



## The effects of overstocking Holstein dairy cattle during the dry period on cortisol secretion and energy metabolism

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### ABSTRACT

The objective was to determine whether overstocking during the dry period could alter physiological parameters in dairy cattle associated with cortisol secretion and energy metabolism. Four groups of 10 late-gestation, nonlactating Holstein cows (6 multiparous cows and 4 heifers per group) were exposed to both a control [1 lying stall/cow and 0.67 m of linear feed bunk (FB) space/cow] and an overstocked (1 stall/2 cows and 0.34 m of FB space/cow) stocking density treatment in a replicated crossover design with 14-d treatment periods. On d 1, 3, 5, 7, 9, and 11 of each 14-d treatment period, blood and fecal samples were collected from each cow for the determination of plasma nonesterified fatty acids (NEFA), glucose, insulin, and fecal cortisol metabolite (11,17-dioxoandrostane; 11,17-DOA) concentrations. Glucose and ACTH challenges were conducted on d 13 and 14, respectively, of each treatment period. Dry matter intake per cow was greater during the overstocked period than during the control period (15.9 vs.  $14.9 \pm 0.5$  kg/d). Plasma NEFA and glucose concentrations were greater ( $0.11$  vs.  $0.09 \pm 0.006$  mEq/L and  $65.3$  vs.  $64.2 \pm 1.1$  mg/dL, respectively) and 11,17-DOA concentration tended to be greater ( $891$  vs.  $792 \pm 86$  ng/g of fecal dry matter) during the overstocked period than during the control period. Insulin concentration was the same during the overstocked ( $29.0 \pm 2.1$   $\mu$ IU/mL) and control ( $31.2 \pm 2.1$   $\mu$ IU/mL) periods. Overstocking was associated with slightly slower glucose clearance from circulation as evidenced by a greater area under the curve estimate for the glucose response curves ( $2,882$  vs.  $2,657 \pm 165$  mg/dL  $\times$  180 min) but a more attenuated insulin response (insulin area under the curve =  $5,258$  vs.  $6,692 \pm 1,104$   $\mu$ IU/mL  $\times$  180 min for the overstocked and control periods, respectively). Changes in tissue glucose uptake may be mediated by changes in pancreatic in-

sulin secretion or peripheral tissue responses to insulin. The role of glucocorticoids in mediating these changes in energy metabolism is still unclear because stocking density treatment was not associated with changes in adrenal secretion of cortisol following ACTH stimulation.

**Key words:** overstocking, energy metabolism, cortisol

### INTRODUCTION

Industry-recommended best practice with regard to space allowance in dairy barns is to provide 1 lying stall for every cow and provide 60 cm (24 inches) of linear feed bunk space per animal (NFACC, 2009). Despite these recommendations, overstocking is still common; survey data of freestall farms in the United States show that 58% of farms have less than the recommended 60 cm of feeding space and 43% have less than the recommended lying stall availability based on average cow numbers on the farm during the year (USDA, 2010).

The behavioral consequences of overstocking are well documented. Feeding time is decreased and time spent standing idly (i.e., standing not feeding) is increased when cows are overstocked at the feed bunk (Huzzey et al., 2006). Increased aggressive displacements are often observed at the overstocked feed bunk or freestalls (Huzzey et al., 2006; Fregonesi et al., 2007); these competitive environments can make it difficult for some cows to gain access to these resources, particularly when motivation to eat or rest is high, such as after fresh feed delivery (DeVries et al., 2004) or during the overnight hours (Fregonesi et al., 2007), respectively. The relationship between overstocking and DMI is less clear and is likely influenced by feeding rate. Some researchers have reported no effects of overstocking on DMI (Proudfoot et al., 2009), whereas others have reported an increase in DMI (Olofsson, 1999) in response to overstocking.

