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# Opioid Peptides and Behavioral and Physiological Responses of Dairy Cows to Social Isolation in Unfamiliar Surroundings<sup>1</sup>

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**ABSTRACT:** To test whether endogenous opioid peptides are involved in the behavioral and physiological responses of cattle to stress, 12 Holstein cows were either placed in social isolation in unfamiliar surroundings for 15 min or remained in their home stalls, either with or without naloxone treatment, following a Latin square design. Vocalizations (judged as high or low frequency), defecation/urination, and heart rate were recorded, latency to respond to local thermal stimulation of the leg by means of a laser was measured to detect pain sensitivity, and blood was sampled and assayed for cortisol concentrations. Naloxone in the home stall increased cortisol concentrations and tended to reduce response latencies to the laser but did not induce vocalization. Social isolation increased the incidence of high-

frequency vocalization and of defecation/urination, heart rate, cortisol concentrations, and response latencies to the laser. Prior administration of naloxone increased the incidence of low-frequency vocalization in isolation, but it had no effect on heart rate or on responses to the laser and only limited effect on cortisol concentrations when the cows were isolated. Brief periods of social isolation in unfamiliar surroundings seem to be stressful to cows, as indicated by increased heart rate, hypothalamic-pituitary-adrenocortical axis activity, and vocalization. Isolation also reduces pain sensitivity, suggesting a stress-induced analgesia. However, we found no evidence that naloxone-sensitive opioid receptors were involved in these responses.

Key Words: Dairy Cattle, Stress, Hydrocortisone, Vocalization, Opioid Peptides, Naloxone

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## Introduction

Farm animals respond to acute stress with diverse physiological and behavioral changes (Moberg, 1987; Minton, 1994; Grandin, 1997). Cattle socially isolated and/or in unfamiliar surroundings have increased cortisol, prolactin, and  $\beta$ -endorphin concentrations, increased heart rate, increased vocalizations, and reduced oxytocin responses to milking (Veissier and Le Neindre, 1992; Bruckmaier et al., 1993; Bruckmaier and Blum, 1996; Boissy and Le Neindre, 1997; Grandin, 1997). Such integrated changes have been interpreted as resulting from a central emotional state in the animal ("fear") (Boissy, 1995; Grandin, 1997), and there has been considerable interest in finding common neu-

rophysiological mechanisms to explain the diversity of responses (Minton, 1994; Grandin, 1997).

Endogenous opioid peptides may integrate these responses to stress (Boissy, 1995). Opioids are involved in many responses to stress (Przewlocki, 1993), regulate various endocrine systems, including the hypothalamic-pituitary-adrenocortical (HPA) axis, and underlie the phenomenon of stress-induced analgesia. Rushen and Ladewig (1991) detected an opioid-based stress-induced analgesia in restrained pigs and found that naloxone (a generalized opioid antagonist with a strong affinity for  $\mu$ -opioid receptors) increased HPA responses and vocalization in response to restraint.

In cattle, opioids may regulate HPA activity (Nanda et al., 1989, 1992; Gazal and Anderson, 1995) and secretion of anterior pituitary hormones (Barb et al., 1991). However, it is not known whether opioids are involved in behavioral responses of cattle to stress. Furthermore, Schwartzkopf-Genswein et al. (1997, 1998) were not able to detect stress-induced hypoalgesia in cattle.

For this experiment, we hypothesized that naloxone would influence the effects of social isolation in unfamiliar circumstances on dairy cow sensitivity to pain, vocalization, heart rate, and plasma cortisol concentrations.

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## Materials and Methods

### *Animals and Housing*

Cows were housed, fed, and handled according to the appropriate guidelines (Agriculture Canada, 1990). Experimental procedures were approved by the Lennoxville Institutional Animal Care Committee, complying with requirements of the Canadian Council for Animal Care. Twelve multiparous, nonlactating Holstein cows were housed in tie-stalls with straw bedding from at least 4 wk before the experiment began. The cows were fed a total mixed ration for ad libitum consumption once daily. Five days before the experiment began, the cows were nonsurgically fitted with a jugular vein catheter. However, seven cows had to be recatheterized the day before the experiment began. At least 1 h before the treatment began, the cows were fitted with a heart rate monitoring system (Polar Oy, Helsinki, Finland) that was set to record mean heart rates during each 5-s period (Hopster et al., 1995).

