Repeated mixing and isolation: Measuring chronic, intermittent stress in Holstein calves¹

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ABSTRACT

Objectives of this study were to determine the physiological effects of psychological stress applied to dairy calves and to test if molasses consumption could be used to validate that a stressed condition was achieved. Twenty male calves (3 wk old) received jugular catheters and were randomly assigned to control (CTR; $n = 4$ pens of 1 calf per pen) or social stress treatments (STR; $n = 4$ pens of 4 calves per pen). The STR treatment included 5 cycles of 24-h isolation followed by regrouping with unfamiliar animals for 48 h (over 15 d). An ACTH challenge (0.1 IU/kg of body weight) was used to determine adrenal fatigue. Peak and total cortisol concentrations were greater for STR calves until the ACTH challenge. After the ACTH challenge, CTR calf cortisol increased and STR calf cortisol continued to decrease, suggesting adrenal fatigue. The number of calves that became positive for fecal shedding of *Salmonella* after the acute stress of being moved and the number of calves that were positive after the move decreased with each move. Fifty-six percent of STR calves changed from negative to positive for shedding after the first move compared with 18.75% of STR calves remaining negative after the third move. Difference in fecal shedding of *Enterobacteriaceae* from samples taken before and after moving calves on d 6 was less than that on d 2, 3, and 5. Leukocyte counts were not different, but trends for day effects were detected for neutrophil and monocyte percentages. Molasses consumption was greater for STR calves on d 2 and 11, as was total consumption. Latency to lie after eating also increased as the study progressed; STR calves required more time to lie after eating on d 12 than on d 3, and latency to lie was greater for STR than CTR on d 4, 8, 12, and 14. The STR calves also stood more than the

CTR calves in the 4-h afternoon period on d 4, 5, 7, and 14. However, during the 4-h morning observations on d 14 (ACTH challenge), CTR calves stood more than STR calves. This model induced chronic stress, as characterized by adrenal fatigue, which was confirmed by molasses consumption and behavior changes. Therefore, molasses consumption could be used to confirm social stress in experimental models.

Key words: bacteria, cortisol, dairy calf, stress

INTRODUCTION

Dairy calf management, as recommended by the Babcock Institute of the University of Wisconsin (Wattiaux, 2000), includes a period of isolation followed by regrouping. To reduce pathogen load originating from the adult animals or from cross-contamination among calves (McGuirk, 2008), dairy farmers frequently remove neonates from their dams between 1 and 12 h postpartum and rear them in individual hutches or pens (USDA-APHIS, 2008). Following this period of isolation, heifers are typically weaned at 6 to 8 wk of age and placed into groups. Male calves used for dairy beef or veal are now grouped earlier (4 to 6 wk), with emphasis on social needs of calves, and are periodically regrouped according to size.

After weaning, calf management then follows a routine of repeated regrouping to maximize group homogeneity (Garnsworthy, 2005). Each of these activities—weaning (Hickey et al., 2003), transportation (Fazio and Ferlazzo, 2003), isolation (Moberg, 2000), mixing, and change of housing (Veissier et al., 2001)—is known to elevate circulating cortisol concentrations, an indicator of acute stress.

Acute stress is the physiological response to physical or psychological threats to an animal's homeostasis. As the animal perceives a threat, corticotropin-releasing factor is produced in the hypothalamus, initiating a cascade of events along the hypothalamic-pituitary-adrenal (HPA) axis. Stimulation of corticotropin-releasing factor receptors located in the anterior pituitary gland produces a release of ACTH and glucocorticoids such as cortisol from the adrenal gland. As part of the

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allostatic control of stress, cortisol regulates the release of glucose from the liver and inhibits the production of ACTH in the pituitary, ultimately decreasing the production of cortisol (Douglas, 2005). Mild acute stimulation of the HPA axis causes a transient spike in metabolism and the temporary suspension of digestion, growth, and repair (Sapolsky, 2004). If an animal has sufficient reserves, it can recover from these physiological changes.