The physiological consequences of overstocking have still not been thoroughly investigated. Previous work has shown that when cows are regrouped into a high stocking density group (Friend et al., 1977) or

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subjected to overcrowding in the resting area (Friend et al., 1979), they have a greater cortisol response to ACTH challenge compared with cows that are not regrouped or overcrowded, respectively. This work suggests that alterations in adrenal function may occur in response to the stress of overstocking. Changes in stress physiology may be a reflection of the physiological adaptations that occur as cows try to cope with an overcrowded environment, and an increase in plasma cortisol concentration may influence other physiological processes. Glucocorticoids are important regulators of energy metabolism; they help to raise circulating glucose concentrations by increasing hepatic gluconeogenesis and inhibiting peripheral tissue uptake of glucose. They also contribute to the regulation of lipolysis and lipogenesis and facilitate increased plasma NEFA concentrations (reviewed in Parker and Rainey, 2004). Excess glucocorticoid production has also been associated with insulin resistance (reviewed in Andrews and Walker, 1999). To date, no work has explored changes in adrenal activity and energy metabolism in response to the potential stress associated with overstocking dairy cattle.

The hypothesis for this study was that if overstocking can lead to changes in glucocorticoid secretion, observable changes in energy metabolism may occur. The objective of this study was to measure physiological responses to overcrowding during the dry period. The effect of overcrowding on energy metabolism was evaluated by (1) measuring daily NEFA, insulin, and glucose concentrations, and (2) the response of these analytes to an intravenous glucose tolerance test (**GTT**). Changes in stress physiology (adrenal activity) were evaluated by measuring daily fecal cortisol metabolite concentrations and the plasma cortisol response to an ACTH challenge.

## MATERIALS AND METHODS

### *Animals, Housing, and Diet*

This study was conducted between January and April 2010 at the Cornell Teaching and Research Dairy Center. The Cornell University Institutional Animal Care and Use Committee approved all procedures involving animals before the beginning of the study. Forty pregnant, nonlactating Holstein dairy cows [16 heifers and 24 multiparous cows (mean parity  $\pm$  SD;  $1.38 \pm 0.65$ )] were used in this study. Cows were housed in a 2-row freestall barn in groups of 10. Groups were balanced based on parity (4 heifers and 6 multiparous cows per group) and previous 305-d mature-equivalent milk yield among multiparous cows. Each pen had 10

freestalls that were arranged in a 2-row formation and bedded with a mattress and layer of sawdust. A post-and-rail feed barrier was used at the feed bunk. During both treatments, cows were fed the same TMR once daily (approximately 0800 h) with feed push-ups occurring at regular intervals throughout the day. The diet was composed of wheat straw (24.6% of DM), corn silage (41.0% of DM), and dry cow grain (34.4% of DM). Weekly samples of the TMR were collected and combined into a 4-wk composite sample that was sent to a commercial laboratory for wet chemistry analysis (Dairy One Cooperative Inc., Ithaca, NY). The TMR analysis was as follows (% of DM  $\pm$  SD): CP =  $14.5 \pm 0.6$ , ADF =  $31.4 \pm 1.5$ , NDF =  $49.8 \pm 2.8$ , starch =  $17.7 \pm 0.7$ , Ca =  $0.76 \pm 0.07$ , P =  $0.29 \pm 0.01$ , Mg =  $0.23 \pm 0.01$ , K =  $1.06 \pm 0.04$ , and Na =  $0.19 \pm 0.03$ . Group as-fed intake (kg/d) was measured daily using a mixer wagon equipped with FeedWatch (Valley Agricultural Software, Tulare, CA); group intakes were determined by subtracting the weight of the orts from the total weight fed during the previous day. As-fed group intake was corrected for the DM percentage of the TMR and reported on a per cow basis (group DMI/10 cows).

