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A Feedback-Controlled Dynamic Linear Actuator to Test Foot Withdrawal Thresholds in Rat

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Introduction

Many experiments performed in somatosensory research rely upon measuring or estimating the force at which an animal lifts its paw in response to a punctate mechanical stimulus. While mechanical stimulators are now available, the vast majority of methods utilize a series of nylon monofilaments (modern renditions of “von Frey hairs” i.e., Wynnes Symmes filaments, Stoelting Corp), each of which produces a characteristic force (Bove et al 2003). The use of these devices is subject to limitations of the accuracy of the devices, as well as experimenter error and bias (Bove 2006; Bove et al 2003; Fruhstorfer et al 2001). These potential measurement errors make the tests less sensitive, making false negative results more likely. Here we describe studies of nociception in rats, done using a mechanical stimulator that delivers controlled, accurate forces and is not susceptible to operator bias.

Methods

Apparatus

Thresholds for rat foot withdrawal were tested using a mechanical stimulator to advance a probe (either sharp or blunt) against the plantar surface of a hind foot. Rats were studied in a plastic 14 × 14 cm enclosure with a perforated aluminum floor (Pitcher et al 1999; Wallas et al 2003). The openings in the floor were circular, with a diameter of 5 mm. A mechanical stimulator (Figure 1) was positioned underneath the floor, below one of the rat's hind feet. When operated, the stimulator advanced a probe until it just contacted the plantar surface of a hind foot. Once the device made contact with the foot, the vertical position of the foot was monitored continuously by the apparatus. The force of application was linearly increased until the animal withdrew its foot, and the force applied at the moment of foot withdrawal was taken as the threshold.

The stimulator (Figure 1) consisted of a linear motor (Baldor LMNM-1), that was controlled by a Harmonica motion control system (Elmo Motion Control Inc.), and incorporated a linear encoder (MicroE Systems Mercury 2000) for measuring shaft displacements. A linear motor actuates its shaft along a single direction, and the force exerted by the shaft is proportional to the current supplied to the motor. The motor can be operated in a position control mode by

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varying the current so as to maintain a particular linear encoder readout, or in a simulated force control mode by regulating the current supplied to the motor using the "current-limitation" function of the controller. In this latter mode of operation, the motor is run in position control; the motor is set to seek its maximal displacement, but the current to the motor is limited to a particular value. Thus the current (and therefore the force) is under program control.

The linear motor was fitted with a probe on the top of its shaft. Loads applied by the stimulator were estimated by measuring the current passing through the motor. The relationship between current and the resulting force was determined in calibration trials in which the motor pushed against a load cell. In these trials, the current was varied and the resulting loads were measured. The motor was operated only in the range of shaft positions for which this relationship was linear. We express loads (i.e., forces) in units of centiNewtons (cN). This unit was chosen because 1 cN is approximately the weight of a 1 gram mass.

Two stimulating tips were used. One, termed 'sharp', was a tapered steel rod with a spherical tip that had a diameter of 0.1 mm. When pushed against one's skin, it elicited a sensation of sharp pain. The other, termed 'blunt', was a steel cylinder with a spherical tip that had a diameter of 1.0 mm. When pushed against one's skin, the resulting sensation was of indentation but not pain.

The stimulator was controlled using a program written in LabView (National Instruments). This program continuously monitored and recorded in a data file both the current passed through the motor (i.e., the load) and the position of the shaft. The sampling interval was 30 msec.

Animals

Experiments were performed on 12 male Wistar rats. All experiments were performed using protocols that were approved by the Institutional Animal Care and Use Committee at the Beth Israel Deaconess Medical Center.

Capsaicin treatment: In one set of experiments (6 rats) we explored how intradermal injection of capsaicin affected foot withdrawal responses. Capsaicin (Sigma) was dissolved in 95% ethanol and diluted to a final concentration of 3 mg / ml. Animals were briefly anesthetized with isoflurane. The center of both feet was marked with a spot approximately 5 mm diameter. Spots were drawn on both feet so as to blind the tester to the injection site. A 30 gauge needle was advanced intradermally until the tip was directly under the spot. 10 μl of the solution (30 μg of capsaicin) was then injected intradermally. Animals were allowed to recover from the anesthetic and were tested within 30 minutes after recovery. Out of concern that capsaicin would cause desensitization (Gilchrist et al 1996), stimuli were not applied directly to the injection site when the foot was tested.

Experimental Design

We sought to determine the effect of a variety of stimulus parameters on withdrawal thresholds. In separate experiments, we investigated the role of the rate of application of load, of static vs ramped stimuli, and of location of stimulus application on the foot. We also compared thresholds measured with the sharp and the blunt stimulus probes, in normal and capsaicin-injected animals.

Individual trials alternatively stimulated right and left feet. Intertrial intervals were approximately 60 sec. Thus, a particular foot was not stimulated more than once every 2 minutes. Trials were initiated only when the animal was not moving around the cage, when the target foot was on the floor and appeared to be supporting weight.

Each trial consisted of 3 phases (Figure 2).

Phase 1 was trial initiation. Each trial was initiated by pressing a key on either the handle of the device itself, or the computer keyboard, located some distance from the animal. In either event, the animal received no direct cues associated with beginning the trial (the stimulator was silent). The motor was operated in position control, with the current limited to exert just enough force to lift the shaft. Contacting the foot (C in Figure 2) imposed an additional load on the motor, which could not be overcome because the current was limited. Thus the motor did not achieve its target position, and this was used as the signal for foot contact. The force on the foot at contact was approximately 0.1 cN.

