Original Research

Female- and Intruder-induced Ultrasonic Vocalizations in C57BL/6J Mice as Proxy Indicators for Animal Wellbeing

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Female urine-induced male mice ultrasonic vocalizations (FiUSV) are ultrasonic vocalizations produced by adult male mice after presentation of adult female urine, whereas intruder-induced ultrasonic vocalizations (IiUSV) are produced by resident adult female mice when interacting with an intruder female mouse. These affiliative behaviors may be reduced when mice have decreased wellbeing or are in pain and distress. To determine whether FiUSV and IiUSV can be used as proxy indicators of animal wellbeing, we assessed FiUSV produced by male C57BL/6J mice in response to female urine and IiUSV produced by female C57BL/6J mice in response to a female intruder at baseline and 1 and 3 h after administration of a sublethal dose of LPS (6 or 12.5 mg/kg IP) or an equal volume of saline. Behavior was assessed by evaluating orbital tightness, posture, and piloerection immediately after USV collection. We hypothesized that LPS-injected mice would have a decreased inclination to mate or to interact with same-sex conspecifics and therefore would produce fewer USV. At baseline, 32 of 33 male mice produced FiUSV (149 ± 127 USV in 2 min), whereas all 36 female mice produced IiUSV (370 ± 156 USV in 2 min). Saline-injected mice showed no change from baseline at the 1- and 3-h time points, whereas LPS-injected mice demonstrated significantly fewer USV than baseline, producing no USV at both 1 and 3 h. According to orbital tightness, posture, and piloerection, LPS-injected mice showed signs of poor wellbeing at 3 h but not 1 h. These findings indicate that FiUSV and IiUSV can be used as proxy indicators of animal wellbeing associated with acute inflammation in mice and can be detected before the onset of clinical signs.

Abbreviations: FiUSV, female urine-induced male mouse USV; IiUSV, intruder-induced USV; USV, ultrasonic vocalizations

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Assessment of pain in biomedical research is essential for monitoring animal wellbeing and obtaining accurate research results. Recognizing pain or distress in mice is challenging because they typically hide signs of disease or pain until profoundly ill or moribund.⁶ A multimodal approach using multiple assays to measure animal wellbeing is ideal for improving animal welfare in research animals. However, due to the low number of assays available, more knowledge regarding pain, distress, animal wellbeing, and techniques to assess these parameters are urgently needed.¹⁶

The most commonly used tools to measure animal wellbeing used by researchers and veterinarians in laboratory animal medicine are ethograms and clinical observation sheets.¹⁶ Ethogram characterizing behavioral changes such as changes in activity, body posture, or facial expressions can indicate wellbeing in rodents. 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 direct measurement and have been gaining favor in the literature with variable success.^{25,48} Of these assays, the Mouse Grimace Scale has become widely used to measure pain in rodents. However, this method

is subjective and may not indicate pain intensity accurately.^{29,38} An alternative proxy indicator assay that is noninvasive, objective, and quantitative will benefit both researchers and clinicians in assessing and recognizing pain or distress in rodents.

To communicate, mice produce ultrasonic vocalizations (USV) in addition to auditory squeaks.⁴⁷ USV are vocalizations above the frequency that humans can hear (>20 kHz) and can indicate either positive or negative effector states.^{47,56} Mouse USV are predominantly studied as models for memory and neuropsychiatric disorders or for analyzing aggression, playing, and mating.⁵⁹

Over the past couple of decades, USV have been studied as a modality for pain recognition in mice and rats, with mixed results.^{5,8,22,23,26,55,58} Many of these studies induced negative effective states to stimulate spontaneous vocalizations by using invasive acute nociceptive stimulation, such as injecting irritants and applying pinch, air, or incisional provocations or a combination of these. Reflex-based assays have shown more success in rats than mice, because rats produce alarm cries in response to noxious stimuli, whereas mice do not.^{8,23,30,35,55} A reliable, noninvasive, repeatable assay using USV to measure animal wellbeing in mice has not been established.

