Effects of social isolation and restraint on adrenocortical responses and hypoalgesia in loose-housed dairy cows

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ABSTRACT: Effects of social isolation or restraint, applied outside the home pen, on adrenocortical and nociceptive responses were examined in 28 loose-housed dairy cows. Treatments lasted 15 min and consisted of social isolation in novel surroundings or restraint by the head in a test pen. A control treatment was applied in the test pen as well. Each cow was exposed to all treatments in a balanced order, with 3 to 4 d between treatments. Compared with the control treatment, social isolation in novel surroundings led to increased plasma concentration of cortisol \((P < 0.001)\) as well as to indications of hypoalgesia [posttreatment lack of decrease in latency to respond toward nociceptive laser stimulation, a tendency for decreased frequency of kicking in the pauses between laser stimulations \((P = 0.06)\), and an increased proportion of leg moving (least possible active response) after treatment \((P = 0.04)\)]. Indications of hypoalgesia were also observed after restraint (reduced kicking in response to laser stimulation, \(P = 0.04\)); however, the indications were to a lesser extent than after social isolation, and restraint treatment did not lead to increased plasma concentration of cortisol. For control and restraint treatment, an initial increase \((P < 0.02)\) in plasma concentration of cortisol was found, suggesting effects of pretreatment factors such as handling. No correlations between adrenocortical and nociceptive responses toward social isolation were found. The results confirm earlier reports stating that nociceptive changes induced by environmental challenges can be shown in dairy cows, even when they are kept in groups and removed from the home pen during the study of stress responses. However, testing outside the home pen seemed to affect the nociceptive and adrenocortical responses, thereby suggesting that care should be taken to avoid effects of pretreatment situational factors.

Key words: cattle, isolation, laser, nociception, restraint, stress

INTRODUCTION

The capacity to cope with environmental challenges is an important part of the welfare of individuals (Broom, 1988). In modern dairy production, cows are kept loose in complex environments and exposed to potentially stressful challenges such as novelty, social stimuli, or physical restraint.

Changes in nociception are a component of the fundamental and integrated behavioral and physiological response toward stressful challenges (Kavaliers, 1988), facilitating the coordinated expression of adaptive defensive behaviors (Bolles and Fanselow, 1980). Increase in nociceptive thresholds (hypoalgesia) has been found in many animal species after exposure to stimuli associated with stress or aversion (Yamada and Nabeshima, 1995). Herskin et al. (2004) reported that cows kept in tie-stalls and exposed to acute stressors such as social isolation in novel surroundings, restraint in the home environment, or exposure to novel neighbors and stall showed indications of hypoalgesia, thereby suggesting that nociceptive changes are part of the acute stress response in dairy cows. However, the nociceptive laser test validated for cattle by Veissier et al. (2000) and Herskin et al. (2003) has not been used on cows kept in large groups.

During development of tests of behavioral reactivity, it has become evident that responses of animals toward challenging events are affected by both the stimulus in question and situational factors such as handling or behavior of group mates (Manteca and Deag, 1993; Herskin, 2004). This complicates interpretation of observed
responses in terms of stress and emotion (Munksgaard and Jensen, 1996), especially in production systems with loose housing.

The aim of this experiment was to investigate whether a method used to quantify changes in nociception induced by potentially stressful challenges—a method that until now has been used in cows kept in tie-stalls—is sufficiently robust to be used in production systems, where effects of situational factors are difficult to avoid.

**MATERIALS AND METHODS**

**Animals and Feeding**

All procedures involving animals were evaluated and approved by the Danish Animal Experiments Inspectorate.

Twenty-eight Danish Holstein-Friesian cows from the resident herd at the Danish Cattle Research Center (Tjele, Denmark) were used as experimental animals. At the beginning of study, the cows were 32.0 ± 1.4 d postpartum (range, 21 to 41 d), with a BW of 612 ± 12 kg, and all were in their second lactation.

