral nociception, or into the abdomen to study systemic disease. LPS activates Toll-Like Receptor-4 (TLR-4) which induces TNF-α, IL-1β, and

KC/CXCL1 (keratinocyte-derived chemokine). The increases in these cytokines have been shown to be associated with LPS-induced hyperalgesia [Calil et al, 2014].

Systemic administration of LPS can induce sickness behaviors in mice [Bassi et al, 2012]. Sickness behaviors include lethargy, anorexia, anhedonia, hunched posture, and piloerection. The degree of these behaviors are dose dependent [Bassi et al, 2012; Szentirmai and Krueger, 2014]. LPS dose and survival times of mice vary greatly. One study showed C57BL/6 mice injected with up to 0.85 mg/kg LPS showed no visual signs of sickness (piloerection, orbital tightness, and hunched posture) for at least 24 h, while mice injected with 1.2 or 4 mg/kg showed clinical signs of a sick mouse 5 h after injection [Thomas et al, 2014]. Another study found roughly 50% of C57BL/6 mice injected with 27 mg/kg died by 48 h, while only 16% of mice injected with 25 mg/kg died within the same time period [Li et al, 2018]. Survival after LPS injection also varies between strains of mice. For example, 50% of BALB/c mice injected with 25 mg/kg LPS succumbed to the disease in less than 20 h after injection [Fermino et al, 2011].

3.2 Postoperative pain models

Postoperative pain models are useful for studying the pathophysiology of surgical pain and also for determining the efficacy of analgesics used to mitigate postoperative pain in humans and animals. There are many postoperative pain models described in the literature. The model selected for use depends on the research goal and desired outcome.

The plantar incision model is one of the most common models used to study the neurophysiology of incisional pain [Pogatzki-Zahn et al, 2017]. The model was originally described in rats in the mid-1990s where surgical pain was induced by cutting and suturing a 1-cm incision on the plantar surface of the hindlimb. A few hours after surgery, pain was assessed

by using mechanical nociceptive tests or measuring spontaneous pain behaviors (wound guarding and limping). More recently, the plantar incision model was developed in mice [Pogatzki and Raja, 2003]. Both rats and mice produce spontaneous pain behaviors for up to 2 d after surgery and have increased nociception for up to 7 d [Pogatzki and Raja, 2003; Zahn and Brennan, 1999]. Incisional pain models can incorporate muscle if prolonged duration of increased nociception is needed. For example, the skin/muscle incision and retraction (SMIR) model in rats showed increased mechanical hypersensitivity up to 22 d postoperatively [Flatters, 2008].

Incisional models, such as SMIR and the plantar incision model, are commonly used when studying the pathophysiology of surgical pain or assessing therapeutics intended for human use. To study the efficacy of analgesics and degree of postoperative pain for specific species, more practical and clinically relevant models are typically used. For example, studies assessing postoperative analgesic efficacy in mice have performed common surgeries completed in mice, such as laparotomy, vasectomy, splenectomy, and ovariectomy surgical models [Clark et al, 2004; Goecke et al, 2005; Kendall et al, 2016; Tubbs et al, 2011]. Results from these studies vary and depend on the methods used to measure postoperative pain, the surgical procedure performed, and the postoperative analgesics used. A previous study assessed multiple parameters (activity, vertical rises, grimace score, body weight, posture, orbital tightness, hair coat appearance, and the use of enrichment) in rats post ovariohysterectomy and showed many of the parameters were poor indicators of postoperative pain [Nunamaker et al, 2018]. They found hair coat, posture, and eye appearance were the most reliable indicator of postoperative pain [Nunamaker et al, 2018].

Cage-side behavior assessments for postoperative pain studies are problematic due to reliance on subjective opinions and poor reproducibility. Real-time scoring creates bias specifically in postoperative pain models due to the observers seeing the surgical incision. Studies have shown still photographs or videos are not as reliable as real-time scores [Nunamaker et al, 2018]. Hedonic behavior assays, such as FiUSV or IiUSV, could potentially mitigate these problems due to objective measurements without visualization of the observer.

CHAPTER II.

FEMALE- AND INTRUDER-INDUCED ULTRAONIC VOCALIZATIONS IN C57BL/6J MICE AS A PROXY INDICATOR FOR ANIMAL WELL-BEING[1]

1. Introduction

Assessment of pain in biomedical research is essential for animal well-being and obtaining accurate research results. Recognizing pain or distress in mice is challenging as they typically hide signs of disease or pain until drastically ill or near moribund [Carstens and Moberg, 2000]. A multimodal approach using many different assays to measure animal well-being is ideal to improve animal welfare in research animals. However, due to the low number of assays available, there is a need for more knowledge and techniques to assess pain, distress, and animal well-being [Hawkins, 2002].

The most commonly used tools to measure animal well-being used by researchers and veterinarians in laboratory animal medicine are ethograms and clinical observation sheets [Hawkins, 2002]. Ethogram characterizing behavioral changes such as changes in activity, body posture, or facial expressions can indicate well-being. Proxy indicator assays such as nest building, time to integrate to nest test, and burrowing behaviors indirectly measure an animal's behavior in the absence of a direct measurement, and have been gaining favor in the literature with variable success [Kendall et al, 2016; Rock et al, 2014]. Of these assays, the mouse grimace scale has become widely used to measure pain in rodents. However, this method is subjective

[1] A version of this manuscript will be published in Comparative Medicine: **Smith BJ, Bruner KE, Kendall LV**. 2019. Female- and intruder-induced ultrasonic vocalizations in C57BL/6J mice as a proxy indicator for animal well-being. Comp med.

and may not indicate pain intensity accurately [Leach et al, 2012; Miller et al, 2016]. An alternative proxy indicator assay that is non-invasive, objective, and quantitative will benefit both researchers and clinicians in assessing and recognizing pain or distress in rodents.

Mice produce ultrasonic vocalizations (USV) in addition to auditory squeaks to communicate [Portfors, 2007]. USV are vocalizations above the frequency humans can hear (>20 KHz) and can indicate either positive or negative effector states [Portfors, 2007; Wang et al, 2008]. Mice USV are predominantly studied as models for memory and neuropsychiatric disorders or analyzing aggression, playing, and mating [Wöhr and Schwarting et al, 2013].

Over the past couple decades, USV have been studied as a modality for pain recognition in mice and rats with mixed results [Calvino et al, 1996; Chisholm et al, 2013; Jourdan et al, 1997; Jourdan et al, 1998; Kurajova et al, 2010; Wallace et al, 2005; Williams et al, 2008]. Many of these studies induced negative effective states to induce spontaneous vocalizations by using invasive acute nociceptive stimulation, like injecting irritants and applying pinch, air, incision, or a combination of these. Reflex based assays have shown more success in rats than mice since rats produce alarm cries in response to noxious stimuli while mice do not [Chisholm et al, 2013; Jourdan et al, 1998; Levine et al, 1984; Martino and Perkins, 2008; Wallace et al, 2005]. A reliable, non-invasive, repeatable assay using USV to measure animal well-being in mice has not been found.

Mice produce USV during many non-aggressive situations [Heckman et al, 2017; Holy and Guo, 2005; Neunuebel et al, 2015; Portfors, 2007]. Although USV are produced by mice during numerous different same-sex and heterosexual interactions [Matsumoto and Okanoya, 2016; Portfors, 2007; Roullet et al, 2011; Zala et al, 2004], there are three mouse USV that are easily reproducible and well described for studying autism spectrum disorder [Portfors, 2007].

These three types of USV can be termed female urine-induced male mice USV (FiUSV), pup isolation-induced USV, and intruder-induced female USV (IiUSV). FiUSV are 70-KHz USV produced by adult males in high rates in the presence of female urine [D'Amato, 1991; Roullet et al, 2011]. Pup isolation-induced USV occur in male and female neonatal pups. When separated from the nest they will produce vocalizations that elicit a search and retrieval behavior from the dam [Blumberg and Sokoloff, 2001; Portfors, 2007]. IiUSV are USV produced by resident female mice in response to an intruder female mouse [Moles et al, 2007; Portfors, 2007].

A recent study showed evidence that male, wild house mice injected with LPS produced fewer USV compared to healthy, untreated mice when housed with a female mouse overnight [Lopes and König, 2016]. The study showed LPS-injected male mice produced zero regular frequency courtship USV compared to control mice. In addition, the study showed LPS-injected mice produced rare higher-than-normal frequency USV that were not seen in the control mice. The paper concluded that the absence of the regular frequency USV or the production of the higher-than-normal frequency USV may be an indicator of decreased animal well-being [Lopes and König, 2016]. Since USV production is dependent on mouse strain [Maggio and Whitney, 1985; Sugimoto et al, 2011], we wanted to assess whether USV can be used as a proxy indicator of decreased animal well-being associated with LPS injection in a commonly used laboratory mouse strain, C57BL/6J.

FiUSV have been extensively studied regarding behavior and mating research [Portfors, 2007]. FiUSV could serve as a proxy indicator for pain assessment due to being a natural behavior and a positive effector state. Pain, being a negative effector state, could counter the positive effector state induced by female urine, resulting in less FiUSV produced by the male. FiUSV would be advantageous because it is an objective, quantitative, and less invasive assay.

There are many studies describing how to maximize FiUSV production [Chabout et al, 2017; Maggio and Whitney, 1985; Nyby et al, 1983; Nyby et al, 1976; Roullet et al, 2011; Schmeisser et al, 2012] and characterizing USV syllable structure [Hanson and Hurley, 2012; Holy and Guo, 2005]. The quantity of FiUSV are dependent on strain [Maggio and Whitney, 1985; Sugimoto et al, 2011], social status [D'Amato, 1991; Nyby et al, 1976], previous heterosexual experience [Dizinno et al, 1978; Maggio and Whitney, 1985l Malkova et al, 2012; Michetti et al, 2012; Nyby et al, 1983; Nyby et al, 1976; Roullet et al, 2011; Schmeisser et al, 2012], stimulus [Malkova et al, 2012; Schmeisser et al, 2012], freshness of urine stimulus [Hurst et al, 2001; Hurst, 1990; Kavaliers et al, 2003; Roullet et al, 2011], and length of interaction [Roullet et al, 2011]. For example, previous studies showed C57BL/6J mice and BALB/c mice have a high prevalence of producing FiUSV, with almost 100% of C57BL/6J male mice producing vocalizations in response to a female or female urine compared to other strains with fewer than 40 % of males producing USV [Sugimoto et al, 2011]. Male mice produce most of the FiUSV in the first 3 min after urine stimulus and the number of vocalizations is significantly decreased if old urine is used or if no previous heterosexual experience occurred before testing [Roullet et al, 2011].

IiUSV associated with female-female interactions are significantly less described in the literature compared to FiUSV [Portfors, 2007]. Historically, it was thought that female mice produced few USV [Nyby et al, 1976]. Recent research has shown female mice produce equivalent number of vocalizations to males using a resident-intruder paradigm [Gourbal et al, 2004; Moles et al, 2007], Evidence suggests that only the resident female mouse produces IiUSV when an intruder mouse is present [Gourbal et al, 2004; Moles et al, 2007].