Mice produce USV during many nonaggressive situations.^{17,19,42,47} In particular, mice produce USV during numerous different same-sex and heterosexual interactions,^{36,47,49,60} and 3 mouse USV are easily reproducible and well described for

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studying autism spectrum disorder.⁴⁷ These 3 types of USV are 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 at high rates in the presence of female urine.^{10,49} Pup isolation-induced USV occur in male and female neonatal pups. When separated from the nest, pups produce vocalizations that elicit search-and-retrieve behavior from the dam.^{1,47} IiUSV are USV produced by resident female mice in response to an intruder female mouse.^{39,47}

A recent study showed evidence that male, wild house mice injected with LPS and housed with a female mouse overnight produced fewer USV than untreated mice.³² The study showed LPS-injected male mice produced no regular-frequency courtship USV compared with control mice. In addition LPS-injected mice produced rare higher-than-normal frequency USV that were not seen in the control mice. The authors concluded that the absence of the regular frequency USV and the production of the higher-than-normal frequency USV may be indicators of decreased animal wellbeing.³² Because USV production is dependent on mouse strain,^{33,52} we wanted to assess whether USV can be used as a proxy indicator of decreased animal wellbeing associated with LPS injection in a commonly used laboratory mouse strain, C57BL/6J.

FiUSV have been extensively studied in regard to behavior and mating research.⁴⁷ FiUSV might serve as a proxy indicator for pain assessment because they are a natural behavior and indicate a positive effector state. Being a negative effector state, pain might counter the positive effector state induced by female urine, resulting in fewer FiUSV produced by male mice. FiUSV would be advantageous in terms of monitoring mouse wellbeing because they are an objective, quantitative, and minimally invasive measure.

Many studies describe how to maximize FiUSV production^{7,33,44,45,49,51} and characterize USV syllable structure.^{15,19} The quantity of FiUSV are dependent on strain,^{33,52} social status,^{10,45} previous heterosexual experience,^{11,33,34,37,44,45,49,51} stimulus,^{34,51} freshness of urine stimulus,^{20,21,24,49} and length of interaction.⁴⁹ For example, previous studies⁵² showed that C57BL/6J and BALB/c mice have a high prevalence of producing FiUSV, with almost 100% of C57BL/6J male mice producing vocalizations in response to female mice or urine, compared with fewer than 40% of males producing USV in other strains. Male mice produce most of the FiUSV during the first 3 min after urine stimulus, and the number of vocalizations is significantly decreased when old urine is used or if no heterosexual experience occurred before testing.⁴⁹

Compared with FiUSV, IiUSV associated with female–female interactions are significantly less described in the literature.⁴⁷ Historically, female mice were thought to produce few USV.⁴⁶ However, recent research using a resident–intruder paradigm has shown that female mice produce an equivalent number of vocalizations to males.^{14,39} Evidence indicates that only the resident female mouse produces IiUSV when an intruder mouse is present.^{14,39}

LPS is a inflammogen commonly used to induce inflammatory disease and mimic sepsis.^{13,41,54} Inflammatory pain is associated with LPS-induced inflammatory disease, due to increased pain sensitivity, such as allodynia and hyperalgesia, in many animal species including humans.^{2,4,9,57} LPS-injected mice have reduced production of chemical signals (scent markings), show sick rodent behaviors, have decreased activity, and demonstrate decreased interest in mating.^{32,41,53,54,60}

The goals of the current study were to assess FiUSV in male mice and IiUSV in female mice in an acute inflammatory pain

model using LPS. We hypothesized that, due to a decreased inclination to mate, male mice experiencing LPS-induced sickness behavior would produce fewer FiUSV than mice experiencing no sickness behaviors. In addition, we hypothesized that, due to a decreased inclination to interact with an intruder mouse, female mice experiencing LPS-induced sickness behaviors would produce fewer IiUSV than healthy, control female mice.