### *Treatments*

Each cow was subjected to four treatments in a balanced order following a Latin square design. There was a period of 2 to 4 d between consecutive treatments. Throughout,  $t = 0$  refers to the time when the cows were released from their stalls to be moved to the isolation chamber or to the room containing the isolation chamber.

1. *Novelty/Isolation + Physiological Saline (I)*. The cow was placed in a small isolation chamber in visual and olfactory isolation from other cows for 15 min and was injected i.v. through the jugular catheter with physiological saline at  $t = -10$  min.

2. *Novelty/Isolation + Naloxone (IN)*. The cow was injected i.v. through the jugular catheter with .5 mg/kg BW of naloxone (Sigma Chemical Co., St. Louis, MO) at  $t = -10$  min prior to being moved to the isolation chamber at  $t = 0$  min as in Treatment, 1. This dose is sufficient to affect HPA axis activity in cattle (Nanda et al., 1989) and to antagonize opiate-induced analgesia in cattle (D. Colwell, personal communication, 1995).

3. *Control (C)*. The cow was injected i.v. through the jugular catheter with physiological saline at  $t = -10$  min, walked to the door of the room containing the isolation chamber at  $t = 0$  min, but then returned to its stall for 15 min, after which it was again walked to the door of the room containing the isolation chamber and then returned.

4. *Control + Naloxone (N)*. The cow was treated as in Treatment 3 but was injected with .5 mg/kg body weight of naloxone at  $t = -10$  min before being led to the door of the room containing the isolation chamber.

The cows were placed in one of two identical isolation chambers ( $4.3 \times 3$  m), which were sound-attenuated with thick concrete walls and which contained a  $2.6 \times 1.5$ -m pen with high wooden sides and door and concrete

floors with no bedding. The bottom of the rear door of the pen was .5 m from the floor of the chamber so that the laser could be applied to the cow's hoof without opening the pen door. The isolation chambers were adjacent to each other and in a separate room of the barn that the cows were housed in. The cows had not been in this room prior to the experiment.

The cows took an average of 2 min to walk to the room. The cows were tethered with a 1-m-long halter to the front of the chamber and inside the wooden pen. The door to the isolation chamber was kept shut during the period of isolation until the laser test was done. A video camera was mounted on the ceiling of the isolation chamber so that the cows were in full view the whole time. Both isolation chambers were used for each cow, once each for Treatments 1 and 2. The chambers were hosed down between tests using water only and all feces were removed.

### *Variables Measured*

*Pain Sensitivity*. The cows' sensitivity to pain was assessed by examining the reaction latency to a source of thermal energy from a 10-W computer-controlled CO<sub>2</sub> laser (MPB Lanscom, Dorval, QC, Canada) applied to the caudal aspect of the metatarsal (see Schwartzkopf-Genswein et al., 1997 and de Passillé et al., 1999, for a description). Animals were prepared by shaving the hair off the hind legs between the dew claws and the hock the day before the experiment. The laser setting used heated the skin to approximately 70°C within 10 s according to infrared thermography (de Passillé et al., 1999). The laser was placed approximately 1 m from the animal. The laser was aimed at the shaved area of the hoof and turned on until the animal responded by lifting its foot, immediately after which the laser was turned off. The laser also turned off automatically if the animal did not respond within 30 s. Four measures were taken during each test, alternating between the left and right legs in a balanced design across treatments, with a pause of 30 s between subsequent measures. A different spot on the leg was used for subsequent measures. The measure was retaken if the cow defecated, urinated, or shifted its weight without lifting its foot. If the animal was lying down, it was made to stand up 15 s before taking any measures. De Passillé et al. (1999) found that this protocol is sufficient to provide reliable measures of pain sensitivity that can differentiate between different power settings and different animals. Examination of the legs throughout the experiment showed no signs of blistering or skin damage on any of the animals.

Tests were carried out at  $t = -20$  min,  $t = -2$  min (just before moving the cows),  $t = 15$  min, and  $t = 40$  min. The test at  $t = 15$  min was done while the animal was still in the pen inside the isolation chamber. To do this, the door of the chamber was opened, but the rear door of the wooden pen within the chamber remained shut so as to keep the animal visually isolated from the

experimenters. All other tests were done while the cows were in their home stalls. During the four measures of each test, we recorded the latency of the animal to lift the leg upon which the laser was aimed so that the hoof was off the ground (leg lift latency). It was apparent that the cows made some preparatory movements before actually lifting the leg. Therefore, a second observer independently recorded the first movements that the cow seemed to make prior to actually lifting its leg off the ground (first response latency). This included a shift of weight, flicking the tail, and lifting the opposite back foot.

