Spontaneous and Evoked Measures of Pain in Murine Models of Monoarticular Knee Pain.
--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59024R2
Full Title:	Spontaneous and Evoked Measures of Pain in Murine Models of Monoarticular Knee Pain.
Keywords:	mouse; arthritis; pain; Animal Models; joint; behavior
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Minneapolis, MN, USA

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Veterans Health Administration
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November 7, 2018

Dear Editors,

Attached please find our revised manuscript "Spontaneous and Evoked Measures of Pain in Murine Models of Monoarticular Knee Pain**"**. We have extensively revised this manuscript according to the excellent comments we received from the reviewers. We feel this paper demonstrates important methods for measuring joint pain in mice and is an excellent topic for your video-journal media.

Thank-you in advance for reviewing our manuscript,

Sincerely,

Hollis Krug MD

Hollis Krug
Chief, Rheumatology, Minneapolis VAHCS
Assoc. Prof. of Medicine, University of Minnesota Medical School

1 **TITLE:**

2 **Spontaneous and Evoked Measures of Pain in Murine Models of Monoarticular Knee Pain.**

3

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18

21

22 **SUMMARY:**

23 We have developed an evoked measure of arthritis pain and coupled it with a standardized
24 method for measuring spontaneous pain in different murine models of chemically induced
25 arthritis. These measures are sensitive and reproducible for different types of joint pain.

26

27 **ABSTRACT:**

28 Pain is the main cause of disability from arthritis. There is currently an unmet need for adequate
29 treatments for arthritis pain. Pre-clinical models are necessary and useful for studying the
30 mechanisms of pain and for evaluating efficacy of arthritis therapies. Measuring pain in animal
31 models of arthritis is challenging. We have developed methods for measuring evoked and
32 spontaneous pain in three models of murine arthritis. We quantitate the evoked pain responses
33 of mice subjected to firm palpation of a painful knee. We also evaluate spontaneous pain by the
34 proportion of weight and the amount of time placed on each of their 4 limbs after induction of
35 arthritis pain in one knee. Joint pain in these mouse models produces a significant increase in
36 evoked pain responses and an alteration in weight bearing. Since mice are quadrupeds, they
37 offload the painful limb to the contralateral limb, to the forelimbs, or some combination. These
38 methods are simple, require minimal equipment, and are reproducible and sensitive for
39 detecting pain. They are useful for studying both disease-modifying arthritis treatments and
40 analgesics in mice.

41

42 **INTRODUCTION:**

43 Pain is the main cause of disability from arthritis[1,2]. Currently available analgesics are often
44 ineffective, toxic, and potentially contribute to the nation-wide narcotic problem[3]. Surgery is not

45 always an option for end-stage arthritis, particularly in the elderly, who may have multiple co-
46 morbidities. Intra-articular steroids and viscosupplementation may be effective in early disease
47 but typically lose their effectiveness in late stage disease[4]. There is a great unmet need for
48 effective analgesics for arthritis pain that cannot be addressed with currently available
49 treatments.
50
51 Pre-clinical models are useful for understanding the mechanism of action of pain therapies and
52 for predicting limiting toxicities, but pain measurement in animals is challenging[5]. Pain measures
53 can be direct and spontaneous (static or dynamic weight bearing, foot posture, gait analysis,
54 spontaneous movement and mechanical or thermal sensitivity) or indirect/evoked (hind limb
55 withdrawal test, knee compression force, or brain imaging)[6]. For mild forms of monoarthritis, gait
56 analysis is insensitive and lacks consistency[7]. Static weight bearing is useful for measuring pain in
57 rat hindlimbs but can be difficult in mice. Mechanical sensitivity measures such as the von Frey
58 test indirectly detect sensitization of the foot pad in the setting of knee arthritis, but do not
59 directly measure knee pain and are cumbersome to perform. Knee compression force as
60 measured with the P.A.M. (Pressure Application Measurement) device can be used in rats, but
61 the large device is difficult to use in mice without producing negative behaviors that do not
62 necessarily reflect pain[8].
63
64 We have developed methods for measuring spontaneous and evoked pain in three different
65 models of murine arthritis that are sensitive and reproducible for measuring pain severity and
66 the analgesic effects of intra-articular therapies[9,10]. We can also correlate pain relief with other
67 histologic markers of pain and screen investigational drugs that would not be possible in humans.
68 We can evaluate synergism of modestly effective analgesics to identify more effective
69 combination therapies. By utilizing proven methods for both evoked and spontaneous pain, we
70 avoid the pitfall of failing to identify pain that is reflected in one type of pain behavior but not
71 the other and more closely mirror pain measurement in humans[11]. By limiting pain evaluation to
72 two methodologies, we minimize stress on the animals, which can affect pain behavior, and
73 maximize productivity of the laboratory.
74
75 **PROTOCOL:**
76 All animal procedures and protocols were approved by the Minneapolis Veterans Affairs Health
77 Care System (VAHCS) Institutional Animal Care and Use Committee and conformed to the "Guide
78 for the Care and use of Laboratory Animals" (The National Academic Press, USA)[12].
79
80 **1. Choice of animals**
81
82 NOTE: C57Bl6 mice are used for all three arthritis models.
83
84 1.1. Use either male or female mice, but note that the response to arthritis pain may vary
85 depending on gender and the pathogenesis of the model being tested.
86
87 1.2. Select mice that have reached sexual maturity, are no longer rapidly growing and are not old
88 enough to have developed spontaneous arthritis that could confound pain measurements.