Cows were kept in a loose housing system in a group of 43 to 50 lactating dairy cows. The unheated barn had 53 straw-bedded cubicles and 24 gated feeding stations. Cows were milked in an automatic milking system with free traffic management and averaged 2.4 ± 0.4 milkings daily (range 2 to 4) and a milk yield of 23.5 ± 1.2 kg/d during the experimental period.

Cows were fed a total mixed ration to meet ad libitum intake consisting of (DM basis): whole-crop barley silage (43%), grass silage (34%), rapeseed cake (11%), rolled barley (11%), and a mineral mix (1%). Water was accessible from 5 drinking troughs situated in the aisles between the lying and feeding areas. Five kilograms of a commercial concentrate product (18.5% CP and 5% fat, DM basis) was provided to the cows in an automatic feeder, situated in the automatic milking system, and using magnetic collars for individual identification. The feed ration was formulated to meet the requirements of cows averaging 9,000 kg of energy-corrected milk/305 d (Strudsholm et al., 1999).

The experiment was conducted from November 2001 to April 2002, with average outdoor temperatures of 5.7 ± 0.9°C and relative humidity of 84.9 ± 1.9% at 1200.

**Experimental Design**

Effects of acute stress on adrenocortical and nociceptive responses were investigated using 3 treatments: 1) control: the cow was standing undisturbed in a feed gate; 2) restraint by the head: the cow was not in a feed gate, but tied by a halter as tightly as possible to the lower bar of fixtures in test pen (height 50 cm), whereby the head was forced downward and with limited freedom of movement; and 3) social isolation in novel surroundings: the cow was led to a novel, adjacent room measuring 7.3 × 15 m and illuminated by natural as well as artificial light, and kept in a solid-sided box measuring 150 cm (height) × 90 cm (width) × 200 cm (length), equipped with gates at both ends (Figure 1).

Experimental cows received all 3 treatments (inter-treatment interval of 3 to 4 d) in a balanced order, thereby ensuring that the treatment effects were not confounded by effects of repeated handling and sampling. Each treatment lasted 15 min, and the order and duration of blood sampling, handling, treatment, and nociceptive testing is shown in Table 1. All blood sampling and nociceptive testing took place while the cow was in a feed gate in a testing pen (measuring 11.2 × 6.0 m) in the same room as the home pen and equipped with 5 feed gates but with no feed available. The control treatment and the restraint treatment were performed in the testing pen as well. To avoid social isolation of the experimental cows while in the testing pen, an unfamiliar companion cow from an adjacent group of cows was placed in a feed gate in the testing pen before the arrival of the first cow to be tested. There was always 1 empty feed gate between the experimental cows and the companion cow.

Cows entered the experiment based on their date of calving, resulting in 1 to 7 cows being tested each day. On each test day, catching and testing of the first cow began at time = 0 min, whereas testing of the second cow began 15 min later. According to the time schedule (Table 1), the first 2 cows were returned to the home pen before testing of further cows was initiated. Testing always began at 1000 and lasted for approximately 1.5 h for each pair of cows being tested on a particular day.

The average interval from milking to initiation of testing was 179 ± 22 min (range, 6 to 625 min) and did not differ between treatments. In 24% of the observations, the experimental cow passed spontaneously through the automatic milking system within 30 min of initiation of catching. To limit the level of human disturbance in the home pen, these cows were not allowed to move back to the group after milking, but were automatically gated into a pen (measuring 4.9 × 6.0 m) situated next to the normal feeding area. In this way, the handler did not enter the home pen to catch the cow. All cows were familiar with the pen, and visual, auditory, olfactory, and tactile contact with group mates was possible. If the cows did not visit the automatic milking system within 30 min of initiation of testing, they were taken quietly from the home pen by 1 of 3 experienced handlers immediately before initiation of testing. In either case, the cow was led by halter for 25 m to the testing pen. The interval from initial contact with the handler (in the home pen or in the separation pen) until the cow was locked in a feed gate in the testing pen was 113 ± 5 s (range, 49 to 290 s).