Phase 2 began at foot contact. The motor was then operated in a virtual force control mode, and the current-limitation setting was increased in a linear fashion. Therefore, the current to the motor increased linearly (D in Figure 2). Operating in this mode, the stimulator maintained a target force independent of the position of the shaft. Any vertical movement of the foot was matched by movement of the motor shaft, as the device moved the shaft so as to maintain the desired force against the foot. Thus, vertical movements of the foot were tracked exactly by the motor shaft and were measured by the linear encoder. Displacements were differentiated to determine vertical velocity of the foot.

Phase 3 began with detection of foot withdrawal (F in Figure 2) and ended when the trial was ended. The criterion for foot withdrawal was an upward displacement with a velocity greater than 4000 encoder counts in a single sampling period (i.e., 26.7 mm/sec). When the velocity exceeded this criterion value, the trial was ended. The motor shaft was retracted to its home position and data collection was stopped.

In many trials, animals exhibited pre-withdrawal behaviors. These took the form of brief downward movements of the foot, or upward movements that were that were slower than the withdrawal criterion. Examples of these behaviors are identified in several figures (e.g., E in Figure 2; Figures 3 and 7).

Excluded trials

Data from some trials were excluded because of behaviors exhibited by the animal. The behaviors differed depending on which probe tip was being used.

a. Sharp probe—When stimulated with the sharp probe, animals typically adopted a hunched, guarded posture and did not move around the cage very much. Typical withdrawal responses from these stimuli were sharp retractions of the foot. However, there were occasions when, during a trial, the animal engaged in behaviors (e.g. scratching or licking), apparently unrelated to the stimulus application. Sometimes, the trial would be terminated because the animal lifted its foot to lick it, and the motor shaft would fully extend, ending the trial. When withdrawals appeared to be clearly unrelated to the stimulus, the data from that trial were excluded.

b. Blunt probe—In trials with the blunt probe, we observed a different type of behavior. Rather than adopting a guarded, immobile posture, during blunt trials the animals simply walked off the probe. In many cases this made it difficult to assess what constituted a withdrawal response. We only counted trials in which the animal stayed in the same place for the duration of the trial and clearly lifted its foot.

Data Analysis

The data from each trial were analyzed to determine the threshold for foot liftoff. In some trials, complex behaviors were observed in which the foot was slowly raised, put back down on the floor, and subsequently lifted off. In order to determine whether these behaviors indicated thresholds lower than the standard liftoff criterion (i.e., 27 mm / sec), we analyzed the data file for each trial using several different criteria for liftoff. Since trials were terminated when foot velocity exceeded 27 mm/sec, these post-hoc analyses involved using only lower velocities. In the event that no withdrawal was observed during a trial (this occurred when using the blunt stimulator tip), the maximum load applied (44 cN) was entered for that trial.

Results

1. Reaction time measured in static indentation trials

In 4 rats we explored the relationship between reaction time (i.e., latency of foot withdrawal responses) and magnitude of static indentation stimuli, using the sharp stimulator tip. The stimulus magnitudes that we used were: 5.8, 6.7, 7.5, 8.3, 10, 11.6, 13.3, 15, and 16.6 cN. Stimuli were presented in sets of 9, in which each stimulus value was presented once, in a random order. Each animal was tested using 5 repeats of the 9-stimulus set, so that each stimulus was presented 5 times in each animal. The stimulus was applied and maintained constant until the animal lifted its foot (Figure 3). Each trial resulted in a withdrawal response, and the latency of the response was measured for each trial. An example of the response to a single trial is shown in Figure 3. Foot withdrawal was often preceded by an anticipatory response, seen as a downward movement in Figure 3. Mean response latencies (also referred to as reaction times) varied with the static load (Figure 4).

Reaction time decreased as static loads were increased up to 10 cN. However with static loads of 10 cN or greater, reaction time did not vary with the magnitude of static load. A one-way analysis of variance done on the data for the trials with loading between 10 and 16.7 cN revealed there was no significant effect of static load on reaction time ($F(4,108) = 0.39$, $P = 0.81$). Because the values did not differ significantly, they were averaged together to determine a mean reaction time, which was 1.58 sec.

2. Trials with Ramped Loads: Effect of Rate of Application of Load

In trials in which the load of application was ramped up, we wished to determine the effect of the rate at which the applied load was increased (i.e., the load rate). In 3 rats, we applied ramped stimuli using the following rates of load application: 3, 6, 9, and 12 cN/sec. In each trial we measured the latency of the withdrawal response and the load present at the time of withdrawal. Unexpectedly, higher rates of loading resulted in greater withdrawal thresholds (Figure 5). Threshold for withdrawal was about 11 cN in trials with load rate = 3 cN/sec and was 16 cN in trials with load rate = 12 cN/sec. We believe (see Discussion) that this counterintuitive finding happens because the stimulus continues to increase during the (rather long) reaction time. The greater the load rate, the more the stimulus grows during the reaction time.

3. Effect of location on foot

In order to explore variations in threshold at different regions of the plantar surface of the foot, ramped stimuli were applied to the heel, midfoot and foot pad regions for 3 rats (Figure 6). All trials were done using the sharp stimulator tip and a load application rate of 6 cN/sec. Thresholds were highest on the heel and on the foot pads. Thresholds were relatively lower in the mid foot region.