During acute stress, elevated circulating cortisol concentrations activate glucocorticoid receptors in the liver that increase the expression of glucose-6-phosphatase, which in turn increases the production and secretion of glucose. Acute stress also increases the secretion of adrenaline, which stimulates the production of insulin. During chronic stress, biological reserves become depleted, leading to adrenal fatigue and hypoglycemia.

Another physiological consequence of prolonged ACTH production and chronic elevation of plasma cortisol concentration is the disruption of immune system functions. Cytoplasmic glucocorticoid receptors are found in almost all mammalian cells. The percentages of neutrophils and eosinophils increase with acute stress. In contrast, chronic elevation of ACTH concentration reduces the production of neutrophils (Bilandzić et al., 2006). Chronic suppression of the immune system shifts the leukocyte differential profile, rendering even the humoral immune system inefficient (Moberg, 2000).

In addition to the benefits of elevating the humoral immune response at the expense of cell-mediated immunity during acute stress (Bilandzić et al., 2006), changes in behavior during acute stress are beneficial for the animal. Increased serum cortisol increases the production and secretion of noradrenaline, which heightens attention. However, repeated stimulation of the hypothalamus and activation of amygdala neuronal cells modifies the morphology of dendrites in these brain regions. This altered morphology changes the sensitivity of neurons and may cause anxiety, increased aggression, and other behavioral changes associated with chronic stress (Cook and Wellman, 2004).

One behavioral change noted in studies of chronic stress in mice and rats is a change in voluntary consumption of sucrose (Pothion et al., 2004). Change in sucrose consumption is typically measured as a percentage of total liquid intake in a 2-bottle preference test (pure water vs. 10% sucrose solution; Pothion et al., 2004). This change, relative to baseline or control animals, is accepted as an indicator of stress and is used in rodents to validate experimental stress protocols (Brenes-Sáenz et al., 2006). It is unknown if dairy calves will modify their consumption of high-sugar feedstuffs as a reaction to stress, as seen in mice and rats. However, a preliminary study conducted in our laboratory dem-

onstrated that upon experimentally inducing greater glucose demand with phlorizin, calves increased their molasses consumption (Wilcox et al., 2008). The observation that endogenous glucose demand is reflected in a change in sucrose consumption behavior suggested that stress may also modify the sucrose consumption behavior of dairy calves.

Changes in behavior, shifts in immune system profile, and HPA axis resistance to ACTH challenge are indicative of chronic stress and are evident in animals that have been exposed to repeated, intermittent, mild stress. For example, weekly regrouping of calves during an 11-wk study resulted in a diminished reaction to novel acute stressors and attenuated cortisol response to ACTH challenges, compared with group-stable controls (Raussi et al., 2004). Social isolation and regrouping are mild psychological stressors that may occur frequently over the life of a calf under current management recommendations. The objectives of this study were to determine physiological effects of psychological stress of isolation and regrouping applied to dairy calves and to test if molasses consumption could be used to validate that a stressed condition was achieved.

MATERIALS AND METHODS

Animals

All animal-related protocols were approved by the Animal Care and Use Committee of Purdue University. Twenty 3-wk-old male calves were fed 2 L of colostrum twice within 24 h of birth. Calves with 24-h plasma protein concentrations of >5.5 mg/dL (measured by refractometer, Brix, Atago, Bellevue, WA) were transported from a commercial herd (Strauss Veal Feeds Inc., North Manchester, IN) for 2 h to the Farm Animal Behavior Laboratory of the Livestock Behavior Research Unit (US Department of Agriculture Agricultural Research Service, West Lafayette, IN). Upon arrival of calves, BW and rectal temperature were recorded. Calves were housed for 3 d in individual pens that converted to group pens by removal of partitions. Calves were then catheterized with jugular venous catheters (Cook Veterinary Products, Bloomington, IN), which were maintained with sterile heparinized saline (4 U of heparin/L of saline; Abbott Laboratories, Abbott Park, IL). After 3 d, calves were assigned randomly to pens of group or individually housed calves. Four pens of 1 control (**CTR**) calf each and 4 pens of 4 stressed (**STR**) calves each were used. Two extra calves were kept in individual pens as replacements to maintain group size if STR calves had to be taken off study. Calves were fed a continuation of the diet and standard operating procedures from the veal farms from which

they came were continued. Calves were fed calf milk replacer in buckets (20% CP and 20% fat, nonmedicated; Strauss Veal Feeds Inc.) reconstituted at 12.5% solids and at 10% of BW twice daily (0700 and 1800 h; 20% of BW daily) and adjusted over time per the producer's protocol, and calves were allowed access to fresh water in buckets at all times. Because these were veal calves, no dry feed (starter grain) was offered.