**Laser Equipment**

The nociceptive responses of cows were assessed by examining the reactions to a laser beam applied to the
Figure 1. Schematic drawing of the experimental barn. Cows were kept in the home pen except during exposure to the 3 treatments: control (CON), which stayed undisturbed in feed gate; restraint (RES), which was not in feed gate, but tied by the head to lower bar of fixtures in test pen; and isolation (ISOL), which was socially isolated in a box in novel surroundings in an adjacent room, behind closed doors. The cows were led by halter from the home pen (or if they had been milked within the last 30 min, from the separation pen) to the test pen, where a companion cow was always present, and placed in a feed gate (a feed gate is indicated by the arrow). The pretreatment nociceptive laser test and blood sample was collected in the feed gate, after which the cow was subjected to CON, RES, or ISOL. After treatment, the cow was led back to the feed gate in the test pen, where the remaining measurements took place. The thick line on the 2 sides of the testing pen indicates solid wooden walls 2.5 m in height, which were put up for safety reasons because of the use of the laser equipment.

Skin on the caudal aspect of the metatarsal (Herskin et al., 2003). An adjustable, 15-W, computer-controlled (by modifying the voltage input to the control box using a digital-to-analog card mounted in a PC) CO₂-laser, with a wavelength of 10.6 μm, a beam diameter of 6 mm, and a power output of 1.8 W, was used as the heat source (Model 48-1, Synrad, Mukilteo, WA). Attached to the CO₂-laser was a visible, cold He-laser pointer (Bantex, Denmark), which was used to aim the laser.

Test of Nociception. At least 24 h before the initial test, the hair on each hind leg was trimmed from the tarsal to the pastern joint, leaving approximately 0.5 cm of hair. The test was validated and carried out before and after exposure to the treatments (Table 1), as de-

<table>
<thead>
<tr>
<th>Time, min</th>
<th>Action</th>
<th>Duration, s</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Catching in home pen or in separation pen</td>
<td>113 ± 5</td>
</tr>
<tr>
<td>3</td>
<td>Pretreatment nociceptive laser test in feed gate in test pen</td>
<td>155 ± 8</td>
</tr>
<tr>
<td>9</td>
<td>First blood sample in feed gate in test pen</td>
<td>59 ± 4</td>
</tr>
<tr>
<td>11</td>
<td>Release from feed gate, handling in order to initiate treatment</td>
<td>92 ± 6</td>
</tr>
<tr>
<td>13</td>
<td>Exposure to treatment (CON, RES, or ISOL)</td>
<td>921 ± 5</td>
</tr>
<tr>
<td>28</td>
<td>Handling bringing the cow to feed gate in test pen</td>
<td>77 ± 5</td>
</tr>
<tr>
<td>30</td>
<td>Posttreatment nociceptive laser test in feed gate in test pen</td>
<td>135 ± 8</td>
</tr>
<tr>
<td>35</td>
<td>Second blood sample in feed gate in test pen</td>
<td>52 ± 2</td>
</tr>
<tr>
<td>55</td>
<td>Third blood sample in feed gate in test pen</td>
<td>58 ± 3</td>
</tr>
<tr>
<td>70</td>
<td>Fourth blood sample in feed gate in test pen</td>
<td>55 ± 2</td>
</tr>
</tbody>
</table>

1Control (CON), which stayed undisturbed in feed gate; restraint (RES), which was not in feed gate but tied by the head to lower bar of fixtures in test pen; and isolation (ISOL), which was socially isolated in a box in novel surroundings in an adjacent room, behind closed doors.
2Mean ± SE.
scribed in Herskin et al. (2003). During testing, the laser equipment was situated in the testing pen, approximately 2 m behind the experimental cows. Initially, the hind legs were brushed briefly to remove manure, after which the experimental cow was allowed a 2-min adaptation period before the beginning of laser stimulation.

Each test consisted of 6 single laser stimulations, 3 on each hind leg in a balanced order. In the case of no response, the maximum exposure time was 25 s. Otherwise, the laser was turned off as soon as the cow responded by moving, lifting, or kicking the stimulated leg. The 6 single laser stimulations were applied immediately after each other (overall mean interval, 12.5 ± 0.7 s; range, 6 to 35 s), avoiding stimulation of the same area of skin more than once, and allowing the cow to stand quietly before the next exposure. If the cow began to urinate, defecate, or perform any other spontaneous movement of 1 of the 3 nonstimulated legs, the laser was turned off and the stimulation was repeated.