89

90 NOTE: Twelve weeks is an ideal age for pain measurement in the models described but this
91 depends on the study design. Age at initiation of arthritis depends on the duration of time the
92 model takes to develop. Acute inflammatory arthritis (carrageenan model) requires 3 h to
93 develop, therefore animals are 12 weeks of age at the time of induction. Chronic inflammatory
94 arthritis (CFA model) requires 3 weeks to develop, therefore animals are 9 weeks old at the time
95 of induction. Chronic degenerative arthritis (collagenase model) requires 4 weeks to develop,
96 therefore animals are 8 weeks at the time of induction. A more chronic model of degenerative
97 arthritis can be used by inducing arthritis at 6 weeks of age. This allows 6 weeks of development
98 of joint damage. However, chronic degenerative arthritis in mice does not produce significant
99 evoked pain.

100

101 1.3. Obtain animals from a national commercial breeder source.

102

103 1.4. House animals in groups of up to 8 animals in standard polycarbonate cages with ad libitum
104 rodent diet and water.

105

106 1.5. Maintain environmental conditions at 72 °C ± 1 °C and 33% ± 1% humidity with 12 h
107 light/dark cycles.

108

109 **2. Intra-articular injections into the knee**

110

111 2.1. Create a 30 G 2.5 mm needle by covering the length of a 0.5 inch needle with a sheath made
112 of polyethylene tubing with an inside diameter of 0.38 mm and outside diameter of 1.09 mm,
113 leaving only 2.5 mm of the needle protruding. This prevents inserting the needle too deeply and
114 exiting the joint posteriorly.

115

116 2.2. Choose a single knee to inject, shave it using any commercially available electric facial hair
117 trimmer, and sterilize with providone-iodine scrub.

118

119 2.3. Anesthetize the mouse with 3% isoflurane in 100% O2 at 1 L/min for 3 min ± 1 min. Maintain
120 anesthesia using a nosecone during the procedure. Confirm anesthesia depth by toe pinch. Take
121 care to monitor respiratory status and minimize anesthesia exposure to maintain adequate
122 respirations.

123

124 2.4. Enter the joint at the midline through the patellar tendon just inferior to the lower edge of
125 the patella using the sheathed needle once the mouse is fully anesthetized. This anterior midline
126 approach maximizes accuracy for entering the joint and injecting the substance of interest.

127

128 2.5. Use the same approach for injecting both arthritis-inducing substances and analgesic
129 substances. Sham controls are produced by inserting the needle as above but without injecting
130 any substance.

131

132 **3. Production of arthritis**

133

134   3.1. Anesthetize animal as in step 2.3 above.

135

136   3.2. Weigh the appropriate amounts (see below) of carrageenan, Complete Freund's Adjuvant
137   and Type IV Collagenase and dilute to the appropriate concentration with normal saline in a
138   laminar flow hood for safety and sterility.

139

140   3.3. Inject 10 μl of 2.5% carrageenan diluted in 0.9% sterile saline into the knee as above using a
141   Hamilton syringe to produce acute inflammatory arthritis. Arthritis peaks 3 h after injection.

142

143   3.4. Inject, 30 μL (30 μg) of Complete Freund's Adjuvant (CFA) into the knee to produce chronic
144   inflammatory arthritis. There is an initial acute inflammatory phase that peaks at 1 week then a
145   chronic phase that peaks at 3 weeks following injection.

146

147   3.5. Inject 10 μL containing 10 IU Type IV Collagenase to produce chronic degenerative arthritis.
148   Arthritis pain peaks 4 weeks after injection.

149

150   **4. Measurement of evoked joint pain**

151

152   4.1. Always examine the normal comparator knee first to minimize the cross-over pain response
153   (windup) from the painful knee[13].

154

155   4.2. Train the examiner to apply consistent and firm pressure of 1100 gf/cm^2 = 15.6 psi using a
156   Palpometer level 4; the device will emit 4 beeps when this level of pressure is applied. This
157   amount of pressure will consistently elicit a greater pain response from an arthritic joint than
158   from a normal joint.

