Quantitative assessment of the nociceptive withdrawal reflex
in healthy, non-medicated experimental sheep

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Running title: Nociceptive withdrawal reflex in conscious sheep

Abstract

This study aimed to characterize the nociceptive withdrawal reflex (NWR) and to define the nociceptive threshold in 25 healthy, non-medicated experimental sheep in standing posture. Electrical stimulation of the dorsal lateral digital nerves of the right thoracic and the pelvic limb was performed and surface-electromyography (EMG) from the deltoid (all animals) and the
femoral biceps (18 animals) or the peroneus tertius muscles (7 animals) was recorded. The behavioural reaction following each stimulation was scored on a scale from 0 (no reaction) to 5 (strong whole body reaction). A train-of-five 1 ms constant-current pulse was used and current intensity was stepwise increased until NWR threshold intensity was reached. The NWR threshold intensity ($I_t$) was defined as the minimal stimulus intensity able to evoke a reflex with a minimal Root-Mean-Square amplitude ($\text{RMS}_A$) of 20 µV, a minimal duration of 10 ms and a minimal reaction score of 1 (slight muscle contraction of the stimulated limb) within the time window 20 to 130 ms post-stimulation. Based on this value, further stimulations were performed below ($0.9I_t$) and above threshold ($1.5I_t$ and $2I_t$). The stimulus-response curve was described. Data are reported as medians and interquartile ranges.

At the deltoid muscle $I_t$ was 4.4 mA (2.9-5.7) with an $\text{RMS}_A$ of 62 µV (30-102). At the biceps femoris muscle $I_t$ was 7.0 mA (4.0-10.0) with an $\text{RMS}_A$ of 43 µV (34-50) and at the peroneus tertius muscle $I_t$ was 3.4 mA (3.1-4.4) with an $\text{RMS}_A$ of 38 µV (32-46). Above threshold, $\text{RMS}_A$ was significantly increased at all muscles. Below threshold, $\text{RMS}_A$ was only significantly smaller than at $I_t$ for the peroneus tertius muscle but not for the other muscles.

Data achieved in this study serve as reference for experimental or clinical applications of the conscious sheep model.

**Keywords**

Nociceptive withdrawal reflex, sheep, electromyogram, stimulus-response curve

1. **Introduction**

In the last few years, various in-vivo research models involving laboratory animals have been successfully replaced by in-vitro models, but so far their utility is limited to biomedical
investigations. Among laboratory animals, the sheep is one of the most used species in experimental orthopaedic research. Analogies between ovine and human joints as well as a high comparability of bone morphology [1, 2] and easy handling turns this species into a very popular resource for this purpose [3]. However, orthopaedic research is often invasive and can only be ethically justified with the concurrent application of adequate peri- and post-operative pain management.

Pain can only be successfully controlled if correctly recognized and quantified. Various pain scoring systems have been applied to sheep [4] and goats [5] to evaluate the degree of post-operative pain and to be able to determine an eventual need of additional analgesics. In multidimensional pain scores, the general behavior of the animal, interactions between animals or between the animal and the observer as well as the sensitivity to wound palpation are evaluated. The sensitivity of the surgical wound can be further assessed by mechanical stimulation (algometry) to quantify the nocifensive pressure threshold at the surgical site [5] as well as at other parts of the limb [6]. Furthermore, the application of a heat source to the ear has been used to assess the sensitivity to increasing temperatures to determine changes in the nocifensive thermal threshold [6]. The end point of these models of acute nociception is determined by monitoring the evoked gross behavioural reaction or the thresholds at which the behavioural aversive response is elicited [7]. Unfortunately, the conclusiveness of these behavioral scores is limited by the stoic behavior of the ovine species and often the nociceptive threshold cannot be determined as cut-off stimulation levels to prevent tissue damage occurs before a behavioral reaction can be observed.

Therefore, there is a need for a non-invasive, technically simple, sensitive, specific, repeatable standardized model to objectively quantify nociception in conscious sheep. The nociceptive withdrawal reflex (NWR), first described by Sherrington at the beginning of the last century, has been extensively used for the study of experimental nociception in conscious animals and humans [8-13]. In humans, the NWR threshold was found to be the threshold of a pain sensation [14] and the NWR and its modulation have been widely used in experimental,
clinical, and pharmacologic studies as a noninvasive neurophysiologic tool to objectively assess spinal nociceptive processing in conscious and anaesthetized humans and animals [15-18]. At stimulation intensities above threshold, nociceptive reflexes of increasing sizes are obtained. If multiple stimuli of increasing intensity are applied a stimulus response curve can be described. Analgesic drugs might modify the steepness of the curve without changing the NWR threshold [19]. So far, the NWR characteristics have not been described in sheep.