Behavioral data were collected by direct observation and organized using PC-software written for this purpose for keyboard operation by the observer. An ethogram of behavioral responses recorded during testing is shown in Table 2.

**Blood Sampling and Assay for Cortisol**

Blood was sampled by puncture of a jugular vein while the cow was standing in the feed gate in the testing pen. The procedure was performed by 2 trained technicians according to the sampling schedule outlined in Table 1. Approximately 10 mL of blood was collected in vacuum tubes coated with heparin (Vacuette, Greiner Labortechnik, Poitiers, France). Blood was centrifuged (2,000 \( \times \) g, 4°C, 20 min), and plasma was separated and frozen at −20°C.

Plasma concentrations of total cortisol were determined using a commercial assay based on time-resolved fluorescence (TR-FIA, Wallac OY, Turku, Finland), modified for use with cattle samples by dissolving the calibrators in charcoal-stripped cattle plasma. The modified assay used a standard curve based on the ratio of counts for the sample to the maximum bound in the zero-calibrator, fitted to the log-value of the concentration using smoothed spline curves in MultiCalc software (Wallac OY). Serial dilution of plasma samples in the zero calibrator gave counts parallel to the standard curve. Recovery of cortisol added to plasma samples averaged 91% (range 81 to 107%). The TR-FIA had a sensitivity of 1 ng/mL and a working range between 2.5 and 500 ng/mL at the same volume of plasma. According to the manufacturer, cross-reactivity of the antiserum with other steroids was less than 0.7%. Samples from the current experiment were analyzed in a single assay comprising 3 plates, where the CV was 10.7% for low (8.6 ng/mL) and 3.6% for high (48.1 ng/mL) controls.

**Variables**

**Tests of Nociception.** For the tests of nociception performed before and after treatment and for each animal, the following behavioral variables were calculated: (1) median latency to move the leg (any leg movement), seconds; (2 to 5) percentage of stimulations terminated after 1 of the 4 response types described in Table 2 (without a leg response, or moving, lifting, or kicking of the leg), %; (6) frequency of kicking/second in the 5 pauses between stimulations; and (7) the proportion of animals observed kicking in the pauses. For all variables, differences between pre- and posttreatment nociceptive tests were calculated. Furthermore, the proportion of animals responding with decreased kicking in the posttreatment nociceptive laser test compared with the initial test was also calculated.

**Adrenocortical Responses.** The plasma concentration of cortisol (ng/mL) was calculated for each animal and blood sample, and the area under the cortisol curve (from sample 1 through sample 4, no baseline deduction) was calculated for each animal and treatment using PROC EXPAND (SAS Inst. Inc., Cary, NC).

**Statistical Analysis**

**Tests of Nociception.** The MIXED procedure of SAS was used, and normal distribution and homogenous variances was confirmed using PROC CAPABILITY and the Bartlett test, respectively. Treatments were compared within the pre- and posttreatment nociceptive laser tests as well as for differences between the 2 tests, using a model including treatment (control, restraint, and social isolation), the order that each treatment was applied to each cow (1, 2, or 3), as well as their interaction, as fixed effects. The identity of the cows was treated as a random factor. For the pretreatment nociceptive test, time since calving and the dura-

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**Table 2. Ethogram of the behavior recorded during the pre- and posttreatment tests of nociception**

<table>
<thead>
<tr>
<th>Behavioral variable to laser stimulation</th>
<th>No response</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Moving</td>
<td>The exposed leg is moved horizontally, but the hoof is not lifted from the floor.</td>
</tr>
<tr>
<td></td>
<td>Lifting</td>
<td>The hoof of the exposed leg is lifted from the floor in a calm manner.</td>
</tr>
<tr>
<td></td>
<td>Kicking</td>
<td>The hoof of the exposed leg is thrust against the floor or withdrawn at high speed.</td>
</tr>
</tbody>
</table>

| Behavior between exposures              | Kicking     | The hoof of one exposed leg is thrust against the floor or withdrawn at high speed. |

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tion of handling before arrival of the cow to the testing pen were included as covariates if $P < 0.10$. For the posttreatment nociceptive test, as well as the differences between the 2 tests, duration of the first blood sample and the interval between the nociceptive tests were included as covariates as well. Results are given as means ± SE as well as $F(df_{treatment}, df_{error})$ and the $P$-values.