159

160   NOTE: The device is calibrated at the factory to elicit 4 beeps when the appropriate level of
161   pressure is applied. Use the device for training only; do not use on the animals but when
162   examining the animals, apply the amount of pressure learned through the training.

163

164   4.3. Restrain the mouse tightly enough to prevent casual movement by holding the scruff of the
165   neck with the index finger and thumb and using the 4th and 5th fingers to restrain the tail. The
166   mouse should be able to make discrete, brief movements with effort and when stimulated, but
167   should not be able to move freely or continuously.

168

169   4.4. Administer repeated firm (Palpometer level 4) palpations concurrently to the lateral and
170   medial sides of each knee at approximately the joint line with the thumb and index finger of the
171   free hand once per second for one minute while restraining the mouse. Do not use the
172   Palpometer for the mouse examination; it is used only for training.

173

174   4.5. Have a second observer count the number of times the animal vocalizes and fights to escape
175   the restraint during this one-minute time-period. The sum of fights and vocalizations is the
176   Evoked Pain Score.

177
178 4.6. Always us the same examiner throughout the study and confirm the examiner and observer
179 are blinded as to experimental condition.
180
181 5. **Measurement of spontaneous joint pain**.
182
183 NOTE: The Advanced Dynamic Weight Bearing (ADWB) device calculates the percent of total time
184 the animal spends on each limb and the percent of total weight borne on each limb.
185
186 5.1. Fill a plastic beaker with 2 L of water. Place the foam pad and medium density fiberboard
187 that are included with the equipment along with the filled beaker on a laboratory scale and
188 record the combined weight.
189
190 5.2. Open the ADWB program and select **Mice** sensor type.
191
192 5.3. Calibrate the system prior to each experiment:
193
194 5.3.1. Select **Full calibration** under the **Calibration** menus. Enter 0.00 as a **Tare value** and the
195 weight of the foam pad, medium density fiberboard, plastic beaker, and water as the **Calibration**
196 **weight**, then select **Next**.
197
198 5.3.2. Select **Tare** before placing anything on the sensor. Before selecting **Gain preview**, cover
199 the sensor pad with the provided foam pad, medium density fiberboard, and plastic beaker filled
200 with 2 liters of water, in that order. Select **Gain preview** once the calibration weight is on the
201 sensor.
202
203 5.3.3. Adjust the calibration weight so it is evenly distributed across the individual sensors and
204 when the value of the **Calibration indicator** is above 95, select **Gain definition**. This process
205 produces a normalized mean for the individual sensors and confirms the integrity of the
206 individual sensors. Finally, select **Next**.
207
208 5.3.4. Save the calibration file into the calibration folder.
209
210 5.4. Select **New** and where provided enter the **Project** name and the **Animal** name for the first
211 mouse to be tested.
212
213 5.5. Select **Browse** and open the calibration file that was created for the project. Select **Next**,
214 ensure **Enable video capture** is selected, and select **OK**.
215
216 5.6. Weigh the animal, select **Capture**, and record the weight in the software. Select **OK**.
217
218 5.7. Do not acclimate mice to the chamber before the collection of data because this device
219 measures spontaneous behavior.
220

5.8. Place mouse in the chamber for a period of 5 min of testing. When the animal is in the chamber select **Launch**. The software will record the animal's activity during this time, capture a video and synchronize with the sensor data, and automatically partition this video into analyzable and non-analyzable segments.

NOTE: The software considers segments analyzable when at least one stable zone is detected. A stable zone is created when sensors detect ≥2 g on one sensor with a minimum of one adjacent sensor detecting ≥1 g and when the weight distribution of a zone is stable for more than 0.38 s.

5.9. Before the first analysis in a series select **Easy scoring** and **Independent paws setting**.

NOTE: Easy scoring allows iteration validation of segments where the stable zone does not change based on the validation of the first stable zone of that segment. Independent paws setting provides the observer with access to the keyboard shortcuts that allow individual identification of each paw. The observer assigns zones that meet minimal criteria as right hind, left hind, right fore, left fore, both forepaws, other body part, or ignore zone by using the keyboard shortcuts while holding the cursor over the segment per the video and scaled map of activated sensors.

5.10. Select **>>** to move to the first analyzable segment. If a stable zone does not have the correct paw identified then hold the cursor over that zone and press the shortcut (right hind, **s**; left hind, **a**; right fore, **w**; left fore, **q**; both forepaws, **e**; other section of the body, **z**, ignore zone, **x**) that corresponds with the correct body part.

NOTE: A minimum of 1.5 min of analyzable time is required to meet analysis thresholds. Analyzed segments are added and averaged automatically for output per paw(s) by the software.

