Published in final edited form as:

Physiol Behav. 2016 December 01; 167: 132–144. doi:10.1016/j.physbeh.2016.09.005.

Mouse preferential incising force orientation changes during jaw closing muscle hyperalgesia and is sex dependent

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Abstract

Introduction—Mouse incising is controlled by a central pattern generator and this activity can change in the presence of pain. The incising frequency and maximum force generation decreases with pain. In this study, we used repetitive acidic injections in the left masseter muscle of male and female mice to determine differences between baseline and jaw muscle pain conditions and the effect of sex on preferential incising direction.

Methods—A within subject design was used to evaluate data previously acquired using multi-axis force data (X, Y and Z) from the 4th baseline recording day and day 7 post-injection (day of maximal pain response) for each mouse of each sex. A total of 34 female and male (age 3–9 months) CD-1 mice were evaluated. After mathematically rotating the X and Y axes to align the Y axis to be parallel to the wire struts of the cage top, data were analyzed to determine incising direction preference during baseline (non-pain) and pain (day 7) conditions and between sex. Radar plots of X–Y, X–Z and Y–Z axes depicted the average direction of incising preference between baseline and pain conditions for each sex. Statistical differences among groups were tested using a mixed model ANOVA.

Results—Similar to previous findings, female mice had a more robust difference in incising direction preference when comparing male and female pain conditions and this was most evident in the X–Z axes. The incising frequencies most commonly affected were 5.3, 6.2 and 7.6 Hz. Male mice varied little in their incising direction preference between the baseline and pain conditions. In addition, statistical comparison of ratios of the percent of time spent incising in the Z versus X axes for each incising frequency found that the incising preference was not different when comparing 5.3 and 7.6 Hz frequencies. Finally, female mice used a novel approach to minimize

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Conflict of Interest

The authors have no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

pain while incising by rotating their head and body nearly 180 degrees while males did not use this strategy as frequently.

Conclusions—The preferred incising direction changes in a jaw muscle pain condition and this information can be used to further characterize functional pain in the masticatory muscle system. The changes were dependent on the incising frequency generated by the central pattern generator for incising.

Keywords

central pattern generator; mastication; incising forces; pain

1. Introduction

Masticatory muscle force production during mastication and incising is the result of the cumulative effect of descending cortical inputs onto neurons comprising the central pattern generator (CPG) for mastication as well as integration of sensory inputs from jaw closing muscles, periodontium, temporomandibular joints and intraoral mucosa [1]. Sensory feedback refines masticatory muscle activity to generate sufficient magnitude and direction of forces to accomplish the task of food breakdown prior to deglutition. The breakdown of different types and hardness of foods is important to maintain the necessary food intake for survival. Assessing relative contributions from non-noxious or noxious sensory inputs on masticatory motor control has been a topic of research for many years [2–10]. However, little is known regarding the effect of noxious inputs such as muscle or joint pain on freely motivated behavior because the effects of pain on masticatory behavior have been difficult to evaluate in a non-invasive approach and over an extended period of time. Pain during mastication or incising can affect the ability to efficiently allow adequate food intake by decreasing maximum masticatory force magnitude and decreasing cycle frequency through the central pattern generator for mastication located in the brainstem [11]. Both animal and human studies have focused on the effects of acute pain and these studies may be limited in the interpretation regarding the effects of persistent pain that lasts for weeks to months to years. Animal studies of experimental jaw muscle pain and temporomandibular joint (TMJ) pain have been conducted using algescic substances injected into the muscle and have been commonly evaluated using reflex studies such as mechanical stimulation or thermal stimulation [12–15]. However, some of these algescic substances, such as Complete Freund's Adjuvant (CFA), cause a prolonged pain and extensive muscle necrosis that does not reflect the typical muscle pain presentation of human subjects. The use of these substances more closely approximates an acute inflammatory pain with deafferentation of peripheral nerves innervating the muscle. Human studies have evaluated the effects of short-term jaw closing muscle pain on masticatory behavior by injection of such algescic substances as hypertonic saline, glutamate or NGF to create masticatory muscle pain [16–18]. The results have been varied possibly due to small sample sizes, presence of a needle or catheter in the jaw closing muscle during active chewing or the high variation observed among the participants [19–23].