The aim of the present study was to demonstrate the feasibility of evoking the NWR from the thoracic and pelvic limb in conscious non-medicated standing sheep, to investigate the species-specific characteristics of the NWR (latency and duration) in relation to gross behavioural reaction and to describe the stimulus-response curve.
2. Methods

2.1. Animals

Twenty-five adult purpose-bred Swiss Alpine Sheep were included in the study (mean weight: 63.1 kg ± 6.1(SD); age: 2-3 years). All sheep were part of an orthopaedic study starting after this experiment. Prior to the experiment, the animals were clinically examined and kept under close clinical surveillance during the entire experimental period. The experiment was approved by the Committee for Animal Experimentation of the Canton Graubuenden, Switzerland. The sheep were housed together in one stable with individual boxes. The room temperature was kept constant at 16°C. A maintenance diet consisting of a mixture of straw, silage, maize and salt was fed twice daily while water was available in an automatic water drinker. Food but not water was withheld in the morning prior to the experimental session. All measurements were performed in the morning and took place in the stable where the animals had been housed for 4 to 6 weeks. The day before the experiment the sheep were placed into purpose-made suspension slings to guarantee restrain and avoid recumbency during the experiment. Normal standing and limited walking were always possible.

2.2. Electrical stimulation and electromyography

Nociceptive withdrawal reflexes were evoked by electrical stimulation and quantified by electromyography (EMG) and a subjective score for the behavior. The behavioural reaction to each stimulation was observed and scored by the same investigator (HR) on a scale from 0 (no reaction) to 5 (strong whole body reaction; Table 1). The occurrence of maximal behavior scores (5) led to immediate interruption of the stimulations.

After clipping, shaving and degreasing of the sites for stimulation and recording, self-adhesive stimulation electrodes (Neuroline 700; Ambu®; Parsenn Produkte, Kueblis, Switzerland) were placed over the lateral digital dorsal nerves of the right thoracic and the pelvic limb on the lateral metacarpal and metatarsal trochlea, respectively. A distance of 2 cm
was kept between 2 electrodes and the anode was always placed distally. At the thoracic limb, the recording electrodes were positioned on the deltoid muscle. At the pelvic limb, the recording electrodes were placed on the biceps femoris muscle in 18 animals and on the peroneus tertius muscle in 7 animals. A minimal distance of 2 cm was kept between the recording electrodes (diameter: 32 mm; PALSplatinum®, Parsenn Produkte, Kueblis, Switzerland). A ground electrode (50x50 mm; PALSplatinum®, Parsenn Produkte, Kueblis, Switzerland was placed on the back. Flexible leads were connected to the electrodes. Mean distance between digital nerves and withers was 70 cm, 25 cm between withers and recording sites of the deltoid muscle as well as lumbar spine and recording sites of the biceps femoris muscle. Mean distance between lumbar spine and the peroneus tertius muscle was 33 cm. The resistance of each pair of stimulation electrodes had to be <5 kOhm before the beginning and was checked again at the end of each experimental session. On completion of the experiments the electrodes were removed, the skin was washed and a dermatological cream was applied.

Electrical stimulation and EMG recordings were performed by use of a specially designed computerized system [11]. The nerves were transcutaneously stimulated using a standard, constant-current electrical stimulus consisting of a train-of-five square-wave 1 ms pulses at 200 Hz, and the reflex response was recorded by surface EMG. The EMG activity was recorded during 500 ms with 512 sampling points from 100 ms prior to the stimulus until 400 ms after the stimulus. The root-mean-square amplitude (RMS$_A$) and the peak-to-peak amplitude (PP$_A$) of the reflex were calculated for the epoch 20 to 130 ms after stimulation onset (NWR interval).