### *Treatments and Experimental Design*

In sets of 2, all 4 groups were exposed to 2 stocking density treatments using a crossover experimental design (i.e., replicated crossover). The stocking density treatments were defined as follows: (1) control: 1 lying stall per cow and 0.67 m of linear feed bunk (**FB**) space per cow, and (2) overstocked: 1 lying stall per 2 cows and 0.34 m of linear FB space per cow. To simulate conditions of overstocking, access to the 4 freestalls facing (nearest) the FB and one additional freestall along the back wall of the pen were roped off to restrict resting space, and access to the FB was restricted using plywood bolted across the feeding area. The first set of groups were formed between d -74 and -61 relative to the cows' expected calving dates, and the second set of groups were formed between d -81 and -67 relative to the cows' expected calving dates. Cows were given 10 d to adapt to their respective groups before the first experimental treatment period began. Cows averaged 214 d in gestation at the beginning of the first experimental period. Each of the 2 treatment periods lasted 14 d, and these periods were separated by a 3-d washout period during which time both groups were housed at the control stocking density. After the first set of 2 groups had been exposed to both treatments, the second set of 2 groups was formed and the crossover design was repeated.

### **Behavior Data Collection**

Behavior at the FB was monitored using video cameras (Sony CCD Digital ULTRA Pro Series, Hi-Resolution BW CCD Camera with Auto-Iris, Sony Corp., New York, NY) connected to a digital recording system (DiGiCam H.264, 120 and 240 FPS, DVR PC Version; Central Alarms Systems Inc., Littleton, CO). A camera was positioned directly above the feeding area to continuously record behavior at the FB. Hair dye was used to create unique alphanumeric symbols on the backs of the cows so that individuals could be identified on the video recordings. Daily feeding time, time to the FB after fresh feed delivery, and proportion of total daily feeding time during the 3-h period after fresh feed delivery was estimated from 10-min time scans of the video recordings over 4 consecutive days (d 7 to 10 of the 14-d overstocked period). A cow was considered to be feeding when its neck collar was visible beyond the top rail of the feed barrier on the feed alley side of the pen. To assess competitive behavior, 3 d of continuous (24-h; d 7 to 9) video recordings were reviewed and each competitive displacement that occurred at the FB was recorded. A displacement was recorded when a cow's head (actor) came in contact with a cow that was feeding (reactor), resulting in the reactor withdrawing its head from the FB.

### **Blood and Fecal Collection and Analysis**

On d 1, 3, 5, 7, 9, and 11 of each 14-d treatment period, blood and fecal samples were collected from each cow. Blood was collected from the coccygeal vessel into 10-mL sterile tubes coated with sodium heparin (BD Vacutainer, Franklin Lakes, NJ), and plasma was harvested after centrifugation ( $2,800 \times g$  for 15 min at 4°C). Plasma samples were stored at -20°C for later laboratory analysis. Plasma concentrations of glucose and NEFA were measured by enzymatic analysis (glucose oxidase, P7119, Sigma Chemical, St. Louis, MO; NEFA-C: Wako Pure Chemical Industries, Osaka, Japan). Spectrophotometric measurements were conducted using a Versa<sub>max</sub> tunable microplate reader (Molecular Devices, Sunnyvale, CA). The intra- and interassay CV for the NEFA assay were 3.7 and 4.4%, respectively, and for the glucose assay were 2.9 and 6.1%, respectively. Plasma insulin concentration was measured by RIA (Porcine Insulin RIA Kit #PI-12K, Millipore Corp., Billerica, MA). The intra- and interassay CV for the insulin assay were 3.3 and 3.2%, respectively.

Fecal samples were collected fresh, sealed within plastic bags, and placed immediately under ice. Within

2 h of sample collection, steroids from the fecal samples were extracted using the wet extraction method described by Palme and Möstl (1997). Briefly, 0.5 g of each raw fecal sample was weighed and mixed by vortex with 5 mL of 80% methanol for 30 min. Samples were then centrifuged for 15 min at  $2,800 \times g$  and the supernatant was divided into aliquots and stored at -20°C until further analysis. The DM percentage of each fecal sample was obtained by weighing samples before and after drying in a hot oven (105°C) for 24 h. Concentrations of fecal cortisol metabolites (11,17-dioxoandrosterone; **11,17-DOA**) were measured using a competitive enzyme immunoassay developed by Palme and Möstl (1997) and validated for use in cattle (Palme et al., 1999). The intra- and interassay CV for the 11,17-DOA assay were 3.2 and 3.4%, respectively.