The comparison of responses between the pre- and posttreatment nociceptive tests was analyzed within treatment using a model including the order of nociceptive tests (pre-, post-), the order that each treatment was applied to each cow (1, 2, or 3), as well as the interaction, as fixed effects. The order of nociceptive tests was treated as a repeated factor.

In case of nonnormality, the variables were analyzed using either the Fisher exact test for pairwise comparison of proportions, the signed rank test for comparison of pre- and posttreatment nociceptive laser test variables, or the Friedman test for comparison of the 3 treatments (SigmaStat, Jandel Inc., Systat Software GMBH, Erkrath, Germany). Data are presented as means ± SE and the $P$-value.

Adrenocortical Responses. Plasma concentration of cortisol in each of the 4 samples was transformed to natural log and analyzed using the MIXED procedure of SAS with the same basic model as for the treatment comparisons of the nociceptive data. A similar model was used to analyze areas under the cortisol curves.

Effects of sample number were analyzed within treatment using a model including sample number (1, 2, 3, or 4) and order of treatment (1, 2, or 3), as well as the interaction between these effects, as fixed effects. Furthermore, time since calving and the duration of handling before arrival of the cow to the testing pen were included as covariates if $P < 0.10$. Sample number was included as a repeated factor. Because of the non-equal time spacing between samples, a spatial power covariance structure was chosen (Littell et al., 1996).

Correlations Between Nociceptive and Adrenocortical Responses. Correlations between nociceptive and adrenocortical responses were calculated using Spearman correlation analysis of SAS. For the isolation treatment, the only treatment that led to an increase in the plasma concentration of cortisol, the following variables were correlated: (1) adrenocortical responses, expressed as the area under the cortisol curve and the changes in plasma concentration of cortisol between the first and second sample; and (2) nociceptive responses, expressed as differences between the pre- and post-treatment nociceptive tests for the latency to move the leg after laser stimulation, the percentage of stimulations without a leg response, the percentage of stimulations leading to moving, and lifting or kicking the leg.

RESULTS

Tests of Nociception

Because of technical difficulties (nonfunctioning laser equipment), only 9 to 11 cows per treatment were tested successfully in the nociceptive laser tests. For the remaining animals, sham laser tests were performed with a nonfunctional laser. Twenty-five of the 372 single laser stimulations (6.7%) had to be repeated because of spontaneous body movements.

Pre- and Posttreatment Nociceptive Laser Tests. For nociceptive results obtained before treatment, no differences between treatments were found (Table 3).

Comparison among the 3 treatments showed that the control led to a tendency to a greater frequency of kicking/min during the 5 pauses between posttreatment laser stimulations than the 2 other treatments ($P = 0.06$; Table 3).

Differences Between the Pre- and Posttreatment Nociceptive Laser Tests. Comparison between the 3 treatments showed that the decrease in kicking from pre- to posttreatment laser test tended to be less for the control than for the restraint treatment ($P = 0.06$; Table 3). Changes between the pre- and posttreatment nociceptive tests were observed within each of the 3 treatments (Table 3). Measurements made after control treatment had an average decrease of 3.9 s in the latency to move the stimulated leg than measures collected before treatment ($P = 0.008$). Furthermore, the percentage of stimulations without a leg response decreased from the pre- to the posttreatment nociceptive test for control animals ($P = 0.006$). Restraint led to decreased percentage of responses seen as kicking ($P = 0.04$), increased percentage of lifting leg in response to laser stimulation ($P = 0.04$), as well as reduced latency to a leg response (on average 3.4 s; $P = 0.04$). Social isolation led to an increase in the percentage of responses observed as moving the leg ($P = 0.04$), as well as a weak tendency for a decrease in the proportion of kicking in response to laser stimulation ($P = 0.13$; Table 3).