Materials and Methods

Animals. Male ($n = 33$) and female ($n = 36$) C57BL/6J mice (stock no. 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 IVC (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-accredited facility.

Experimental design. To assess FiUSV in male mice and IiUSV in female mice as proxy indicators of animal wellbeing in C57BL/6J mice, 9-wk-old male mice and 5-mo-old female mice were randomized into groups and injected intraperitoneally with LPS or saline. Male mice were split into 4 groups ($n = 8$ per group) and treated with 12.5 mg/kg IP LPS or equal volume of saline and tested at 1 or 3 h after injection. Female mice were split into 6 groups ($n = 6$ per group) and treated with 12.5 mg/kg IP LPS, 6 mg/kg IP LPS, or equal volume of saline and tested at 1 or 3 h after injection. To determine whether a dose-dependent change in USV occurred, groups of female mice received a lower dose (6 mg/kg) of LPS in addition to the larger dose (12.5 mg/kg). Baseline values were collected 24 h before the 1- or 3-h time points. To maximize USV production, male mice were sexually primed to a female at 1 wk before experimentation.^{7,49} Mice were euthanized through CO₂ inhalation immediately after their respective 1- and 3-h time points.

Sexual priming of male mice. Male mice were sexually primed similarly to the methods previously described.⁷ Briefly, male mice were housed 2 per cage. At 7 d before collecting baseline data, one female mouse was added to each male cage at 1600. At 16 h afterward, female mice were removed from the males' cage and returned to their original home cage. Female and male mice were the same age.

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, St Louis, MO). LPS was prepared in aqueous sterile saline, aliquoted 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 the injection was administered. LPS samples remained at room temperature for less than 30 min and were not refrozen or reused.

Testing chamber. Testing was conducted in a quiet room, away from noisy equipment and activities. All animal handling was completed when USV were not actively recorded. A recording chamber was used to detect activity and USV in a sound-attenuated and visually isolated environment.⁷ Briefly, a beach cooler (internal dimensions, 27 × 23 × 47 cm) was configured with a 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) was centered 30 cm above the cage bottom. Recording software (RECORDER USGH Software, Avisoft Biocoustics) was configured with a sampling rate of 250,000 Hz, Fourier fast transform 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 daily for 2 d before collection of baseline values. The testing chamber was cleaned with 70% ethanol after each animal.

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). The clean testing cage containing the mouse was then placed into the testing chamber for 10 min before data were collected. Recordings (5 min each) were collected before and after urine stimulus.

Urine was collected from unfamiliar female mice as described.⁷ Briefly, female mice in estrus or proestrus, as determined by visual examination, were used for urine collection.³ By using physical restraint and gentle palpation, urine samples were collected on a cotton-tipped applicator from 2 female mice from separate cages. Urine was collected immediately before the urine stimulus was added 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.

IiUSV data collection. Resident-intruder tests were used to collect IiUSV from female mice, according to similar methods previously described.³⁹ Briefly, female mice were individually housed for 3 d preceding baseline measurements and acted as the resident female on test days. Intruder female mice remained in social groups of 4 mice per 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 mouse, housed in her home cage, was then moved into the testing chamber for USV recordings.

Resident females were habituated to the chamber for 10 min before a 5-min background recording was collected. After the 5-min background recording, an unfamiliar, unanesthetized intruder female mouse was added to the resident female's home cage. Resident and intruder female mice were allowed to have contact with each other. An additional 5-min recording was collected, then the intruder female mouse was returned to her home and enrichment items replaced.

USV quantification. The 5-min spectrographic recordings were analyzed by using Avisoft-SASLab Pro (Version 5.2.07, Avisoft Biocoustics). USV syllables were counted individually by examining the entire 5-min spectrogram by hand, determining the call latency (time until the 1st vocalization after the stimulus), and then counting all syllables for an additional 2 min after the first USV occurred.