**Blood Samples.** Blood samples were taken at  $t = -20$  min,  $t = -10$  min,  $t = -1$  min (just before moving the animals),  $t = 20$  min (just after the cows were returned to their stalls), and at  $t = 40, 60, 80,$  and  $100$  min. Blood was put immediately on ice and centrifuged no later than 20 min after collection. Plasma was stored at  $-20^{\circ}\text{C}$  until it was assayed for cortisol. Thawed samples were analyzed in duplicate using a RIA method described in Schwartzkopf-Genswein (1997). The antiserum used was F36314 5 Endocrine Sciences (Calabasas Hills, CA), with 1, 2, 6, and 7  $^3\text{H}$  tracers (New England Nuclear Corp., Boston, MA). Cross-reactivity of the antiserum was as follows: prednisolone 52%, cortisone 30%, prednisone 26%, 21-deoxycortisol 6.8%, and deoxycortisol 4.5%, but aldosterone, corticosterone, deoxycorticosterone, dexamethasone, estradiol, and estriol were  $< 3\%$ . Interassay CV were 14.63%, and intraassay CV were 12.13%. Samples were reassayed if the duplicates differed by more than 2%.

**Behavior.** During the period in the isolation chamber, the cow was observed on the television screen and was observed directly during the equivalent 15-min period in the control conditions. All incidences of defecation/urination and of vocalization were recorded using a hand-held Psion computer running the Observer program (Noldus Information Technology, Wageningen, The Netherlands). Instances of vocalization were recorded as either high-frequency or low-frequency vocalizations, based on the judgment of the observer. High-frequency vocalizations were guttural calls, usually performed with the mouth open, and involved an increase in frequency during the call. Low-frequency vocalizations were nasal calls that were of a constant frequency and were usually performed with the mouth kept closed (for a description of cattle vocalizations, see Kiley, 1972).

**Heart Rate.** Mean heart rate was recorded continuously during each 5-s interval for 2 h, which included the entire treatment period.

### Statistics

Frequency of high- and low-frequency vocalizations and of defecation/urination by each cow were log-transformed because the distribution of values was positively skewed. The frequency per animal of each behavior was tested with the GLM procedures of SAS (1988) with a

model that included cow identity, block, and treatment as main factors. The effect of block was tested using the cow's identity as the error term, and the effect of treatment was tested using the residual error. Planned paired contrasts were made between treatments C and N (effects of naloxone), C and I (effects of isolation), and between I and IN (effects of naloxone on responses to isolation). Because the data were not normally distributed, paired comparisons were also done between each treatment using a nonparametric Kruskal-Wallis test. However, this analysis produced the same pattern of results, and so we report on the results of the GLM. For cortisol, surface under the curve and maximum values following treatment were calculated correcting for pretreatment baseline by subtracting the mean for  $t = 0$  min,  $t = -10$  min, and  $t = -20$  min. Mean heart rates during the 15-min period immediately prior to moving the cows were taken as baseline values and subtracted from the mean heart rates during the period of isolation. Heart rates during the first 3 min after the animals were taken from their stalls were excluded; these reflected the locomotion of the cow. Heart rate and cortisol measures were tested using the same GLM model as the behavioral data. Median latencies for first response and leg lift were tested with the same GLM model, except that time was included as a repeated measures factor and the response at each time was contrasted with the response at the previous time. Because the response latencies were positively skewed, the median latency for the four measures taken at each time was used rather than the mean latency.

## Results

### Responses to the Laser

The latency of the first movement in response to the laser was highly correlated with the leg lift latency at all times in all treatments ( $r > .9$   $P < .01$ ).

**First Response Latency.** There was a significant overall time  $\times$  treatment interaction for the contrast between C and I ( $P < .001$ ). No other contrasts were significant ( $P > .10$ ). However, when each time period was examined separately, there was a significant time  $\times$  treatment interaction for the change from  $t = -20$  min to  $t = -2$  min for the contrast C vs N: naloxone led to a slight decrease in first response latencies relative to C (Figure 1). There was a significant change in latency between  $t = -2$  min and  $t = 15$  min with a significant effect for the contrast between C and I ( $P < .001$ ). In C, first response latencies did not change between  $t = -2$  min and  $t = 15$  min. In I, initial movement latencies increased at  $t = 15$  min (Figure 1). No other contrasts were significant ( $P > .10$ ). There were no significant changes or treatment effects between  $t = 15$  min and  $t = 40$  min.