### **GTT and ACTH Challenge**

Between d 11 and 12 of each treatment period, each cow was fitted with an indwelling, sterilized jugular catheter (30 cm  $\times$  1.78 mm o.d., Tygon S-54-HL Medical tubing, Saint-Gobain Performance Plastics, Akron, OH). The catheter was secured within a fabric pouch that was attached to the neck of the cow using a topical adhesive approved for use in animals. The entire neck was wrapped with elastic bandages to prevent the cow from dislodging the catheter while in the group pen. Body weights of all animals were measured on their catheterization day to determine glucose and ACTH doses for the GTT and ACTH challenge, respectively. The GTT was performed on all cows on d 13 of each treatment period, and the ACTH challenge was performed on all cows on d 14 of each period. Approximately 1 to 1.5 h before the start of the both the GTT and ACTH challenge, cows were moved a short distance to a tiestall barn, where each could be tethered in an individual stall to facilitate sample collection.

On d 13, the GTT was administered to one group of 10 cows in the morning (0900 to 1200 h) and to the second group during the afternoon (1300 to 1600 h). Feed was removed from the cows 2 h before the start of the GTT. This test involved administering 0.25 g/kg of BW of glucose i.v. (dextrose 50% wt/vol., Butler Animal Health Supply, Dublin, OH) and then collecting jugular blood samples at -15, -5, 0, 5, 10, 15, 20, 25, 30, 45, 60, 75, 90, 120, 150, and 180 min relative to glucose administration. After the 180-min sample was collected, catheters were flushed with 5 mL of heparinized saline (500 IU/L) and stored in the fabric pouch protected by the neck wrap, and the cows were returned to their freestall group pen. Feed was not withheld from cows before the start of the ACTH

challenge, and the test was administered to all 20 cows during the morning of d 14 (0900 to 1300 h). The ACTH challenge involved administering 0.125 IU/kg of BW of ACTH i.v. (Porcine, A6303, Sigma Chemical) and then collecting jugular blood samples at -60, -15, 0, 15, 30, 45, 60, 90, 120, 150, 180, and 240 min relative to the administration of ACTH. During both the GTT and ACTH challenge, samples were immediately centrifuged after collection and the plasma was harvested and stored at -20°C. At the end of the ACTH challenge, catheters were removed, cows were returned to their respective group pens, and the stocking density barriers were taken down to signal either the beginning of the 3-d washout period or the end of the experiment. Plasma cortisol concentration was determined for samples collected during the ACTH challenge using an RIA (Coat-A-Count Cortisol RIA Kit, Siemens Medical Solutions Diagnostics, Los Angeles, CA). The intra- and interassay CV for the cortisol assay were 3.2 and 1.9%, respectively.

### Calculations and Statistical Analysis

All statistical analyses were performed using SAS version 9.1 (SAS Institute, 2009). Pen was considered the experimental unit for all analyses, with measures from all cows in the group (10 cows/group) averaged to create one overall observation (by time where appropriate) per pen and treatment. To explore how primiparous cows responded to the stocking density treatments, measures from only the primiparous cows in each group (4 cows/group) were averaged to create one primiparous observation per pen and treatment; multiparous cow data were excluded for the calculation of primiparous group means. To explore how multiparous cows responded to the stocking density treatments, measures from only the multiparous cows in each group (6 cows/group) were averaged to create one multiparous observation per pen and treatment; primiparous data was excluded for the calculation of multiparous group means.

Differences in plasma NEFA, glucose, insulin, 11,17-DOA, and DMI measured every 2 d (daily for DMI) and the behavioral measures between stocking density treatments were analyzed as a replicated crossover design using the MIXED procedure in SAS. The statistical model included the fixed effects of treatment, sequence, day, and the treatment  $\times$  day interaction, and the random effects of period and pen within sequence. Day was identified as a repeated measure and an autoregressive covariance structure was used in each model based on best fit using the Bayesian information criterion. The DMI on d 13 and 14 of each treatment

period were excluded from this analysis because feed was withheld for approximately 5 h per day during the GTT and ACTH challenge. Logarithmic transformation was required for the 11,17-DOA data to comply with the model assumptions and improve fit. Least squares means and SEM for the 11,17-DOA data were estimated from untransformed values, whereas *P*-values reflect statistical analysis of transformed data.