LPS is a commonly used inflammogen to induce inflammatory disease and mimic sepsis [Fink, 2014; Nemsek et al, 2008; Thomas et al, 2014]. Inflammatory pain has been shown to be associated with LPS-induced inflammatory disease due to an increase in pain sensitivity, such as allodynia and hyperalgesia, in many animal species including humans [Boucher et al, 2018; Calil et al, 2014; Cunha et al, 2007; Wegner et al, 2015]. Evidence suggests LPS-injected mice have reduced production of chemical signals (scent markings), show sick rodent behaviors, have decreased activity, and have a decreased interest in mating [Lopes and König, 2016; Nemsek et al, 2008; Swiergiel and Dunn, 2007; Thomas et al, 2014; Zala et al, 2004].

The goals of this study were to assess FiUSV in male mice and IiUSV in female mice in an acute inflammatory pain model using LPS. Male mice experiencing LPS-induced sickness behavior would produce fewer FiUSV than mice experiencing no sickness behaviors due to a decreased inclination to mate. Additionally, female mice experiencing LPS-induced sickness behaviors would produce fewer IiUSV than healthy, control female mice due to a decreased inclination to interact with an intruder mouse.

2. Materials and Methods

2.1 Animals

Male (n = 33) and female (n = 36) C57BL/6J mice (JAX stock #000664, The Jackson Laboratory, Bar Harbor, ME) were free of Sendai virus, mouse hepatitis virus, minute mouse virus, mouse parvovirus, mouse norovirus, Theiler murine encephalitis virus, rotavirus, *Mycoplasma pulmonis*, pinworms, and ectoparasites according to dirty bedding sentinel testing and vendor health reports. Mice were housed in same sex groups of 2 male or 4 female per individual ventilated cage (catalog no. CG09B01, Thoren Caging Systems, Hazleton, PA). Mice were housed with unrestricted access to chow (Teklad Irradiated Diet 2918, Envigo,

Madison, WI) and filter-sterilized water. Mice were maintained on a 12:12-h light:dark cycle at a temperature of 21 to 24 °C. All experimental procedures were approved by the IACUC and conducted at an AAALAC International accredited facility.

2.2 Experimental design

To assess FiUSV in male mice and IiUSV in female mice as proxy indicators of animal well-being in C57BL/6J mice, 9-wk-old male mice and 5-mos-old female mice were randomized into groups and injected with LPS or saline intraperitoneally. Male mice were split into four groups (n = 8/group) and treated with 12.5 mg/kg LPS or equal volume of saline intraperitoneally and tested at 1 or 3 h postinjection. Female mice were split into six groups (n = 6/group) and treated with 12.5 mg/kg LPS, 6 mg/kg LPS, or equal volume to the 12.5 mg/kg LPS of saline intraperitoneally and tested at 1 or 3 h postinjection. A lower dose (6 mg/kg) of LPS was given to the female mice in addition to the larger dose (12.5 mg/kg) to determine if there was a dose-dependent change in USV. Baseline values were collected 24 h preceding the 1- or 3-h time points. Male mice were sexually primed to a female 1 wk before experimentation to maximize USV production [Chabout et al, 2007; Roullet et al, 2011]. Mice were euthanized via CO2 inhalation immediately after their respective 1- and 3-h time points.

2.3 Sexual priming of male mice

Male mice were sexually primed similarly to the methods previously described [Chabout et al, 2007]. Briefly, male mice were housed 2 per cage. Seven days before collecting baseline data, one female was added to each male cage at 16:00. Sixteen hours later, females were removed from the male’s cage and returned to their original cage. Females were the same age as males.

2.4 LPS injection

Male mice and resident female mice were injected intraperitoneally with bacterial LPS at 6 or 12.5 mg/kg (E. coli, serotype O111:B4, Sigma-Aldrich, Inc. St. Louis, MO). LPS was prepared in aqueous sterile saline, alliquoted into 0.75 mL microcentrifuge tubes, and stored at -80 °C. On injection days, LPS was removed from storage, warmed to room temperature, and vortexed before administering the injection. LPS samples remained at room temperature for less than 30 min and were not refrozen or reused.

2.5 Testing chamber

Testing was conducted in a quiet room away from noisy equipment and activities. All animal handling was completed when not actively recording ultrasonic vocalizations. A recording chamber was used to detect activity and USV in a sound attenuated and visually isolated environment [Chabout et al, 2007]. Briefly, a beach cooler with internal dimensions of L 27 x W 23 x H 47 cm was used and configured with a small, 2.5 cm, hole drilled in the top to allow the ultrasonic-microphone wire to exit the chamber and connect to the recording device (UltraSoundGate 116Hb, Avisoft Biocoustics, Glienicke, Germany). The ultrasonic microphone (UltraSoundGate CM16/CMPA, Avisoft Biocoustics, Glienicke, Germany) was centered 30 cm above the cage bottom. Recording software (Avisoft, RECORDER USGH Software, Avisoft Biocoustics, Glienicke, Germany) was configured with a sampling rate of 250,000 Hz, FFT-length of 256 points, time window overlap of 50%, FlatTop window, and 16 bit format. Mice were acclimated to the testing chamber for 10 min/day for two days before collecting baseline values. The testing chamber was cleaned with 70% ethanol after each animal.

2.6 FiUSV data collection

On acclimation and testing days, a single male mouse was removed from their home cage and placed into an empty and clean testing cage. The testing cage was made up of an empty cage bottom (catalog no. CG09B01, Thoren Caging Systems, Hazleton, PA) and a clean wire cage cover (catalog no. CC01B01 1B, Thoren Caging Systems, Hazleton, PA). The clean testing cage containing the mouse was then placed into the testing chamber for 10 min before collecting data. Five-minute recordings were collected before and after urine stimulus.

Urine was collected from unfamiliar female mice as described [Chabout et al, 2007]. Briefly, female mice in estrus or proestrus, as determined by visual examination, were used for urine collection [Byers et al, 2012]. Urine samples were collected on a cotton tipped applicator from two females from separate cages by using physical restraint and gentle palpation. Urine was collected immediately before adding the urine stimulus to the testing chamber. The urine soaked cotton tipped applicator was placed in the middle of the testing cage in the same location for every mouse.

2.7 IiUSV data collection

Resident-intruder tests were used to collect IiUSV in female mice similar to methods previously described [Moles et al, 2007]. Briefly, female mice were individually housed for 3 days preceding baseline measurements and acted as the resident female on test days. Intruder female mice remained in social groups of 4 mice/cage. Resident female mice remained in their home cage on acclimation and testing days. Before testing, the wire cage cover on the home cage containing food and water was replaced with a clean, empty wire cover and all enrichment was removed from the resident female cage. The female, while housed in her home cage, was then moved into the testing chamber for ultrasonic recordings.

Resident females were habituated to the chamber for 10 min before collecting a 5 min background recording. An unfamiliar, unanesthetized intruder female was added to the resident female's home cage after the 5 min background recording finished. Resident and intruder female mice were allowed to have contact with each other. An additional 5 min recording was collected followed by removal of the intruder female and return of the enrichment.

2.8 USV quantification

Five-minute spectrogram recordings were analyzed using Avisoft-SASLab Pro (Version 5.2.07, Avisoft Bioacoustics, Glienicke, Germany). USV syllables were individually counted by examining the entire 5-min spectrogram by hand, determining the call latency (time it took for initial vocalization post stimulus), and then counting all syllables for an additional 2 min after the first USV occurred.

2.9 Activity quantification

Using the Avisoft-SASLab Pro program, background recordings were used to measure activity by quantifying the number of bouts of increased noise from the mouse moving in the testing chamber (activity bouts). Activity bouts were detected according to a threshold of 4 % FS (Full Scale), and a hold time of 0.05 s. Overloaded (saturated events greater than 100 % FS) events were excluded. This means activity bouts greater than 4 % FS that lasted longer than 0.05 s were assigned section labels. Section labels were saved as a text file and total number of activity bouts and total activity duration were determined.

2.10 Behavior assessment

Male and female behavior assessments were conducted immediately after USV recordings. Mice were placed into an empty cage bottom and wire cover and allowed 1 min to acclimate. Cage side pictures were collected from each mouse and scored by a blinded individual

based on orbital tightness (0-2, 0 = normal, 1 = slightly squinted, 2 = squinted), body posture (0-2, 0 = normal, 2 = hunched posture with abdomen raised), and piloerection (0-2, 0 = normal smooth appearance, 2 = hair raised).

The camera was placed roughly 15 cm adjacent to the behavior assessment cage. After the 1-min acclimation period, multiple pictures (15-20) were taken within 1 min period. Pictures of the mouse rearing up, out of focus, not facing the camera, or eyes not visible were excluded. One picture per mouse was randomly selected, and placed in random order with respect to time point and group into a power point presentation for the blinded observer. In addition to scoring mice on orbital tightness, piloerection, and hunched posture, the blinded observer was also asked to give overall opinion on whether the mouse looked sick or not. Mice that were considered to have sick behaviors by the blinded observer had a summation of the three attributes (behavior score) ≥ 3.

2.11 Statistical analysis

Statistical analysis was completed using GraphPad Prism 7.00 for Windows (GraphPad Software, La Jolla, California). Data were not normally distributed as determined by visual examination of diagnostic plots. Wilcoxon matched-pairs signed rank test (W = test statistic) was used for within group comparison of the number of USV and number of activity bouts comparing baseline to post-injection data. Mann Whitney tests (U = test statistic) were used for between group comparisons comparing relative change data. Correlations between activity bouts and activity duration were determined by Spearman correlation. P values less than 0.05 were considered statistically significant.

3. Results

3.1 FiUSV

Male mice were treated with 12.5 mg/kg LPS or saline and FiUSV were collected at 1 or 3 h postinjection. FiUSV results were normalized for each mouse by determining the relative change ([final-baseline]/baseline) and the results shown in Figure 2.1 A.

Male mice produced zero vocalizations before the urine stimulus at any time point. Thirty-two of 33 male mice exposed to fresh, unfamiliar, female urine produced 149 ± 127 USV/2 min (Mean ± SD) with a call latency of 46.8 ± 55.3 s at baseline. The male mouse that did not produce any USV at baseline had a preputial gland abscess on physical examination. This mouse was removed from study due to not producing any USV at baseline and did not undergo any injection.

All LPS-injected mice produced zero FiUSV at the 1- and 3-h time points while 7 of 8 saline-injected mice produced vocalizations at the 1-h time point (93 ± 75 USV/2 min) and 8 of 8 saline-injected mice produced vocalizations at the 3-h time point (212 ± 156 USV/2). There was a significant difference in the number of vocalizations from baseline for LPS-injected mice (W = -36, P = 0.0078 at both 1- and 3-h time points) but not saline-injected mice (W = 8, P = 0.64 and W = -4, P = 0.84 at the 1- and 3-h time points, respectively). There was a significant difference in FiUSV relative change between LPS-injected mice and saline-injected mice at 1- and 3-h time points (U = 4, P = 0.0014 and U = 0, P = 0.0002, respectively).