Adrenocortical Responses

Before exposure to treatments, no differences were found in the plasma concentration of cortisol (blood sample 1). Samples 2 and 3 were affected by treatment as shown by an increased concentration of cortisol after social isolation compared with the 2 other treatments ($P < 0.003$ for sample 2 and 3; Figure 2). By sample 4 these treatment differences were no longer apparent. Within treatment, differences between samples were found for all 3 treatments. For control cows, concentrations of cortisol in samples 2 and 3 were less than sample 1 ($P = 0.02$). For cows to be restrained, a higher concentration of cortisol was found for sample 1 compared with sample 2, 3, and 4 as well as a higher concentration of cortisol at sample 2 compared with sample 3 ($P = 0.007$). Social isolation led to an increase in concentration of cortisol at sample 2 compared with levels measured at all other sample times as well as a decreased concentration at sample 4 compared with sample 1 and 3 ($P < 0.001$; Figure 2). Social isolation in novel surroundings led to a larger area under the
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Table 3. Behavior of dairy cows during tests of nociception performed before and after exposure to each of the 3 treatments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Variable</th>
<th>Pretreatment</th>
<th>Posttreatment</th>
<th>P-value</th>
<th>Pretreatment</th>
<th>Posttreatment</th>
<th>P-value</th>
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<th>Posttreatment</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Latency to move leg, s</td>
<td>15.0 ± 2.2</td>
<td>15.7 ± 1.8</td>
<td>0.04</td>
<td>27.3 ± 7.9</td>
<td>28.5 ± 5.6</td>
<td>0.04</td>
<td>38.2 ± 6.9</td>
<td>25.7 ± 6.9</td>
<td>0.05</td>
</tr>
<tr>
<td>Control</td>
<td>Type of response</td>
<td>11.1 ± 1.8</td>
<td>16.3 ± 1.8</td>
<td>0.04</td>
<td>9.1 ± 4.1</td>
<td>10.8 ± 4.5</td>
<td>0.04</td>
<td>18.2 ± 7.6</td>
<td>12.0 ± 4.9</td>
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</tr>
<tr>
<td>Control</td>
<td>Lifting leg, %</td>
<td>13.2 ± 3.4</td>
<td>12.5 ± 1.8</td>
<td>0.04</td>
<td>9.1 ± 4.1</td>
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<tr>
<td>Control</td>
<td>Kicks/min</td>
<td>0.15 ± 0.10</td>
<td>0.59 ± 0.25</td>
<td>0.06</td>
<td>0.10 ± 0.05</td>
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<td>0.06</td>
</tr>
<tr>
<td>Control</td>
<td>Animals kicking, %</td>
<td>18.2 ± 2.2</td>
<td>45.4 ± 4.5</td>
<td>0.02</td>
<td>5.9 ± 0.5</td>
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<tr>
<td>Restraint</td>
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<td>15.0 ± 2.2</td>
<td>15.7 ± 1.8</td>
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<td>Isolation</td>
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<td>Isolation</td>
<td>Kicks/min</td>
<td>0.15 ± 0.10</td>
<td>0.59 ± 0.25</td>
<td>0.06</td>
<td>0.10 ± 0.05</td>
<td>0.10 ± 0.05</td>
<td>0.06</td>
<td>0.10 ± 0.05</td>
<td>0.10 ± 0.05</td>
<td>0.06</td>
</tr>
<tr>
<td>Isolation</td>
<td>Animals kicking, %</td>
<td>18.2 ± 2.2</td>
<td>45.4 ± 4.5</td>
<td>0.02</td>
<td>5.9 ± 0.5</td>
<td>45.4 ± 4.5</td>
<td>0.02</td>
<td>5.9 ± 0.5</td>
<td>45.4 ± 4.5</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Correlations Between Nociceptive and Adrenocortical Responses

There were no significant correlations among any of the 10 adrenocortical and nociceptive responses evaluated in animals subjected to the isolation treatment.