2.3. Nociceptive threshold definition

The current intensity of the stimulus was initially set at 1 mA and gradually increased in steps of 1 mA up to a maximum of 40 mA. Once the animal started to react, adjustments in steps of 0.2 mA were made until a precise threshold was determined. This protocol was applied to minimize the number of stimulations and therefore to reduce the stress for the
animals. Threshold intensity (It) was defined as the minimal stimulus intensity able to evoke an EMG response of 20 µV (RMSA) with a duration of at least 10 ms within the NWR interval and a minimal reaction score of 1/5 (Table 1). If no reflex response was elicited, the current was gradually increased. Two additional stimulations at threshold intensity were performed to verify reproducibility of the response. If the reflex was not clearly elicited by both stimulations, the search for a stable threshold was continued. At least 20 s elapsed between stimulations. Once It was defined and confirmed, stimulations below (0.9It) and then above threshold intensity (1.5 and 2It) were applied once to describe the stimulus-response curve. Each reflex response was quantified by its latency, duration and amplitude in the electromyographic response. The reflex latency was defined as the time elapsing from the stimulus onset to the reflex onset (EMG deflection) while the duration of the reflex was determined as the time elapsed from the reflex onset to the end of a continuous EMG deflection by visual inspection of the records.

2.4. Statistical Analysis

Data analysis was performed using statistical software (Sigma Stat, Version 3.5, Systat Software). Nonparametric analysis of data was chosen on the basis of tests for normality of distribution. Friedman repeated measures were applied to compare the effects of stimulation intensity levels on the reflex characteristics. Post-hoc Wilcoxon signed rank test was used to compare It values with the values at different stimulation intensities (0.9It, 1.5It and 2It).

A chi square test was used to evaluate the portions of animals showing reflexes at 0.9It and Spearman rank correlation was performed to evaluate the degree of correlation between RMSA and behaviour scores. Significance was set at p<0.05.

3. Results

The sheep were judged to be healthy at the physical examination. They tolerated the experiments well, as the observed nocifensive reactions stopped immediately after stimulation, and as they were not accompanied by any persistent behavioural abnormalities or tissue damage.
With the current intensities used to elicit the NWR in the present study, none of the sheep appeared severely distressed. All results are reported in Table 2.

Nociceptive withdrawal reflexes could be evoked and recorded from all muscles under study (deltoid, biceps femoris and the peroneus tertius muscles). Intensities necessary to evoke a NWR varied among individuals and were muscle-specific.

Determination of the nociceptive threshold ($I_t$) was followed by stimulation at $0.9I_t$. Out of 25 recordings from the deltoid muscle 4 had no deflection from the baseline, 6 had subthreshold EMG activity bursts while 15 had a NWR ($p < 0.01$). Out of 18 recordings from the biceps femoris muscle 5 had no deflection from baseline, 2 had subthreshold EMG activity bursts and 11 had a NWR ($p < 0.01$). Out of 7 recordings at the peroneus tertius muscle 6 had subthreshold EMG activity and only 1 had a NWR ($p = 0.7$). Compared to $I_t$, latency was significantly increased at $0.9I_t$ for the deltoid muscle while for the muscles of the pelvic limb median latency was only slightly but non-significantly increased below threshold. Above threshold ($1.5I_t$ and $2I_t$), latency was significantly decreased for the deltoid muscle and for the biceps femoris muscle if compared to $I_t$ while for the peroneus tertius muscle only a non-significant reduction could be detected.

Compared to $I_t$, reflex duration was significantly shorter at $0.9I_t$ for the deltoid muscle but not for the muscles of the pelvic limb. Above threshold, reflex duration increased significantly for the deltoid muscle but not for the muscles of the pelvic limb.

Compared to $I_t$, median RMS$_A$ was significantly smaller at $0.9I_t$ for the deltoid muscle and the peroneus tertius muscle but not for the biceps femoris muscle. Above threshold, RMS$_A$ and PPA were significantly increasing for all muscles.

Compared to $I_t$, the behavioural reaction score was reduced at $0.9I_t$ for the forelimb but not for the hind limb. Above threshold, increasing stimulation intensities were accompanied by increasing reaction scores at the deltoid and at the biceps femoris muscle but not at the peroneus tertius muscle. A correlation between RMS$_A$ and the corresponding reaction scores could be detected at for the deltoid ($R=0.71; p<0.001$) and the biceps femoris muscles ($R=0.50; p=0.012$).
at 0.9I, but neither for the peroneus tertius muscle at 0.9I, nor for any muscle at I, or above

threshold.
4. Discussion

The present study demonstrates the feasibility of evoking the NWR by electrical stimulation from both thoracic and pelvic limbs in conscious non-medicated standing sheep. Individual NWR thresholds were determined, the effects of stimulus intensity increase were assessed and the neurophysiological species-specific characteristics of the reflex were described. Reflex recording was accompanied by a scoring of the behavioural reaction following each stimulation.