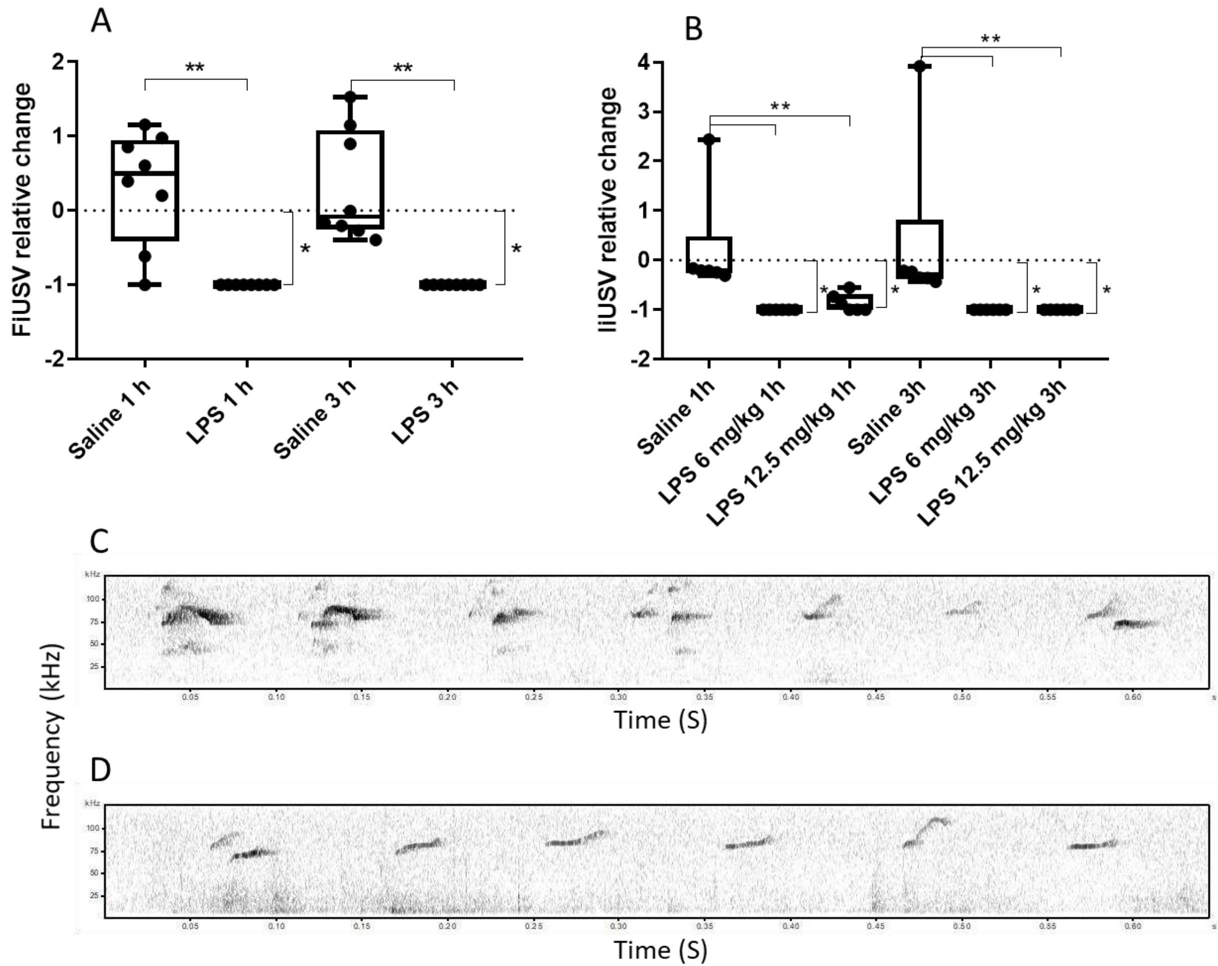

Figure 2.1. FiUSV and IiUSV results. (A) FiUSV relative change scatter plot data with box and whisker plot overlay from male mice treated with 12.5 mg/kg LPS or saline and collected at 1 or 3 h post injection. (B) IiUSV relative change scatter plot data with box and whisker plot overlay from female mice treated with 6- or 12.5 mg/kg LPS or saline and collected at 1 or 3 h post injection. LPS-injected mice have significantly decreased USV production at 1 and 3 h postinjection compared to saline-injected mice. Significant difference between groups represented by ** and significant difference from baseline represented by *. Representative spectrograms (FFT-length = 256 points, overlap of 50%, and FlatTop window) of recorded vocalizations produced by male mice elicited with female urine (C) and vocalizations produced by female mice elicited by an intruder female (D).

3.2 IiUSV

Female mice were treated with 6 mg/kg or 12.5 mg/kg LPS or equal volume of saline and IiUSV were collected at 1 or 3 h postinjection using a resident-intruder paradigm. IiUSV results were normalized for each mouse by determining the relative change ([final-baseline]/baseline) and the results shown in Figure 2.1 B.

Female mice did not produce any vocalizations before the intruder stimulus at any time point. All 36 resident female mice produced vocalizations at baseline when exposed to an unfamiliar intruder female (370 ± 156 USV/2 min with a call latency of 32.9 ± 32.3 s). All saline-injected mice produced IiUSV postinjection (317 ± 119 and 266.7 ± 109.9 USV/2 min at the 1- and 3-h time point, respectively) and were not significantly different from baseline at either time point ($W = -9$, $P = 0.44$ at both time points).

All mice treated with 6 mg/kg LPS produced zero IiUSV at both time points postinjection. At the 1-h time point, 3 female mice treated with 12.5 mg/kg LPS produced zero IiUSV while the other three mice produced vocalizations (132 ± 92 USV/2 min). All 12.5 mg/kg LPS-injected mice produced zero IiUSV at the 3-h time point. LPS-injected groups' number of USV were significantly different from baseline at both time points ($W = -21$, $P = 0.03$). There was a significant difference in IiUSV relative change between 6 mg/kg LPS-injected mice and saline-injected mice at both time points ($U = 0$, $P = 0.002$) as well as between 12.5 mg/kg LPS-injected mice and saline-injected mice at both time points ($U = 0$, $P = 0.002$).

3.3 Activity

There was a large difference in the appearance of the background ultrasonic recordings taken before adding urine or intruder stimuli in LPS-injected mice compared to saline-injected mice (Figure 2.2). Mice did not produce any vocalizations before the urine or intruder stimulus.

Since all recordings were completed in a quiet room away from animals being handled, the background recordings only consisted of sound produced by the mouse moving in the testing chamber and the approach described here was used as a metric for quantifying movement. The total activity duration and total number of activity bouts produced during the 5 min recordings were quantified using the software. Activity bouts and activity duration for each mouse were correlated as shown in Figure 3 A and 3 B. The activity duration findings between groups and baseline were the same as the findings for activity bouts. Thus, only activity bouts were used to assess activity. Activity bouts were normalized for each mouse by determining the relative change and the results are shown in Figure 2.3 C for male mice and Figure 2.3 D for female mice.

Male mice had 437 ± 150 activity bouts at baseline. Male LPS-injected mice had 94 ± 45 and 16 ± 17 activity bouts at 1- and 3-h time points, respectively and activity bouts were significantly decreased at both time points compared to baseline ($W = -36$, $P = 0.08$). Saline-injected male mice had no difference in activity at either time point compared to baseline ($W = 8$, $P = 0.6$ and $W = 5$, $P = 0.8$ at the 1- and 3-h time points, respectively). There was a significant difference between activity relative change for LPS-injected male mice compared to saline-injected male mice at both time points ($U = 0$, $P = 0.002$).

Female mice had 568 ± 152 activity bouts at baseline. Female mice treated with 6 mg/kg LPS had 52 ± 36 and 9 ± 9 activity bouts while 12.5 mg/kg treated mice had 35 ± 27 and 14 ± 18 activity bouts at 1- and 3-h time points, respectively. 6 mg/kg and 12.5 mg/kg LPS-injected female mice had significantly lower activity bouts at both time points compared to baseline ($W = -21$, $P = 0.03$ for all comparisons). Saline-injected female mice had no difference in number of activity bouts at either time point compared to baseline ($W = -7$, $P = 0.5$ and $W = -9$, $P = 0.4$ at

the 1- and 3-h time points, respectively). There was a significant difference between activity bouts relative change for LPS-injected female mice (6 and 12.5 mg/kg groups) compared to saline-injected female mice at the both time points ($U = 0$, $P = 0.002$).

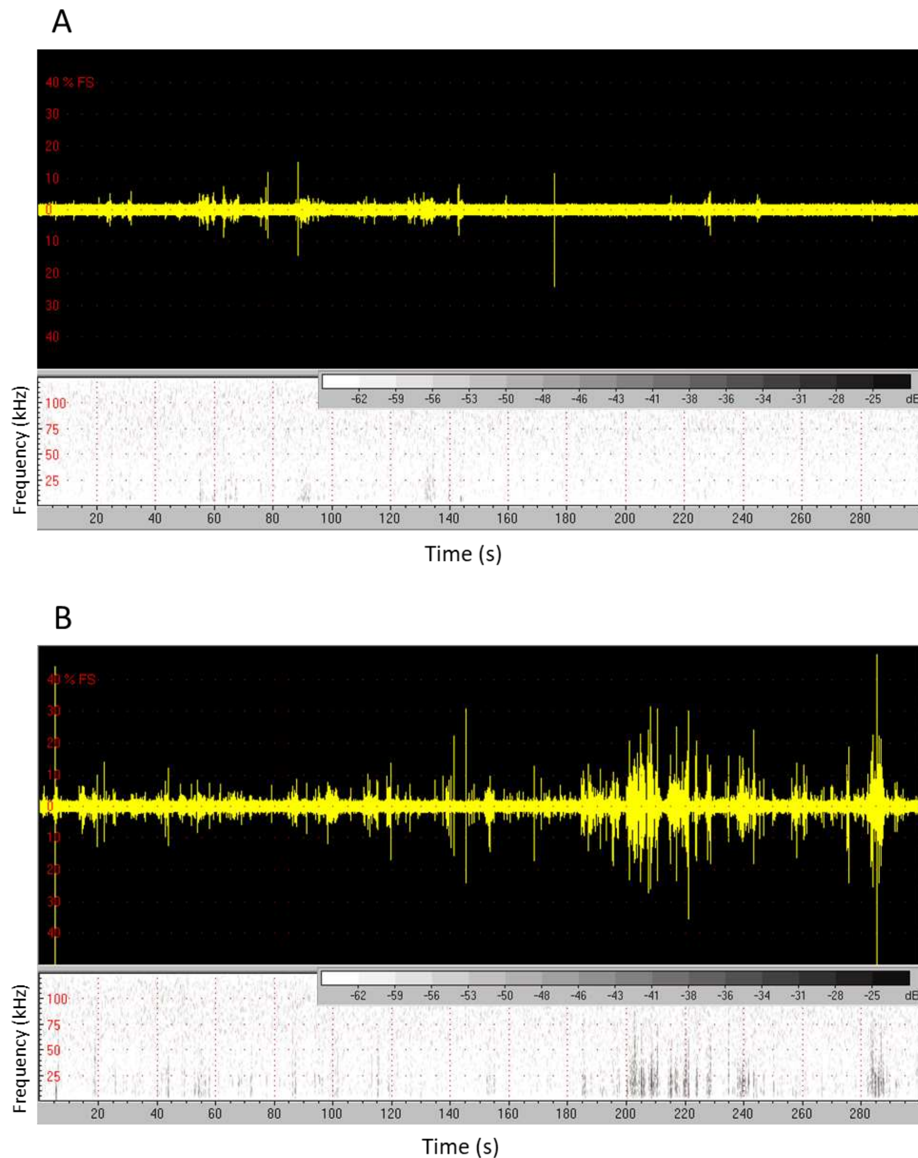

Figure 2.2. Background noise quantification. Representative voltage trace (top) and spectrogram (bottom) examples from 5-min background recordings showing decreased activity in LPS-injected mice (A) compared to saline-injected mice (B). Spikes in the voltage trace represent increased noise and correlate with mouse movement within the testing chamber, as seen in the spectrograms.