Recently, we described a technique to non-invasively record incising forces without experimenter intervention and for long periods of time (24 hrs) in the home cage environment [10]. In an experimental jaw closing muscle pain condition, female mice were

found to respond more robustly to repetitive acidic saline injections into the masseter muscle than male mice with a longer duration of the response and a shift to lower incising frequencies. Assessment of the preferred direction of rhythmic force production during incising during non-noxious and noxious afferent inputs can provide novel information on the effects of peripheral afferent activation on the central pattern generator. In addition, sex differences in response can be assessed during these varied inputs. In this study, two hypotheses were tested. The first hypothesis, based on our previous findings, was that jaw muscle pain would cause a deviation from the normal, non-noxious incising direction preference and that the magnitude of deviation would be sex dependent. A second hypothesis was also based on our previous findings that, of the five incising frequencies identified, there was a high, negative correlation ($CC = -0.85$) found specifically for the 5.3 and 7.6 Hz incising frequencies. One explanation for this high correlation was the possibility of the bistability of neurons in the incising CPG network. Bistable neuronal activation should elicit a similar mechanical action but at different incising frequencies. Thus, our second hypothesis to be tested was that incising frequency pairs, such as the 5.3 and 7.6 Hz frequencies, would have similar preferred incising directions but would be statistically different from other incising frequencies. Male and female mice were evaluated using a within comparison design during baseline (no pain), repetitive acidic saline injections (pain) and repetitive normal saline control mice (minimal pain) to determine the influence of jaw muscle pain on masticatory muscle motor control during incising.

2. Materials and Methods

2.1 Animals

Animals that were used in this study were the same ones as described in a previous report [10]. A total of 34 CD-1 mice (Charles Rivers) consisting of 17 males and 17 females (age 3–9 months) were housed in the same animal room with four male or female mice residing in the same home cage. Mice were individually assessed in a sound attenuation chamber (ENV-022V, Med Associates, Inc.) in the same room as they were housed. All mice had ad libitum access to water and food and were exposed to a 12 hr light/dark cycle with the room maintained at an average temperature of 25°C. Mice were maintained in the sound attenuation chamber with the same 12 hr light/dark cycle schedule as the animal room. The University of Florida Institutional Animal Care and Use Committee approved the animal protocol for this study.

2.2 Data Acquisition

The data acquisition procedures for this study have already been described in a previous publication [10]. Briefly, audio, video and incising force recordings were simultaneously acquired from four cages during a 24 hr period. Video recordings provided an assessment of behavior that could be correlated to the recordings of incising forces. Incising forces were assessed in three dimensions using a multi-axis force transducer (NANO17-E, ATI Industrial Automation) and the transducer was attached to three pieces of standard mouse chow (Harlan Laboratories). Mice had access to the chow through the wire top of the cage in the area of the chow bin (Fig. 1). Custom-written software (LabView 2013, National Instruments, Inc.) detected episodes of incising forces after the analog signal was digitized

using an A/D converter at a 500 Hz acquisition rate converter (PCIe-6343 X Series Multifunction DAQ, National Instruments, Inc.). Episodes of incising forces were recorded when the resultant of the X, Y and Z forces exceeded a pre-determined threshold and continued until no force peaks were detected for more than 4 sec. The digital force recordings for each axis (X, Y and Z) were post-processed to remove DC bias and to digitally filter the recordings (bandwidth 0–31.25 Hz) using custom written software (LabView 2013, National Instruments, Inc.).

2.3 Pain Model Description

As previously described [10], mild to moderate masseter muscle hyperalgesia was elicited in 13 males and 13 females by repetitive injections (20µl, separated by five days) of acidic saline (pH = 4.0) into the center of the muscle on the left side. The pain that was elicited was a persistent pain that lasted at least 3–4 weeks depending on the sex of the mouse. A control group consisted of repetitive injections of neutral saline (pH = 7.0) into the left masseter of 4 males and 4 females. Prior to injections of either acidic or neutral saline, mice were anesthetized using xylazine (15 mg/kg) and ketamine (75 mg/kg).