Chronic, Intermittent, Mild Stress Model

Calves were subjected to 5 cycles of isolation and regrouping (STR) or remained in individual calf pens (CTR). After completion of all 5 cycles, all calves were challenged with 0.1 IU of ACTH/kg of BW (Sigma Aldrich, St. Louis, MO).

After preconditioning to the Livestock Behavior Research Unit facility, 5 groups of 4 calves consisting of 1 CTR group and 4 stressed groups were placed in 7.31-m² group pens with straw bedding. These 5 pens were visually and physically isolated from one another by a solid plastic wall (Agri-Plastics Corp., Grassie, ON, Canada). The pen for the CTR calves was divided into 4 equal pens with a 3-rail iron fence. This allowed for auditory, visual, and limited physical contact between calves. However, it also allowed monitoring of individual molasses consumption. After 48 h, the 16 calves from the 4 stress-treated pens were moved (by calf cart; Wiggins, Gersham, OR) into isolation pens. After 24 h, ST calves were moved out of isolation pens and into group pens consisting of 4 unfamiliar calves. This cycle was repeated 5 times, with new groups of unfamiliar calves formed each time.

Isolation pens, constructed of solid plastic walls that created visual and physical isolation, were 1.4 m² and bedded with straw. Isolation pens were located in a room adjacent to the room with CTR calf and group-housing pens within the same facility. Calves were placed in these pens such that members who formed the previous group were not near each other and members of the next group to be formed were not near each other. Between each move, both group and isolation pens were cleaned, wiped with disinfectant cleaning solution (0.4% vol/vol Rocal D-plus, Pfizer Inc., New York, NY), and bedded with fresh straw to rid pens of odors from familiar calves. Control calves were moved in the calf cart on the same day for an equal time as STR calves and returned to their pen each time. Calves were given access to liquid molasses (Evolved Habitats, New Roads, LA) in standard-size feed buckets while in isolation pens. Control calves also were given access to molasses in their home pens at that time (24 h). All calves had a 12-h light and 12-h dark daily cycle throughout the study.

Collection of Peripheral Blood

Blood samples were collected by a single person trained to restrain and collect blood via jugular catheters into 2-mL tubes containing 25% K₂-EDTA and placed on ice. Catheters were flushed with 12 mL of saline followed by 3 mL of heparinized saline (100 g/dL). Blood samples were collected at 15 and 0 min before calves being moved (baseline). After each move, blood samples were taken from calves immediately and then 15, 30, 60, 90, 120, and 300 min after calves had been moved.

ACTH Challenge

Blood samples were taken in a similar manner for the ACTH challenge: 2 baseline samples before injection of ACTH and then 15, 30, 60, 90, 120, and 300 min after ACTH challenge. Initial blood samples were collected 48 h after the last cycle in group pens. Two milliliters of blood was collected 15 min and just before injection of ACTH (0.1 IU/kg of BW in 3 mL of normal saline; Sigma-Aldrich), which was delivered through the jugular catheter and followed by 6 mL of normal saline. This dose has been used previously in our laboratory to create a significant and predictable cortisol response (Lay et al., 2008). Stress-treated calves were in group pens and CTR calves were in single calf pens throughout the ACTH challenge.

Fecal Sample Collection and Bacterial Evaluation

Fecal samples were collected before and after each move. Individual fecal samples were collected directly from the rectum using sterile swabs (Johnson and Johnson, Langhorne, PA). One-gram samples were processed for isolation of *Salmonella enterica*. An additional 1-g sample was processed for enumeration of total *Enterobacteriaceae*.