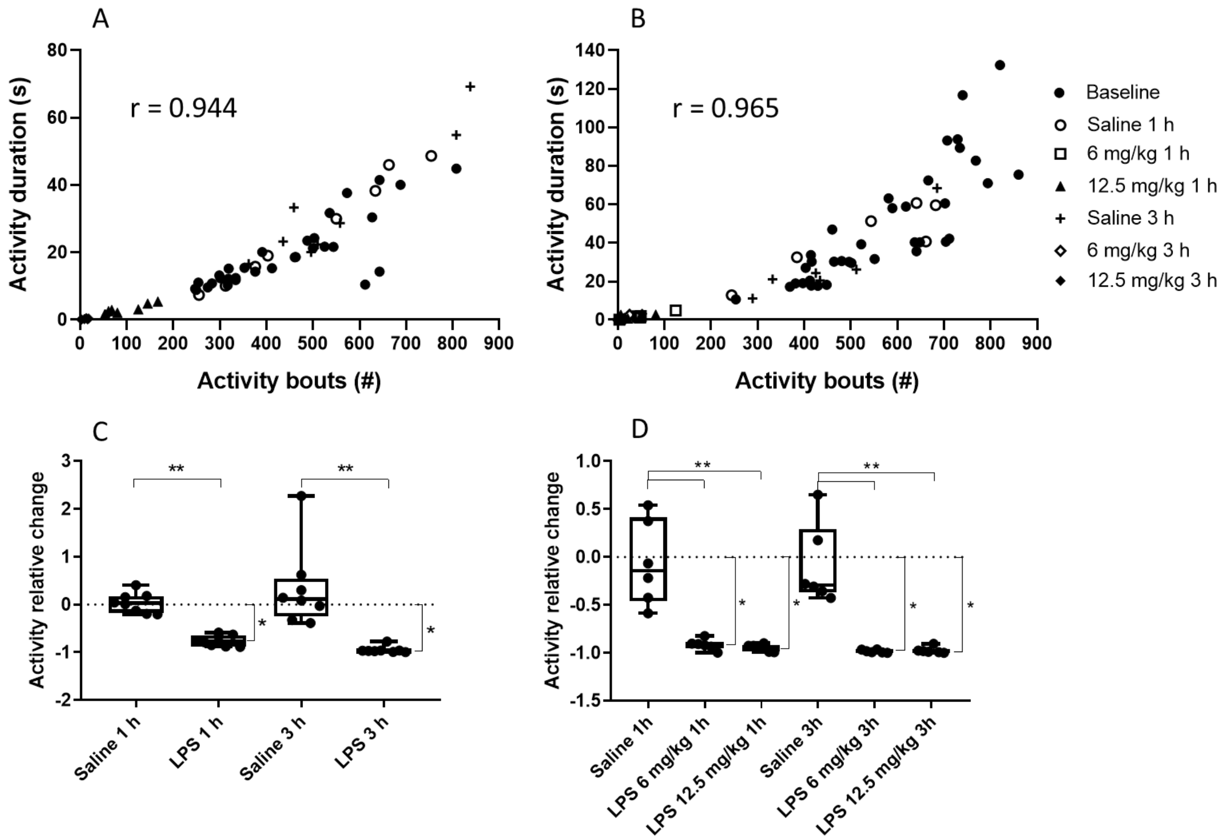

Figure 2.3**.** Activity results. Correlation between activity bouts and activity duration in male mice (A) and female mice (B) injected with LPS or saline and measured at 1 or 3 h after injection. Activity bouts and activity duration were correlated in both male (r = 0.944) and female mice (r = 0.965). Thus, Activity only relates to Activity bouts. Activity relative change scatter plot data with box and whisker plot overlay from male (C) and female mice (D). Male mice were injected with 12.5 mg/kg LPS or saline while female mice were injected with 6- or 12.5 mg/kg LPS or saline. Activity measured at 1 and 3 h after injections. Significant difference between groups represented by ** and significant difference from baseline represented by *.

3.4 Behavior assessment

Behavior was assessed using photographs taken immediately after USV recordings and compiled into a power point presentation for a blinded individual to score based on orbital tightness, piloerection, and posture (Figure 4 A for male mice and Figure 4 B for female mice). Mice were considered to have poor well-being if the total behavior score was ≥ 3. Only one of 8 LPS-injected male mice was considered to have poor well-being at 1 h and all LPS-injected male

mice were considered to have poor well-being at 3 h, while none of the saline-injected male mice were considered to have poor well-being at either time point. There was a significant difference between saline- and LPS-injected male mice at 3 h ($U = 0$, $P = 0.0002$) but not at 1 h ($U = 29$, $P = 0.9$) post-injection. One of 6 female mice treated with 6 mg/kg LPS was considered to have poor well-being while none of the female mice treated with 12.5 mg/kg LPS was considered to have poor well-being at 1 h post-injection. All LPS-injected female mice were considered to have poor well-being at 3 h. Similar to males, there was a significant difference between saline and LPS-injected female mice at 3 h ($U = 0$, $P = 0.02$) but not at 1 h postinjection ($U = 18$, $P > 0.99$).

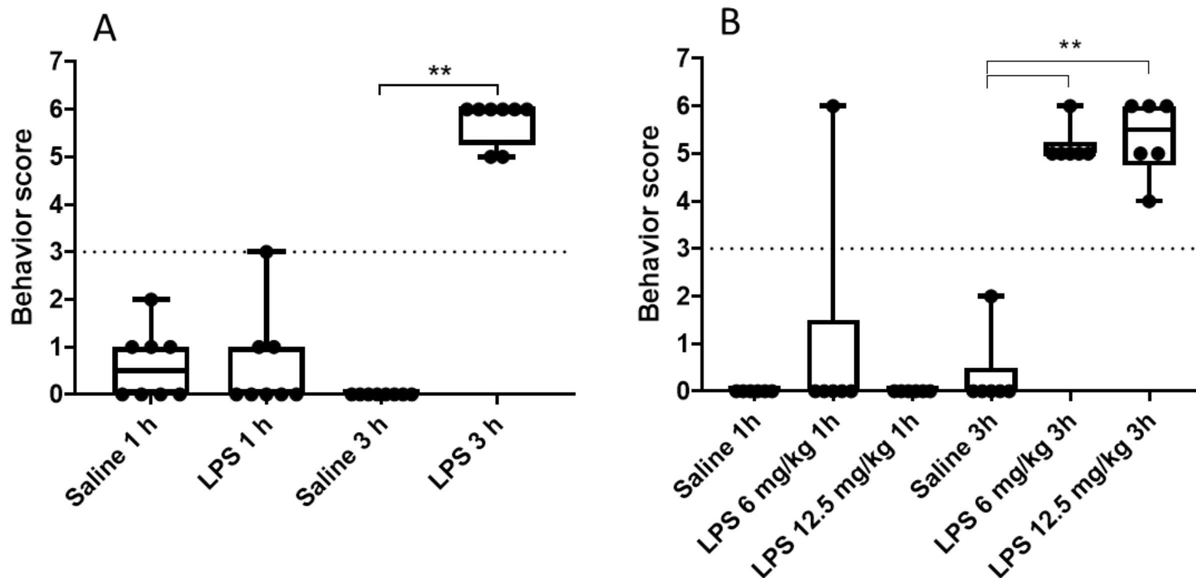

Figure 2.4. Behavior assessment results. Behavior score scatter plot data with box and whisker plot overlay from male (A) and female mice (B). Male mice were injected with 12.5 mg/kg LPS or saline while female mice were injected with 6- or 12.5 mg/kg LPS or saline. Behavior scores assessed at 1 and 3 h post injection. LPS-injected mice were considered to have poor well-being (behavior score ≥ 3, dotted line) only at the 3-h time point. Significant difference between groups represented by **.

4. Discussion

The findings from this study showed that C57BL/6J mice undergoing LPS-induced acute inflammatory disease do not produce FiUSV or IiUSV while control, healthy mice have no change in FiUSV or IiUSV. Furthermore, the absence of these vocalizations occurs before the onset of visual clinical signs of orbital tightness, hunched posture, and piloerection. The data showed most of the LPS-injected mice produced zero USV and lacked visual signs of a sick mouse at the 1-h time point while LPS-injected mice at the 3-h time point produced zero USV and showed visual signs of a sick mouse.

Baseline USV data from this study were consistent with previously published papers [Chabout et al, 2012; D'Amato, 1991; Hoffmann et al, 2009]. All LPS-injected male mice produced zero USV and all but 3 LPS-injected female mice produced zero USV postinjection. The three females that produced vocalizations post injection were treated with 12.5 mg/kg LPS and produced IiUSV at the 1-h time point. The number of vocalizations were less than their respective baseline values. Pain perception varies between individuals [Latasch et al, 1984]. The production of USV in these three mice could be due to varying degrees of pain perception. Alternatively, although previous studies showed only the resident female produced USV [Gourbal et al, 2004; Moles et al, 2007], the intruder could have been producing vocalizations as well. Using anesthetized intruder mice or injecting intruder mice with the same volume of LPS could eliminate this variable.

Findings from this study coincide with findings seen in wild house mice injected with LPS [Lopes and König, 2017]. Lopes and König study showed LPS-injected male mice produced zero regular frequency USV when housed with a female mouse overnight. The lack of regular frequency USV were also seen in this study when female urine was introduced to LPS-injected

C57BL/6J mice. Lopes and König detected high frequency USV in LPS-injected male mice when housed with the female mouse. These high frequency USV were not seen in this study. These vocalizations might not have been seen in this study due to the short duration the male was exposed to the female urine or due to the female not being present during recordings.