Activity quantification. By using the Avisoft-SASLab Pro program, background recordings were used to assess activity by counting the number of bouts of increased noise due to the mouse moving in the testing chamber (activity bouts). Activity bouts were detected according to a threshold of 4% full scale, and a hold time of 0.05 s. Overloaded events (that is, saturated events longer than 100% full scale) were excluded; activity bouts above 4% full scale that lasted longer than 0.05 s were assigned section labels. Section labels were saved as a text file, and the

total number of activity bouts and total activity duration were determined.

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 to acclimate. A blinded researcher obtained cageside pictures of each mouse and scored them according to orbital tightness (0 to 2; 0, normal; 1, slightly squinted; 2, squinted), body posture (0 to 2; 0, normal; 2, hunched posture with abdomen raised), and piloerection (0 to 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 photos (15 to 20) were taken within 1 min. Pictures of the mouse rearing up, out of focus, not facing the camera, or with eyes not visible were excluded. One photo per mouse was selected and placed in a PowerPoint (Microsoft, Redmond, WA) presentation in random order with regard to time point and group for the blinded observer. In addition to scoring mice on orbital tightness, piloerection, and hunched posture, the blinded observer was asked to give an overall opinion regarding whether the mouse looked sick. Mice that were considered by the blinded observer to have sick behaviors had a total behavior score (sum of the attribute subscores) of 3 or greater.

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

Results

FiUSV. Male mice were treated with 12.5 mg/kg LPS or saline, and FiUSV were collected at 1 or 3 h after injection. FiUSV results were normalized for each mouse by determining the relative change [(final – baseline) / baseline; Figure 1 A).

None of the male mice produced any USV before the urine stimulus at any time point. However, at baseline, 32 of 33 male mice exposed to fresh urine from an unfamiliar female mouse produced 149 ± 127 USV within 2 min (mean $\pm$ 1 SD), with a call latency of 46.8 ± 55.3 s. The single male mouse that did not produce any USV at baseline had a preputial gland abscess on physical examination and was therefore removed from the study.

None of the LPS-injected mice produced any FiUSV at the 1- and 3-h time points, whereas 7 of 8 saline-injected mice produced vocalizations at the 1 h time point (93 ± 75 USV/2 min) and all 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 the 1- and 3-h time points) but not saline-injected mice (at 1 h: $W = 8$, $P = 0.64$; at 3 h: $W = -4$, $P = 0.84$). The FiUSV relative change between LPS-injected mice and saline-injected mice at the 1- and 3-h time points was statistically significant ($U = 4$, $P = 0.0014$ and $U = 0$, $P = 0.0002$, respectively).

IiUSV. Female mice were treated with 6 mg/kg or 12.5 mg/kg LPS or an equal volume of saline, and IiUSV were collected at 1 or 3 h after injection by using a resident-intruder paradigm.

Figure 1. (A) FiUSV relative change scatter plot with box-and-whisker plot overlay of data from male mice treated with 12.5 mg/kg LPS or saline and collected at 1 or 3 h after injection. (B) liUSV relative change scatter plot with box-and-whisker plot overlay of data from female mice treated with 6 or 12.5 mg/kg LPS or saline and collected at 1 or 3 h after injection. Compared with saline-injected mice, LPS-injected mice have significantly decreased USV production at 1 and 3 h after injection. **, Significant ($P < 0.05$) difference between groups; *, significant ($P < 0.05$) difference from baseline. Representative spectrograms of recorded vocalizations (C) produced by male mice elicited with female urine and (D) vocalizations produced by female mice elicited by an intruder female.

liUSV results were normalized for each mouse by determining the relative change $[(\text{final} - \text{baseline}) / \text{baseline}]$; Figure 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 mouse (370 ± 156 USV during 2 min, with a call latency of 32.9 ± 32.3 s). All saline-injected mice produced liUSV after injection (1 h, 317 ± 119 USV during 2 min; 3 h, 266.7 ± 109.9 USV during 2 min); neither value differed significantly from baseline at either time point ($W = -9$, $P = 0.44$ at both time points).