For *Salmonella* isolation, individual 1-g fecal samples were initially enriched in tetrathionate broth (1:10 dilution; 37°C for 24 h; Neogen Corp., Lansing, MI), transferred (0.1 mL) to 10 mL of Rappaport-Vassiliadis broth (Neogen Corp.), incubated at 42°C for 24 h, and streaked on 4 selective solid media xylose-lysine-tergitol-4 (Neogen Corp.) agar plates and incubated at 37°C for 24 h. Red to yellow colonies with black centers were picked from xylose-lysine-tergitol-4 agar plates, streaked on Rambach agar (DRG International Inc., Mountainside, NJ) and incubated at 37°C for 24 h. Red colonies were considered positive for *Salmonella*.

To enumerate total *Enterobacteriaceae*, individual samples were serially diluted (10-fold) in buffered peptone water (Neogen Corp.). One milliliter of each

dilution was plated in duplicate on a 20-cm² all-in-one plating system (Petrifilm *Enterobacteriaceae* Count Plates, 3M Microbiology, St. Paul, MN) and incubated at 37°C for 24 h. The number of colony-forming units (cfu/g) for each count plate was recorded.

Environmental Measures

The ambient temperature and relative humidity were recorded every 30 min for the 14 d of the study with data loggers (Hobo, MicroDAQ, Warner, NH). Data loggers were placed about 1 m above the floor at each end of the room with isolation pens. Another set of data loggers was placed about 1.3 m above the floor at the end of the room that housed CTR and group calves.

Behavioral Measures

Behavior of calves in group pens was recorded using ceiling-mounted cameras (VW-BP330, Panasonic Secaucus, NJ) and a digital video recording system (Xenon; Interpacific, Northbrook, IL). Behaviors were recorded continuously during the 12-h light period throughout the study. Animal posture of laying or standing and activities, including walking, running, eating, drinking, resting, being alert, socially interacting, and self-grooming, were measured using a 10-min instantaneous scan sample technique (behaviors that occurred at the end of each 10-min period over the 12-h light cycle for each of the days). Latency to lie down after eating in the morning and evening was determined each day using continuous behavior observations. The percentage of observations in which calves were exhibiting play behavior was calculated.

Molasses was weighed in buckets and buckets were placed on hooks in each isolation pen so that molasses was available as soon as the calf entered the pen. At the same time, molasses in buckets was placed in CTR pens in place of milk buckets. The next morning just before feeding, each bucket with remaining molasses was weighed again. Consumption of molasses was calculated as the difference of those 2 weights.

Enumeration and Composition of Blood Leukocyte Population

Jugular blood collected into the EDTA tubes just before the calf being moved each time was set on a tube rocker and allowed to come to room temperature. The 5-part blood cell differential counts consisting of hematocrit percentages, total white blood cell counts, and counts and relative percentages of neutrophils, lymphocytes, monocytes, eosinophils, and basophils

were determined using an automated cell counter (Hemavet HV950FS, Drew Scientific, Dallas, TX).

Cortisol Assay

Total circulating plasma cortisol concentration in each of the blood samples was measured by RIA (Coat-a-Count RIA Cortisol kit, Diagnostic Products Corp., Los Angeles, CA). The protocol has been previously validated in this laboratory for measuring bovine plasma cortisol concentrations (Eicher et al., 2007). Briefly, 500 μ L of buffer, 10 μ L of the calibrators, CTR and STR samples in duplicate, and 1.0 mL of ¹²⁵I cortisol was added to 4 uncoated 12 $\times$ 75 mm polypropylene tubes (for total counts and nonspecific binding in duplicate) and cortisol-specific antibody-coated tubes. After vortexing each tube and incubating for 45 min at 37°C, tube contents were decanted thoroughly and counted for 1 min using a gamma counter (2470 Wizard, Perkin Elmer, Waltham, MA). Sample concentrations were calculated from the logit-log representation of the calibration curve and were presented as the average nonspecific binding-corrected counts per min. Interassay CV was 2.12%, and intraassay CV was $\leq$ 1.7%. Samples with CV >5% were rerun.