2.4 Incising Recordings

In a previous study, eleven recordings were made including four baseline and seven post-injection recordings to assess the effect of acidic saline vs normal saline on incising behavior [10]. In this study, a within subject design was used. Multi-axis force data (X, Y and Z) from the 4th baseline recording day and day 7 post-injection (day of maximal pain response) for each mouse were analyzed to determine incising direction preference during baseline (non-pain) and pain (day 7) conditions.

2.5 3D Rotation of Force Data and Calculation of Preferential Force Direction

The Z axis was oriented perpendicular to the chow bin with the positive direction towards the floor of the cage while the X and Y axes were oriented at 45 degrees to the direction of the bars of the cage top (due to orienting the lead of the transducer away from the cage). The placement of the X and Y axes that was used during data collection was not optimal to allow interpretation of the direction of force (Fig. 1). Ideally, it would have been optimal to orient the axes so that the X axis was parallel to the wire bars and the Y axis perpendicular to the bars. Therefore, the X and Y axes were rotated in space using the following formula:

$$x' = x \cos \theta - y \sin \theta$$

$$y' = x \sin \theta + y \cos \theta$$

where $\theta = 45$ degrees expressed in radians. This orientation had the X axis oriented parallel to the wire mesh of the cage top with positive towards the side with the mounted chow and the Y axis at 90 degrees to the X axis with positive pointing away from the water access bottle (Fig. 2). Since the wire bars limited movement in the Y axis, the incising direction preference generated along this axis was small compared to the X and Z directions.

Preferential incising force direction was calculated in three planes (X–Y, X–Z and Y–Z) and in six degree increments over the 360 degree range for each axis.

2.6 Statistical Analyses

In a previous study, no statistically significant differences were determined for various incising parameters across the age group that was studied (3–9 months) so the results from these animals were pooled [10]. Non-responding animals to the acidic saline injections were removed from the analyses as previously described. Preferred incising directions for each discrete incising frequency (4.6, 5.3, 6.2, 7.6 and 10.4 Hz) were evaluated separately. Medians of incising direction for each sex, condition and incising frequency were calculated. Significant differences among the medians for each sex/condition were evaluated using the non-parametric Wilcoxon matched pair signed rank test for within comparisons (condition) and the Mann-Whitney U test for between comparisons (sex). Radar charts representing incising preference direction at each frequency were constructed to demonstrate the baseline and pain conditions for males and females. Radar charts of control animals (baseline and repetitive neutral saline injections) were also constructed to evaluate the effects of repetitive injections. Statistical differences between sexes for preferred incising direction between non-pain and pain conditions were evaluated using ANOVA for repeated measures and, when significant, post-hoc testing (LSD Test) using a probability level of $p < 0.05$. Statistically significant differences among groups were indicated on the radar charts.

Preferred incising direction was evaluated across the different frequencies using data from the X and Z axes only because the Y axis was physically constrained by the narrow wire spacing at the cage top. Preferred incising direction was determined by summing the counts of incising events that occurred within a given range for each axis. For the X axis, this range was between 246–294 degrees (Fig. 3). For the Z axis, counts for the negative and positive components were summed from 180–204 degrees, and 336–360 degrees, respectively (Fig. 3). A ratio of the counts of Z to X (i.e., Z/X) was then calculated for each animal at each of the five incising frequencies and tested for significant differences between sex, Hz and condition (baseline or pain) using a repeated-measures ANOVA.