DISCUSSION

This is the first report to present nociceptive testing outside the home pen in loose-housed dairy cows before and after exposure to social isolation in novel surroundings or restraint by the head. Social isolation in novel surroundings increased plasma cortisol concentrations and led to hypoalgesia, but no evidence for a simple relation between the adrenocortical and nociceptive responses was found. Restraint by the head led to indications of hypoalgesia but did not affect the plasma concentration of cortisol.

The present control treatment (standing in the feed gate in the testing area for 15 min combined with blood cortisol curve than either of the 2 other treatments (P = 0.002 and areas of 448 ± 45 for the control, 595 ± 99 for the restraint treatment, and 831 ± 103 for the social isolation treatment, respectively).

Figure 2. Concentrations of cortisol (ng/mL) in plasma before and after exposure to 1 of the 3 treatments: control (CON), which stayed undisturbed in feed gate (n = 26); restraint (RES), which was not in feed gate but tied by the head to lower bar of fixtures in test pen (n = 26); and isolation (ISOL), which was socially isolated in a box in novel surroundings in an adjacent room, behind closed doors (n = 26). The black bar above the x-axis indicates the 15-min duration of the treatment. The asterisks refer to statistical comparison between treatments (**P < 0.01; ***P < 0.001), whereas the letters refer to the statistical comparison of samples within the control treatment (a,bP = 0.02), restraint (c–eP = 0.007), and isolation (f–hP < 0.001).
sampling and nociceptive testing) led to nociceptive changes comparable with previous experiments (Herskin et al., 2003, 2004) and indicative of hyperalgesia; cows were shown to respond more strongly to the posttreatment laser test—had a shorter latency to respond and fewer laser stimulations without leg response. Furthermore, the control treatment did not lead to increased plasma concentration of cortisol. On the contrary and not similar to our previous experiment (where cortisol remained unchanged for the whole period) the plasma concentration of cortisol decreased from the first to the second blood sample. This might suggest that the first blood sample was not expressing the basal level of cortisol in the blood. The elevated cortisol levels in the first blood sample were found for all treatments and are likely due to handling of animals as well as the 2 min adaptation period allowed in the test pen before the pretreatment nociceptive test. In this study 9 min passed from the initial human contact until the first blood sample was collected, and such an interval from activation of the hypothalamus-pituitary-adrenal axis is considered enough for cortisol to be present in plasma of mammals (Sapolsky, 2002).

In the present experiment, not only the first blood sample, but also the pretreatment nociceptive laser test seemed to be affected by the experimental conditions. Before treatment, the overall latency to respond was 15.0 ± 1.2 s (n = 31 observations), whereas Herskin et al. (2004) found a pretreatment latency of 7.1 ± 0.2 s having 96 observations of dairy cows tested in their home tie-stall, and Herskin et al. (2003) found 6.9 ± 0.4 s for 36 similar observations. Thus, in line with the adrenocortical results, the responses of the present cows in the pretreatment nociceptive laser test might have been affected by handling bringing them to the test pen (lasting 115 ± 5 s and followed by a 2 min adaptation period). Presently, the lower temporal limit of stressors capable of inducing nociceptive changes in cattle is not known, but in other animal species, stressors lasting less than 1 min have induced hyperalgesia (Rodgers and Randall, 1988). It can, therefore, be hypothesized that nociceptive responses of dairy cows are sensitive toward potential stressful situational factors such as handling or testing outside the home pen. Additional experiments comparing different experimental designs are needed to clarify this.

Effects of the restraint treatment on nociceptive responses might be another example of effects of pretreatment situational factors. Restraint by the head led to indications of hypoalgesia because some effects of the control treatment were counteracted (less kicking in response to the posttreatment nociceptive laser test and increased leg lifting in response to laser stimulation). Furthermore, the restraint treatment led to less kicking in the pauses between laser stimulations than the control treatment. From other animal species, it is known that physical restraint or immobilization is one of the most potent methods of inducing hypoalgesia (Porro and Carli, 1988). Herskin et al. (2004) found evidence of stress-induced hypoalgesia in dairy cows after similar restraint treatment performed in the home stall. Effects of restraint found here are less obvious than those reported in the previous experiment. There appear to be 2 major reasons for these differences: (1) the observed low level of response in the pretreatment nociceptive laser test in the current study; and 2) the fact that the control and restraint treatments did not differ as much as in the previous experiment, where the control treatment was performed in the home environment and the cows were kept in tie-stalls. The same reasons might explain that the present restraint treatment did not lead to increased plasma concentration of cortisol.