Statistical Analysis

Two calves were kept in reserve as replacement stressed animals in the event that they were needed. The replacements were used before d 5 and maintained the integrity of the experimental design so that at all times group pens could be regrouped with a total of 4 calves each unfamiliar to the other. However, blood and behavior samples were not collected from these replacement calves, so data analysis reflects a sample size of 14 for the stressed group after d 5. Animal numbers were determined for each study using variation in previous work and $\alpha = 0.05$ in SAS Power test. All statistical analyses were conducted using the MIXED procedure of SAS, version 9.1 (SAS Institute Inc., Cary, NC). Calves nested within treatment were considered to be random. Normality of the data was checked using the UNIVARIATE procedure and residuals were examined using PROC GLM. When necessary, data were transformed to achieve normality and the statistical ANOVA and *P*-values reported were calculated using transformed data. Graphs are presented using least squares means of untransformed data. Treatment, day, and move were considered fixed effects for variables, except for cortisol data, where time and the interactions treated $\times$ day and treated $\times$ move were considered fixed effects. The area under the curve, determined by the concentration of cortisol found in each of the 9 blood samples taken

on each of the move days, was calculated using the SAS macro published by Yeh (2002). Cortisol data required an inverse square root transformation, whereas the leukocyte data were nontransformed and molasses consumption data required a square root transformation. A lambda of -1 was the optimum transformation for normalizing the behavior data as found by the BOXCOX procedure of SAS. Probability values reported reflect statistical analysis of transformed data, and the more conservative Tukey adjustment was used. Correlation analysis was performed for 3 d of molasses consumption and latency to lie and for 4 d of molasses consumption and peak cortisol.

RESULTS AND DISCUSSION

Environment

On d 3 of the study, ambient temperatures increased to 37°C with relative humidity varying between 80.5 and 94.8% (every 15 min samples, 24 h/d, similar to environmental humidity). Barn temperatures rose briefly to 36.2°C until an effective cooling system maintained the barn temperature at 29.5°C or below with a mean daily temperature 27.1°C or below. Calf rectal temperatures

did not differ between treatments, but varied by day (data not shown). The shedding of *Enterobacteriaceae*, neutrophil percentages, and percentage of observations spent standing reflect that temperature increased in STR and CTR calves.

Cortisol

Area under the curve of cortisol concentrations over the 315-min experimental period and peak cortisol and baseline cortisol concentrations (Figure 1) had a treatment $\times$ day interaction ($P < 0.01$). Area under the curve and peak cortisol concentrations for STR calves on d 1 to 5 were greater than those of CTR calves and lower than those of CTR calves after ACTH injections. Baseline concentrations also had a treatment $\times$ day effect. However, for baseline values, CTR calves had greater cortisol concentrations on d 2 and 3 but lesser concentrations throughout the remainder of the study.

Decreased cortisol concentration in response to a repeated stress stimulus can indicate adrenal fatigue or habituation (Moberg, 2000). Social stress of calves by isolation and regrouping in this study not only increased peak and total cortisol secretions but baseline concentration as well. When ACTH was injected, STR

Figure 1. Mean area under the curve (AUC; A), peak (B), and baseline (C) cortisol concentrations by day for control (CTR) and social stress-treated calves (STR). The mean of the AUC derived from the cortisol concentration in blood samples taken after treatment, peak cortisol concentrations, and baseline cortisol concentrations had a treatment by day effect ($P = 0.001$). Graph means are nontransformed data. ^{a,b}Means with differing superscripts within a day are different ($P < 0.05$). Injections of ACTH are indicated by arrows.

Table 1. Fecal shedding of *Salmonella* for stress-treated (STR; n = 16) and control (CTR; n = 4) calves before and after moving to new groups

Move number	Treatment	Shedding pre-move	Shedding post-move	Change: – to + (%)
1	STR	4	11	9 (56)
	CTR	3	3	1 (25)
2	STR	0	3	3 (19)
	CTR	2	4	2 (50)
3	STR	3	6	3 (19)
	CTR	1	1	0 (0)
4	STR	0	1	1 (6)
	CTR	1	0	0 (0)
Overall	STR	7 ^{a,A}	21 ^{b,A}	16 ^A
	CTR	7 ^{a,A}	8 ^{a,B}	3 ^B

^{a,b}Statistical comparison within the same row by χ^2 ($P < 0.05$).