4. Effect of sharp and blunt tips, right vs left side, and animals

The effects of sides (right vs left foot), probe geometry (sharp vs blunt) and individual animals were studied in 6 rats. We measured thresholds in both feet, in separate experimental sessions that were separated from each other by intervals ranging from several days to a week. These trials were the control runs prior to capsaicin injections. Each rat was studied at the same time of day (morning) and all were studied using a load rate of 6 cN/sec. Altogether, approximately 40 measures of threshold were made on each foot in each animal. The applied load rate was 6 cN/sec, and stimuli were applied to the mid foot region. Figure 7 shows data, collected during typical single trials, using the sharp (Figure 7A) and blunt (Figure 7B) stimulator tips.

a. Sharp vs Blunt tip—Mean threshold measured with the sharp tip was 13.5 cN, vs 33.7 cN measured using the blunt tip. The difference was highly significant ($P < 0.001$). Given the geometry of the two tips, compressive stresses were on the order of 17.5 mPa for the sharp tip and 440 kPa for the blunt tip.

b. Right vs Left foot—Thresholds measured with the sharp tip were not significantly different ($P = 0.10$) between right and left feet (14.3 vs. 12.8 cN). Neither were the differences between feet different when measured with the blunt probe (33.9 cN vs 33.5 cN for right and left feet, respectively). This difference was not statistically significant ($P > 0.50$).

c. Animals—There were significant differences in withdrawal thresholds between individual rats. In the 6 rats that were tested, sharp tip thresholds ranged from 10.9 to 15.4 cN (Figure 8). The differences were statistically significant ($P = 0.003$).

5. Changes in thresholds with repeated trials

Each trial conducted with the sharp tip can be thought of as a single trial in a classical conditioning paradigm. When the probe first touches the foot, the touch sensation can be thought of as a conditional stimulus (CS). It is inexorably followed by an unconditional stimulus (UCS), namely the same probe pushing with sufficient intensity as to cause pain and elicit an unconditional response (withdrawal). With repeated trials, an association develops between the CS and the UCS, and eventually the CS alone can elicit the withdrawal response. Observations made in 6 rats support the idea that this happened in the present experiment. In these animals, thresholds were measured when the animals were naïve. Over the next 3 weeks, the animals were used to test the effect of rates of stimulus application, to test the effect of location on the feet, and to test responses to static indentations. At the end, thresholds were again measured. In the second set of measurements, mean thresholds were lower (Figure 9A), and in many trials the rats were observed to withdraw their foot almost immediately upon being contacted by the probe. The likelihood of observing an immediate withdrawal upon contact was related to the number of trials that were interjected between the 2 threshold sessions (Figure 9B).

6. Capsaicin and hyperalgesia and allodynia

Intradermal capsaicin injections were performed in 6 rats, in order to demonstrate the use of the ramped-stimulus method to test for changes in thresholds. Each animal was studied using both the sharp and the blunt stimulator tips. All stimuli were ramped up at 6 cN/sec. Both injected and control feet were stimulated alternately.

Thresholds were measured up to a week before the capsaicin injections, immediately prior to the injections, within 30 minutes after the injections, and 12 days after the injection.

Following capsaicin injection, thresholds using the blunt probe were significantly lowered. In contrast, those measured with the sharp probe did not change (Figure 10). Thresholds measured

with the sharp tip, after injection, were not significantly different from those measured prior to injection. After a 12 day recovery period, all thresholds were the same as prior to injection.

7. Power analyses: Number of observations needed to reliably detect changes in threshold

Power analyses were performed to estimate the numbers of animals and trials that might be needed in order to observe significant changes in thresholds. These analyses were done using the sharp tip data from the 6 animals in the capsaicin experiments. The data used for these analyses were the 240 trials from the 2 pre-injection control sessions. The data were collected in just 2 sessions, and on day 1 the animals were naïve. Thus, the potential confounding effect of repeated learning trials was minimized. Power analyses revealed, for a particular number of observations per animal, the number of animals that would be needed to determine a significant (at $P = 0.05$) difference 90% of the time, for effects of 10% - 50% (Figure 11).

Discussion

Our goal in conducting these experiments was to explore how to optimally study nociception thresholds in rats, using a computer-driven stimulator in place of manually-applied von Frey filaments. Stimulators that are generally similar to ours are now commercially available (Gibbs et al, 2006), so it is of interest to systematically demonstrate how they can be used. In addition, we describe how such a stimulator can be built. The device we describe appears to be similar in some ways to that used by Gibbs et al (2006) and is a simplified version of that described by Schneider et al (1995). The stimulator described by Schneider et al (1995) is based on a feedback-controlled linear motor. However their device operates in force feedback by using an array of 3 load cells to measure the applied force. The array of load cells allows for detection of non-normal components of force. In contrast, we control force by regulating the current passed through the motor, thus simplifying the design by eliminating the load cell(s). Our device measures only the vertical component of force.

Biases are introduced when manually-applied von Frey filaments are used to evaluate nociception thresholds (Bove 2006). Filaments of increasing force are applied sequentially until a withdrawal response is observed. Bias is introduced because it is the experimenter who manually determines the load rate, decides how long to apply the stimulus, and subjectively determines what constitutes a withdrawal. The mechanical stimulator improves on this, inasmuch as load rate and trial duration are program-controlled, and withdrawal responses are judged by a consistent criterion. On the other hand, we do not completely eliminate bias since the investigator must still make judgments as to whether trials should be excluded because of extraneous motor behavior on the part of the animal.

A second important feature of the computer-controlled stimulator is that it can improve experimental throughput: i.e., speed up experiments by evaluating thresholds more quickly., and by reducing variability in the data, reduce the number of trials and animals that are needed. It is much faster to estimate threshold in a single trial in which the load is ramped up than by using a series of trials in which the foot is indented sequentially with different filaments. Furthermore, the high degree of consistency obtained with the stimulator means, as shown by our power analysis, that, it is possible to use relatively few observations in small numbers of animals (see also Gibbs et al, 2006).