Under the present circumstances, the social isolation treatment, however, led to indications of hypoalgesia such as counteracting the decrease in the latency to respond seen in the control treatment, increasing the proportion of moving the leg in response to laser stimulation, decreasing kicking in the pauses between laser stimulations, as well as tending to decrease the proportion of kicking in response to laser stimulation. However, as was the case for the restraint treatment, these differences were of a lesser magnitude than reported by Herskin et al. (2004) and Rushen et al. (1999) in tie-stalls.

In the present experiment, the social isolation treatment led to the clearest indications of hypoalgesia compared with the restraint treatment, as shown by the changed latency to respond, and increased response by moving the leg (the least possible leg response) after social isolation, whereas the restraint treatment led to increased lifting legs (intermediate leg response). These results suggest that the present design is able to show that social isolation leads to increased adrenocortical and hypoalgetic responses compared with the restraint treatment, thereby confirming results reported by Herskin et al. (2004) using dairy cows kept in tie-stalls.

The only treatment leading to significant adrenocortical responses was social isolation. The capacity of social isolation in novel surroundings to induce adrenocortical responses has been described earlier in dairy cows (Munksgaard and Simonsen, 1996; Rushen et al., 2001; Herskin et al., 2004), and the present cortisol concentrations correspond with previous findings from similar treatments (Rushen et al., 1999; Herskin et al., 2004). Social isolation in novel surroundings can therefore be considered as the most stressful of the applied treatments. Even though no simple correlations between the increase in the plasma concentration of cortisol and the nociceptive responses toward social isolation were found (similar results were reported by Herskin and Munksgaard, 2004), it is not surprising that the isolation treatment, being the most stressful, led to the clearest signs of hypoalgesia. Herskin et al. (2004) also showed that social isolation led to greater plasma concentration of cortisol and clearest indications of hypoalgesia as compared with restraint or exposure to novel neighbors and stall. The present results suggest that social isolation in novel surroundings is applicable as
an acute stressor for dairy cows kept in large groups, even though, in future studies, steps should be taken to avoid effects of pretreatment situational factors, e.g., by using feed gates inside the home pen (preferably after spontaneous visits) for adrenocortical and nociceptive sampling.

In our previous work using the nociceptive laser test, originally described for calves by Veissier et al. (2000), we have tested dairy cows in the home stall (Herskin et al., 2003, 2004) or just outside a group pen of only 12 animals (Herskin et al., 2003). In the current study, the dairy cows were tested outside the home pen before and after exposure to social isolation or restraint. In this way, the nociceptive data are independent of the context of the challenging events because the nociceptive tests were not performed during stressor exposure, as done by Rushen and Ladewig (1991) in pigs, or while the cows were still in the stressor environment as done by Rushen et al. (1999). Furthermore, based on results of the present as well as previous studies (Herskin et al., 2003, 2004), it seems to be important for interpretation of the observed nociceptive responses to be aware of possible effects of pretest situational factors, such as handling. This limitation also applies to studies of basal levels of pain sensitivity, such as when examining nociceptive responses of diseased animals (Whay et al., 1997). When testing nociceptive responses of dairy cows toward laser stimulation, it is therefore recommended to 1) perform the initial test as quickly and after as little handling as possible; and 2) to use the same environment for pre- and posttreatment tests.

In conclusion, results of the present experiment confirm earlier reports stating that nociceptive changes are part of stress responses in dairy cows and suggest that the nociceptive laser test is sufficiently robust to show nociceptive changes induced by environmental challenges, even when the animals are kept in groups and are removed from their home pen during the study of stress responses.

LITERATURE CITED