**Leg Lift Latency.** There was an overall time  $\times$  treatment interaction for the contrast between C and I ( $P < .001$ ). The other contrasts were not significant ( $P > .10$ ).

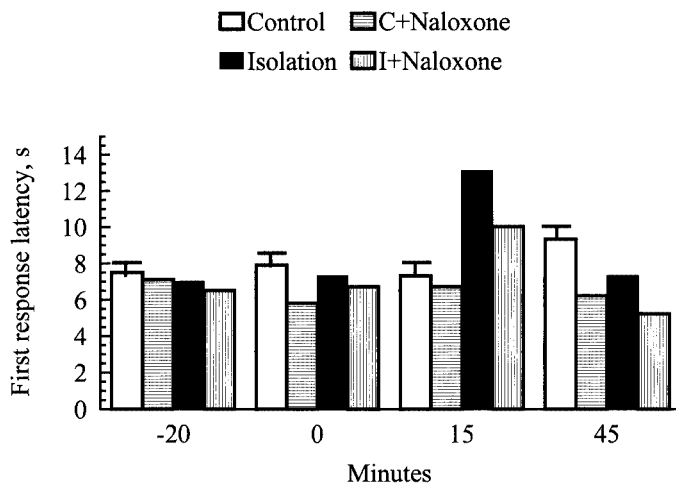


Figure 1. Mean (+ pooled SE) of first response latency at each time.

There was no change in latency from  $t = -20$  min to  $t = -2$  min and no significant effect of treatment during this time. There was a significant change in latency between  $t = -2$  min and  $t = 15$  min with a significant effect for the contrast between C and I ( $P < .001$ ). In C, leg lift latencies did not change between  $t = -2$  min and  $t = 15$  min. In I, leg lift latencies increased at  $t = 15$  min (Figure 2). No other contrasts were significant ( $P > .10$ ). There were no significant changes or treatment effects between  $t = 15$  min and  $t = 40$  min.

### Behavior

For high-frequency vocalization, there was an overall treatment effect for the contrast C vs I ( $P = .002$ ), with a higher incidence of vocalization in isolation (Figure 3). Other contrasts were not significant. For low-frequency vocalization, there were significant contrasts between C and I ( $P = .001$ ) and between I and IN ( $P = .003$ ), with more frequent vocalization in isolation than in control and more frequent vocalization in isolation following naloxone injections (Figure 3). The contrast between C and N was not significant. Defecation/urination occurred only rarely, but there was a significant treatment effect for the contrast C vs I ( $P = .019$ ), with a higher frequency in isolation (mean  $\pm$  SE =  $.82 \pm .21$ ) than in the control condition ( $0 \pm .21$ ). The other contrasts were not significant ( $P > .10$ ).

### Cortisol

Following isolation and naloxone treatment, cortisol levels increased, reaching maximum values at  $t = 20$  min and then decreasing until baseline levels were reached at  $t = 60$  min (Figure 4). Both the surface under the curve and maximum values showed an overall treatment effect for the contrast C vs N and for the contrast C vs I ( $P < .001$ ). Both surface under the curve and maximum values of cortisol were higher in N and I than

in C (Figure 5). The contrast between I and IN was not significant, although there was a clear trend for higher cortisol concentrations in the IN condition (Figures 4 and 5).

### Heart Rate

There were no differences ( $P > .10$ ) between treatments in mean heart rates during the 15 min before the cows were moved (Figure 6). During the 15-min treatment periods, heart rates increased (beats per minute; **bpm**) during the period of isolation (Figure 6; change in heart rate I:  $11.3 \pm 1.4$  bpm, IN:  $13.0 \pm 3.1$  bpm) but not when the cows were left in their stalls (Figure 6; change in heart rate C:  $.3 \pm .9$  bpm, CN:  $2.0 \pm 1.4$  bpm). The contrast between C and I was significant ( $P < .01$ ), but other contrasts were not significant.

