Pre-test habituation improves the reliability of a handheld test of mechanical nociceptive threshold in dairy cows

CrossMark

P.M. Raundal [a,e,*], P.H. Andersen [b], N. Toft [c], M.S. Herskin [d], B. Forkman [e], L. Munksgaard [d], A.M. de Passillé [f], J. Rushen [f]

[a] Dairy and Cattle Farming, SEGES, Agro Food Park 15, DK-8200 Aarhus N, Denmark
[b] Department of Clinical Sciences, Swedish Agricultural University, Ulls väg 26, Box 7054, 750 07 Uppsala, Sweden
[c] National Veterinary Institute, Technical University of Denmark, Bülowsvej 27, DK-1870 Frederiksberg C, Denmark
[d] Department of Animal Science, Aarhus University, AU-Foulum, Blichers Allé, Postbox 50, DK-8830 Tjele, Denmark
[e] Department of Large Animal Science, University of Copenhagen, Grønnegårdsvej 8, DK-1870 Frederiksberg C, Denmark
[f] University of British Columbia, PO Box 202, 6947 Highway 7, P.O. Box 1000, Agassiz, British Columbia V0M 1A0, Canada

ARTICLE INFO

ABSTRACT

Mechanical nociceptive threshold (MNT) testing has been used to investigate aspects of painful states in bovine claws. We investigated a handheld tool, where the applied stimulation force was monitored continuously relative to a pre-encoded based target force. The effect on MNT of two pre-testing habituation procedures was performed in two different experiments comprising a total of 88 sound Holsteins dairy cows kept either inside or outside their home environment. MNT testing was performed using five consecutive mechanical nociceptive stimulations per cow per test at a fixed pre-encoded target rate of 2.1 N/s. The habituation procedure performed in dairy cows kept in their home environment led to lowered intra-individual coefficient of variation of MNT ($P < 0.001$), increased MNT ($P < 0.001$) and decreased the discrepancy between applied and target force during stimulations ($P < 0.001$). Pre-test habituation improved the reliability of the handheld tool when used in dairy cows kept in their home environment.

© 2015 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

The nociceptive threshold can be identified as the intensity of a noxious stimulation which activates the pain sensory system. Persistent injury or intense stimulation may sensitize the pain processing system leading to a decreased nociceptive threshold and/or an exaggerated pain perception to stimuli at the site of the painful lesion and potentially at locations distant from the primary painful lesion (Anderson and Muir, 2005; Basbaum et al., 2009). Nociceptive threshold testing can be used to determine the presence of hyperalgesia, which is clinically characterized by an increased avoidance response, compared to baseline, elicited upon application of a noxious stimulus (Love et al., 2011). In dairy cattle mechanical (Chambers et al., 1994; Whay et al., 1997, 1998; Laven et al., 2008; Tadich et al., 2013) and thermal (laser) (Veissier et al., 2000; Herskin et al., 2003) devices have been used to apply controlled nociceptive stimulations as ramped mechanical forces or constant radiant heat until a behavioural avoidance response occurs. The use of a laser device has the advantage of applying constant nociceptive heat

stimulation remote from the subject, but its use in commercial herds is restricted due to safety issues. In bovine orthopaedic research, the application of a ramped mechanical stimulation has usually been performed by pressing a rounded steel pin against the skin of the dorsum of the cannon using an actuator to ensure gradual increase of the force at a constant rate (Ley et al., 1996; Whay et al., 1997, 1998, 2005; Laven et al., 2008; Tadich et al., 2013). The cuff-mounted actuator has been attached to the stimulation limb, which required handling and restraint of the animals, potentially affecting their responses to the stimulations (Veissier et al., 2000). Hence, the use of a handheld tool to measure mechanical nociceptive threshold (MNT) in loose housed animals kept in their home environment is of interest.

Methods to determine MNT by handheld tools have been described in sheep (Stubsjøen et al., 2010), pigs (Janczak et al., 2012; Di Giminiani et al., 2013, 2014) and cattle (Raundal et al., 2014), where large variabilities in the animals' responses to the nociceptive mechanical stimulations are typically reported. Some of these variations are reported to be related to other factors than hyperalgesia, for example fear or stress in the testing situation. As a consequence, inclusion of habituation procedures, aimed to reduce the variability, has been suggested by Raundal et al. (2014). Importantly, the habituation procedure should be aimed only at the test procedure and the initial non-noxious part of the

---

* Corresponding author at: Dairy and Cattle Farming, SEGES, Agro Food Park 15, DK-8200 Aarhus N, Denmark.
E-mail address: pra@seges.dk (P.M. Raundal).

ramped stimulation. Learning effects based on repeated ramped stimulations (Le Bars et al., 2001) or habituation or sensitization of the nervous system by repeated noxious stimulations (Bergadano et al., 2009; Mouraux et al., 2012) should be avoided.

It has been recommended to apply a ramped mechanical stimulation at a constant rate (Dundee and Moore, 1960; Janczak et al., 2012). However, when handheld tools are used, the operator's influence may attribute to variation arising from discrepancy between the actual applied force and the ramped target force, given by a predefined constant rate and stimulation time. Operator induced variation is among the main technical challenges of this methodology. Only a few reports exist from MNT studies in large animals using handheld devices, where the rate of stimulation can be controlled (Di Giminiani et al., 2013, 2014), but no studies have been described in cattle.