3.0 Results

3.1 Radar Plots – General Observations

Radar plots of each incising frequency (4.6, 5.3, 6.2, 7.6 and 10.4 Hz) for each of the 2D planes (X–Y, X–Z and Y–Z) were constructed. Obvious differences in the preferential direction of force during incising could be observed (Fig. 4–6). First, consistent with the limited range of movement due to the wire slats in the cage top that separated the mouse from the chow, the incising direction preferences in the Y axis were limited and this restriction can be seen in the X–Y and Y–Z plots. The plots confirmed that the X and Y axes were correctly rotated mathematically to align the axes with the long axis of the wire top.

A second observation was that the number of incisions in the Z axis at the highest and lowest incising frequencies differed from the number at the middle range frequencies; this resulted in a different shape of the radar plot at each frequency. A much lower preference to incise in

the Z axis and a higher preference to incise in the X axis was observed at these two frequencies. Thus, the presence of multiple distinct incising frequencies with different preferential directions of incising suggests that there may be functional differences during the incising effort. This observation was tested by evaluating the ratio of incising preference predominantly in the direction of the negative Z axis (range 246–294 degrees) compared to the X axis (negative or positive (180–204 degrees and 336–360 degrees) (see Fig. 2 for axes reference). Table 1 shows the repeated-measures ANOVA results for the main effects of sex (male or female), experimental condition (baseline or pain) and incising frequency (five different frequencies). Only the main effect of incising frequency was found to be statistically significant. No interactions of the main effects were statistically significant. Significant differences between the different frequencies are shown in Fig. 7. Interestingly, the Z/X ratio for the incising frequencies of 5.3 Hz and 7.6 Hz were not significantly different and these frequencies were highly correlated in a previous study [10].

Lastly, visual comparison of the acidic saline responses can be made to the neutral saline responses in the panels on the right side of each figure (Fig. 4–6). No statistically significant differences were found among the baseline or pain groups or between sex ($n = 4$ in each group) for the neutral saline injection condition.

3.2 Sex Differences for Incising Direction Preference During Baseline Conditions

Statistically significant differences were found between male and female mice for their preferential incising direction during the baseline recording (ANOVA, $p < 0.05$; LSD test, $p < 0.05$). These differences were found in the X–Z and Y–Z plots with preference on the Z axis. Unexpectedly, female mice had more incisions in the positive Z axis (jaw opening direction when the mouse is facing the chow normally – see Fig. 2) compared to males. Examination of video recordings of these mice found that a small proportion of the time incising was spent with the head turned almost 180 degrees to the left side with the incisions exerted in a positive Z direction (Fig. 8). The average baseline incising preference percentage between 50 to 144 degrees (the range of significant differences) in the X–Z plots at 6.2 Hz was 22% for females compared to 9% for males. At the incision frequency of 7.6 Hz and between the range of 72 to 174 degrees, females preferred to incise in this range 22% of the time compared to 6% for males. These data support sex differences in the incising orientation with females having a preference to turn their heads almost 180 degrees to achieve their incising goals.

3.3 Sex Differences for Incising Direction Preference During Pain Conditions

Comparing the same animal between the baseline and pain condition found several differences in the incising direction preference between males and females. First, female mice following the pain treatment (black thick line) differed from the baseline condition (thin dark line) but this was not the case for the males (Figs. 4–6; left panels). Statistical comparisons of the incising directions between male and female mice are shown as dark (female) or light (male) lines (thin lines, neutral saline condition; thick lines, pain condition) around the edge of the radar plot (ANOVA $p < 0.05$; LSD Test $p < 0.05$). Due to the limitation of jaw movement in the Y axis, X–Y and Y–Z plots have a limited range of incising directions that were statistically different. However, in the X–Z plots, a broader

range of statistically significant differences can be observed, especially at the incising frequencies of 6.2 and 7.6 Hz. The differences occur in the region of the radar plots where baseline differences were also observed, but had a broader range of incising directions. Relatively more incisions were made with the female mice orienting their heads nearly 180 degrees from their normal posture. The opposite direction in this plane (+X, -Z) had fewer occurrences of incising compared to baseline.