5.11. The ADWB software then automatically calculates the percentage of analyzed time spent and percentage of body weight (g) placed on each of the four limb options. Export these values into a new spreadsheet by selecting **Results**, **Export**, selecting **Yes** to the prompt question asking if only the first five summary lines should be exported, and selecting the location of the output.

5.12. Euthanize animals by CO_2 inhalation followed by thoracotomy at the end of the study period.

5.13. For each additional animal in the same group, select **New** and enter a unique animal name but do not change the project name. Select **Next**, ensure **Enable video capture** is selected, and select **OK**. Follow steps 5.5 to 5.12 as for the first animal.

REPRESENTATIVE RESULTS:
Acute and chronic inflammatory and chronic degenerative joint pain was produced by IA injection into the left knee of C57BL/6J male and female mice prior to pain assessment using the protocol outlined above. Joint pain was measured as evoked pain scores (EPS) and spontaneous pain behaviors measured with an advanced dynamic weight bearing (ADWB) device. For the purposes

264   of this report, individual comparisons were made using Student's t tests. For experiments where
265   analgesic doses are being compared, ANOVA may be a more appropriate statistical comparison.
266
267   EPS (the sum of tallied fights plus vocalizations) increased with all three types of joint pain (**Figure
268   1**). ADWB measures of percent weight bearing on the left hind limb compared to naïve mice were
269   reduced in acute and chronic inflammatory joint pain. Females but not males with chronic
270   degenerative joint pain (COL) showed similar reductions in ADWB measures (**Figure 2A**). Males
271   with acute inflammatory joint pain transferred more weight to the forelimbs than naïve animals.
272   Weight bearing on forelimbs increased in female mice compared to naïve but not in males with
273   chronic degenerative joint pain (COL) (**Figure 2B**). ADWB measures of percent time on the
274   affected left hind limb decreased compared to naïve in mice with acute and chronic inflammatory
275   joint pain, but this was not statistically significant. These animals did spend significantly less time
276   on the left hind limb than on the right hind limb. In both males and females with chronic
277   degenerative joint pain, there was no difference in time spent on the left hind limbs compared
278   to naïve. Females with chronic degenerative joint pain spent more time on the nonarthritic right
279   hind limb compared to naive (**Figure 3A**). Mice with acute inflammatory joint pain and females
280   with chronic degenerative joint pain spent significantly more time on the forelimbs than naïve
281   animals (**Figure 3B**).
282
283   **FIGURE LEGENDS:**
284   **Figure 1. Evoked pain scores for left (blue column) and right (red column) hind limbs.** Right hind
285   limbs served as internal controls and did not undergo any IA injections. Measurements were
286   taken in the following conditions: uninjected naïve (male and female), acute inflammatory
287   carrageenan-induced arthritis (CAR), chronic inflammatory Complete Freund's Adjuvant-induced
288   arthritis (CFA) and chronic noninflammatory Collagenase-induced arthritis (COL – male and
289   female). In naïve animals the EPS of the left hind knee was not statistically different from the
290   right in males and only minimally different in females (**$P = 0.038$) likely due to some increased
291   pain response in the knee examined second (the left) due to crossover pain response. In all
292   arthritic groups the arthritic left knee EPS was significantly greater than the nonarthritic
293   contralateral right knee. When comparing the EPS of the left knee of arthritis groups to naïve
294   animals, all arthritis groups had significantly greater EPS scores in the arthritic left knee compared
295   to naïve left knees. This was true in both males and females. *$P < 0.05$ compared to male naïve,
296   #$P < 0.05$ compared to female naïve. All error bars are SEM in all figures.
297
298   **Figure 2: Spontaneous pain behavior measured by percent body weight borne on limbs**. (**A**)
299   Spontaneous pain behavior measured by total percent body weight on the left (blue column) and
300   right (red column) hind limbs. In both male and female naïve animals, there was no significant
301   difference between percent weight borne on the left and right hind limbs. Male mice with CAR
302   and CFA induced joint pain and females with COL induced joint pain bore significantly less weight
303   on the left hind limb compared to the naive. *$P < 0.05$ compared to male naïve, #$P < 0.05$
304   compared to female naïve. (**B**) Spontaneous pain behavior measured by total percent body
305   weight placed on the forelimbs. In males with CAR induced acute joint pain and females with COL
306   induced chronic joint pain, there was a significant increase in the amount of weight borne on the
307   forelimbs. This was not seen in males with CFA or COL induced joint pain. Naïve females tended

308 to bear more weight on the forelimbs than naïve males (P = 0.02). Additional weight may be
309 borne by other body parts such as the tail or rump. These were usually minimal and did not
310 contribute significantly to the understanding of the pain response and are therefore not shown.
311 *P < 0.05 compared to male naïve, #P < 0.05 compared to female naïve and to male 4 week COL
312 in forelimb weightbearing.