The objective of the present study was to investigate effects of two different pre-test procedures aiming at habituating dairy cows in the presence of the observer and to the initial tactile phase of the ramped mechanical stimulations on the reliability of a handheld methodology for MNT testing in dairy cows. The effect of habituation on the intra-individual coefficient of variation (CV) of MNT, on MNT and on the discrepancy between the actual applied force and the target force during stimulations, was evaluated.

2. Materials and methods

This study comprised two experiments. Experiment 1 tested the cows outside their home environment. It was carried out at the University of British Columbia (UBC) Dairy Education and Research Centre, Agassiz, Canada, and was approved by the institutional Animal Care Committee at UBC. Experiment 2 tested the cows in their home environment and was carried out at the Danish Cattle Research Centre, Tjele, Denmark. The animal procedures and housing complied with the Danish Animal Experiments Inspectorate, according to the Danish Ministry of Justice Act no. 1306 (November 23rd, 2007), as procedures that do not require specific approval. The habituation and testing procedures as well as visual inspections and lameness scorings (based on Thomsen et al., 2008) of the dairy cows were performed by the same observer (trained veterinarian) in both experiments.

2.1. Experiment 1: testing outside of the home environment

2.1.1. Study design

The purpose of this experiment was to conduct an initial investigation of the effect of a habituation procedure. The experiment was conducted as a matched pair design (Ersbøl et al., 2004) and consisted of a baseline test followed by a retest of all cows. The two test sessions were separated by a period where the treatment cows received a habituation procedure and the matched control cows did not. The experiment was carried out on workdays between 0900 and 1500 h during October and November 2011.

2.1.2. Animals and housing

Experimental cows were selected from the 260 cow research dairy herd at the UBC Dairy Research and education Centre in Agassiz, British Columbia, Canada. Cows were loose housed with sand-bedded cubicles, fed daily at 0700 and 1600 h, with a fresh total mixed ration formulated for high producing dairy cows and milked in a milking parlour at 0800 and 1700 h.

Eighty-five cows met the inclusion criteria: Lactating, non-lame (lameness score below 3, using a 1 (non-lame)–5 (severely lame) point scale, Thomsen et al., 2008) Holsteins, more than 30 days in milk (DIM), and more than 60 days to due date, out of which forty-six cows were chosen from pens closest to the testing area. Experimental cows were blocked in pairs by parity, DIM and state of pregnancy. Within each pair, the cows were randomly allocated to either habituation or control group. Finally pairs were randomly assigned to one of

three experimental weeks. Health status was assessed by information from the herdsmen, visual inspection by the observer and rectal temperature (based on the average of two measurements taken by a technician at the end of each nociceptive test session), within 38.0–39.0 °C. Eighteen cows were excluded at the end of the experiment (12 became lame, during the experimental weeks, two were excluded to balance number of habituation and control cows and four cows due to technical difficulties during the familiarization or testing procedures).

2.1.3. Test area and familiarization

The test area (Fig. 1) had concrete flooring which was thinly covered with sand in the mornings of each habituation and test day. Cows were restrained by a head lock on a weigh scale (Pacific Industrial Scale Co. Ltd., Richmond, British Columbia, Canada), that afforded a safe and comfortable space for testing.

Each experimental cow was familiarized during the week prior to the first test week, by walking them through the scale three times. They were restrained for 2 min on the scale during the last two passages. Cows were scored for lameness at the last passage. An extra familiarization session was given on Fridays to cows to be tested the following week. Handlers and the observer wore blue overalls.

2.1.4. Habituation procedure

Cows were baseline tested on Monday mornings and retested on Thursday mornings in the same order. Between the two test sessions, the cows received either one habituation or one control procedure. Since cattle can use the colour of clothes worn by people as a cue to discriminate between humans (Munksgaard et al., 1997), and to increase the difference between the habituation and the control procedure, the observer wore blue overalls for the baseline test and the control procedure, and wore a red coat for the habituation and retest procedures.
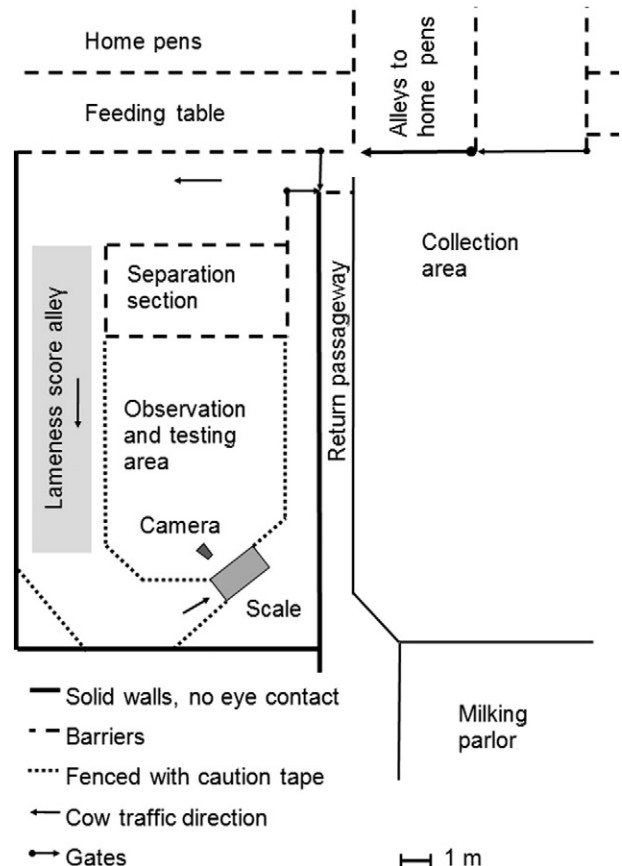

Fig. 1. Outline of testing area in Exp. 1.