3.4 Comparison of Median Incising Direction for Baseline and Pain Conditions for Each Sex

Median baseline and pain incising directions were calculated for each sex and are shown in tables 2, 3, 4, 5, 6, 7 representing each incising frequency during control (saline) or pain conditions for each set of axes (X-Y, X-Z or Y-Z). Pairs of statistical comparisons are designated in the tables with the results of each of the statistical tests. In the X-Y plots, no differences were found between the baseline and pain conditions (Wilcoxon Matched Pair Signed Rank test, $p > 0.05$, n.s.) or between sex (Mann-Whitney U test, $p > 0.05$, n.s.) at each incising frequency. Similar findings were observed for all incising frequencies represented by the Y-Z plots except for the 7.6 Hz incising frequency. A significant difference was detected between the male and female pain medians (Mann-Whitney U test, $p < 0.05$) and was due to the greater number of incisions in the + Z direction and lower number in the -Z direction in the female compared to the male. The X-Z plots had the highest variation in incising direction due to the freedom of jaw movement in these planes. Significant differences in incising direction between the female pain median and the male pain median were observed during incising frequencies 5.3, 6.2 and 7.6 Hz. In addition, female pain medians were different from all other groups at 6.2 Hz incising frequency and were different from the male baseline and pain groups at 5.3 Hz incising frequency.

4.0 Discussion

4.1 Incising Direction Preference Can Be Determined in the Home Cage Environment

Using a previously described approach which allows the non-invasive acquisition of incising force data using chow mounted on a 3D force transducer, the preferential direction of incising could be analyzed in relation to the frequency of incising [10]. The advantage of this approach is that it minimizes experimenter bias and allows long term recording (24 hrs) to maximize the ability to interpret differences due to such factors as sex or pain. In this study, we have examined baseline and pain related recordings of incising within the same animal to maximize the ability to determine differences between these two conditions.

4.2 Incising Direction Preference is Different Between Male and Female Mice

A surprising finding was that female mice utilize a different strategy for incising compared to males. Females achieved incising a fixed piece of chow by the usual frontal approach where their incisors produced an upward force in the -Z axis. Females also used an approach that was nearly 180 degrees from this norm by turning their heads clockwise to position their incisors where the incising produced a downward direction of force in the +Z axis. This produced an asymmetrical pattern of preferred incising directions in the X-Z plane. Males rarely used this alternative positioning for incising chow and the pattern of

preferred incising direction was relatively symmetrical. Females may use this novel strategy of head positioning to minimize pain during incising since they experience more pain after a repetitive masseter muscle injection of acidic saline compared to males [10], and in humans, muscle pain is enhanced during function [24]. The female asymmetry may also have been influenced by a higher anterior force (-X axis) secondary to gnawing on the chow rather than incising that would produce more closing (-Z axis) force. Gnawing can be accomplished by rostral-caudal movement of the incisors across the chow and would predominantly be due to activation of the lateral pterygoid muscles and, to a lesser extent, activation of the masseter muscles. Another statistically significant difference between sexes was found in the Y-Z plane. A statistically significant difference was observed between female pain and male pain for preferential incising along the Z axis (closing) and along the X axis (preferred incising towards the water bottle as the mouse is facing the chow holder). During female muscle pain, a fewer number of incisions were made in the jaw closing direction (-Z axis) compared to the male pain condition (see Fig. 6). This change during muscle pain may be due to the reduced ability to generate high force during function in muscles with persistent pain [11] or may be due to the avoidance of functional activities that cause an increase in pain. Smaller, but statistically significant differences were also found between female non-pain and female pain conditions. A greater percentage of incisions were made towards the side of the water bottle (the left side of the mouse where the masseter muscle was injected with acidic saline) when the predominant incising direction was more parallel to the X axis and has a smaller Z axis (closing) direction. This effect is subtle since the wire of the cage top minimized movement in the Y axis, but this preferential direction of incising occurred consistently to produce a statistically significant effect.