## Discussion

Our results show that, for dairy cows, social isolation in an unfamiliar environment resulted in a number of behavioral and physiological changes that are generally indicative of stress (Grandin, 1997). These were increased plasma concentrations of cortisol, increased heart rates, increased vocalization, especially high-frequency, open-mouth vocalizations, and increased incidence of defecation/urination during the periods of social isolation. The occurrence of vocalizations was increased with prior administration of naloxone, although this mainly involved low-frequency vocalizations. We also found evidence of a reduced sensitivity to pain, which is the first report of stress-induced hypoalgesia in cattle. However, this did not seem to involve naloxone-sensitive opioid receptors.

Previous research has shown that short-term social isolation of dairy cattle or exposure to unfamiliar surroundings can increase cortisol secretion (Bruckmaier et al., 1993; Bruckmaier and Blum, 1996, 1998), as well

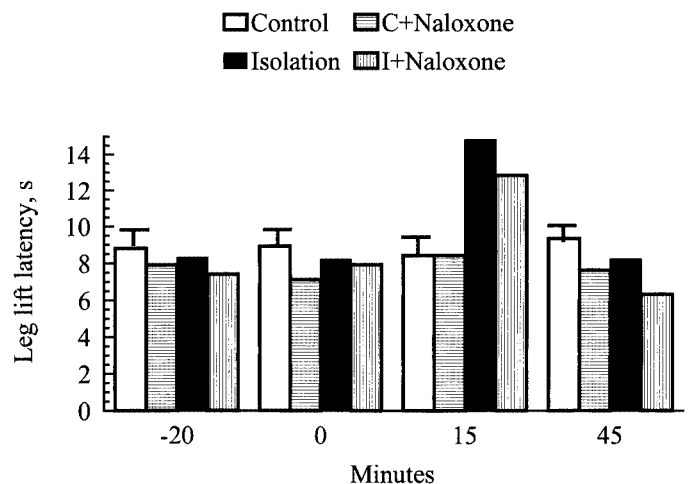


Figure 2. Mean (+ pooled SE) of leg lift response latency at each time.

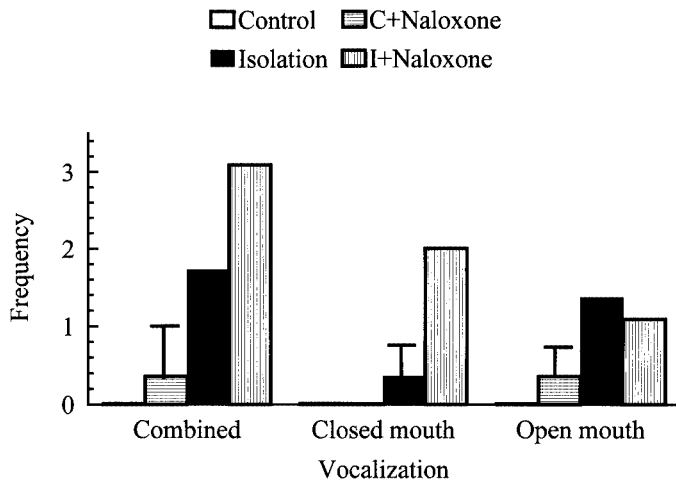


Figure 3. Mean (+ pooled SE) frequency of vocalizations during the period of isolation.

as heart rates and the incidence of vocalization and defecation/urination (Veissier and LeNeindre, 1992; Boissy and LeNeindre, 1997). When social isolation is prolonged, however, many of these effects seem to disappear (Munksgaard and Simonsen, 1996). Stress-induced hypoalgesia has been found repeatedly in rats (Amit and Galina, 1986) and more recently in pigs (Rushen and Ladewig, 1991) and poultry (Sufka et al., 1994). However, stress-induced reductions in pain sensitivity have not been found previously in cattle (Schwartzkopf-Genswein et al., 1997). We found that this reduced sensitivity was apparent in both the first movements that the animals made in response to the laser and the full leg lift response. From our results, we cannot determine the relative importance of the social isolation vs the unfamiliar environment in bringing about these changes. Novelty alone can be a potent stressor for cattle (Grandin, 1997), and Boissy and LeNeindre (1997) found that social isolation alone was sufficient to increase heart rates and cortisol concentrations and vocalization in cattle. However, there is likely to be an interaction between novel surroundings and social isolation, because the presence of conspecifics can reduce cattle responses to novel environments (Boissy and LeNeindre, 1990).