None of the mice treated with 6 mg/kg LPS produced any liUSV at either time point after injection. At the 1-h time point, 3 female mice treated with 12.5 mg/kg LPS produced 0 liUSV, whereas the other 3 mice produced 132 ± 92 USV during 2 min. None of the mice injected with 12.5 mg/kg LPS produced any liUSV at the 3-h time point. For LPS-injected mice, the number of USV differed significantly from baseline at both later time points ($W = -21$, $P = 0.03$). The liUSV relative change differed significantly between 6 mg/kg LPS- and saline-injected mice

at both time points ($U = 0$, $P = 0.002$) and between 12.5 mg/kg LPS- and saline-injected mice at both time points ($U = 0$, $P = 0.002$).

Activity. Ultrasonic recording was used as a measure of activity. Because all recordings were completed in a quiet room away from animals being handled, the background recordings consisted only of sound produced by the mouse moving in the testing chamber. The appearance of the background ultrasonic recordings taken before adding urine or intruder stimuli in LPS-injected mice differed significantly ($W = -36$, $P = 0.08$) as compared with saline-injected mice (Figure 2). Mice did not produce any vocalizations before the urine or intruder stimulus.

The total activity duration and total number of activity bouts produced during the 5-min recordings were quantified by using a software program. For each mouse, the number of activity bouts and activity duration for each mouse were correlated (Figure 3 A and B). The activity duration differences between groups and baseline were the same as the differences in the number of activity bouts ($r = 0.94$, $P < 0.0001$). Therefore, we opted to use only activity bouts to assess activity. The number

Figure 2. Representative (top) voltage trace and (bottom) spectrogram from 5-min background recordings showing decreased activity in (A) LPS-injected mice compared with (B) saline-injected mice. Spikes in the voltage trace represent increased noise and correlate with mouse movement within the testing chamber.

Figure 3. Correlation between activity bouts and activity duration in (A) male mice and (B) female mice 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 ($r = 0.965$) mice. Activity relative change scatter plot with box-and-whisker plot overlay of data from (C) male and (D) female mice. Male mice were injected with 12.5 mg/kg LPS or saline; female mice were injected with 6 or 12.5 mg/kg LPS or saline. Activity measured at 1 and 3 h after injections. **, Significant ($P < 0.05$) difference between groups; *, significant ($P < 0.05$) difference from baseline.

of bouts was normalized for each mouse by determining the relative change (Figure 3 C and D).

Male mice had 437 ± 150 activity bouts at baseline. Male LPS-injected mice had 94 ± 45 and 16 ± 17 activity bouts at the 1- and 3-h time points, respectively, and activity bouts were significantly decreased at both time points compared with baseline ($W = -36$, $P = 0.08$). Saline-injected male mice had no difference in activity at either time point compared with baseline (1 h: $W = 8$, $P = 0.6$; 3 h: $W = 5$, $P = 0.8$). Activity relative change differed significantly between LPS-injected and 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, whereas those treated with 12.5 mg/kg mice had 35 ± 27 and 14 ± 18 activity bouts at 1- and 3-h time points, respectively. In addition, both 6- and 12.5-mg/kg LPS-injected female mice had significantly lower activity bouts at both time points compared with baseline ($W = -21$, $P = 0.03$ for all comparisons). Saline-injected female mice had no difference in the number of activity bouts between baseline and either later time point (1 h: $W = -7$, $P = 0.5$; 3 h: $W = -9$, $P = 0.4$). There was a significant difference in activity bouts relative change for LPS-injected female mice (6- and 12.5-mg/kg groups) compared with saline-injected female mice at the both time points ($U = 0$, $P = 0.002$).