314 **Figure 3: Spontaneous pain behavior measured by percent time spent on limbs.** (**A**)
315 Spontaneous pain behavior measured by total percent time spent on the left (blue column) and
316 right (red column) hind limbs. There was no significant difference between right and left hind
317 limbs in male and female naïve mice with respect to time spent on the limb. Male mice with CAR
318 and CFA induced arthritis appeared to spend less time weightbearing with the arthritic left hind
319 limb compared to naïve, but this was not statistically significant and likely due to increased
320 variability in this measure in these mice. There was a significant difference in these animals
321 comparing right to left as the right hind limb spent more total time weightbearing than the left.
322 In females with COL induced arthritis, the right hind limb was weightbearing a greater proportion
323 of time than in naïve females. *P < 0.05 comparing right to left, #P < 0.05 compared to female
324 naïve and to male 4 week COL in time spent on forelimbs. (**B**) Spontaneous pain behavior
325 measured by total amount of time spent on the forelimbs. In males with CAR induced arthritis
326 and females with COL induced arthritis the mice spent significantly more time weightbearing on
327 the fore limbs than did the naïve animals. Female naïve mice spent more time on the forelimbs
328 than male naïve animals. *P < 0.05 compared to male naïve, #P < 0.05 compared to female naïve.

330 **DISCUSSION:**
331 Measurement of joint pain in preclinical models of inflammatory arthritis and degenerative joint
332 disease is an increasingly valuable tool to evaluate new treatments for arthritis pain and for
333 restoring joint function[9,10]. Measuring pain behaviors in mice with chronic degenerative joint
334 disease is challenging. As quadrupeds, they can offload a painful limb to any combination of the
335 other three limbs or the tail. Decreased function in one limb may not be displayed as obviously
336 as it is in bipedal humans. As prey animals, mice have a survival benefit to minimize pain
337 behaviors.

339 This report demonstrates that joint pain from different pathologic mechanisms can be measured
340 in mice with simple and relatively inexpensive methods. Inflammatory joint pain causes bigger
341 changes in weight bearing time and distribution and therefore may be a more sensitive model
342 for the detection of analgesia. However, other models of joint pain will be important when
343 evaluating new potential analgesics since mechanisms of pain initiation and maintenance are
344 likely different with different pathology. We found differences in offloading patterns between
345 males and females and among different models of arthritis pain.

347 It is important to recognize that there can be strain as well as gender differences in pain behavior
348 in mice[14-16]. It is also known that there are distinct strain differences in pain thresholds. We found
349 that in C57/Bl6 mice, females demonstrated significantly more spontaneous pain behaviors
350 (increased weight and time on forelimbs) than male mice with chronic degenerative joint pain
351 (**Figure 2** and **Figure 3**3). We did not determine the estrus phase for these female mice, so we

352   cannot say whether this had any effect on our results, but others have determined that estrus
353   phase did not explain observed sex differences in C57/Bl6 mice[17].

355   Evoked pain behavior is sensitive to all types of joint pain tested and has good reproducibility. It
356   is crucial that the examiner is trained to deliver consistent, standardized palpation pressure and
357   use consistent restraint methods that allows the range of pain behavior to detect both pain and
358   analgesia. It is important to always examine the normal knee first to minimize the pain response
359   in the normal knee due to a crossover effect and maximize the difference in pain response
360   between the normal and painful knee. Using a second observer to tally the pain responses that
361   make up the EPS is necessary so that the examiner focuses on consistent palpation and so that
362   vocalizations and fights are consistently tallied. It may be necessary to practice the restraint
363   method in particular to ensure the animal can react to pain adequately but does not react too
364   easily so as to dilute the pain response. An advantage of the EPS measurement is that it is
365   analogous to measuring joint tenderness in human patients.

367   We feel these methods are superior to existing methods in that they directly measure pain
368   coming from the knee joints of mice, they can be used in different models of monoarthritis pain,
369   they include both spontaneous and evoked pain responses, and are relatively inexpensive but
370   reliable and reproducible. As therapies are developed to more effectively treat arthritis, these
371   methods will be useful for confirming whether treatments are actually effective for the most
372   disabling outcome of arthritis, pain.

374   Our methods have some limitations. Primarily, the EPS measure is limited by the requirement for
375   a single examiner and observer and the need for very consistent technique. The evoked pain
376   measures are more automated and are not as subject to inter-observer differences. Further
377   refinement of the EPS measure might include automated methods for counting vocalizations in
378   response to palpation, such as using an ultrasonic bat detector to count vocalizations. This
379   method would need to be compared to our current method to confirm sensitivity and specificity
380   before it could be widely implemented.