For all tests, cows were walked through the scale twice. The cows were lameness scored at the last passage and restrained on the scale for approximately 5 min in total. After a 1 min break, the stimulation procedure (described in Section 2.3) was initiated.

The habituation sessions were repeated five times (from Monday to Wednesday afternoons) in the same order as for the baseline tests. Each session followed the same procedure as the baseline test, except for the colour of the observer's clothes and except that the ramped force was not applied. Instead, the backside of the handle of the testing device, opposite to the transducer head, served as a rounded surface used to gently touch the stimulation site without increasing the force. In order to decrease the risk of negative reinforcement (Sankey et al., 2010), the handle was removed from the stimulation site just prior to an impending behavioural avoidance response, visually assessed by a shift of weight between the hind limbs of the cow. Control cows were brought to the scale in an identical way, but the observer (in blue overalls) did not approach the cows.

2.2. Experiment 2: testing in home environment

2.2.1. Study design

The purpose of this experiment was to evaluate two quick habituation methods operable in the home environment of loosed housed dairy cows. Two habituation groups (H1 and H2) and one control group (C) in a parallel group design were used (Ersbøl et al., 2004). A baseline test was not included in the design to avoid a potential habituation effect by performing the baseline test. Hence, the observer wore the same coloured overalls (blue) during the experiment. The experiment was performed on three successive workdays between 0900 and 1500 h during January 2012.

2.2.2. Animals and housing

Experimental cows were selected from the 140 Danish Holsteins of the dairy herd at the Danish Cattle Research Centre (Tjele, Denmark). Here, lactating cows were loose housed on slatted, concrete flooring, fed a total mixed ration for ad libitum in take in individual feeders (Roughage Intake Control (RIC), Insentec B.V., Marknesse, The Netherlands) four times a day and milked in two Automatic Milking System units (VMS, De Leval A/S, Vejle, Denmark). Cubicles were 120×225 cm, mattress-bedded with saw dust and animals were tested in the cubicles.

Seventy cows met the inclusion criteria: more than 30 DIM, more than 60 days to expected calving and a lameness score below 3 within four weeks prior to the experimental period. Sixty-three of these cows were randomly selected as experimental animals. Seven cows were selected as reserves.

Experimental cows were blocked in groups of three by parity, lactation stage (early: 30–90, mid: 91–210 and late: >210 DIM) and due date. Within each block, the cows were randomly allocated to one of two habituation groups (H1 or H2) or to a control group (C), each consisting of 21 cows. The blocks were randomly allocated to one of three experimental days. Assessment of health status was based on information from the herdsmen, and visual inspection and rectal temperature measurements between 38.0 and 39.0 °C both performed by the observer. Nine cows were excluded during the experimental period (three cows kicked fiercely at testing, five were diseased or lame and one slaughtered) of which six could be replaced by reserve cows of the same parity and lactation stage.

2.2.3. Habituation procedure

Habituation cows were kept in the cubicle by a rope behind and a 30 s pause was provided to accommodate the cows to the restraining. The observer then began the habituation procedure by stroking the cow by hand from the base of the tail towards the stimulation site (described in Section 2.3). Stroking was stopped whenever the cow attempted to lift its legs and was then repeated beginning at the base of the tail. Stroking by hand was done repeatedly until the cow could be touched at the stimulation site for approximately 3 s without lifting its legs. The procedure was repeated with the backside of the handle of the testing device until acceptance of being touched for approximately 3 s. Hereafter, each stroke was followed by rotating the device, bringing its tip into skin contact. This was repeated until the tip could be in skin contact for 3 s without initiating a leg lift. However, a maximum duration of the total habituation session was set to 3 min in accordance with the purpose of the experiment. Cows in group H1 received one habituation session immediately before the test session, and cows in the H2 group received two habituation sessions, the first one 1–4 h before the test session, and the second one immediately before the test session.

2.3. Mechanical nociceptive threshold testing

To apply the mechanical nociceptive stimulations, a handheld Pressure Application Measurement device (PAM, Ugo Basile Comerio, Italy) was modified to bovine use by adding a 0.8 mm diameter pin with a rounded tip, attached to a custom made protective fitting (MBRose, Faaborg, Denmark) to avoid abaxial forces on the transducer during use. The device was encoded with a predetermined rate of loading force of 210 g/s (2.1 N/s). To guide the operator to increase the force in accordance with encoded rate during stimulations, the device displayed the actually applied force relative to the target force which the PAM calculated based on the encoded rate and stimulation time. A safety end point at 1500 gf (14.7 N) was determined to avoid tissue damage. For control cows, each stimulation session was initiated by the observer approaching the left hind limb of the cow and pausing for 30 s at a distance of approximately 50 cm, allowing the experimental cow to notice his presence. The tip of the device was then gently brought into contact with the skin and the ramped stimulation force was applied immediately, as close to the rate as possible. For habituation cows in Exp. 2, the last habituation procedure was succeeded by the testing procedure, so the last tip-skin contact continued into loading the ramped force. The stimulation was terminated by a behavioural avoidance response, defined as any leg movements, that abruptly terminated the contact between the tip of the device and the testing site, or if the safety end point was reached. The PAM recorded the applied force at 20 time points per s as well as the end-point given as a peak force (in gf) and a stimulation time (s) at the time point of the behavioural avoidance response. A test session consisted of five consecutive stimulations with approximately 30 s between stimulations. The stimulation site was a 2×5 cm skin area along the dorsal aspect of the middle part of the left cannon bone. Based on visual inspection, stimulations were applied approximately 1 cm apart within the stimulation site.