The NWR model is based on the use of a short lasting, reproducible electrical stimulus to evoke the reflex and on the possibility to quantify the withdrawal reaction using electromyography in addition to the observation of gross behaviour. The stimulation of A-beta fibres with a conduction velocity of 96 m s\(^{-1}\) would lead to a reflex latency of 10 ms in adult sheep [20]. With afferent nerve conduction velocities in the A-delta range of 4-36 m s\(^{-1}\) [21], some delay in the spinal cord and an efferent velocity of 100 m s\(^{-1}\) [22] a nociceptive reflex can be expected between 19 and 175 ms in sheep. In our recordings, most of the EMG activity was observed between 20 and 130 ms following onset of the stimulus, and therefore this epoch was chosen as a fixed interval to quantify reflex activity. A comparable post-stimulation window of 20-100 ms has been previously selected in dogs [9].

Beside the temporal aspect the NWR was quantified by the evaluation of its energy using the methods of the root mean square amplitude (RMS\(_A\)) and of the peak to peak amplitude (PP\(_A\)). While the PP\(_A\) value corresponds to the dimension of the maximal spike within the reflex burst, the RMS\(_A\) provides a measure of the integrated energy over the predefined interval including the whole reflex burst and is therefore typically used in NWR experimental studies [23].

An individual reflex threshold could be defined for each muscle. In some cases, slight alterations in stimulus intensity led to significant changes in reflex characteristics and behavioural reaction. If the evoked reflex was too large, the stimulation intensity was decreased until the NWR threshold could be precisely defined [25].
Even though reflex characteristics could not only be recognized at $I_t$ and at suprathreshold intensities but also sporadically at $0.9I_t$, the reflex was lost when the nociceptive threshold was to be confirmed at this intensity with 2 additional stimuli. During the experiment a learning effect of the animal was avoided as they could not relate any noise or event to the stimulus. Measurements were only performed when the sheep was standing on 4 limbs to avoid any possible influence of weight distribution on the evoked limb reflex.

In horses, stimulus intensity necessary to evoke a nociceptive threshold was comparable to NWR thresholds achieved in this study [26]. In dogs, the stimulus intensity needed to evoke a NWR was lower [9]. Both, sheep and horses were standing during the measurements and therefore the limbs were weight bearing while the dogs were positioned in lateral recumbency and any muscle activity was avoided. A silent background EMG was mandatory in all studies to start the stimulation process and any reflex facilitation due to active muscle contraction prior to stimulation could be excluded [27]. Interestingly, when Rossi et al. [28] stimulated electrically the sole of the foot of human volunteers at pain threshold intensity in standing position the reflex response decreased with increasing weight on the stimulated limb. In contrary, unloading induced a generalised enhancement of NWR excitability [29].

Recordings from the deltoid, the biceps femoris or the peroneus tertius muscles allowed a determination of a muscle specific NWR latency and a quantification of the extent of muscular activity in response to the stimulus. Differences in latencies and NWR thresholds among muscles appear to be a result of a muscle-specific response pattern [30, 31]. The main function of the deltoid muscle of the thoracic limb is the flexion of the shoulder joint. In this muscle the most linear correlation between stimulus intensity and behavioral expression could be observed (Fig. 2). Electrical stimulation of the lateral aspect of the thoracic digit strong enough to provoke actual withdrawal was consistently evoking a visible immediate flexion of the shoulder and the elbow joint. This was accompanied by EMG activity at the deltoid muscle.

After electrical stimulation of the pelvic limb inducing a withdrawal, an immediate flexion of the knee joint accompanied by a partial extension of the hip joint could be seen. In sheep, the
contraction of the biceps femoris muscle leads to extension of the hip joint and flexion of the
knee joint due a division of its structure into a cranial and a caudal part while in humans a
flexion of the knee is the only function of this muscle where it has been previously used in
experimental NWR studies in humans [8]. The peroneus tertius muscle is a flexor of the tarsus
and an extensor of the knee joint thereby mainly acting as a stabilizer of the lower pelvic limb
[32]. The activation of this muscle following the stimulation of the lateral digital nerve has not
been described before. In equine studies, electromyographic measurements were performed at
the cranial tibial muscle [12, 16]. In dogs, the cranial tibial muscle and the biceps femoris
muscle were used but no difference in activation threshold between muscles could be detected
[9]. In this study the peroneus tertius muscle or the biceps femoris muscle were used since the
cranial tibial muscle is very small and the structure is tendon-like while the peroneus tertius
muscle is more prominent and the size is comparable to the cranial tibial muscle in horses and
dogs [33].