A threshold is the quantitative point at which some action is triggered. The threshold we refer to is the lowest applied load that triggers a withdrawal response under the conditions of the experiment. There are a number of factors that influence the values that we obtained for threshold. Many of these factors are arbitrary. For example, the parameters of the stimuli are arbitrary, and we defined threshold using a withdrawal response of a particular (arbitrary) velocity. Stimulus velocity, in particular, affects the value of thresholds. For stimuli that were

clearly superthreshold, there was a latency of 1.6 sec for foot withdrawal. Some fraction of that latency is reaction time, i.e., the interval between detection of a stimulus and the eventual withdrawal of the foot. Therefore in trials in which the stimulus is ramped up, the stimulus continuously increases during the reaction time interval. Thus the greater the loading rate is, the greater the apparent threshold is.

As a result of the above, our measures of thresholds are referable to the particular conditions of the experiment, and should not be taken as absolute values. Nonetheless, changes in the measured threshold are very useful in evaluating the effect of experimental treatments. For example, we showed differences in thresholds along the plantar surface of the foot. Perhaps most revealing were the results of the capsaicin injection experiments. The nociception threshold, as measured with the sharp probe, was not changed by capsaicin, while thresholds measured with the blunt probe were dramatically lowered after capsaicin injection. This implies that capsaicin injection did not cause hyperalgesia but did cause allodynia.

Observing the animals during the tests revealed several aspects of their behavior that are relevant. The animal's degree of attentiveness to stimuli differed when using the sharp vs. the blunt tips. After animals had experienced several stimuli using the sharp tip, they adopted a crouching posture and moved relatively little. This made it easy to position the stimulator, and in general the tip contacted the foot at the intended location. In contrast, when studied using the blunt tip, animals moved around the cage a lot. Foot withdrawals using the blunt tip had long response latencies, on the order of 6 sec, and it was often the case that the animals did not stand still long enough to finish a trial; i.e., they simply walked off the stimulator.

A second behavior related to the posture of the hind foot. Animals often did not spread their foot out on the floor of the cage. Rather they supported themselves on their heels, and the front of the foot contacted the floor only with the footpads. This was particularly true in the case of the capsaicin-injected foot. Animals contacted the floor with the heel of the injected foot. The front half of the foot was held off the floor with the toes held protectively together. This made it very difficult to visualize where the tip contacted the foot.

The reason we did the experiments with static stimuli (as opposed to ramped stimuli) was to mimic the approach used with nylon monofilaments (von Frey hairs). Using von Frey filaments, threshold is usually defined using the probability that a particular stimulus will elicit a withdrawal response. Stimuli whose magnitude is below the detection threshold should never yield a withdrawal response, while very intense stimuli always should. We show that with sharp stimuli, such methods are confounded by application duration: using the sharp tip, all of our static loads eventually resulted in a withdrawal response. Thus, the definition of threshold varied not only with the stimulus magnitude, but also with duration of the stimulus. We did not systematically explore this approach further because it seemed an inefficient way of evaluating thresholds.

Our finding that thresholds vary across the plantar surface of the foot were not unexpected, given the apparent differences in skin thickness at the locations studied. Sensory receptors lying in the footpads and the heel would be expected to be shielded from external loads by the relatively thick skin at those locations, resulting in higher indentation thresholds. Similar findings have been reported for human feet by Perry (2006): similar to our findings, the heel had the highest threshold in that study.

The finding that capsaicin caused mechanical allodynia was consistent with other studies (Simone et al 1991; Gibbs et al 2006) reporting that finding. In contrast, our failure to demonstrate hyperalgesia was surprising in light of the many studies (LaMotte et al 1991; Simone et al 1989) that report hyperalgesia following capsaicin injections.

In light of the apparent similarity of our stimulator to that used by Gibbs et al (2006), it is of interest to note the similarity in both results i.e. thresholds and the effect of capsaicin. Gibbs et al used a 1 mm diameter rod as a probe, similar to our 1 mm diameter blunt probe. They ramped up force at 3 grams / sec while we (Figure 10) used 6 cN/sec (essentially 6 grams / sec). Before capsaicin treatment they reported thresholds of around 28 grams. We obtained a threshold of 32 grams. Since our stimulus rate was faster, it is expected that our threshold value would be greater (see Figure 5). Furthermore the effect of capsaicin treatment was essentially identical in both studies: mechanical allodynia took the form of a reduction of the threshold to about half of its initial value.