Naloxone alone resulted in increased plasma concentrations of cortisol, suggesting that endogenous opioids are involved in tonic control of the HPA axis in cattle, supporting previous findings (Nanda et al., 1992). In other species, naloxone influences HPA activity primarily by influencing the secretion of corticotropin-releasing hormone and ACTH rather than by a direct action on the adrenal gland (Estienne et al., 1988). Naloxone also tended to increase plasma concentrations of cortisol during periods of social isolation/novelty stress, although this effect was not statistically significant. In contrast to previous research (Nanda et al., 1989), our results show that the effect of naloxone on cortisol concentrations was not increased during the stress. This

suggests that opioid control of the HPA axis does not increase in times of stress, as it seems to do in rats (Buckingham and Cooper, 1986) and pigs (Rushen and Ladewig, 1991; Rushen et al., 1993; Janssens et al., 1995).

We found no evidence that naloxone affected heart rates, as it seems to do in other farm animal species (e.g., Schouten et al., 1991). This was so for animals both in their home stalls and when isolated. This suggests that there is little naloxone-reversible opioid regulation of the cardiovascular system in cattle.

Naloxone alone resulted in a slight decrease in response latencies to the laser, indicating that endogenous opioids can influence basal pain sensitivity in cattle and that the dose of naloxone used was sufficient to affect pain sensitivity. However, the noticeable decrease in pain sensitivity that followed social isolation/novelty stress was clearly not blocked by naloxone. This suggests that this form of stress-induced hypoalgesia either involves opioid receptors that have a low binding for naloxone or is not opioid-based. In most species, naloxone binds primarily to  $\mu$ -opioid receptors, and  $\mu$ -opioid receptor agonists are potent analgesics (Zadina et al., 1997). Non-opioid stress-induced analgesia has been found repeatedly in rats (Chance, 1980; Watkins and Mayer, 1982; Yamada and Nabeshima, 1995), although it is still not clear what factors determine whether hypoalgesia is opioid- or non-opioid-based. At this point, we do not know whether the animals were less responsive only to painful stimuli, or whether social isolation in an unfamiliar environment affected their attention to other, non-painful stimuli. Research in humans suggests that anxiety can reduce responses to painful stimuli by influencing the focus of attention (Arntz et al., 1994).

Cattle respond to a variety of stressors with increased vocalization (Grandin, 1997, 1998). The present results

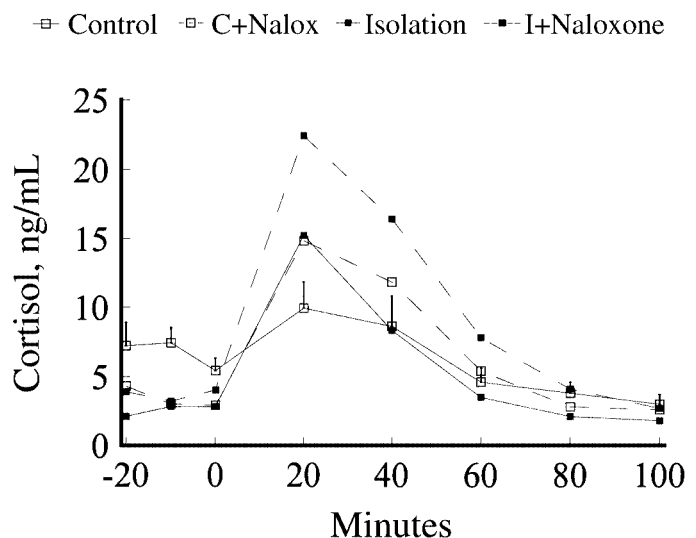


Figure 4. Mean (+ pooled SE) concentrations of cortisol at each time of sampling.

show that social isolation in unfamiliar environments increases the incidence of high-frequency vocalizations, with little influence on the occurrence of low-frequency vocalizations. High-frequency calls are also seen when animals are socially isolated in familiar environments, and the incidence of such calls is correlated with the degree of social contact that occurs before social separation (Boissy and LeNeindre, 1997). This suggests that their main function is to restore social contact. Barfield et al. (1994) found that cattle can use vocalizations to distinguish between individuals, although they did not distinguish between the different types of calls. Neither we nor Boissy and LeNeindre (1997) found an effect of social isolation on the incidence of low-frequency calls. Vocalizations of cattle seem to lie along a continuum rather than representing different categories of calls (Kiley, 1972), in which case the low-frequency calls may reflect a lower intensity of arousal.