382   In summary, we describe two different pain behavior measures for quantifying joint pain in mice.
383   Gender and strain differences must be considered before choosing an experimental model.
384   Measures of evoked pain require careful training and consistent implementation by the
385   examiner. Both spontaneous and evoked pain behaviors are recommended to evaluate pain
386   severity and functional limitation and should be utilized to fully evaluate the effects of new
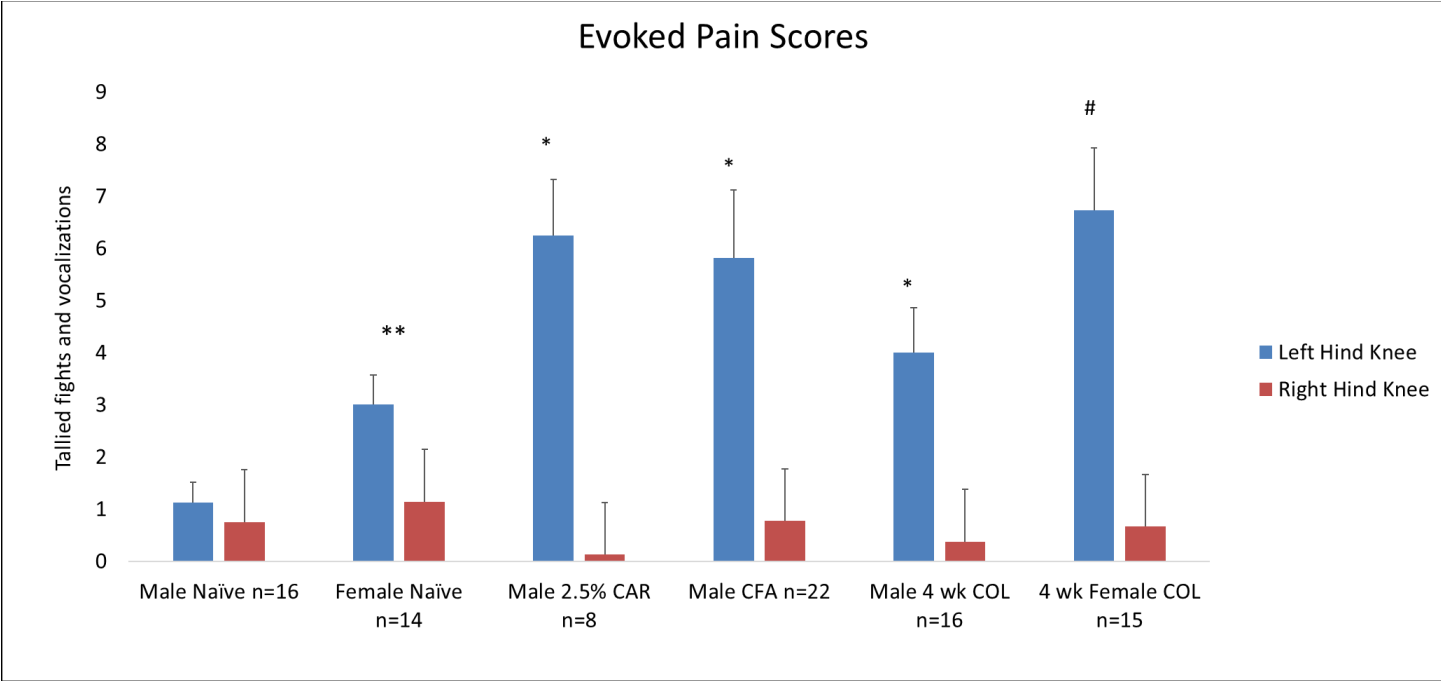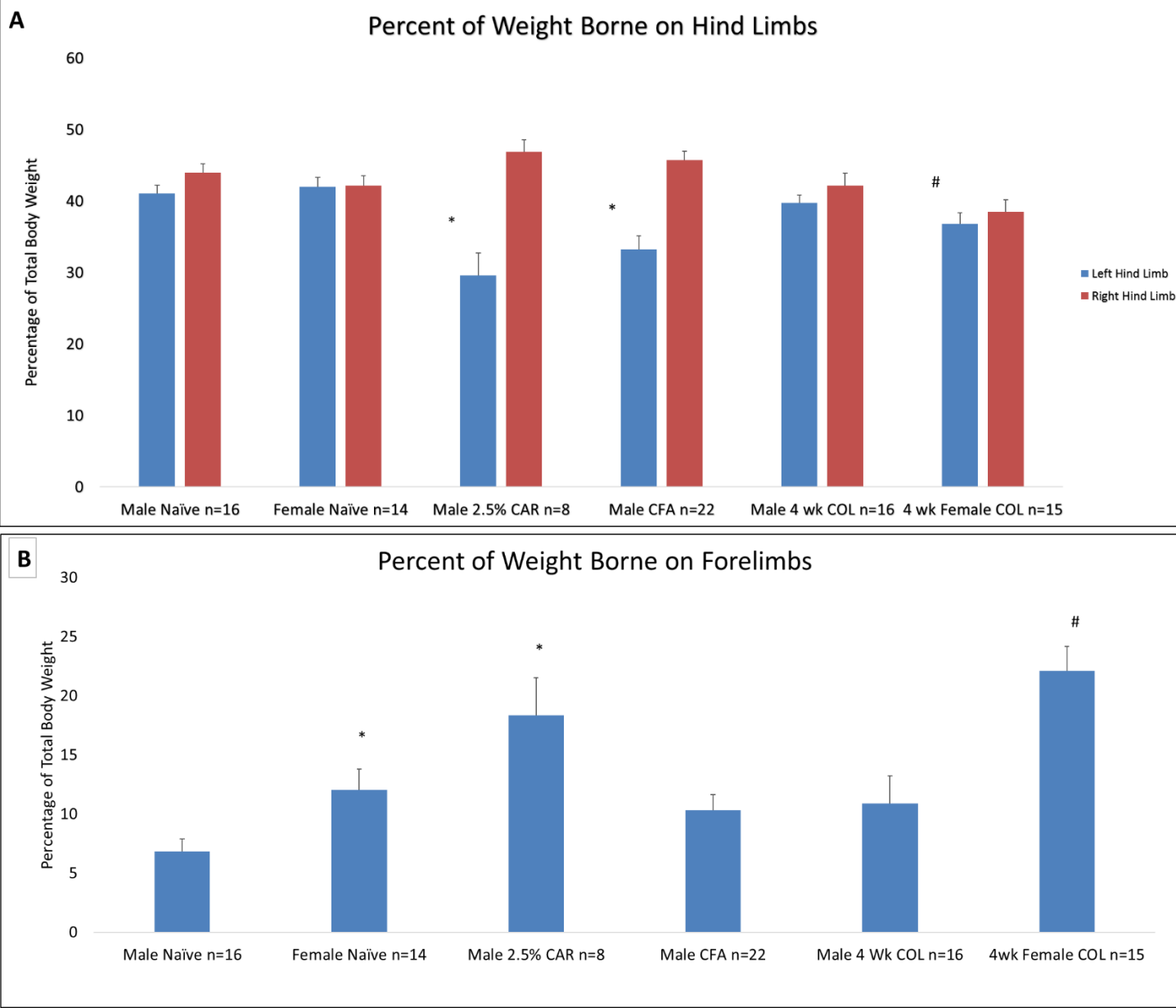387   potential analgesics.