2.4. Outcomes and statistical methods

The recorded gf at the behavioural avoidance response was converted to Newtons: $1 \text{ gf} = 101.98^{-1} \text{ N}$. The safety endpoint, i.e. 1500 gf, was assigned as the MNT value, when no behavioural avoidance response to stimulation was observed.

The effect of the habituation method on the variation in MNT within cows was evaluated by using the intra-individual coefficient of variation (CV) based on the five stimulations per cow per test-session as the outcome. The CVs were analysed using linear mixed models and presented as estimated mean $\pm$ standard error (se).

The effect of the habituation method on the level of MNT was evaluated using the median of the five nociceptive stimulations per cow as the outcome. In Exp. 1, the difference between the MNT at baseline and at retest was calculated per cow and analysed in a linear model. In Exp. 2, the MNT data was analysed in a linear mixed effect model. For both experiments, the estimated effects on MNT were presented as estimated means $\pm$ se.

During each stimulation with the ramped loading force the PAM measured actual applied force at 20 time points per s. For each

stimulation the discrepancy between the applied force and the target force was calculated for every time point as the numerical difference between the applied and the target force divided by the target force. The discrepancy for each stimulation was then calculated as the average discrepancy for all the time points as the sum of the discrepancies divided by the number of time points minus one (as the force was applied continuously but only recorded 20 times/s) and multiplied by 100%:

$$\text{Discrepancy}(\%)$$
$$= \sum_{i=1}^{n}(|\text{applied force}_i - \text{target force}_i|/\text{target force}_i)/(n-1) * 100 \%,$$

where n was the total number of time points per stimulation. The effect of habituation on the discrepancy was analysed in a linear mixed effect model and presented as estimated means ± se.

In the statistical analysis, the maximal models were reduced by stepwise backwards elimination, with significant effects and interactions accepted at $P < 0.05$ (Crawley, 2007). Blocking factors were initially included as random effects and subsequently excluded if the variance contribution was found not to contribute significantly (using Akaike's Information Criteria). Contrasts were used to compare means within fixed effects with a pre-specified significance level of $P < 0.05$. All

analyses were made using R version 3.0.0 (R Development Core Team, 2013).

3. Results

Due to technical problems in Exp. 1, none of the measured MNTs at retest in week three was stored in the PAM device. The retest was repeated on the following day and end-point MNTs were recorded manually, and therefore the discrepancy could not be calculated on retest in week three.

In total, 28 (14 control and 14 habituation) and 60 (20 in each of the three groups) cows were included in the analyses of Exp. 1 and 2, respectively. Mean parity (± se) was 2.6 (± 0.3) and 1.6 (± 0.1), and mean days in milk (± se) were 146 (± 12) and 155 (± 12) for Exp. 1 and 2, respectively. Visual inspection of the data indicated that the number of low MNTs was reduced by habituation in both experiments, indicating fewer avoidance responses to tactile sensations (Fig. 2). Safety end point was reached three times and once in Exp. 1 and 2, respectively.

3.1. Intra-individual variation

The intra-individual coefficient of variation (CV) of MNT was calculated for each cow for each test session. Model analysis revealed group
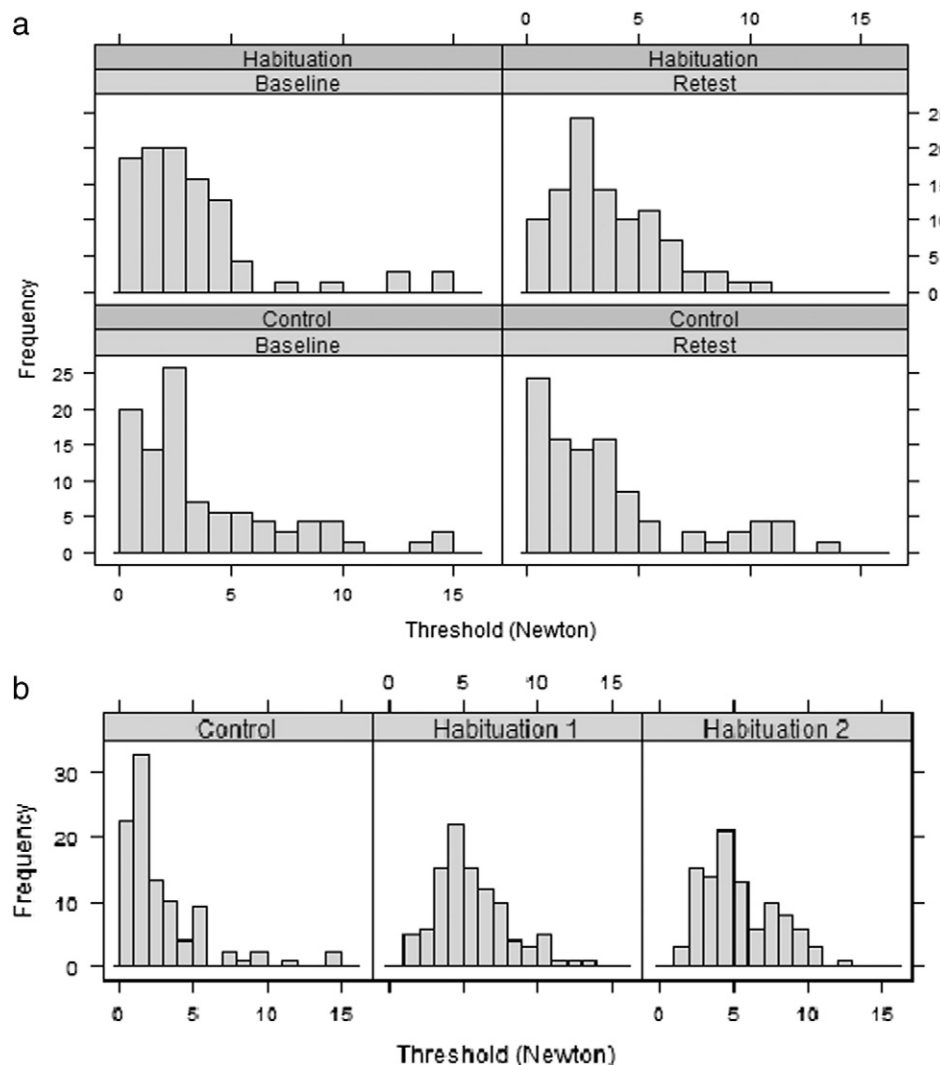