Clear nocifensive behavioral reactions accompanied the appearance of the biceps NWR, but
not of the peroneus NWR, suggesting that the peroneus tertius might have more a balance
preparatory movement than a withdrawal function. This finding confirms that species and
muscle specific reference values are of high importance for a further use of the model [34].

After definition of the nociceptive threshold stimuli at suprathreshold intensity were
applied. The stimulus-response curve revealed a strong stimulus intensity dependence of the
NWR characteristics latency, reflex duration and behavioural reaction score in the deltoid and
the biceps femoris muscles. In the peroneus tertius muscle these reflex characteristics altered
less when the stimulus intensity was increased. Even though the reflex amplitude increased
with increasing stimulus intensity in all muscles the changes were more prominent in the deltoid
and in the biceps femoris muscles.

The lack of consistent recruitment of the EMG activity observed for the peroneus tertius
muscle suggests that this muscle is not adequate to describe the stimulus-response function,
while it provides interesting information at low stimulation intensities, being the first muscle to
be activated, before any other nocifensive reaction can be observed.

Additionally to the evaluation of the electromyographic recordings the behavioural
reactions following stimulation were evaluated by use of a specifically designed reaction score
(Table 1). The focus of the score was the extent of withdrawal of the stimulated limb as well as
the involvement of the whole body. Until now, the visual detection of a withdrawal movement
of the limb following electrical, thermal or mechanical stimulation was used as an endpoint in
various studies evaluating analgesic drugs [4, 35]. As already determined in previous studies in
other species, the combination of electromyographic and behavioural evaluations by use of the
NWR model allows quantitative determination of changes in nociception in conscious animals
[16].

Conclusion

The nociceptive withdrawal reflex following a train-of-five standard stimulus in healthy
non-medicated sheep could be determined and species- and muscle-specific characteristics of
this reflex and the corresponding stimulus-response curve could be defined. These values can
now serve as baseline data for the evaluation of analgesic drugs and techniques in this species.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people
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References


Table 1: Reaction scores used to evaluate the behavioural reaction following electrical stimulation of the thoracic and the pelvic limb

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No reaction</td>
</tr>
<tr>
<td>1</td>
<td>Slight, muscular contraction at the stimulated limb</td>
</tr>
<tr>
<td>2</td>
<td>Muscular reaction and short lifting of the stimulated limb</td>
</tr>
<tr>
<td>3</td>
<td>Extended lifting of the stimulated limb</td>
</tr>
<tr>
<td>4</td>
<td>Lifting of the stimulated limb with involvement of other parts of the body</td>
</tr>
<tr>
<td>5</td>
<td>Lifting of the stimulated limb and strong whole body reaction</td>
</tr>
</tbody>
</table>

Table 2: After definition of the nociceptive threshold, stimulations were performed at intensities below and above threshold

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0.9It</th>
<th>1It</th>
<th>1.5It</th>
<th>2It</th>
<th>P value #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulation intensity (mA)</td>
<td>4.0*</td>
<td>4.4*</td>
<td>6.6</td>
<td>8.8*</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Latency (ms)</td>
<td>39.1** (27.4-71.0)</td>
<td>31.3* (25.4-69.0)</td>
<td>25.4** (21.5-36.2)</td>
<td>24.5** (21.3-30.6)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Duration (ms)</td>
<td>65.6** (41.4-84.3)</td>
<td>66.5 (61.1-99.9)</td>
<td>100.8** (84.9-139.5)</td>
<td>116.5** (88.3-139.7)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>RMSa (µV)</td>
<td>24** (12-51)</td>
<td>62* (30-102)</td>
<td>197** (85-363)</td>
<td>260** (138-439)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>PPa</td>
<td>159** (81-385)</td>
<td>488** (221-1002)</td>
<td>1287** (551-2609)</td>
<td>1746** (1152-2926)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Reaction score</td>
<td>1** (1-1)</td>
<td>1* (1-2)</td>
<td>2* (2-2)</td>
<td>2** (2-4)</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