392   **DISCLOSURES:**
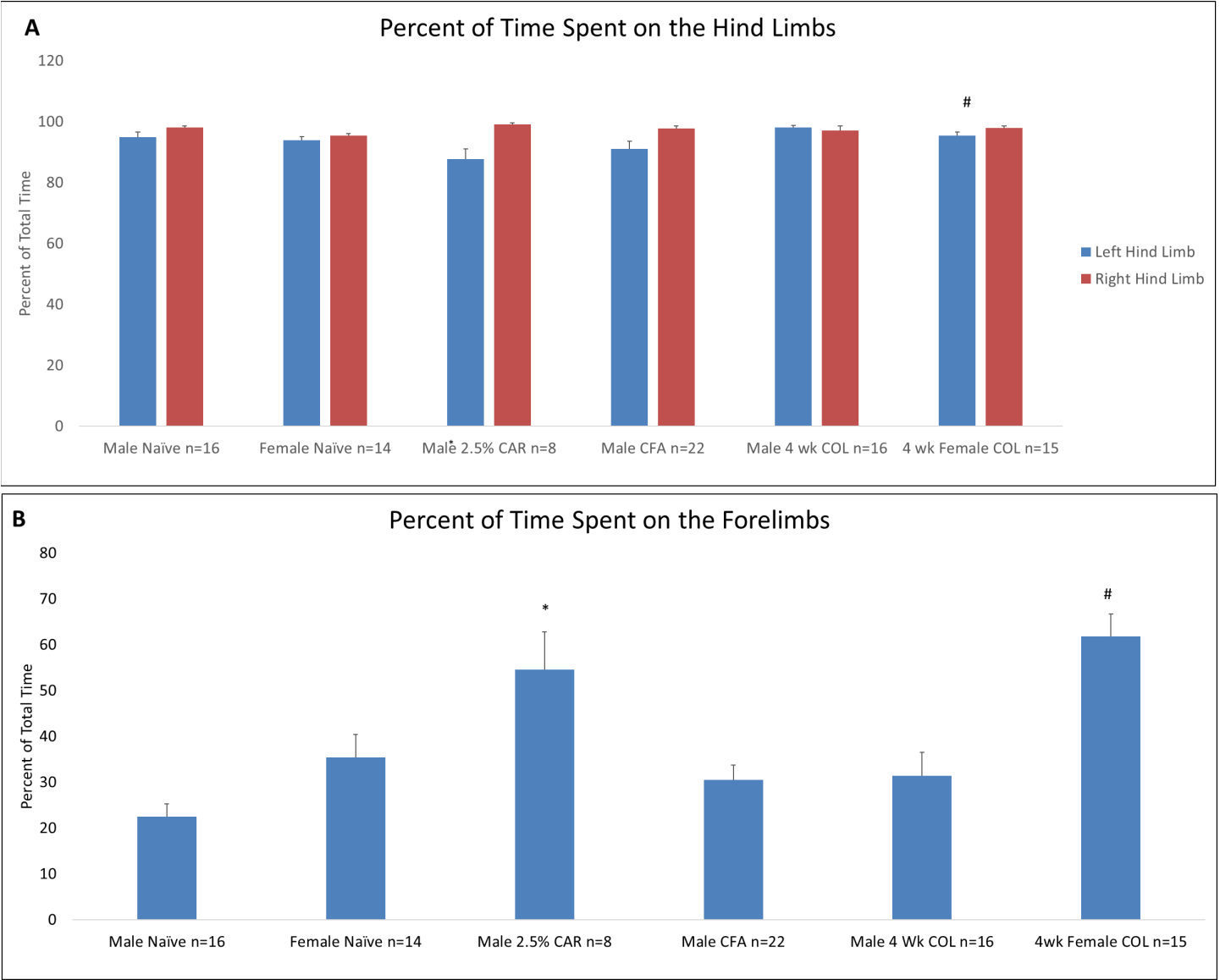393   The authors have nothing to disclose.