4.3 Incising Direction Preference Varies by Incising Frequencies

Using a ratio of the frequency of incising in the Z and X axis as a measure of the preferential direction of incising for each condition and sex, it was possible to identify differences across five previously reported incising frequencies [10]. In the previous study, a high negative correlation coefficient (-0.85) was found between incising frequencies 7.6 Hz and 5.3 Hz and this finding suggested that these two frequencies of incising may represent bistability found with reciprocally inhibitory neuronal networks [25]. If the same mechanical actions occur at two different incising rates, then this evidence would support the concept of bistability. In this study, the Z/X ratio was not found to be statistically different between these two frequencies while most other pairs were significantly different. In addition, no statistically significant differences between these ratios were observed between condition (baseline or pain) or sex (male or female) similar to the analysis of correlation coefficients in our previous study. Therefore, the incising force preferential direction evidence supports the observation of bistability of incising and activation of motoneurons at certain frequencies during incising. To our knowledge, this is the first evidence of mechanical actions in an in vivo model during bistable neuronal activation. Chandler and colleagues have demonstrated bistable firing of rhythmic activation of trigeminal motoneurons after serotonin exposure in an in vitro model [26, 27]; thus the evidence generated in this study is consistent with known properties of the trigeminal system.

Acknowledgments

This work was supported by the National Institute of Dental and Craniofacial Research/National Institutes of Health (DE021849).

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Highlights

- A multiaxis force transducer was used to assess incising direction preference in a mouse model
- Sex differences were identified for preferred incising direction during baseline (non-pain) recordings
- Incising direction preference differed among the discrete incising frequencies
- Female incising direction preference was more affected than males in a pre-clinical model of pain

Fig. 1.

Position and orientation of multi-axis force transducer mounted on the home cage with mouse chow attached to the transducer. Top view (A) and side view (B) show the orientation of the X, Y and Z axes. White arrows represent X and Y axes and black arrow represents the Z axis. Positive is represented by the direction of the arrowhead. The X axis represents the anterior-posterior direction parallel to the wire slats of the cage top (as the animal faces the food tray normally); the y axis represented the left-right direction; and the z axis represented the opening-closing jaw movement.

Fig. 2.
Image of a female mouse incising chow in the standard frontal position with the 3D force transducer in front of the mouse.

Fig. 3. Radar chart with predominant X axis regions (a and b) delineated as black and predominant Z axis regions (c and d) delineated as gray. Ratios were calculated by the formula $(c+d)/(a+b)$ representing Z/X for each incising frequency.

Fig. 4.

Radar plots for each of the five incising frequencies (4.6, 5.3, 6.2, 7.6 and 10.4 Hz) with the percent of preferred direction of incising shown in the X–Y plane in 6 degree increments. The value plotted on a radar plot at each increment represents the percentage of time the mouse incised in that direction. The preferred directions of incising are shown for the two different conditions (acidic saline condition, left side, $n = 13$ F and 13 M; neutral saline condition, right side, $n = 4$ F and 4 M). Within each radar graph, overlays of the preferred incising directions for baseline and acidic/neutral saline conditions are shown for each sex. Statistically significant differences between pairs of traces are shown around the periphery of each graph at their statistically different locations over the range of 360 degrees.

Significantly different pairs are represented by the intensity (light = male, dark = female) and size of the line (thin = baseline, thick = pain/neutral condition) as shown in the legend.

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Fig. 5.

Radar plots for each of the five incising frequencies (4.6, 5.3, 6.2, 7.6 and 10.4 Hz) with the percent of preferred direction of incising shown in the X-Z plane in 6 degree increments. The value plotted on a radar plot at each increment represents the percentage of time the mouse incised in that direction. The preferred directions of incising are shown for the two different conditions (acidic saline condition, left side, $n = 13$ F and 13 M; neutral saline condition, right side, $n = 4$ F and 4 M). Within each radar graph, overlays of the preferred incising directions for baseline and acidic/neutral saline conditions are shown for each sex. Statistically significant differences between pairs of traces are shown around the periphery of each graph at their statistically different locations over the range of 360 degrees.

Significantly different pairs are represented by the intensity (light = male, dark = female) and size of the line (thin = baseline, thick = pain/neutral condition) as shown in the legend.