LPS, a microbial cell wall component, is an endotoxin that results in a dose dependent elevation of proinflammatory cytokines that facilitates or enhances pain and is commonly used as a model of sepsis and inflammatory pain [National Research Council, 2009; Thomas et al, 2014]. Clinical signs associated with LPS induced sepsis include hyperalgesia, malaise, and inappetence [National Research Council, 2009]. LPS was used in this study to mimic a systemic acute illness and subsequent pain induction. Previous studies have shown C57BL/6 and BALB/c mice can survive over 20 h after being injected with 12.5 mg/kg or higher doses of LPS [Fermino et al, 2011; Laubach et al, 1995; Liu et al, 2006]. Survival curves were not completed in this study due to euthanizing mice after the 1- and 3-h time points. LPS-injected mice, regardless of dose, had signs of a sick mouse at 3 h post injection. Mice were not moribund, but had decreased activity, hunched postures, piloerection, and obvious orbital tightness.

Male mice did not show a time-dependent decrease in USV when measured at 1- and 3 h post LPS injection due to a complete absence in USV production in mice at both time points. Female mice similarly did not have a time or dose depedent decrease in USV when measured at 1 or 3 h post injection given 6 and 12.5 mg/kg LPS. Lowering LPS doses or assessing shorter time points postinjection could be used to determine if USV production is dependent on animal well-being, LPS dose, or time.

Ages of male and female mice used in this study were chosen based on previous studies assessing FiUSV and IiUSV [Chabout et al, 2007; Moles et al, 2007; Scattoni et al, 2008]. Mice

of varying ages have been shown to produce FiUSV and IiUSV. Male mice produce low numbers of FiUSV if less than 7 wks old, [Chabout et al, 2007] but can produce FiUSV once older than that age and still produce FiUSV at 300 d of age [Hoffmann et al, 2009]. Previous studies have shown female mice produce IiUSV between 3-5 mos and 12-mos old, but the number of IiUSV produced by 12-mos-old female mice were less than the number of IiUSV produced by 3-5-mos-old mice [Moles et al, 2007]. Further studies could assess the effect different ages of mice have on USV production associated with animal well-being.

LPS-injected mice have decreased testosterone in plasma compared to control mice [Lopes and König, 2017]. Decreased testosterone has been linked to decrease production of courtship USV in mice [Dizinno et al, 1978; Nunez and Tan, 1983]. The reduction in testosterone did not completely abolish USV production. LPS-induced decreases in plasma testosterone may attribute to the reduction in FiUSV in this study. However, since all of the male mice and most of the female mice completely lacked production of USV after LPS-injection, the change in USV production in this study is most likely due to decreased animal well-being.

Although FiUSV and IiUSV are likely impractical for cage-side assessment of sick mice across an entire vivarium, USV assessment using the methods described could be used in studying the pathology of pain, studying analgesic efficacy, or assessing animal well-being in well-controlled infectious disease studies. The most common method for determining study end-point criteria is a decrease in weight or a change in a cage-side behavior assessment score using an ethogram or score sheet [Hawkins, 2002]. In this study, a cage-side ethogram measuring orbital tightness, piloerection, and posture was used to quantify animal well-being. Behavior ethograms are based on subjective criteria and can vary depending on the observer. This is evident in this study. For example, 4 saline-injected males and 1 saline-injected female, although

considered to have normal well-being, had behavior scores of 1 and 2. The elevated behavior scores seen in these mice could be due to the blinded individual misinterpreting the animal's position, piloerection, or orbital tightness and is a fault of subjective assessments. This example shows how subjective score sheets and behavior ethograms are not ideal for assessing animal well-being. Objective criteria, such as USV and activity described in this sudy, can improve how animal well-being and study end-points are decided.

The ultrasonic microphone could detect noise associated with mouse movement within the chamber. Ultrasonic recordings were collected in a sound attenuating chamber in a room separated from noisy equipment and activities, and animal handling was conducted when not actively recording ultrasonic vocalizations. Also, mice did not produce any vocalizations before the urine or intruder stimulus. Thus, 5-min background ultrasonic recordings collected prior to adding urine stimulus or intruder stimulus only consisted of sound produced by the experimental mouse moving in the testing chamber. Using the Avisoft-SASLab Pro program, we were able to quantify the noise associated with this movement and is represented as activity bouts.

The activity data from this study correlated with the USV data. LPS-injected mice had significantly less activity compared to saline-injected mice at both time points. There was no significant difference in activity between the LPS concentrations or between the 1- and 3-h time points. Future studies could assess activity at shorter time points postinjection or at lower LPS concentrations to determine if the ultrasonic microphone is sensitive enough to detect dose-dependent and time-dependent changes. Further studies could also compare the ultrasonic microphone to different modalities of measuring activity, like running wheels, telemetry, treadmills, or video recordings to determine the functionality of the ultrasonic microphone at detecting activity.

A design limitation to this study was that USV were collected in an enclosed chamber, thus mice were visually isolated from the observer, while the behavior scores were obtained from images taken with an observer present. Mice producing zero USV but appearing non-painful at the 1-h time point could have been exaggerated since mice hide their visual signs of pain in front of predators. An alternative method for collecting images could be to have a camera mounted in the chamber, allowing for simultaneous USV and behavior data collection. In this experiment, the observer was present during the behavior assessment to mimic real-world cage-side assessments.

The results of the current study suggest that FiUSV in male mice and IiUSV in female mice can be used as a proxy indicator of animal well-being associated with acute inflammation as absence of vocalizations occur before the onset of clinical signs of pain. Additionally, the ultrasonic microphone can detect noise from mouse movement within the testing chamber allowing for simple and non-invasive quantification of mouse activity. The combination of measuring activity and USV production in mice is a powerful method for assessing animal well-being. Future studies should assess the functionality of these modalities in other mouse pain models.

CHAPTER III.

FEMALE URINE-INDUCED MALE MICE ULTRASONIC VOCALIZATIONS IN C57BL/6J MICE AS A PROXY INDICATOR FOR POSTOPERATIVE PAIN[2]

1. Introduction

Appropriate postoperative analgesia is essential in minimizing pain and maintaining optimal animal welfare in laboratory animals. Mice are the most common animal model used in biomedical research, often undergoing surgical procedures that require postoperative analgesia. Pain recognition is difficult in mice due to the limited number of objective pain recognition assays available, and mice typically hide visual signs of pain until drastically ill or nearly moribund [Carstens and Moberg, 2000]. It is difficult to determine if mice are receiving appropriate postoperative analgesia due to the limited number of modalities to recognize postoperative pain. A non-invasive and objective method for measuring postoperative pain would be advantageous in determining appropriate analgesic regimens in mice and improving animal welfare.

In addition to audible vocalizations, mice produce ultrasonic vocalizations (USV) above the frequency humans can hear (> 20 KHz) [Moles et al, 2007; Nyby et al, 1983; Neunuebel et al, 2015; Portfors, 2007; Roullet et al, 2011; Williams et al, 2008]. USV have been studied as a modality for pain recognition in mice and rats with mixed results [Calvino et al, 1996; Chisholm et al, 2013; Jourdan et al, 1997; Jourdan et al, 1998; Kurajova et al, 2010; Wallace et al, 2005;

[2] A version of this manuscript will be published in Comparative Medicine: **Smith BJ, Bruner KE, Hess A, Kendall LV**. 2019. Female urine-induced male mice ultrasonic vocalizations in C57BL/6J mice as a proxy indicator for postoperative pain. Comp Med.

Williams et al, 2008]. Previous attempts to use USV to detect pain in mice have shown mice do not spontaneously emit USV in response to handling, restraint, acute pain, or noxious stimulation such as tail snips or ear punches [Wallace et al, 2005; Williams et al, 2008]. This is because mice more commonly produce USV during nonaggressive same-sex and heterosexual interactions and not spontaneously [Heckman et al, 2017; Moles et al, 2007; Neunuebel et al, 2005; Portfors, 2007; Smith et al, 2019]. There are three types of USV that are easily reproducible and heavily described in the literature which can be termed female urine-induced male mice USV (FiUSV), pup isolation-induced USV, and intruder-induced USV [Roullet et al, 2011]. FiUSV are produced by adult males in the presence of adult females or their urine and are associated with courtship and mating [D'Amato, 1991; Portfors, 2007; Roullet et al, 2011]. Pup isolation-induced USV are produced by male and female neonatal pups when separated from the nest [Blumberg and Sokoloff, 2011; Portfors, 2007]. Intruder-induced USV are produced by resident female mice in response to an intruder female mouse [Moles et al, 2007; Portfors, 2007]. Previous research has shown that C57BL/6J mice do not produce FiUSV when experiencing LPS-induced inflammatory sickness, and the absence of these vocalizations occurred before mice showed visual signs of sickness [Lopes and König, 2016; Smith et al, 2019].

FiUSV could be advantageous as a proxy indicator of postoperative pain because they are objectively measured, non-invasive, quantifiable, and may be more sensitive than visual examination. To determine if FiUSV can be used as a proxy indicator for postoperative pain, FiUSV, activity, and behavior scores were measured in male mice once daily for 5 days before and after vasectomy or sham surgery (anesthesia only) and with or without postoperative analgesia of sustained-release buprenorphine (Bup-SR).

2. Materials and Methods

2.1 Animals

Male (*n* = 38) and female (*n* = 36) C57BL/6J mice (JAX stock #000664, The Jackson Laboratory, Bar Harbor, ME) were housed in same sex groups of 2 males or 4 females per individual ventilated cage (catalog no. CG09B01Small Mouse II Cage, Thoren Caging Systems, Hazleton, PA). According to dirty bedding sentinel testing and vendor health reports, mice were free of Sendai virus, mouse hepatitis virus, minute mouse virus, mouse parvovirus, mouse norovirus, Theiler murine encephalitis virus, rotavirus, *Mycoplasma pulmonis*, pinworms, and ectoparasites. Mice had unrestricted access to chow (Teklad Irradiated Diet 2918, Envigo, Madison, WI) and filter-sterilized water. Animal rooms were kept at a temperature of 21 to 24 °C and maintained on a 12:12-h light:dark cycle. Experimental procedures were approved by the IACUC and conducted at an AAALAC International accredited facility.

2.2 Experimental design

9-wk-old C57BL/6J male mice were randomly separated into four groups to assess FiUSV as a proxy indicator of surgical pain: surgery with analgesia (Sx-Tx), surgery without analgesia (Sx-No Tx), sham surgery with analgesia (Sham-Tx), and sham surgery without analgesia (Sham-No Tx). Postoperative analgesia consisted of a single injection of Bup-SR (0.06 mg/kg, 1 mg/mL, Zoopharm, Windsor, CO). To maximize FiUSV production by male mice, female mice were used to sexually prime male mice 1 wk before collecting baseline values. Sexual priming was done by placing one female into each male cage for 16 h [Chabout et al, 2017, Smith et al, 2019]. Baseline values (FiUSV and activity) were collected once daily for 5 d before undergoing surgery and averaged (mean) together to determine each mouse's baseline data. Postoperative pain, activity, and FiUSV production were assessed at 4, 28, 52, 76, and 100

h after surgery. Baseline and postoperative time points for each mouse were collected at the same time every day. Male mice were euthanized immediately after the 100 h time point.