^{A,B}Statistical comparison between STR and CTR (within a column) by χ^2 ($P < 0.05$).

calves did not respond, but CTR calves responded with concentrations greater than or equal to those of STR calves during the stress events. These data show that adrenal fatigue was achieved using this model over 2 wk.

Characterization of Blood Leukocyte Populations

Hematocrit percentage (data not shown) tended to have ($P = 0.08$) a treatment $\times$ day effect. Hematocrit percentages were greater ($P = 0.05$) for CTR than for STR calves on d 9 (28.0 and 21.48%, respectively). No treatment or treatment $\times$ day effects were found for any of the leukocyte counts (data not shown). However, a day effect was found for percentages of neutrophils ($P = 0.01$), lymphocytes ($P = 0.03$), and monocytes ($P = 0.02$).

Because one focus of this study was on the effect of repeated isolation and mixing on the leukocyte profile, blood samples designated for this analysis were taken just before each move. The effect on the leukocyte profile of acute stress caused by the calf cart ride is, therefore, not evident from this data set. Increased cortisol concentrations are known to cause depressed lymphocyte counts (Riondato et al., 2008) and neutrophilia (Madsen-Bouterse et al., 2006). In this study, total leukocyte concentration did not change due to the calf being stressed. Because changes in neutrophil numbers are a later event than increases in cortisol concentrations after a stressor and can be observed at 24 h after the stressor, samples were collected only once on each day of blood collections.

Salmonella and *Enterobacteriaceae*

During the first move, 4 and 3 calves (STR and CTR, respectively) were positive for fecal shedding of *Salmonella* before the move and 11 and 3 were positive

after the move. During the second move, 0 and 2 (STR and CTR, respectively) were positive for fecal shedding of *Salmonella* before the move, whereas 3 and 4 (STR and CTR, respectively) were positive after the move. Thereafter, the number of CTR calves that were positive after the move decreased with each move (Table 1). However, more STR calves (6) were positive after the third move and did not decrease until the fourth move. After the first move of group to isolation, 56.25% of STR calves and 50% of CTR calves changed from negative to positive for shedding. After the second move of isolation to group, 18.75% of stressed calves and 50% of CTR calves changed from negative to positive for shedding. After the third move from group to isolation, 18.75% of STR calves and no CTR calves changed from negative to positive for shedding of *Salmonella*.

A small number of calves (12.5% for STR group and 25% for the CTR) changed from positive to negative on the first move (data not shown). Twenty-five percent of the CTR calves also changed from positive to negative for fecal shedding of *Salmonella* on the fourth move. Further analysis of *Salmonella* shedding was not done because the calves were treated for pneumonia.

The number of *Enterobacteriaceae* (cfu) found in fecal samples taken before each move (Figure 2A) showed treatment ($P = 0.038$) and day ($P = 0.017$) effects. The mean number of *Enterobacteriaceae* in the STR group before the move on d 3 tended ($P = 0.095$) to be greater than that on d 2, and was greater than on d 5 ($P = 0.007$) and d 6 ($P < 0.001$). Difference in the mean number of *Enterobacteriaceae* from fecal samples taken before and after moving calves, whether into or out of isolation pens (Figure 2A), followed the same pattern. Mean difference was significantly affected by day ($P = 0.001$), and a treatment $\times$ day interaction ($P = 0.023$) was detected. On d 3, difference in fecal shedding of *Enterobacteriaceae* for STR calves was greater ($P < 0.001$) than for CTR calves.

Figure 2. The mean number (cfu) of *Enterobacteriaceae* in fecal samples taken before each move (A) and the mean difference in number of *Enterobacteriaceae* in fecal samples taken before and after moving calves (B). Graph means are nontransformed data. CTR = control, STR = social stress treated. ^{a,b}Means within a day with different letters differ ($P < 0.05$).