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Fig. 6.

Radar plots for each of the five incising frequencies (4.6, 5.3, 6.2, 7.6 and 10.4 Hz) with the percent of preferred direction of incising shown in the Y-Z plane in 6 degree increments. The value plotted on a radar plot at each increment represents the percentage of time the mouse incised in that direction. The preferred directions of incising are shown for the two different conditions (acidic saline condition, left side, $n = 13$ F and 13 M; neutral saline condition, right side, $n = 4$ F and 4 M). Within each radar graph, overlays of the preferred incising directions for baseline and acidic/neutral saline conditions are shown for each sex. Statistically significant differences between pairs of traces are shown around the periphery of each graph at their statistically different locations over the range of 360 degrees.

Significantly different pairs are represented by the intensity (light = male, dark = female) and size of the line (thin = baseline, thick = pain/neutral condition) as shown in the legend.

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Fig. 7. Bar graph of the mean ($\pm$ st. err.) Z/X ratios for each incising frequency. Statistical differences of pairs of incising frequencies are shown by an asterisk (*) and represents $p < 0.05$.

Fig. 8.
Image of a female mouse incising chow in inverted (nearly 180 degree rotation of head) frontal position with the 3D force transducer in front of the mouse.

Repeated Measures Analysis of Variance - Test for Z/X Ratio Differences Between Sex, Experimental Condition and Incising Frequencies.

Table 1

Effect	SS	Degr. of Freedom	MS	F	p
Sex	5.067	1	5.067	0.08	0.77
Hz	221.55	4	55.39	8.09	.000016*
Condition	43.02	1	43.01	1.36	0.26
Condition*Sex	20.49	1	20.49	0.65	0.43
Hz*Condition	17.47	4	4.37	1.11	0.36
Hz*Condition*Sex	17.81	4	4.45	1.13	0.35

Table 2

X-Y Axis – Repetitive Neutral Saline Injections

Incising Frequency	Male or Female	Baseline or Neutral	Median Force (deg.)	Statistical Comparisons for Each Incising Frequency							
4.6 Hz	Male	Baseline	270	x							
	Male	Neutral	276								
	Female	Baseline	270	x							
	Female	Neutral	264								
5.3 Hz	Male	Baseline	270	x							
	Male	Neutral	270								
	Female	Baseline	264	x							
	Female	Neutral	270								
6.2 Hz	Male	Baseline	270	x							
	Male	Neutral	276								
	Female	Baseline	264	x							
	Female	Neutral	270								
7.6 Hz	Male	Baseline	276	x							
	Male	Neutral	276								
	Female	Baseline	270	x							
	Female	Neutral	270								
10.4 Hz	Male	Baseline	270	x							
	Male	Neutral	270								
	Female	Baseline	270	x							
	Female	Neutral	270								

n.s., not significant.

Table 3

X-Y Axis – Repetitive Acidic Saline Injections

Incising Frequency	Male or Female	Baseline or Pain	Median Force (deg.)	Statistical Comparisons for Each Incising Frequency							
4.6 Hz	Male	Baseline	270	x	n.s.	n.s.	x	n.s.	x	n.s.	
	Male	Pain	270				x	n.s.			
	Female	Baseline	270	x					x		
	Female	Pain	270				x				
5.3 Hz	Male	Baseline	270	x			x				
	Male	Pain	270				x				
	Female	Baseline	270	x					x		
	Female	Pain	270				x				
6.2 Hz	Male	Baseline	270	x							
	Male	Pain	270				x				
	Female	Baseline	270	x					x		
	Female	Pain	270				x				
7.6 Hz	Male	Baseline	270	x							
	Male	Pain	270				x				
	Female	Baseline	270	x					x		
	Female	Pain	270				x				
10.4 Hz	Male	Baseline	270	x			x				
	Male	Pain	270				x				
	Female	Baseline	270	x					x		
	Female	Pain	264						x		

n.s., not significant.