The experiment was conducted over four cohorts (8-10 mice per cohort). Due to performing multiple cohorts, females were older than the males for cohorts 2, 3, and 4. Female mice were 9-wk-old at the start of cohort 1, 19-wk-old for cohort 2, 20-wk-old for cohort 3, and 23-wk old for cohort 4.

2.3 Vasectomy procedure

A vasectomy was performed via an abdominal approach as previously described [Bermejo-Alvarez et al, 2014]. Briefly, mice were anesthetized with isoflurane and placed on a heating pad throughout the procedure and recovery. Ophthalmic ointment was applied to the eyes. Mice were aseptically prepared for a ventral abdominal surgery by shaving the abdomen and wiping with 10% chlorohexidine scrub and 70% isopropyl alcohol sequentially three times. Treatment (Bup-SR 0.6 mg/kg, SQ or equal volume saline) was administered immediately once anesthetized.

A 1-cm longitudinal, midline abdominal skin incision was made 1 cm cranial to the prepuce followed by a 5-10 mm incision made along the *linia alba*. Forceps were used to expose the testis, vas deferens, and epididymis. Micro dissecting forceps, heated by a bead sterilizer, were used to cut and cauterize the vas deferens, removing roughly a 1 cm portion of tissue. The exposed tissues were replaced back into the abdomen and the same procedures were repeated to remove a 1-cm portion of the contralateral vas deferens. The muscle was closed with one horizontal mattress stitch with 4/0 absorbable suture. The skin was closed with 2 wound clips and the mouse was returned to its cage for recovery. Sham-surgery mice were prepped the same

way as vasectomized mice. They remained under anesthesia for relatively the same duration (15 ± 2 min) as vasectomized mice but received no incision.

2.4 Testing chamber

A sound-attenuating recording chamber was used to measure activity and USV similarly to the methods previously described [Chabout et al, 2017, Smith et al, 2019]. Briefly, a beach cooler with internal dimensions of L 27 x W 23 x H 47 cm was constructed with a small hole drilled in the top to allow the ultrasonic-microphone wire to exit the chamber. The ultrasonic microphone (UltraSoundGate CM16/CMPA, Avisoft Biocoustics, Glienicke, Germany) was located inside the testing chamber, centered 30 cm above the cage bottom. The recording device (UltraSoundGate 116Hb, Avisoft Biocoustics, Glienicke, Germany) was located outside the testing chamber. The recording software (Avisoft, RECORDER USGH Software, Avisoft Biocoustics, Glienicke, Germany) was configured with a sampling rate of 250,000 Hz, FFT-length of 256 points, time window overlap of 50%, FlatTop window, and 16 bit format.

Before collecting baseline values, mice were acclimated to the testing chamber for 10 min/day for two days. All recordings were completed in a quiet, secluded room. To decrease background interference, animal manipulation occurred when the ultrasonic microphone was turned off.

2.5 FiUSV and activity data collection

Data collections were conducted similarly to the methods previously described [Chabout et al, 2017, Smith et al, 2019]. Briefly, male mice were placed individually into a clean, empty cage bottom with a clean wire cage cover. The cage containing the male mouse was placed into the testing chamber for 10 min before collecting data. Five-min ultrasonic recordings were collected before and after the addition of the urine stimulus. The recordings collected before the urine

stimulus were used to measure activity, and the 5-min recordings after the urine stimulus were used for FiUSV quantification. The testing chamber was cleaned with 70% alcohol between each mouse.

The female urine was collected immediately before adding it to the testing chamber using methods previously described [Chabout et al, 2017, Smith et al, 2019]. Urine was collected from the same cohort of female mice used to sexually prime males. However, the female urine was never used more than once for the same male mouse. Female mice in estrus or proestrus, as determined by visual examination [Byers et al, 2012], were used for urine collection. A cotton tipped applicator was used to collect urine from two females from two separate cages by using physical restraint and gentle palpation. The urine-soaked cotton-tipped applicator was placed in the middle of the testing cage in the same location for every mouse.

2.6 USV quantification

Spectrogram recordings were analyzed using Avisoft-SASLab Pro (Version 5.2.07, Avisoft Bioacoustics, Glienicke, Germany). A blinded individual examined each 5-min spectrogram completely by hand. The call latency (time it took for initial vocalization post stimulus) was determined and then all syllables were counted for an additional 2 min after the first USV occurred. The total number of FiUSV produced during the 2-min period were recorded and used for comparisons. Mice that produced 0 FiUSV for more than one of the five baseline time points did not undergo surgery and were removed from the study due to not producing FiUSV reliably.

2.7 Activity quantification

Using the Avisoft-SASLab Pro program, 5-min ultrasonic recordings collected prior to adding the urine stimulus were used to measure activity as previously described [Smith et al,

2019]. Briefly, activity bouts (bouts of increased noise from the mouse moving in the testing chamber surrounded by silence) were assigned a section label according to a threshold of 4 % FS (full scale) and a hold time of 0.05 s. Overloaded (saturated) events were excluded. This means sound that was above 4 % FS that lasted at least 0.05 s was counted as an activity bout. If vocalizations occurred prior to urine stimulus, the ultrasonic vocalizations were excluded by removing the section label associated with each vocalization. Section labels were saved as a text file and total number of activity bouts were determined.

2.8 Behavior assessment

Male behavior assessments were conducted immediately after USV recordings as previously described [Smith et al, 2019]. Mice were moved from the testing chamber and into a new empty cage covered by a wire top. Mice were allowed 1 min to acclimate to the new cage. Cage-side pictures were collected from each mouse by placing a camera roughly 15 cm adjacent to the cage. Multiple still photographs (15-20) were taken within a 1-min period of time. Pictures of the mouse rearing up, out of focus, not facing the camera, or with eyes not visible were excluded. One picture per mouse was randomly selected, and placed in random order with respect to time point into a power point presentation for a blinded observer to score each mouse. The mice were scored based on orbital tightness (0-2, 0 = normal, 1 = slightly squinted, 2 = squinted), body posture (0-2, 0 = normal, 2 = hunched posture with abdomen raised), piloerection (0-2, 0 = normal smooth appearance, 2 = hair raised), and whether the blinded observer thought the mouse looked painful or not. Mice that were considered painful by the blinded observer had a summation of the three attributes (behavior score) ≥ 3.

2.9 Statistical analysis

Analysis of baseline data were done using R 3.4.3 for windows with the lme4 package. Analyses were only completed on mice that underwent surgery or sham surgery and excluded mice that did not produce USV more than one of the five baseline days. Model fitting was done separately for each variable (FiUSV and Activity). Specifically, a random effects model was used to estimate variance attributable to mouse (mouse variance), days within mouse (residual variance), and cohort (cohort variance).

Repeated-measures analyses were done using GraphPad Prism 8.00 for Windows (GraphPad Software, La Jolla, California). Analyses were only completed on mice that underwent surgery or sham-surgery and excluded mice that did not produce FiUSV more than one of the five baseline days. Data were collected once daily for 5 days before surgery and the 5-day baseline means were determined for each variable (FiUSV and activity bouts). Two-way ANOVA with Dunnett's multiple comparison post hoc test compared the FiUSV and activity bouts produced during the 5 baseline time points from cohorts 2-4 to the baseline values from mice in cohort 1. Two-way ANOVA with Tukey's multiple comparison post hoc test was used to analyze postoperative FiUSV and activity bouts between groups. Factors included time (4, 28, 52, 76, and 100 h) and experimental group (Sx-Tx, Sx-No Tx, Sham-Tx, Sham-No Tx). Two-way ANOVA with Dunnett's multiple comparison post hoc test was performed for FiUSV and activity bouts to compare each postoperative time point (4, 28, 52, 76, and 100 h) to the 5-day baseline mean of each mouse within each experimental group (Sx-Tx, Sx-No Tx, Sham-Tx, Sham-No Tx). A P value less than 0.05 was considered to be statistically significant for all analyses.

3. Results

3.1 Baseline FiUSV

Baseline FiUSV were collected from male mice once daily for five days before undergoing surgery. Male mice did not produce any spontaneous USV during the 5-min ultrasonic recordings before adding the urine stimulus to the testing chamber at any baseline time point. Male mice that produced 0 FiUSV after the addition of the urine stimulus for more than one of the five baseline time points did not undergo surgery and were removed from the study due to not producing reliable FiUSV.

A noticeable difference in the number of mice that reached the cutoff criteria for surgery was seen between the four cohorts (Table 3.1). Eight of eight mice from cohort 1, seven of ten mice in cohort 2, four of ten mice in cohort 3, and six of ten mice in cohort 4 passed the cutoff criteria. Thus, 25 of the 38 mice (66%) produced FiUSV during four of the five baseline time points and underwent vasectomy or sham surgery.

Table 3.1. FiUSV comparison between cohorts. Comparison of how many mice met surgical cutoff criteria (FiUSV produced at least 4 of 5 baseline days) and the number of FiUSV produced by mice that met that cutoff for each cohort. Fewer mice in cohorts 2-4 met the surgical cutoff criteria compared to cohort 1. Mice that did meet the cutoff criteria in cohorts 2-4 produced significantly fewer FiUSV compared to cohort 1. The only difference between cohorts was the age of the females used for sexual priming and urine collection.

Table 3.1. FiUSV comparison between cohorts

Cohort #	**# of mice**	**Met cutoff criteria (%)**	**FiUSV (Mean ± SD)**	**Female age (wk)**
Cohort 1	8	100	229 ± 145	9
Cohort 2	10	70	117 ± 99	19
Cohort 3	10	40	109 ± 107	20
Cohort 4	10	60	85 ± 112	23

The average number of FiUSV from the 5 baseline time points for mice in cohorts 2-4 were significantly different than the average number of FiUSV produced by mice in cohort 1 (Table 1). The overall baseline average of all 25 mice that underwent surgery was 143 ± 93 FiUSV (mean ± SD). According to the random effects model, the estimated cohort variance for FiUSV was estimated to be 17%, while the number of FiUSV between mice (mouse variance) was estimated to be 24%. The variance between days produced by each mouse (residual variance) was 59%. This means the variance in the number of FiUSV produced between baseline time points (day to day variance) for each mouse was higher compared to the variance in the number of FiUSV produced between mice.

3.2 Postoperative FiUSV

FiUSV were recorded at 4, 28, 52, 76, and 100 h after surgery in vasectomized or sham-surgery male mice treated with Bup-SR or saline. Postoperative data were normalized to baseline by calculating the relative change ([final – baseline]/baseline, where baseline is the mean of the 5 time points collected once daily for 5 days before surgery) to visualize the FiUSV changes between groups and from baseline over time (Figure 3.1).