Acknowledgements

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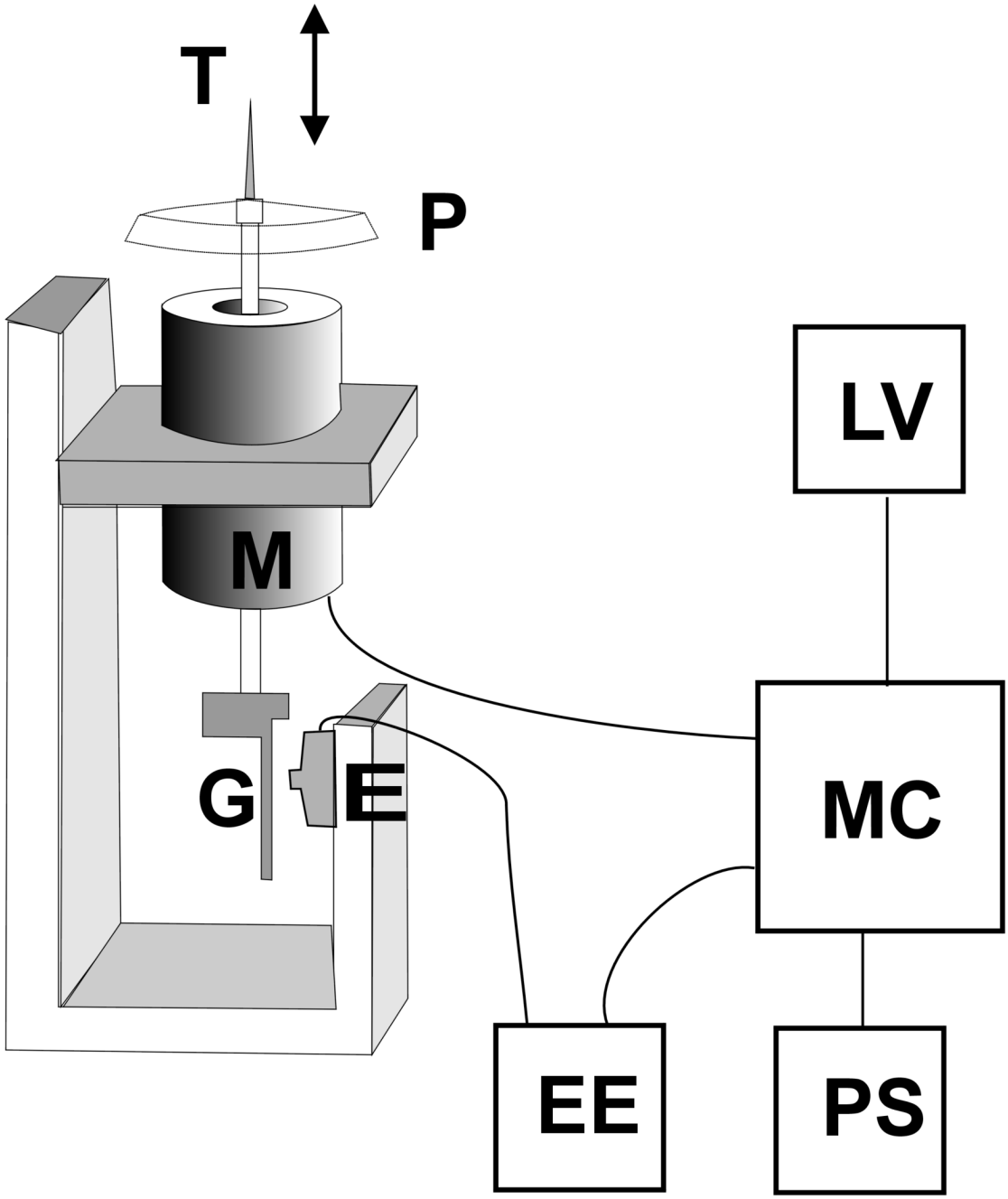

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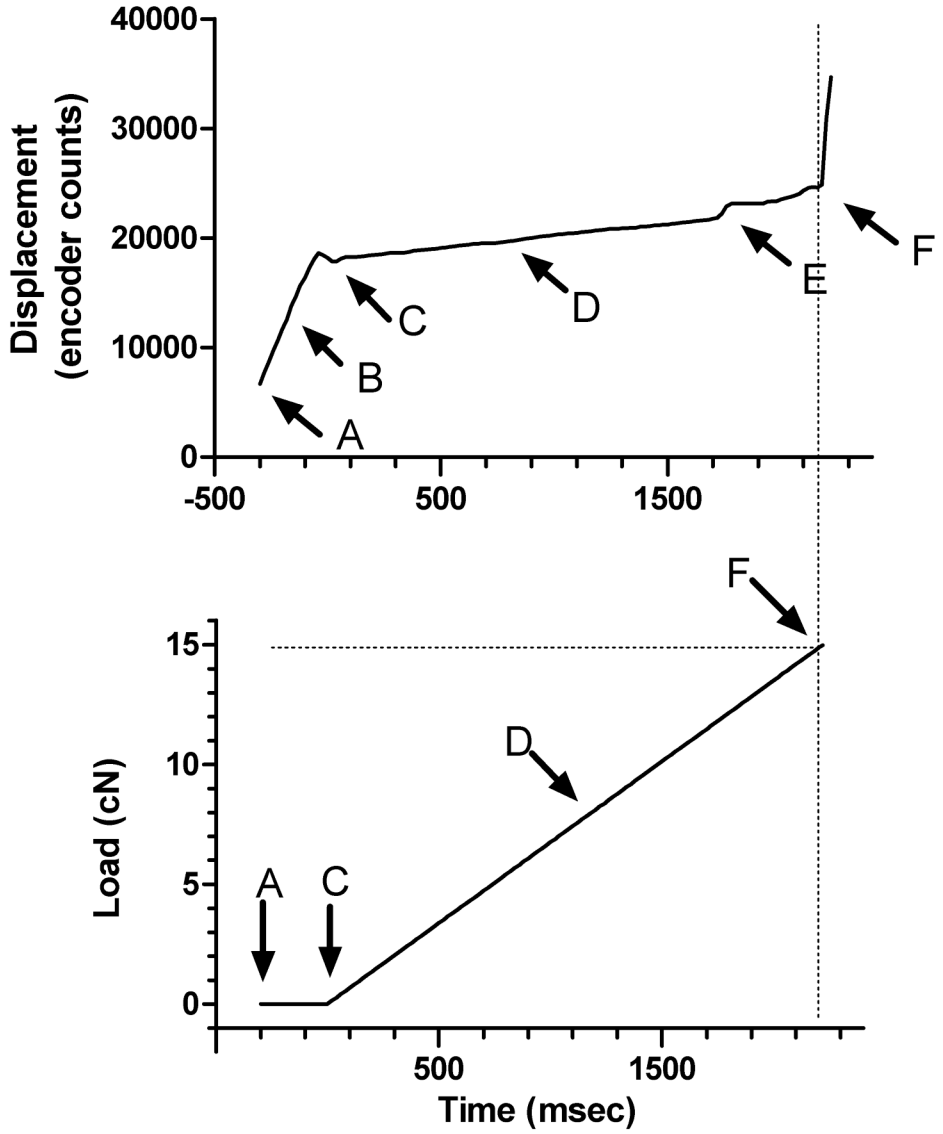