Thoracic limb deltoid muscle (25 animals)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0.9It</th>
<th>1It</th>
<th>1.5It</th>
<th>2It</th>
<th>P value #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulation intensity (mA)</td>
<td>6.4** (3.6-9.0)</td>
<td>70 (4.0-10.0)</td>
<td>10.4 (6.5-15.5)</td>
<td>14.0** (8.6-20.0)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Latency (ms)</td>
<td>38.2** (29.2-46.7)</td>
<td>37.2* (29.4-70.5)</td>
<td>28.8** (25.4-38.7)</td>
<td>27.4** (24.9-32.6)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Duration (ms)</td>
<td>77.3 (49.7-101.7)</td>
<td>79.3 (64.5-96.9)</td>
<td>105.7 (66.1-134.1)</td>
<td>111.6 (78.3-140.9)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>RMSa (µV)</td>
<td>25** (13-55)</td>
<td>43* (34-50)</td>
<td>88* (33-115)</td>
<td>123** (70-178)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>PPa</td>
<td>164** (81-349)</td>
<td>245* (234-303)</td>
<td>495.6* (208-705)</td>
<td>627** (429-1107)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Reaction score</td>
<td>1* (1-1)</td>
<td>1* (1-2)</td>
<td>2* (1-2.25)</td>
<td>4** (1.75-4)</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

Pelvic limb biceps femoris muscle (18 animals)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0.9It</th>
<th>1It</th>
<th>1.5It</th>
<th>2It</th>
<th>P value #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulation intensity (mA)</td>
<td>6.4** (3.6-9.0)</td>
<td>70 (4.0-10.0)</td>
<td>10.4 (6.5-15.5)</td>
<td>14.0** (8.6-20.0)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Latency (ms)</td>
<td>38.2** (29.2-46.7)</td>
<td>37.2* (29.4-70.5)</td>
<td>28.8** (25.4-38.7)</td>
<td>27.4** (24.9-32.6)</td>
<td>P &lt; 0.001</td>
</tr>
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<td>111.6 (78.3-140.9)</td>
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<td>627** (429-1107)</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Reaction score</td>
<td>1* (1-1)</td>
<td>1* (1-2)</td>
<td>2* (1-2.25)</td>
<td>4** (1.75-4)</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

Pelvic limb peroneus tertius muscle (7 animals)
Stimulation intensity, latency (onset of stimulation to onset of reflex), duration (onset of reflex to end of reflex), reflex amplitude (root mean square amplitude (RMS_A); root mean square amplitude of the reflex at the NWR interval) and reaction score (1-5) after transcutaneous electrical stimulation of the lateral digital nerve of the forelimb and of the hind limb at the nociceptive threshold intensity (I_t) as well as at intensities corresponding to 90, 150 and 200% of the threshold (0.9, 1.5, 2I_t). Results are reported as median values and interquartile ranges (IQR). a, b, c data with the same superscript letters are significantly different (p < 0.05; Friedman test); * data with a star are significantly different from I_t (p < 0.05; Signed rank test); # p-values represent results for Friedman Repeated Measures Analysis of Variance on Ranks.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0.9I_t</th>
<th>I_t</th>
<th>1.5I_t</th>
<th>2I_t</th>
<th>P value #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stimulation intensity (mA)</td>
<td>3.0³ (2.7-4.0)</td>
<td>3.4² (3.1-4.4)</td>
<td>5¹ (4.7-6.6)</td>
<td>6.4⁵ (6.1-8.7)</td>
<td></td>
</tr>
<tr>
<td>Latency (ms)</td>
<td>26.4 (21.8-35.0)</td>
<td>24.5 (20.8-28.9)</td>
<td>22.5 (21.5-23.3)</td>
<td>21.5 (20.5-22.5)</td>
<td>P = 0.025</td>
</tr>
<tr>
<td>Duration (ms)</td>
<td>29.9³ (20.6-57.0)</td>
<td>46 (35.3-76.3)</td>
<td>61.7 (48.7-93.5)</td>
<td>100.8³ (62.1-131.6)</td>
<td>P = 0.034</td>
</tr>
<tr>
<td>RMS_A (µV)</td>
<td>11³ (9-17) P=0.016</td>
<td>38 (32-46)</td>
<td>116⁵ (75-166) P=0.016</td>
<td>108³ (71.75-165.25) P=0.016</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>PPA</td>
<td>85³ (59-140) P=0.016</td>
<td>278 (233-368)</td>
<td>720⁵ (579-1077) P=0.016</td>
<td>735³ (489-1122) P=0.016</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Reaction score</td>
<td>1 (0-1)</td>
<td>1 (1-1)</td>
<td>1 (1-1)</td>
<td>1 (1-1)</td>
<td>P = 0.029</td>
</tr>
</tbody>
</table>