Fig. 2. Histogram of all measured threshold values given treatment group and test type in Exp. 1 (a) and treatment group in Exp. 2 (b).

(habituation or control) and treatment (H1, H2 or control) as fixed effects in Exp. 1 and 2, respectively. Random effects were found to be week number and individual cow in Exp. 1, and lactation number in Exp. 2. In Exp. 1, cows were baseline tested before and retested after the habituation procedure and no difference was found between the CV of the MNT in the two tests ($P = 0.5$). However, a main effect of group was found as cows in the habituation group had lower CV than cows in the control group (0.43 (± 0.04) vs. 0.58 (± 0.04), $P = 0.005$), at both baseline test and retest. In Exp. 2, the cows were only tested after the treatment procedures. The CV was lower in both habituation groups compared with controls. There was no difference between the two habituation groups (Fig. 3).

3.2. MNT

In Exp. 1, the difference in MNT between baseline and retest was not modified by the habituation procedure ($P = 0.7$). In Exp. 2, treatment (H1, H2 or control) was found to be a significant fixed effect and lactation number and experimental day random effects by model analysis. MNT was significantly higher in the habituation groups compared to control group with no difference between the two habituation groups (Fig. 4). The frequency of low MNTs was reduced by the habituation procedures in both experiments however, most markedly in Exp. 2 (Fig. 2).

3.3. Discrepancy between applied and target force

For a given stimulation, discrepancy indicates the average difference between the applied force and the target force (defined by the rate) in percentage of the target. In Exp. 1, model analysis revealed experimental week number as a categorical fixed effect to be included in the final model as well as test type (baseline or retest) and group (habituation or control). The individual cow was held as random effect. The discrepancy decreased from week 1 to 2 ($P < 0.001$) however, there was a strong interaction ($P < 0.001$) between test type (baseline vs. retest) and week (1 vs. 2) as the discrepancy decreased from baseline to retest in week 1 ($P < 0.001$) but not in week 2 ($P = 1.0$). The interaction was not modified by habituation ($P = 0.3$).

In Exp. 2, treatment (H1, H2 or control) was found to be a fixed effect and individual cow, experimental day and lactation number random effects. The discrepancy was reduced by habituation with no differences between the two habituation groups (Fig. 5).
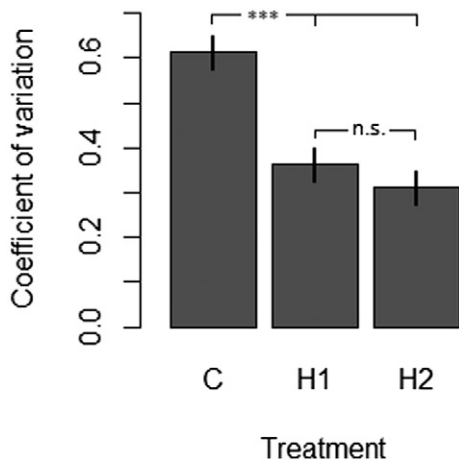

Fig. 4. Median of mechanical stimulations threshold in treatment groups in Exp. 2. Data was subjected to a mixed model for analysing effect of habituation on the median threshold. Error bars indicate standard error. C: Control (n = 20), H 1: Habituation group 1 (n = 20), H 2: habituation group 2 (n = 20). ***$P < 0.001$, n.s. not significant.
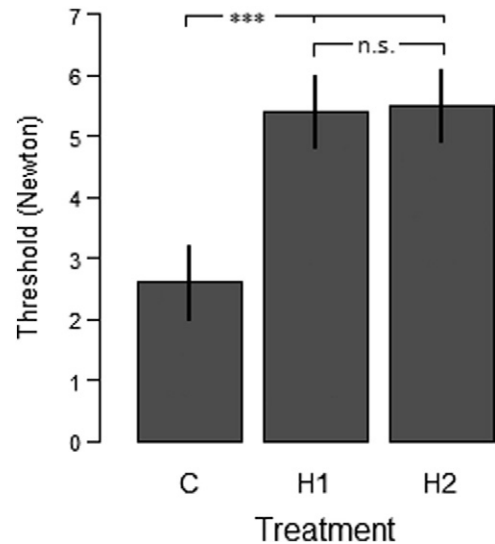
4. Discussion

The effect of two different habituation procedures on the reliability of a handheld methodology used for MNT testing in loose housed dairy cows was evaluated. The intra-individual variation in MNT decreased, MNT increased, and the discrepancy between applied and target force decreased by the habituation method used in Exp. 2 but not in Exp. 1. The habituation procedures used in both experiments reduced the frequency of low MNT values. In Exp. 1, the discrepancy decreased by experimental week influenced by test type (from baseline test to retest). Taken together, the results indicate that pre-test habituation improves the reliability of MNT testing, performed in the home environment of loose housed dairy cows, when a handheld device is used.