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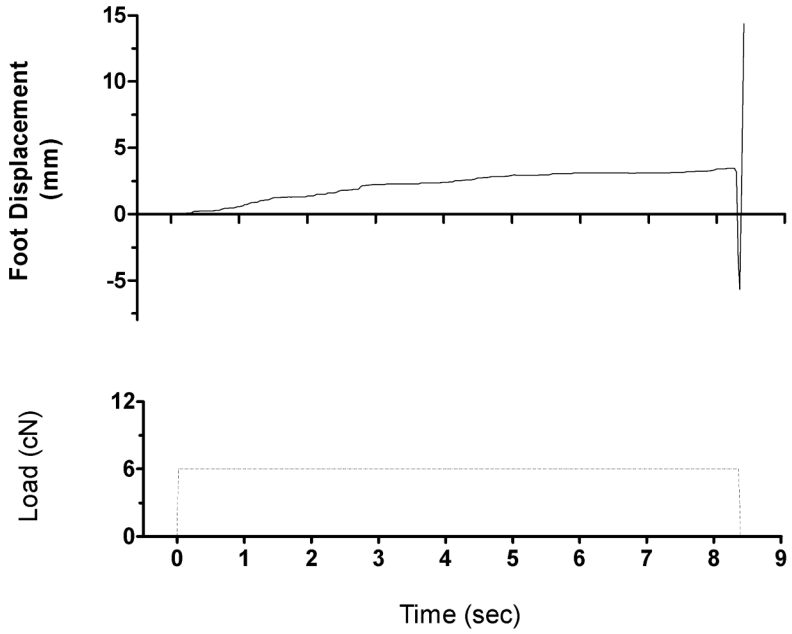

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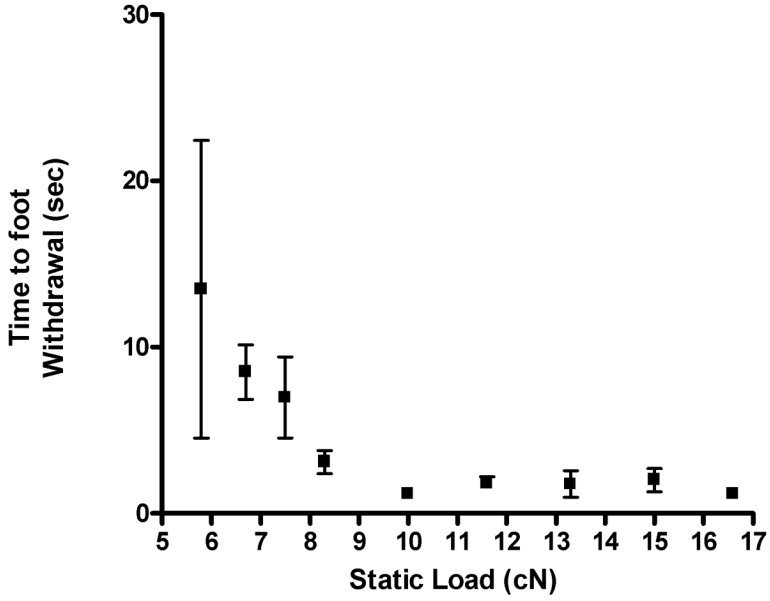

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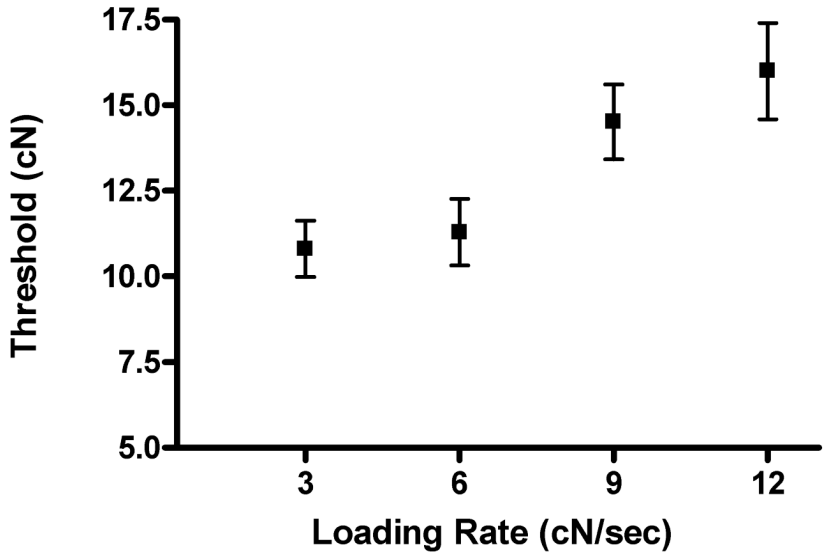

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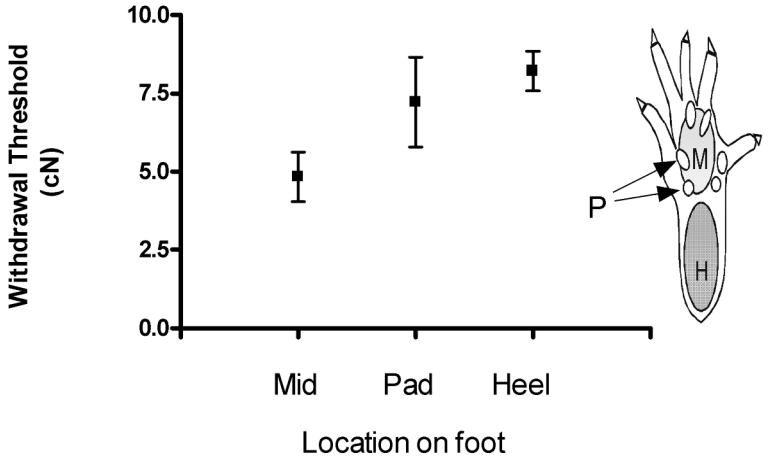