There were significantly fewer FiUSV produced by Sx-No Tx mice at the 4-h time point compared to baseline ($P = 0.03$) with mice in this group producing 212 ± 111 FiUSV at baseline and 59 ± 29 FiUSV at the 4-h time point. All mice in the Sx-No Tx group (n = 6) produced fewer FiUSV at the 4-h time point after surgery compared to baseline, while only three of the seven Sx-Tx mice produced fewer FiUSV at the 4-h time point compared to baseline. The mice that were considered painful 4 h after surgery had fewer number of FiUSV compared to baseline. The two mice from Sx-Tx group that were painful had a FiUSV relative change of -0.39 and -0.88 at the 4-h time point, while the two mice from Sx-No Tx group had a FiUSV relative change of -0.45, -

0.72 at the 4 h time point. Sx-Tx, Sham-Tx, and Sham-No Tx groups had no significant differences in the number of FiUSV from baseline at any postoperative time point. There were differences in the number of FiUSV from baseline at any postoperative time point. There were also no significant differences in the number of FiUSV between groups (Sx-Tx, Sx-No Tx, Sham-Tx, and Sham-No Tx) at any postoperative time point.

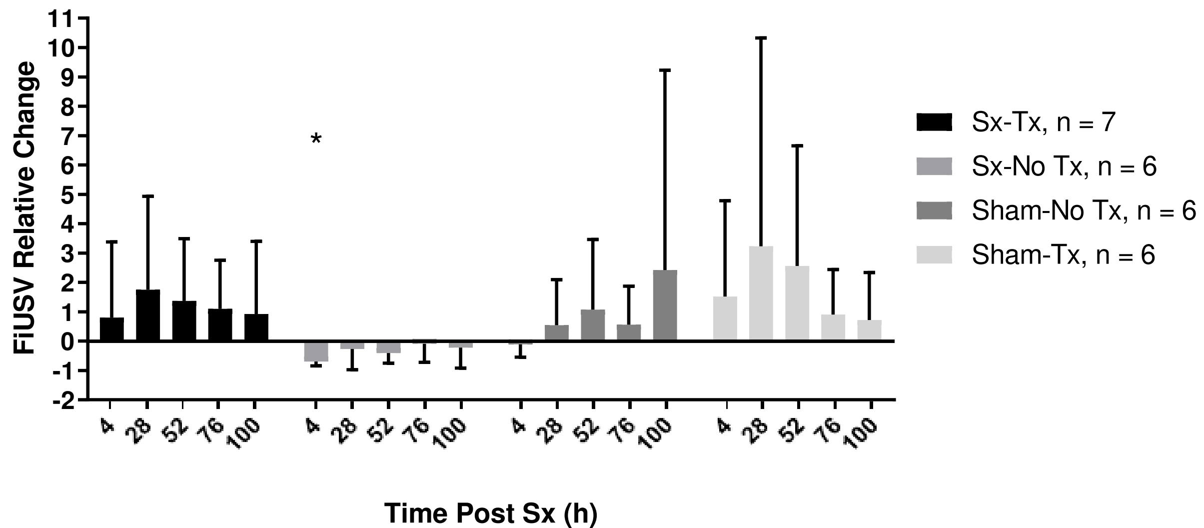

Figure 3.1. FiUSV results. FiUSV relative change ([final – baseline]/baseline, where baseline is the mean of the 5 time points collected once daily for 5 days before surgery; mean ± SD) over time in mice that underwent surgery (Sx) or anesthesia only (Sham) ± postoperative analgesia (Tx). * Significantly different from baseline, P < 0.05.

Six of twenty-five mice that underwent a vasectomy or sham surgery produced USV prior to adding the urine stimulus to the cage. Four mice produced USV prior to urine stimulus at the 52-h time point, three mice at the 76-h time point, and one mouse at the 100-h time point. This was abnormal because previous studies showed no USV were produced prior to the urine stimulus [Nyby et al, 1983; Smith et al, 2019]. The production of these spontaneous USV prior to the urine stimulus were independent of experimental group and time. The USV syllable structure and pattern of these spontaneous vocalizations were similar to FiUSV syllables (Figure

3.2). Three mice did not produce any FiUSV at specific time points postoperatively independent of experimental group or time point. However, the mice that produced 0 FiUSV postoperatively were from all cohorts except cohort 1. One mouse in the Sx-Tx group produced 0 FiUSV at the 28- and 100-h time point postoperatively. Another Sx-Tx mouse produced 0 FiUSV at the 4-h time point postoperatively. One Sx-No Tx mouse produced 0 FiUSV at the 100-h time point postoperatively.

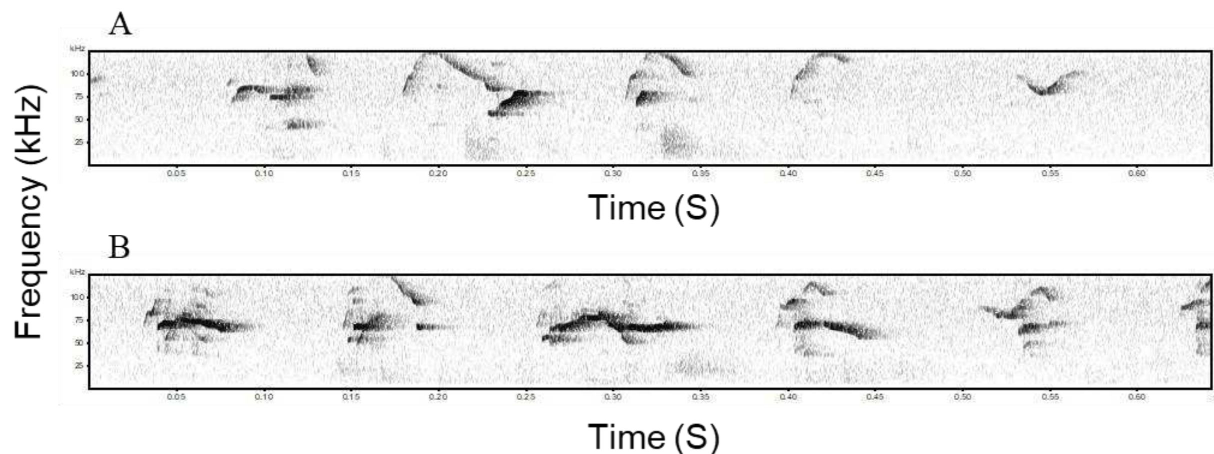

Figure 3.2. FiUSV comparison to spontaneous USV. Representative spectrogram 0.6 s snapshots (FFT-length = 256 points, overlap of 50%, and FlatTop window) of USV (5 syllables) spontaneously produced by a mouse before urine stimulus was added to the testing chamber (A) and FiUSV (6 syllables) produced after addition of the urine stimulus by the same male mouse (B).

3.3 Activity

Individual mouse activity was determined by counting the total number of activity bouts that occurred during the 5-min ultrasonic recordings collected before adding the female urine stimulus to the testing chamber. According to the random effects model, the estimated cohort variance for activity bouts (14%) was similar to FiUSV cohort variance (17%). The number of activity bouts between mice (mouse variance) was estimated to be 42%, higher than FiUSV mouse variance (24%). The variance in activity bouts between days produced by each mouse

(residual variance) was 44%. This means the residual variance for activity bouts was slightly less than FiUSV residual variance (59%).

Activity was normalized to baseline to show the differences in activity between experimental groups and time by determining the relative change for each mouse (Figure 3.3). There were no significant differences in activity between any experimental groups at any time point postoperatively. Sx-Tx mice had significantly decreased activity ($P = 0.02$) at 4 h after surgery (251 ± 107 activity bouts) compared to baseline (401 ± 132 activity bouts), while Sx-No Tx mice had a trend towards having decreased activity ($P = 0.06$) at the 4-h time point after surgery (249 ± 155 activity bouts) compared to baseline (386 ± 106 activity bouts). There were no other significant differences between any time points after surgery compared to baseline for any experimental group.

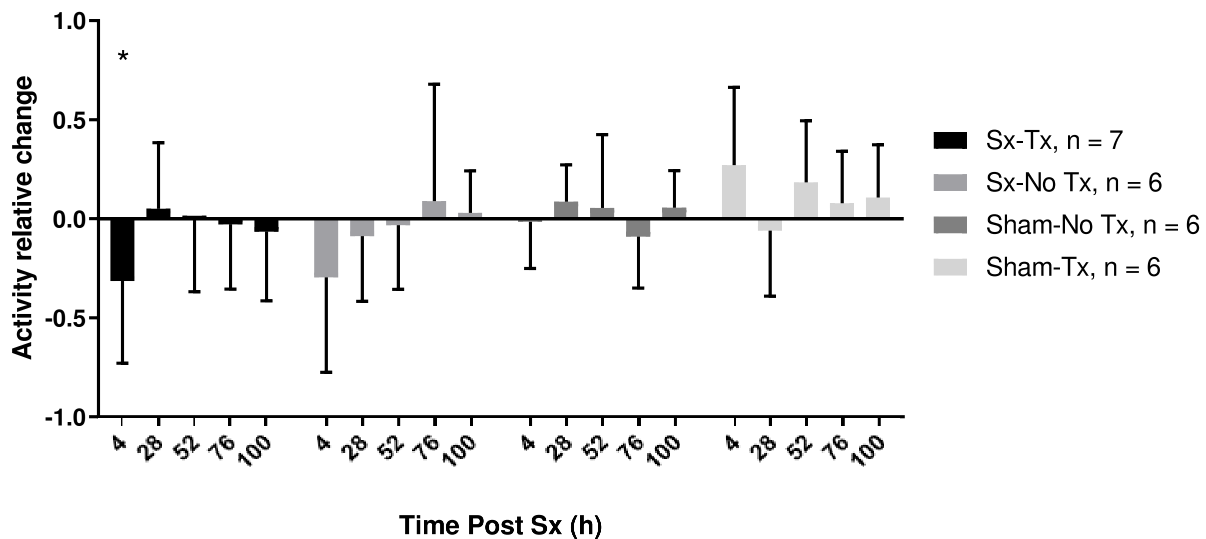

Figure 3.3. Activity results. Activity relative change ([final – baseline]/baseline, where baseline is the mean of the 5 time points collected once daily for 5 days before surgery; mean ± SD) over time in mice that underwent surgery (Sx) or anesthesia only (Sham) ± postoperative analgesia (Tx). *Significantly different from baseline, $P < 0.05$.

3.4 Pain assessment

Pain was assessed immediately after ultrasonic recordings by a blinded individual. Mice were given a behavior score according to orbital tightness, piloerection, and posture (Figure 3.4). Male mice were considered painful if the total behavior score was ≥ 3. Two of seven mice in the Sx-Tx group and two of six mice in Sx-No Tx group were considered painful, each with behavior scores of 4. No other mice from any of the experimental groups were considered painful at any time point.