The habituation procedures in both experiments aimed at habituate the cows to the initial part of the testing procedure including the presence of the observer. Cattle can learn to distinguish between persons (Munksgaard et al., 1997). As a consequence the observer should perform both the habituation and the testing procedure to avoid cows' behavioural responses to a novel observer at the time of testing. However, this also implied that the observer was not blinded to the treatments. To minimize the potentially bias at testing, the cows were habituated and tested in random order and the observer focused on the display of the PAM to keep a constant rate when applying the ramped force.
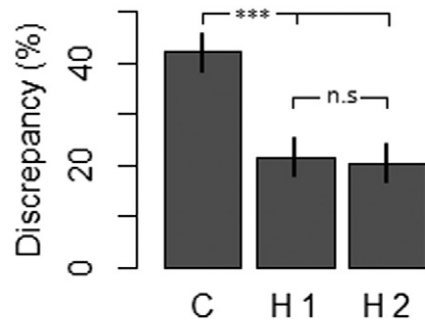

Fig. 3. Estimated means of intra individual coefficient of variation (CV) in the treatment groups in Exp. 2. CV was calculated per cow and the effect of habituation methods analysed by mixed model. Error bars indicate standard errors. C: control (n = 20), H 1: Habituation group 1 (n = 20), H 2: habituation group 2 (n = 20). ***$P < 0.001$, n.s. not significant.

Fig. 5. Estimated mean discrepancy in Exp. 2. The average discrepancy between the measured and the target force was calculated for each stimulation (see text for the calculation method) and the effect of habituation analysed by mixed model. Error bars denotes standard error. C: Control (n = 20), H 1: Habituation group 1 (n = 20), H 2: habituation group 2 (n = 20). *** indicates significance at $P < 0.001$, n.s.: not significant.

4.1. Intra-individual variation in MNT

In Exp. 2, habituated cows had lower CV than control cows. Until now, within-subject coefficient of variation has only been reported in two studies regarding nociceptive responses of cattle. Raundal et al. (2014) showed intra-individual CVs in MNT ranging from 0.34 to 0.52, when investigating MNT using two different handheld devices and two anatomical stimulation sites, in dairy cows kept in their home environment for testing. Veissier et al. (2000) investigated the repeatability of measures of nociceptive thresholds using laser stimulation (from a CO_2-laser), where the observers were not in the proximity of the experimental animals, and reported intra-individual CVs of 0.36. We found similar values for the habituated cows in Exp. 2, suggesting that this habituation procedure may reduce the effects of the presence of the observer on the reliability, to a level comparable to a method using remote laser stimulations. In Exp. 1 we found no effect of habituation on intra-individual CV, despite the attempt to augment the difference between the habituation and the control procedure by using different coloured clothes worn by the observer. The difference in success of the habituation procedures on the CV in our two experiments may be caused by several factors. The habituation procedure was different and the stroking procedure in Exp. 2 may have been more efficient to overcome the cow's fear of people (as discussed by Rushen et al. (1999)) than the habituation procedure in Exp. 1. In Exp. 1, the cows were tested outside their home environment, which may also have affected their thresholds (Herskin et al., 2007) and the restraint in a scale may have produced inconsistent behavioural responses over time (Gibbons et al., 2011). Therefore, we suggest that the habituation procedure and the familiarity of the cows with the test area in Exp. 2 reduced the fear of cows towards the test person and the test area resulting in a more consistent response to the mechanical force stimulations.

4.2. MNT

In Exp. 1 MNT was not affected by habituation, whereas in Exp. 2, the two habituation groups had significantly higher MNT values than the control group. The increase in MNT in Exp. 2 could be caused by a reduced occurrence of very low values in the habituation groups compared to the control group. Initially, ramped mechanical stimulation activates mechanoreceptors, resulting in a tactile sensation, followed by additional activation of nociceptors at the mechanical nociceptive threshold (Le Bars et al., 2001). In our study, we could not distinguish between behavioural avoidance responses induced by the tactile sensations and those induced by nociceptive stimulations. However, the habituation methods in Exp. 2 caused a decrease in the frequency of very low MNT values, which may reflect fewer responses to the tactile part of the stimulations. Given this, we hypothesize that MNTs obtained in the habituation groups in Exp. 2 are closer to the true nociceptive threshold. In Exp. 1 the habituation procedure also reduced the frequency of low MNTs. However, the overall MNT was not affected.

The thresholds found in the habituation groups of Exp. 2 in the present study differ from other studies of mechanical nociceptive threshold testing in dairy cows. For example, Whay et al. (1998) and Tadich et al. (2013) found thresholds of 13.3 N and 13.6 N, respectively, in sound cows, using cuff mounted devices attached to the stimulated limb. The difference between these work and the recent results may be caused by differences in the probe diameter (2 mm (Whay et al., 1998) and 2.5 mm (Tadich et al., 2013)) or the stimulation rate (0.22 N/s (Whay et al., 1998)), as these parameters may have influence on the response (Jensen et al., 1986; Garell et al., 1996; Grigg et al., 2007; Nie et al., 2009). Further studies are needed to examine nociceptive thresholds in dairy cows, including possible separation of the tactile part, as well as the effects of different probe sizes and stimulation rates.