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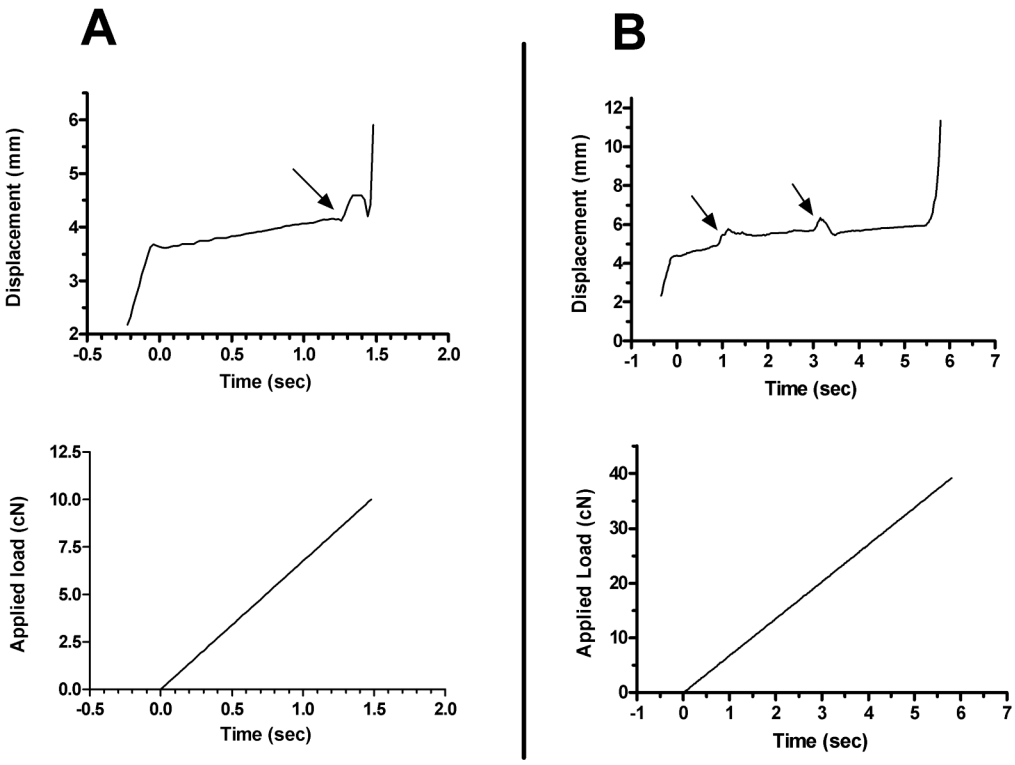

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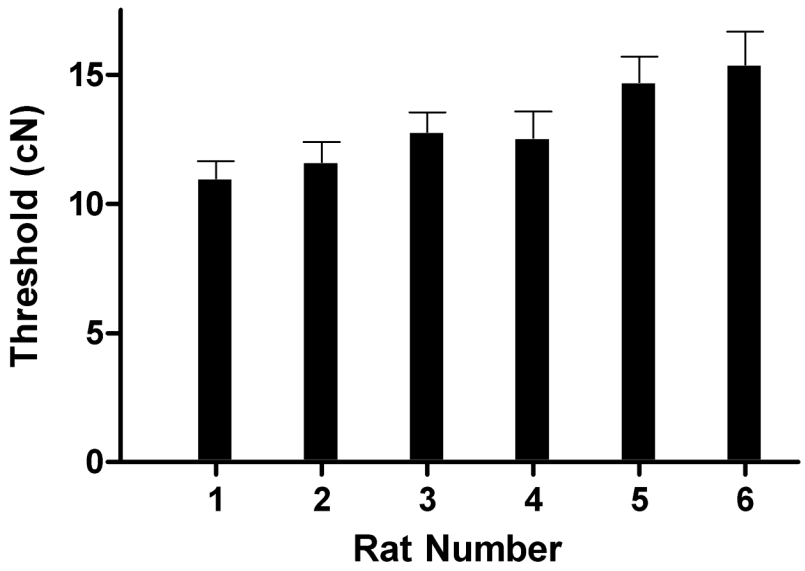

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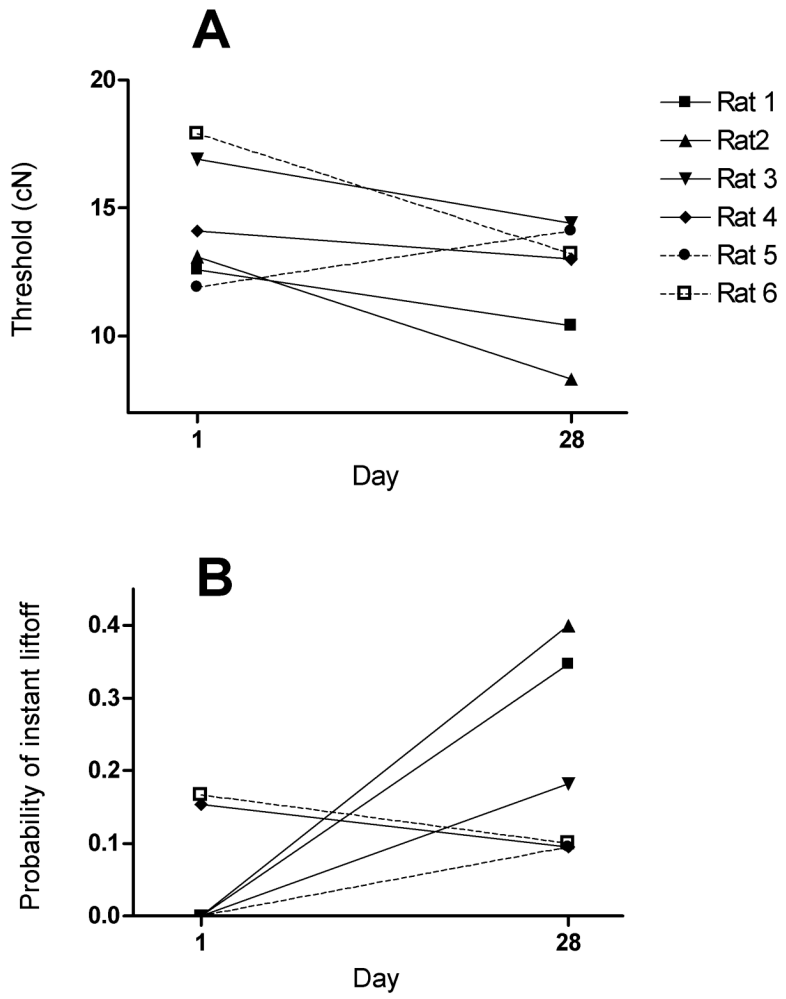