Behavior assessment. Behavior was assessed by using photographs taken immediately after USV recordings and compiled

into a PowerPoint presentation for blind scoring based on orbital tightness, piloerection, and posture (Figure 4 A and B). Mice were considered to have poor wellbeing when the total behavior score was 3 or greater. Only 1 of the 8 LPS-injected male mice was considered to have poor wellbeing at 1 h, but all 8 LPS-injected male mice were considered to have poor wellbeing at 3 h. In comparison, none of the saline-injected male mice were considered to have poor wellbeing at either time point. There was a significant difference in wellbeing between saline- and LPS-injected male mice at 3 h ($U = 0$, $P = 0.0002$) but not at 1 h ($U = 29$, $P = 0.9$) after injection. Only 1 of the 6 female mice treated with 6 mg/kg LPS was considered to have poor wellbeing at 1 h after injection, whereas none of the female mice treated with 12.5 mg/kg LPS was considered to have poor wellbeing at this same time point. All LPS-injected female mice were considered to have poor wellbeing at 3 h. Similar to findings from male mice, wellbeing differed significantly between saline- and LPS-injected female mice at 3 h after injection ($U = 0$, $P = 0.02$) but not at 1 h afterward ($U = 18$, $P > 0.99$).

Discussion

We found that C57BL/6J mice undergoing LPS-induced acute inflammatory disease do not produce FiUSV or IiUSV, whereas control, healthy mice demonstrate the expected FiUSV and IiUSV responses. Furthermore, the absence of these vocalizations in LPS-treated mice occurs before the onset of visual clinical

Figure 4. Behavior score scatter plot with box-and-whisker plot overlay of data from (A) male and (B) female mice. Male mice were injected with 12.5 mg/kg LPS or saline; female mice were injected with 6 or 12.5 mg/kg LPS or saline. Behavior scores assessed at 1 and 3 h after injection. LPS-injected mice were considered to have poor wellbeing (behavior score ≥ 3 , dotted line) only at the 3-h time point. **, Significant ($P < 0.05$) difference between groups.

signs of orbital tightness, hunched posture, and piloerection. The data showed that most of the LPS-injected mice did not produce any USV and lacked overt signs of sickness at the 1-h time point, but at the 3-h time point, LPS-treated mice produced no USV and showed visual signs of a sick mouse.

Baseline USV data from this study were consistent with previously published reports.^{7,10,18} In our study, all LPS-injected male mice and all but 3 LPS-injected female mice produced zero USV after injection; the 3 females that produced vocalizations were

treated with 12.5 mg/kg LPS, produced liUSV only at the 1-h time point, and produced fewer liUSV at 1 h than at baseline. Because pain perception varies between individuals,²⁷ the production of USV in these 3 mice might reflect decreased pain perception. Alternatively, although previous studies showed that only the resident female produced USV,^{14,39} the intruder might have produced vocalizations as well. Using anesthetized intruder mice or injecting intruder mice with the same volume of LPS could eliminate this variable.

Findings from our study are consistent with those seen in wild house mice injected with LPS,³² which showed that LPS-injected male mice produced no regular-frequency USV when housed with a female mouse overnight. The authors of this previous study³² detected high-frequency USV from LPS-injected male mice when housed with a female mouse; however, we did not obtain these same high-frequency USV in our current study. The apparent lack of vocalizations in our current study may reflect the short duration that male mice were exposed to female urine or the fact that the female not being present during recordings.

LPS, a microbial cell wall component, is an endotoxin that dose-dependently increases the concentrations of proinflammatory cytokines that facilitate or enhance pain and that is commonly used as a model of sepsis and inflammatory pain.^{40,54} Clinical signs associated with LPS-induced sepsis include hyperalgesia, malaise, and inappetence.⁴⁰ We used LPS to mimic a systemic acute illness and subsequent pain induction. Previous studies have shown C57BL/6 and BALB/c mice can survive more than 20 h after being injected with 12.5 mg/kg or higher doses of LPS.^{12,28,31} We did not plot survival curves because we euthanized the mice after the 1- and 3-h time points. Regardless of dose, LPS-injected mice had signs of sickness at 3 h after 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 after LPS injection, because of the complete absence in USV production in mice at both time points. Similarly, female mice did not show time- or dose-dependent decreases in USV when measured at 1 or 3 h after injection of 6 and 12.5 mg/kg LPS. Using lower doses of LPS or assessing sooner after injection might reveal whether USV production is dependent on animal wellbeing, LPS dose, or time.