4.3. Discrepancy

In the present report, discrepancy is a measure of how well the predefined rate was followed during the mechanical stimulations. The discrepancy was decreased by the habituation method in Exp. 2, showing that the mechanical nociceptive stimulations performed on habituated cows were closer to the predefined rate than the nociceptive stimulations applied to the control cows. When using a handheld method, the concordance of applied force to the target force relies on, e.g., the ability of the observer to increase the force constantly, movements of the subject's extremity until the expression of the defined behavioural avoidance response as well as ability of the observer to adjust for these movements. Supported by a study by Waiblinger et al. (2004), the habituation procedures in our study aimed at reducing the animals fear of the observer and hence to reduce behavioural fear responses such as restlessness, or freezing. The habituation procedure used in Exp. 2 may have decreased the occurrence of responses unrelated to nociception such as small movements of the stimulated limb until the expression of the defined behavioural avoidance response.

At present, no literature defines acceptable levels of discrepancy for mechanical nociceptive stimulations in large animals. Keeping a constant rate during MNT testing is important for the quality and interpretation of the outcome (Jensen et al., 1986; Love et al., 2011). Hence, we suggest that the pre-test habituation procedure used in Exp. 2 may be one way to improve the reliability of the MNT testing procedure.

In Exp. 1, the habituation procedure did not affect the discrepancy as such. However, a decrease in discrepancy from baseline to retest was found in week 1, and further decreased from week one to week two. This decrease may be related to improvements in the skills of the observer while using the device during the first week, as baseline testing systematically was performed on Mondays and retest on Thursdays. Training and experience of observers are generally recommended to produce repeatable results when using handheld devices for MNT testing (Jones et al., 2007; Walton et al., 2011; Janczak et al., 2012), which then is supported by our results from Exp. 1.

5. Conclusion

In this study, we investigated whether two different pre-testing habituation procedures improved the reliability of a handheld methodology used to quantify MNT in loose housed dairy cows. The habituation procedures, where cows were tested in their home environment and stroked repeatedly from the base of the tail to the stimulation site, decreased the intra-individual variation in MNT, increased the overall MNT and decreased the frequency of low MNTs, and decreased the discrepancy between applied and target stimulation force during stimulations. Habituation in novel surroundings without stroking reduced the frequency of low MNTs however, the overall level of MNT as well as the CV and discrepancy was not affected.

We conclude that pre-test habituation of dairy cows improves the reliability of a handheld methodology used to quantify MNT in loose housed dairy cows. We suggest that pre-test habituation should be performed prior to MNT testing in loose housed dairy cows and that habituation and testing should be performed in their home environment.

References

Anderson, D.E., Muir, W.W., 2005. Pain management in cattle. Vet. Clin. Food Anim. Pract. 21, 623–635.

Basbaum, A.I., Bautista, D.M., Scherrer, G., Julius, D., 2009. Cellular and molecular mechanisms of pain. Cell 139, 267–284.

Bergadano, A., Andersen, O.K., Arendt-Nielsen, L., Spadavecchia, C., 2009. Modulation of nociceptive withdrawal reflexes evoked by single and repeated nociceptive stimuli in conscious dogs by low-dose acepromazine. Vet. Anaesth. Analg. 36, 261–272.

Chambers, J.P., Waterman, A.E., Livingston, A., 1994. Further development of equipment to measure nociceptive thresholds in large animals. J. Vet. Anaesth. 21, 66–72.

Crawley, M.J., 2007. Statistical modelling. In: Crawley, M.J. (Ed.), The R Book. Wiley, West Sussex, England, pp. 323–386.

Di Giminiani, P., Petersen, L.J., Herskin, M.S., 2013. Nociceptive responses to thermal and mechanical stimulations in awake pigs. Eur. J. Pain 17, 638–648.

Di Giminiani, P., Petersen, L.J., Herskin, M.S., 2014. Characterization of nociceptive behavioural responses in the awake pig following UV-B-induced inflammation. Eur. J. Pain 18, 20–28.

Dundee, J.W., Moore, J., 1960. Alterations in response to somatic pain associated with anaesthesia. Br. J. Anaesth. 32, 396–406.

Ersbøl, A.K., Bruun, J., Toft, N., 2004. Data analysis. In: Houe, H., Ersbøll, A.K., Toft, N. (Eds.), Introduction to Veterinary Epidemiology. Biofolia, Frederiksberg, pp. 205–266.

Garell, P.C., McGillis, S.L.B., Greenspan, J.D., 1996. Mechanical response properties of nociceptors innervating feline skin. J. Neurophysiol. 75, 1177–1189.

Gibbons, J.M., Lawrence, A.B., Haskell, M.J., 2011. Consistency of flight speed and response to restraint in a crush in dairy cattle. Appl. Anim. Behav. Sci. 131, 15–20.

Grigg, P., Robichaud, D.R., Bove, G.M., 2007. A feedback-controlled dynamic linear actuator to test foot withdrawal thresholds in rat. J. Neurosci. Methods 163, 44–51.

Herskin, M.S., Müller, R., Schrader, L., Ladewig, J., 2003. A laser-based method to measure thermal nociception in dairy cows: short-term repeatability and effects of power output and skin condition. J. Anim. Sci. 81, 945–954.