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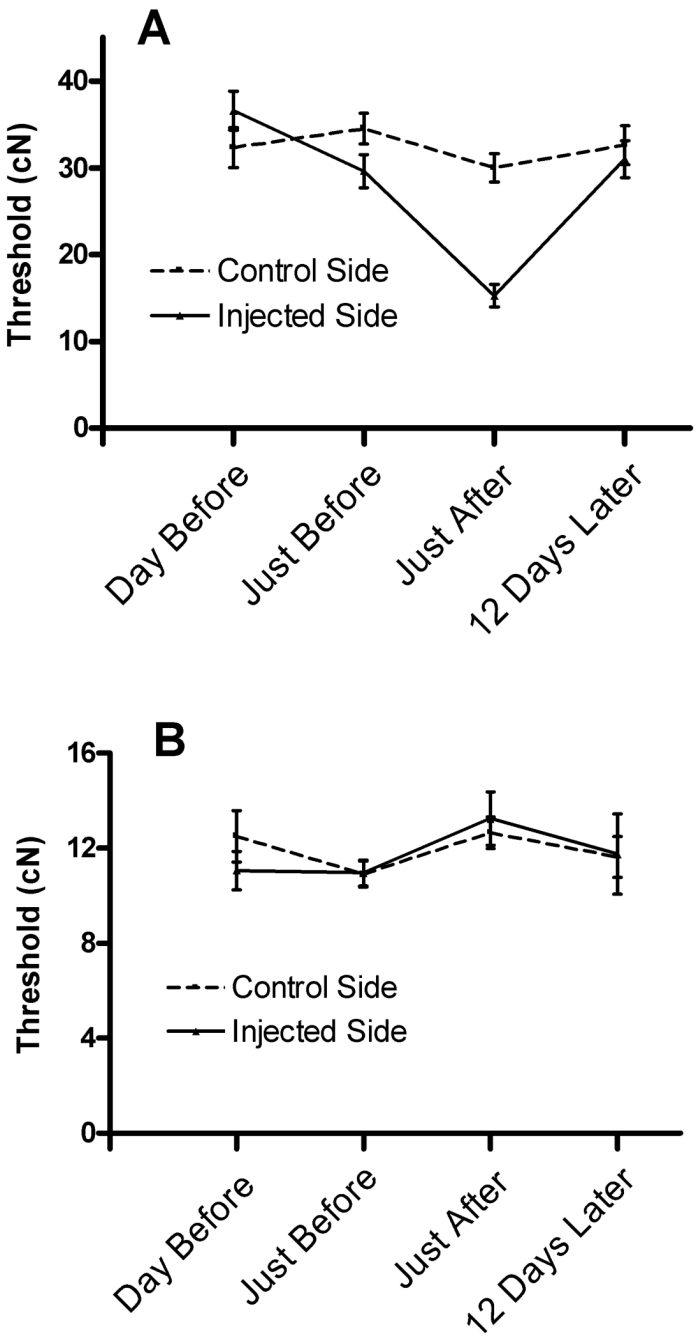

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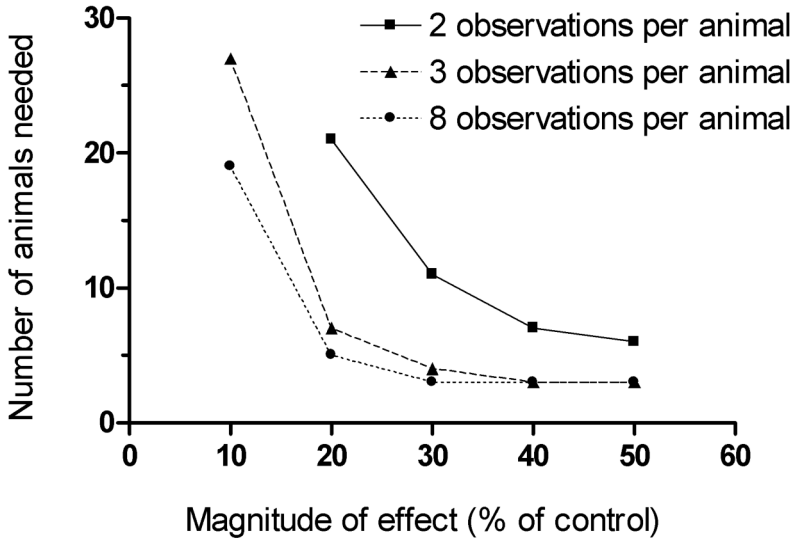

Figure 11.