We selected the ages of male and female mice to be used in this study according to previous studies that assessed FiUSV and liUSV.^{7,39,50} Mice of various ages produce FiUSV and liUSV. Male mice produce low numbers of FiUSV when younger than 7 wk⁷ but can produce when as old as approximately 43 wk (300 d).¹⁸ Previous studies have shown female mice produce liUSV between 3 to 5 mo and 12 mo of age, but 12-mo-old female mice produced fewer liUSV than the younger mice.³⁹ Additional studies could assess the effect of age on USV production associated with animal wellbeing.

LPS-injected mice have less plasma testosterone than control mice.³² Decreased testosterone has been linked to a decrease (but not complete absence) in the production of courtship USV in mice.^{11,43} LPS-induced decreases in plasma testosterone might underlie the reduction in FiUSV in our current study. However, given that 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 our current study is most likely due to decreased animal wellbeing.

Although evaluation of FiUSV and liUSV likely is impractical for cageside assessment of sick mice across an entire vivarium, USV assessment using the methods we described could be

useful in studying the pathology of pain, assaying analgesic efficacy, or assessing animal wellbeing 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 from an ethogram or score sheet.¹⁶ In the current study, we used a cageside ethogram for assessing orbital tightness, piloerection, and posture to quantify animal wellbeing. Behavior ethograms are based on subjective criteria and can vary depending on the observer, as is evident in the current study. For example, 4 saline-injected male mice and 1 saline-injected female were considered to have normal wellbeing even though they had behavior scores of 1 and 2. The elevated behavior scores seen in these mice might be due to misinterpretation of 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 suboptimal for assessing animal wellbeing. Conversely objective criteria, such as USV and activity, can improve how animal wellbeing and study end points are decided.

The ultrasonic microphone detected 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 restricted to times outside of USV recording sessions. In addition, mice did not produce any vocalizations before the urine or intruder stimulus. Therefore, 5-min background ultrasonic recordings collected prior to adding urine stimulus or intruder stimulus comprised only of sound produced by the experimental mouse moving in the testing chamber. Using a specialized software program, we were able to quantify the noise associated with this movement, which is represented as activity bouts.

The activity data from our study correlated with the USV data. LPS-injected mice were significantly less active than saline-injected mice at both time points. Activity did not differ between LPS doses or between the 1 and 3 h time points. Future studies could assess activity sooner after injection or at lower LPS concentrations to determine whether the ultrasonic microphone is sufficiently sensitive to detect dose- or time-dependent changes. In addition, future experiments might compare the ultrasonic microphone with other modalities of measuring activity, such as running wheels, telemetry, treadmills, or video recordings to determine the functionality of the ultrasonic microphone at detecting activity.

A design limitation to our current study was that USV were collected in an enclosed chamber; therefore mice were visually isolated from the observer. In contrast, behavior scores were obtained from images taken with an observer present. The scores of mice producing no USV but appearing nonpainful at the 1-h time point might have been exaggerated, given that mice hide visual signs of pain from predators. An alternative method for collecting images might be to mount a camera in the chamber, thus supporting simultaneous collection of USV and behavior data collection. In our 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 proxy indicators of animal wellbeing during acute inflammation, with the absence of vocalizations occurring before the onset of clinical signs of pain. In addition, the ultrasonic microphone can detect noise from mouse movement within the testing chamber allowing for simple and noninvasive quantification of mouse activity. The combination of measuring activity and USV production in mice

is a powerful method for assessing animal wellbeing. Future studies should assess the functionality of these modalities in other mouse pain models.

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