However, even though naloxone alone did not influence the incidence of vocalization or of defecation/urination, we found that naloxone increased the incidence of vocalization during the period of social isolation in unfamiliar environments, particularly the incidence of the low-frequency vocalizations. Opioid antagonists have been found to increase isolation-induced vocalization in rat pups (e.g., Cardin and Hofer, 1990; Goodwin et al., 1994), puppies (Panksepp et al., 1980), primates (Kalin et al., 1988), and poultry (Sufka et al., 1994) and restraint-induced vocalization in pigs (Rushen and Ladewig, 1991). We can offer no suggestions for the mechanism by which naloxone tended to increase the incidence of low-frequency calls rather than of the higher-frequency calls, which are more usually associated with social isolation. Possibly, these two types of calls are based on different mechanisms rather than lying on a continuum. The results suggest that it may be necessary to distinguish between the different types of calls that cattle do make when using these behaviors

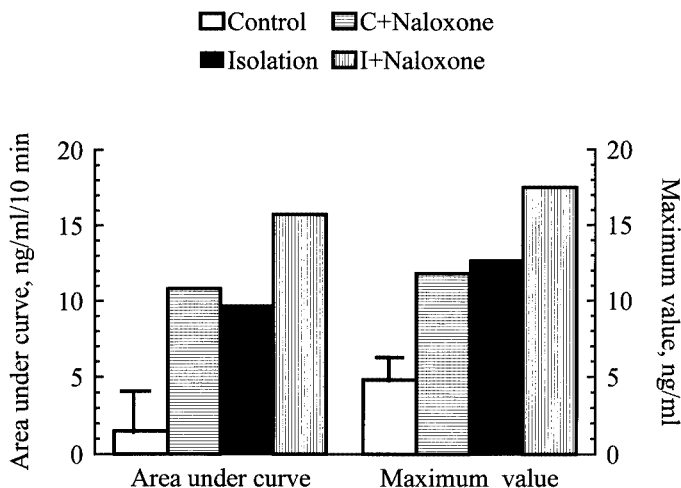


Figure 5. Mean (+ pooled SE) integrated area under the curve and maximum concentrations for cortisol.

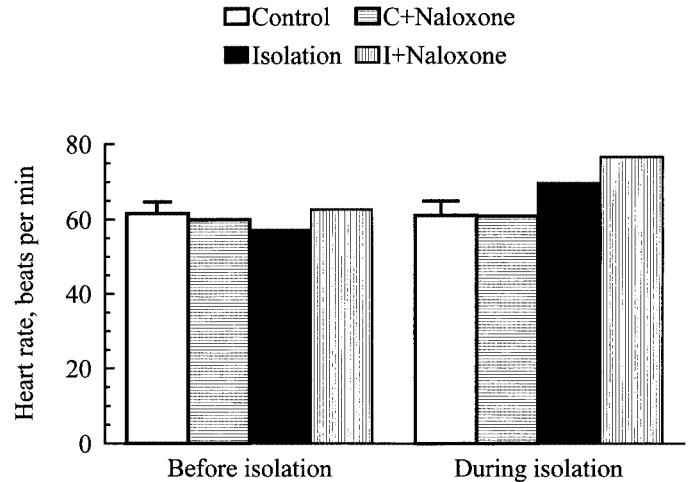


Figure 6. Mean (+ pooled SE) heart rate before and during the period of isolation.

as indicators of stress, which is often not done (e.g., Grandin, 1998).

Panksepp et al. (1980) have suggested that endogenous opioid systems underlie the responses of animals to many forms of stress, including social isolation, although more recent research suggests that a variety of opioid and non-opioid systems underlie the responses of animals to isolation (Goodwin and Barr, 1997). The increased incidence of vocalization in cattle socially isolated in unfamiliar environments following naloxone treatment suggests that endogenous opioids are involved in regulating some responses of cattle to stress.

## Implications

Dairy cattle, despite their docility and adaptation to being handled, are still highly sensitive to psychological stressors, and these stressors result in integrated physiological and behavioral responses. Although during stress naloxone may increase low-frequency vocalizations, the limited effect on cortisol concentrations and the lack of a clear effect on high-frequency vocalizations, heart rate, and pain sensitivity suggest that endogenous opioid peptides play only a limited role in integrating these responses in cattle. The signs of stress-induced hypoalgesia, despite the lack of evidence that this is opioid-based, show that cattle responses to painful stimuli can be affected by their environment. Our data from examining the combined effects of novelty and social isolation, and those from the examination of cattle response to social isolation alone, underline the gregarious nature of cattle.

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