Habituation to acute stress was also evident in the pattern of fecal shedding of total *Enterobacteriaceae*, and of *Salmonella* in particular. Composition and quantity of pathogenic intestinal bacteria is known to increase because of stress (Kanitz et al., 2004; Williams et al., 2008). Fecal shedding of *Salmonella* is directly related to ileal loop concentration (Callaway et al., 2005). Both STR and CTR calves entered the study shedding approximately 107.5 cfu of *Enterobacteriaceae* per gram of fecal matter. This high number might result from the stress of travel, as evidenced by 2 calves testing positive for *Salmonella* at that time, too. By the second move, the number of *Enterobacteriaceae* found in the fecal samples peaked and then steadily decreased over the next 2 moves, as did the difference between the number of *Enterobacteriaceae* before and after each move. Fecal shedding of *Salmonella* also followed this pattern. After the first move, 9 of 16 STR calves that were negative before the move tested positive for *Salmonella* in samples taken after the move. This declined rapidly over the next 3 moves until 1 out of the 16 STR calves changed from testing negative for fecal shedding of *Salmonella* before the move to being positive after the move. This pattern of declining differences of fecal

shedding pre- and post-move confirmed the assumption that declining plasma cortisol concentration was due, at least in part, to habituation to the acute stressors. Fecal shedding samples were not collected after the fourth move because some calves were given antibiotics to combat disease.

Behavior

Molasses Consumption. Molasses consumption while the calves were in isolation (STR) or individual (CTR) pens (Figure 3A) was affected by treatment ($P = 0.006$) and tended to have a day $\times$ treatment interaction ($P = 0.065$). On d 2 ($P = 0.029$) and d 11 ($P = 0.008$), STR calves consumed more molasses than did CTR calves. Total molasses consumed over the experiment (Figure 3B) was greater ($P = 0.02$) for STR than for CTR calves.

Increased anxiety, not seen in the cortisol concentration values, was reflected in increased molasses consumption. Increased plasma concentrations of norepinephrine increase anxiety (Shishkina et al., 2004). Norepinephrine and epinephrine stimulate glucagon release, glycogenesis, and food consumption (Ste. Marie

Figure 3. Mean daily molasses consumption (A) and total molasses consumption (B) of control (CTR) and social stress-treated (STR) calves. Graph means are nontransformed data. ^{a,b}Means within a day with differing letters differ $P < 0.05$.

and Palmiter, 2003). Psychological stress is also known to activate the reward centers and the increased desire for carbohydrate-rich comfort food (Dallman et al., 2003). Therefore, the increased glucose consumption by STR calves may be an indicator not only of HPA axis stimulation but also of sympatho-adrenal medullary axis stimulation.

Correlation coefficients of molasses consumption (during isolation) and latency to lie (in groups or control) were 0.49 ($P = 0.11$) and 0.46 ($P = 0.06$) on d 5 and 11, respectively; however, the correlation coefficient on d 8 was -0.004 ($P = 0.99$). The correlation coefficients for molasses consumption and peak cortisol were 0.03 ($P = 0.92$), -0.08 ($P = 0.81$), 0.34 ($P = 0.19$), and 0.61 ($P = 0.03$) on d 1, 5, 8, and 11. Reber et al. (2006) demonstrated adrenal fatigue and associated BW and thymus weight loss in mice with a 19-d chronic intermittent psychosocial stress of social defeat and overcrowding. Accordingly, they demonstrated that cytokine secretion of inflammatory cytokines was greater in the chronic stress model. In contrast, Finger et al. (2012) found increased weight gain, intake, and caloric efficiency in mice fed a low-fat diet that were under a chronic intermittent psychosocial stress, find-

Figure 4. Latency to lie after eating for control (CTR) and social stress-treated (STR) calves. Graph means are nontransformed data. ^{a,b}Means within a day with differing letters differ ($P < 0.05$).

ings related to circulating leptin. The changes observed in the current study could be related to the increased molasses intake of the STR calves.

Latency to Lie After Eating. A treatment ($P < 0.001$) effect and treatment $\times$ day interaction ($P = 0.04$) were detected for latency to lie after eating (Figure 4). Stressed calves took longer ($P = 0.03$) to lie down after eating compared with CTR calves on d 5 ($P = 0.03$), d 8 ($P < 0.002$), d 12 ($P < 0.003$), and d 14 ($P < 0.001$).