Figure 3.4. Behavior assessment results. Behavior score scatter plot data over time for individual male mice that underwent surgery (Sx) or anesthesia only (Sham) ± postoperative analgesia. Behavior scores ≥ 3 (dotted line) considered to be painful.

4. Discussion

In this study, vasectomy via a midline abdominal incision was performed in male mice to assess whether FiUSV can be used as a proxy indicator of postoperative pain. The results obtained from this study showed that FiUSV can detect postoperative pain at least 4 h after surgery. Vasectomized mice that received no postoperative analgesia produced fewer FiUSV 4 h

after surgery compared to baseline, while vasectomized mice given analgesia and sham-surgery mice, regardless of receiving analgesia or not, had no difference in the number of FiUSV compared to baseline at any postoperative time point.

FiUSV have been described as an objective method to detect decreased animal well-being in male mice [Lopes and König, 2016; Smith et al, 2019]. Previous studies assessing FiUSV as a proxy indicator of animal well-being showed that male mice presenting sickness behaviors (hunched posture, piloerection, and orbital tightness) after injection of LPS produced zero FiUSV [Lopes and König, 2016; Smith et al, 2019]. Mice expected to be painful in this study (Sx-No Tx mice) produced less FiUSV 4 h after surgery compared to baseline, but still produced some FiUSV. The difference in the number of FiUSV produced by mice that are experiencing pain or decreased well-being between this study and the previous studies referenced could be due to differences in pain intensity, pain type, or a combination of the two.

Previous studies assessing FiUSV showed male mice do not tend to produce USV prior to urine being added to the testing chamber [Nyby et al, 1983; Smith et al, 2019]. In this study, some mice started producing USV prior to the urine stimulus. These vocalizations were seen towards the end of the 5 min background recording and the syllables were similar in appearance to FiUSV syllables. The vocalizations appeared during the 52 to 100 h time points, after the male mice have been exposed to female urine at least eight times. Since these vocalizations occurred in mice in all experimental groups after being exposed to urine multiple times, it is suspected that these vocalizations were in anticipation of the female urine. This means that the male mice were potentially behaviorally conditioned to produce these vocalizations due to the study design. Male mice received female urine every time they were placed into the testing chamber, and possibly started to anticipate the female urine. There are mixed opinions whether FiUSV are produced in

response to a positive affective state [Chabout et al, 2012; Portfors, 2007; Rieger and Dougherty, 2016; Wang et al, 2008]. Mice behaviorally conditioned to producing FiUSV prior to the urine stimulus provides some evidence that FiUSV might be associated with a positive affective state.

The production and quantity of FiUSV are dependent on numerous factors like mouse strain [Maggio et al, 1985; Sugimoto et al, 2011], social status [D'Amato, 1991; Nyby et al, 1976], and previous heterosexual experience [Dizinno et al, 1978; Maggio and Whitney, 1985; Malkova et al, 2012; Michetti et al, 2012; Nyby et al, 1983]. Previous studies have shown C57BL/6J mice have a high prevalence of producing FiUSV [Smith et al, 2019; Sugimoto et al, 2011]. In this study, roughly 34% of male mice did not produce FiUSV reliably during the five baseline collections. Mice that produced zero FiUSV during baseline and postoperative time points were in cohorts 2-4. Females used for sexual priming and urine collection were older than the males for cohorts 2-4. Furthermore, there was a significant decrease in baseline vocalizations in the later cohorts compared to cohort 1. Potentially, the decrease in the prevalence and number of FiUSV seen in the later cohorts could be due to using female mice that were older than the male mice. Since the same females were used for sexual priming and urine collection, we cannot determine if the change in the prevalence and number of FiUSV were due to using older female urine or older females for the sexual priming experience. Further studies can determine which factor is decreasing the production of FiUSV by exposing male mice to older females for sexual priming but using urine from 9-wk old female mice and vice versa. Regardless, it would likely be beneficial to use females of the same age for both procedures (sexual priming and FiUSV collection) when eliciting FiUSV.

Assessing postoperative pain and efficacy of analgesics using behavioral tests in rodents have shown mixed results in the literature. The effectiveness of the behavioral tests depends on

many factors such as the surgical procedure, mouse strain, postoperative analgesia, and methods of behavior scoring [Clark et al, 2004; Miller et al, 2016; Nunamaker et al, 2018; Langford et al, 2010; Wright-Williams et al, 2013]. Multiple behavior parameters using a variety of methods were assessed in rats after undergoing laparotomy and ovariohysterectomy [Nunamaker et al, 2018]. The study found cageside observations assessing body posture, activity, and coat appearance were sensitive indicators of postoperative pain while body weight, vertical rises, and still photographs evaluating facial grimace scores were poor indicators of postoperative pain [Nunamaker et al, 2018]. This study assessed posture, coat appearance (piloerection), and orbital tightness using still photographs as previously described [Smith et al, 2019]. Mice that underwent surgery had minimal elevation in their behavior scores compared to mice that received anesthesia only, regardless of receiving analgesia or saline. This could be because the still photographs assessing posture, piloerection, and orbital tightness are not sensitive enough to detect spontaneous behaviors associated with postoperative pain. Photographs were chosen over real-time cageside assessments so the observer would be blinded. An observer performing cageside observations would inevitably become unblinded due to seeing the abdominal incisions on vasectomized mice. The same parameters used in this study were able to detect sickness behaviors associated with systemic inflammation [Smith et al, 2019], but perhaps still photographs are not sensitive enough for localized postoperative pain. Alternatively, the surgical procedure might not be invasive enough to elicit spontaneous painful behaviors at the time points assessed in this study.

Previous studies have shown mice have decreased activity postoperatively [Clark et al, 2004; National Research Council, 2009; Roughan et al, 2009; Tubbs et al, 2011], while some studies have shown no change in activity after surgery [Goecke et al, 2005; Nunamaker et al,

2018]. The level of activity varied depending on the method used to measure activity, the mouse strain, the surgical procedure, and whether postoperative analgesia was administered. For example, mice that received a splenectomy and no postoperative treatment had decreased activity for 48 h after surgery by measuring voluntary running-wheel activity [Clark et al, 2004]. Mice that received a partial hepatectomy and received postoperative analgesia (buprenorphine or meloxicam) had decreased activity compared to baseline 1 day after surgery that returned to normal by day 2, also using voluntary running-wheel activity [Tubbs et al, 2011]. Mice that underwent a mock ova implantation surgery had no change in activity from baseline using radiotelemetry transmitters to monitor home cage activity [Gocke et al, 2005].

It has been shown previously that an ultrasonic microphone can be used to quantify activity [Smith et al, 2019]. Using the same methods as previously described, it was possible to measure USV and activity in tandem to assess postoperative pain. Mice that underwent surgery had decreased activity compared to baseline 4 h after surgery, regardless of receiving postoperative analgesia or saline. The activity returned to baseline in vasectomized mice by 28 h after surgery. Future studies could assess different modalities to measure activity (radiotelemetry, voluntary running-wheel activity, ultrasonic background noise, cage-side scoring) after surgery to determine the most reliable and accurate method to measure postoperative activity. Additionally, increased frequency of time points could give insight on when activity is restored between 4 and 28 h after undergoing a vasectomy.

Bup-SR, at the dose given, has been shown to stay above the therapeutic level in the plasma of mice for 24 – 48 h [Kendall et al, 2014], and efficacious in female mice post laparotomy [Kendall et al, 2016]. In this study, mice that had a vasectomy and received Bup-SR had no change in FiUSV at any time point after surgery but had decreased activity at 4 h after

surgery compared to baseline. Two of the seven vasectomized mice treated with Bup-SR were considered painful at 4 h after surgery. The results from this study suggest Bup-SR provides some degree of pain relief after vasectomy surgery via midline abdominal incision in C57BL/6J mice.

The results of this study suggest that FiUSV produced by male mice in response to female urine can be used as a proxy indicator to assess postoperative pain in C57BL/6J mice following vasectomy surgery. Additionally, the ultrasonic microphone can detect noise made by the mouse's movements within the testing chamber, which can be quantified to objectively measure activity in mice. This is advantageous due to detecting two absolute objective parameters in tandem that can be used to assess postoperative pain with minimal animal handling. Furthermore, Bup-SR given at 0.6 mg/kg can provide postoperative pain relief associated with vasectomy procedure as evident by no change in FiUSV production.

CHAPTER IV.

CONCLUSIONS

The two studies described provide evidence that FiUSV and IiUSV can be used as proxy indicator assays for animal well-being and postoperative pain. Mice injected with LPS lacked production of both FiUSV and IiUSV, and the suppression of these innate behaviors occurred before visual signs of sickness behavior (hunched posture, orbital tightness, and piloerection). Additionally, mice that underwent a vasectomy and received no postoperative analgesia produced fewer FiUSV 4 h after surgery compared to baseline, while mice that underwent a sham surgery or vasectomy and received postoperative analgesia had no change from baseline.

The above studies also showed that the ultrasonic microphone can be used to quantify activity. The microphone was able to detect noise production from nice moving within the testing chamber. Mice injected with LPS were less active at 1 and 3 h after injection compared to baseline, and mice that underwent a vasectomy were less active at 4 h after surgery compared to baseline. Using the methods described, we showed activity and FiUSV/IiUSV can be measured in tandem, and both provide powerful information on the animal’s health status.

Measuring FiUSV/IiUSV and activity have many advantages over other types of pain recognition modalities discussed in Chapter 1. For example, both parameters are objectively measured and have less experimenter bias than cage-side assessments. Compared to operant tests, measuring these parameters are beneficial as they do not require time for behavior conditioning. Furthermore, FiUSV/IiUSV and activity quantification are advantageous over nociceptive tests as they measure the presence of on-going, non-evoked pain.

There are some disadvantages in measuring FiUSV/IiUSV and activity. Their uses are restricted to well-controlled experimenter studies. FiUSV/IiUSV cannot be used across an entire vivarium or animal facility due to variability in USV production between strains, age, and sex. USV and activity quantification need to be compared to baseline for comparisons, which is not practical for assessments across a facility. Another drawback to using these parameters is the dedicated quiet space and specialized equipment (ultrasonic microphone and software) needed for quantification of FiUSV/IiUSV and activity. Facilities may not have the finances or space required to perform these assays.

Future studies can further determine the usefulness of FiUSV/IiUSV as proxy indicator assays for animal well-being by assessing different inflammogens at various doses, routes, or time points. Additionally, the clinical effectiveness of these parameters in regards to animal well-being can be determined by assessing their changes in response to an experimental infection of a bacteria or virus. The functionality of these parameters as proxy indicator assays for pain can be further assessed by using different pain models or different pain types, as discussed in Chapter 1. Additionally, their use as proxy indicator assays for postoperative pain can be further evaluated by comparing FiUSV or IiUSV after performing different surgical procedures, such as ovariohysterectomy, splenectomy, or laparotomy.

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