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Evoked Pain Scores

Figure 2

Click here to access/download;Figure;Fig 2.pdf ⬇

A Percent of Weight Borne on Hind Limbs

B Percent of Weight Borne on Forelimbs

Figure 3

A Percent of Time Spent on the Hind Limbs

B Percent of Time Spent on the Forelimbs

Essiantial Supplies	Company	Catalog Number
Advanced Dynamic Weightbearing Device	Bioseb In Vivo Research Instruments, Vitrolles, France	Model: BIO-DWB-M - For mice
C57/Bl6 mice (male and female)	Jackson Labs, Bar Harbor ME	Stock No: 000664 | Black 6
Carrageenan	Sigma-Aldrich, St. Louis, MO, USA	22049-5G-F
Complete Freund's Adjuvant	Sigma-Aldrich, St. Louis, MO, USA	F5881
Palpometer	Palpometer Systems, Inc. Victoria, B.C	Not available - no longer in business
Type IV Collagenase	Worthington Biomedical Corp, Lakeville, NJ	LS004210

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Hollis E. Krug, Christopher Dorman, Nicole Blanshan , Sandra Frizelle, Maren Mahowald

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Response to reviewers 11-10-18 Responses are in bold following the editorial comments.

Editorial comments:

1. We are concerned about filming section 4 of the protocol, as it apparently involves showing animals in distress/pain (the rest of the protocol is fine). If you think this is essential to show, we may have alternative ways to visualize it, though–e.g., we could use still images (either provided by you or stills from footage captured by the videographer) and overlays of text, arrows, or highlighting to show things like where to palpate and how to locate the important structures; alternatively, we may be able to construct a simple animation using our stock graphics of rodents and anatomy. Please let us know your thoughts on this. **We believe this is essentially the most crucial part of this paper to share with readers as it is technically highly reliant on technique. Please understand that the reason this is difficult is that the pain is very mild so that it is hard to discern when the animals are actually in pain and when they are just uncomfortable being restrained. I think once you see what we are doing, it will not be disturbing to film. But alternatively, demonstrating placement and restraint technique would be helpful. Video graphics depicting the types of pain responses we grade might be an acceptable substitute.**

2. The Figures are currently fairly low resolution (even in the original files). If you are having technical difficulties converting them to tif format, pdf format is acceptable (although, again, all panels for each figure should be combined into one file per figure). **We did have difficulty creating TIFF files without losing resolution. We have combined the figures into pdf files for better resolution.**

3. Figures: Please explicitly define CAR and COL in the legends. **This has been done.**

4. Figures: It's "COL" in the legends/text but "Col" (not capitalized) in the Figures themselves; please make consistent. **This has been done**

5. Figures 2 and 3: Please label panels only as 'A' and 'B' (e.g., not as '2A'). **Done**