Table 4

X-Z Axis – Repetitive Neutral Saline Injections

Incising Frequency	Male or Female	Baseline or Neutral	Median Force (deg.)	Statistical Comparisons for Each Incising Frequency							
4.6 Hz	Male	Baseline	204	x							
	Male	Neutral	198								
	Female	Baseline	192	x							
	Female	Neutral	198								
5.3 Hz	Male	Baseline	204	x							
	Male	Neutral	204								
	Female	Baseline	216	x							
	Female	Neutral	198								
6.2 Hz	Male	Baseline	216	x							
	Male	Neutral	228								
	Female	Baseline	222	x							
	Female	Neutral	210								
7.6 Hz	Male	Baseline	210	x							
	Male	Neutral	228								
	Female	Baseline	192	x							
	Female	Neutral	210								
10.4 Hz	Male	Baseline	192	x							
	Male	Neutral	192								
	Female	Baseline	192	x							
	Female	Neutral	198								

n.s., not significant.

Table 5

X-Z Axis – Repetitive Acidic Saline Injections

Incising Frequency	Male or Female	Baseline or Pain	Median Force (deg.)	Statistical Comparisons for Each Incising Frequency							
4.6 Hz	Male	Baseline	210	x							
	Male	Pain	204								
	Female	Baseline	210	x							
	Female	Pain	192								
5.3 Hz	Male	Baseline	246	x							
	Male	Pain	240								
	Female	Baseline	234	x							
	Female	Pain	192								
6.2 Hz	Male	Baseline	252	x							
	Male	Pain	252								
	Female	Baseline	228	x							
	Female	Pain	192								
7.6 Hz	Male	Baseline	234	x							
	Male	Pain	246								
	Female	Baseline	198	x							
	Female	Pain	186								
10.4 Hz	Male	Baseline	192	x							
	Male	Pain	192								
	Female	Baseline	192	x							
	Female	Pain	186								

n.s., not significant.

* p < 0.05, Mann-Whitney U test

** p < 0.05, Wilcoxon Matched-Pair Signed Rank test

Table 6

Y-Z Axis – Repetitive Neutral Saline Injections

Incising Frequency	Male or Female	Baseline or Neutral	Median Force (deg.)	Statistical Comparisons for Each Incising Frequency							
4.6 Hz	Male	Baseline	258	x							
	Male	Neutral	216								
	Female	Baseline	252	x							n.s.
	Female	Neutral	228								x
5.3 Hz	Male	Baseline	264	x							
	Male	Neutral	240								
	Female	Baseline	258	x							n.s.
	Female	Neutral	240								x
6.2 Hz	Male	Baseline	264	x							
	Male	Neutral	264								
	Female	Baseline	258	x							n.s.
	Female	Neutral	258								x
7.6 Hz	Male	Baseline	270	x							
	Male	Neutral	264								
	Female	Baseline	246	x							n.s.
	Female	Neutral	252								x
10.4 Hz	Male	Baseline	240	x							
	Male	Neutral	240								
	Female	Baseline	234	x							n.s.
	Female	Neutral	246								x

n.s., not significant.

Table 7

Y-Z Axis – Repetitive Acidic Saline Injections

Incising Frequency	Male or Female	Baseline or Pain	Median Force (deg.)	Statistical Comparisons for Each Incising Frequency							
4.6 Hz	Male	Baseline	258	x							
	Male	Pain	258								
	Female	Baseline	252	x							
	Female	Pain	204								
5.3 Hz	Male	Baseline	270	x							
	Male	Pain	264								
	Female	Baseline	264	x							
	Female	Pain	246								
6.2 Hz	Male	Baseline	270	x							
	Male	Pain	270								
	Female	Baseline	258	x							
	Female	Pain	246								
7.6 Hz	Male	Baseline	270	x							
	Male	Pain	270								
	Female	Baseline	252	x							
	Female	Pain	234								
10.4 Hz	Male	Baseline	246	x							
	Male	Pain	240								
	Female	Baseline	228	x							
	Female	Pain	186								

n.s., not significant.

* p < 0.05, Mann-Whitney U test