Herskin, M.S., Munksgaard, L., Andersen, J.B., 2007. Effects of social isolation and restraint on adrenocortical responses and hypoalgesia in loose-housed dairy cows. J. Anim. Sci. 85, 240–247.

Janczak, A.M., Ranheim, B., Fosse, T.K., Hild, S., Nordgreen, J., Moe, R.O., Zanella, A.J., 2012. Factors affecting mechanical (nociceptive) thresholds in piglets. Vet. Anaesth. Analg. 39, 628–635.

Jensen, K., Andersen, H.O., Olesen, J., Lindblom, U., 1986. Pressure–pain threshold in human temporal region. Evaluation of a new pressure algometer. Pain 25, 313–323.

Jones, D.H., Kilgour, R.D., Comtois, A.S., 2007. Test–retest reliability of pressure pain threshold measurements of the upper limb and torso in young healthy women. J. Pain 8, 650–656.

Laven, R.A., Lawrence, K.E., Weston, J.F., Dowson, K.R., Stafford, K.J., 2008. Assessment of the duration of the pain response associated with lameness in dairy cows, and the influence of treatment. N. Z. Vet. J. 56, 210–217.

Le Bars, D., Gozariu, M., Cadden, S.W., 2001. Animal models of nociception. Pharmacol. Rev. 53, 597–652.

Ley, S.J., Waterman, A.E., Livingston, A., 1996. Measurement of mechanical thresholds, plasma cortisol and catecholamines in control and lame cattle: a preliminary study. Res. Vet. Sci. 61, 172–173.

Love, E.J., Murrell, J., Whay, H.R., 2011. Thermal and mechanical nociceptive threshold testing in horses: a review. Vet. Anaesth. Analg. 38, 3–14.

Mouraux, A., Ragé, M., Bragard, D., Plaghki, L., 2012. Estimation of intraepidermal fiber density by the detection rate of nociceptive laser stimuli in normal and pathological conditions. Neurophysiol. Clin. 42, 281–291.

Munksgaard, L., De Passillé, A.M., Rushen, J., Thodberg, K., Jensen, M.B., 1997. Discrimination of people by dairy cows based on handling. J. Dairy Sci. 80, 1106–1112.

Nie, H., Graven-Nielsen, T., Arendt-Nielsen, L., 2009. Spatial and temporal summation of pain evoked by mechanical pressure stimulation. Eur. J. Pain 13, 592–599.

R Development Core Team, 2013. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria 3-900051-07-0 (http://www.R-project.org/. Accessed February 1st, 2014).

Raundal, P.M., Andersen, P.H., Toft, N., Forkman, B., Munksgaard, L., Herskin, M.S., 2014. Handheld mechanical nociceptive threshold testing in dairy cows — intra-individual variation, inter-observer agreement and variation over time. Vet. Anaesth. Analg. http://dx.doi.org/10.1111/vaa.12159.

Rushen, J., Taylor, A.A., de Passillé, A.M., 1999. Domestic animals' fear of humans and its effect on their welfare. Appl. Anim. Behav. Sci. 65, 285–303.

Sankey, C., Richard-Yris, M.-A., Henry, S., Fureix, C., Nassur, F., Hausberger, M., 2010. Reinforcement as a mediator of the perception of humans by horses (*Equus caballus*). Anim. Cogn. 13, 753–764.

Stubsjøen, S.M., Valle, P.S., Zanella, A.J., 2010. The use of a hand-held algometer as a method to measure mechanical nociceptive thresholds in sheep. Anim. Welf. 19, 31–36.

Tadich, N., Tejeda, C., Bastias, S., Rosenfeld, C., Green, L.E., 2013. Nociceptive threshold, blood constituents and physiological values in 213 cows with locomotion scores ranging from normal to severely lame. Vet. J. 197, 401–405.

Thomsen, P.T., Munksgaard, L., Tøgersen, F.A., 2008. Evaluation of a lameness scoring system for dairy cows. J. Dairy Sci. 91, 119–126.

Veissier, I., Rushen, J., Colwell, D., de Passillé, A.M., 2000. A laser-based method for measuring thermal nociception of cattle. Appl. Anim. Behav. Sci. 66, 289–304.

Waiblinger, S., Menke, C., Korff, J., Bucher, A., 2004. Previous handling and gentle interactions affect behaviour and heart rate of dairy cows during a veterinary procedure. Appl. Anim. Behav. Sci. 85, 31–42.

Walton, D.M., MacDermid, J.C., Nielson, W., Teasell, R.W., Chiasson, M., Brown, L., 2011. Reliability, standard error, and minimum detectable change of clinical pressure pain threshold testing in people with and without acute neck pain. J. Orthop. Sports Phys. Ther. 41, 644–650.

Whay, H.R., Waterman-Pearson, A.E., Webster, A.J., 1997. Associations between locomotion, claw lesions and nociceptive threshold in dairy heifers during the peri-partum period. Vet. J. 154, 155–161.

Whay, H.R., Waterman-Pearson, A.E., Webster, A.J., O'Brien, J.K., 1998. The influence of lesion type on the duration of hyperalgesia associated with hindlimb lameness in dairy cattle. Vet. J. 156, 23–29.

Whay, H.R., Webster, A.J.F., Waterman-Pearson, A.E., 2005. Role of ketoprofen in the modulation of hyperalgesia associated with lameness in dairy cattle. Vet. Rec. 157, 729–733.