Standing Behavior. Percentage of observations in which calves were standing was affected by day ($P = 0.015$) and treatment ($P = 0.037$; Figure 5A). When 4-h periods in the a.m. (Figure 5B) and p.m. (Figure 5C) were analyzed separately, treatment ($P = 0.05$ and 0.031 , for a.m. and p.m., respectively) and day ($P = 0.043$ and 0.015 , for a.m. and p.m., respectively) effects were detected.

Standing behavior of STR calves also indicated adaptation to the stress model. Stressed calves clearly stood more than CTR calves during the first full day in group pens, after the first cycle of group to isolation and isolation to group moves. This behavior decreased to the level of the CTR calves for the next cycle but steadily increased in subsequent cycles. Our findings in the 4-h a.m. and p.m. analyses are consistent with findings

in other studies in which behavioral responses of an individual animal to repeated controllable stressors depended on past experiences with that particular stressor (Bear et al., 2001), and responses were attenuated with repetition of the stressor (Vermetten and Bremner, 2002). The ACTH challenge, when analyzed over 12 h, apparently did not produce a change in behavior of the CTR calves despite an elevated cortisol concentration. However, by comparing the 12-h standing activity of CTR and STR calves, a disruption of circadian rhythm (principally in the afternoon) was evident. Thus, it seemed appropriate to analyze standing behavior during particular segments of the day.

During the first 4 h of the ACTH challenge, CTR calves stood more than on previous mornings and more than STR calves on that morning. In the 4 h of the afternoon on the day of ACTH challenge, CTR calves stood less than on previous afternoons, perhaps from the unaccustomed amount of activity earlier in the day. Standing behavior of STR calves indicated not only habituation to acute stress but perhaps also increased anxiety not apparent by analysis of cortisol concentrations.

It is known that the HPA system adapts easily to mild stressors; however, the sympatho-adrenal medullary system does not adapt as easily (Schommer et al., 2003). This may be why, despite an apparent adaptation

Figure 5. Mean percentage of observations of calves spent standing when calves were observed in group pens for 12 h (A), for 4 h in the morning (B), and for 4 h in the afternoon (C) for control (CTR) and social stress treated (STR) calves. Means are non-transformed data. ^{a,b}Means within a day with differing letters differ ($P < 0.05$).

to the stress model, mean afternoon standing behavior and latency to lie after eating in STR calves indicated increasing anxiety. Addition of a startle reaction test with a novel stimulus or measuring circulating norepinephrine or epinephrine (products of the sympatho-adrenal medullary system activation) throughout the study would have helped to confirm increasing anxiety.

Molasses consumption increased in CTR calves and decreased in STR calves on d 5. This may also be due to heat stress of CTR calves and increased handling of STR calves to check and reduce body temperature. The number of *Enterobacteriaceae* in CTR calves showed an erratic pattern. However, analysis within STR calves showed a steady decline, indicating habituation. This pattern of wide variation is repeated in means found for behavior data. Behavior data, both the latency to lie after eating and the percentage of observations when cows were standing, gave a clear indication of habituation to acute stress but increasing anxiety with repeated applications of stress in STR animals.

This model employed 5 cycles of isolation and regrouping repeated at regular intervals. This repeated application of mild psychological stressors produced an initial acute stress reaction. This acute stress reaction diminished beginning with the second cycle of isolation and regrouping. Then, isolation and regrouping cycles 3 and 4 caused a trend of increased but muted acute stress reaction. The regular intervals at which the psychological stressors were applied seemed to habituate calves to the stressors and produced chronic stress, as intended.

CONCLUSIONS

This study was designed to determine if chronic stress could be induced by isolation and regrouping and to investigate whether molasses consumption changed as a result of chronic psychological stress. This model appeared to achieve chronic stress, as characterized by adrenal fatigue, and the stress treatment caused an increase in molasses consumption. The latter finding can be further studied and developed to be used as a noninvasive indicator of acute or chronic stressors in dairy calf research. Refinement of this stress model may be useful to study chronic stress in dairy calves. These results indicate that chronic stress may be achieved and that molasses consumption in calves may be used to verify that stress has occurred, as seen by sucrose consumption in some mouse models.

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