Area under the curve (AUC) for the glucose, NEFA, and insulin responses to the GTT and the cortisol response to the ACTH challenge were calculated using the trapezoidal method and actual concentration values after discounting the basal values. Positive and negative AUC were calculated separately. Positive AUC (+AUC) included only the periods when the actual metabolite concentration was greater than the basal concentration, and negative AUC (-AUC) included only those periods when the actual metabolite concentration was lower than the basal concentration. This differentiation in AUC was done to better describe the differences in the metabolite curves when they dropped below the basal concentrations, as treatment differences were lost when including all the data together.

The NLIN procedure of SAS was used to fit exponential curves for glucose concentration during the first 60 min of GTT, for NEFA during the first 30 min of the GTT, for insulin between 15 and 75 min of the GTT (period following peak insulin concentration), and for cortisol between 60 and 180 min of the ACTH challenge using the following equation:  $F(t) = A \times e^{(-k \times t)}$ , where  $F(t)$  is the analyte concentration at time  $t$ ;  $A$  is the maximum value of the analyte (estimated by model);  $t$  is the time (min); and  $k$  is the regression coefficient. Using this equation, calculated for each cow and treatment period, the following parameters were calculated: clearance rate of glucose, insulin, and cortisol ( $CR_{ta-tb}$ ; %/min) =  $\{(\ln[t_a] - \ln[t_b]) / (t_b - t_a)\} \times 100$ ; rate of NEFA decline from circulation ( $Slope_{ta-tb}$ ; %/min) =  $\{(\ln[t_a] - \ln[t_b]) / (t_b - t_a)\} \times 100$ ; time to reach half maximal glucose concentration ( $T_{1/2}$ ; min) =  $\{[\ln(2)] / CR\} \times 100$ ; and time to reach basal glucose concentration ( $T_{basal}$ ; min) =  $\ln([basal]/A) / (-k)$ , where  $[t_a]$  and  $[t_b]$  are the concentrations of the analyte at time  $a$  and  $b$  respectively and  $[basal]$  is the basal analyte concentration, calculated by averaging the analyte concentration at  $t = -15$  and  $-5$  min for the GTT or  $t = -60$  and  $-5$  min for the ACTH challenge. The lowest NEFA concentration (NEFA nadir) and highest insulin and cortisol concentrations (peak insulin, peak cortisol) of each cow were identified from samples collected following the administration of dextrose or ACTH. These parameters were averaged by group and treatment for statistical analysis. Data were analyzed as a replicated



crossover design using the MIXED procedure in SAS. The statistical model included the fixed effects of treatment and sequence, and the random effects of period and pen within sequence.

## RESULTS

### Overall Results

During the overstocked period, cows took longer to approach the FB following delivery of fresh feed ( $68$  vs.  $37 \pm 9$  min;  $P = 0.02$ ), spent a smaller proportion of their total daily feeding time at the FB during the 3-h period following fresh feed delivery ( $22$  vs.  $28 \pm 3\%$ ;  $P = 0.001$ ), and engaged in more competitive displacements at the FB over a 24-h period ( $50$  vs.  $28 \pm 3$  displacements/d;  $P = 0.003$ ). Stocking density treatment did not affect average daily feeding time ( $241$  vs.  $242 \pm 12$  min/d for the control and overstocked periods, respectively;  $P = 0.91$ ).

The average DMI across all experimental days was  $15.9 \pm 0.5$  kg/d per cow during the overstocked treatment and  $14.9 \pm 0.5$  kg/d per cow during the control treatment ( $P < 0.001$ ). The average DMI of cows in each of the 4 groups during the control and overstocked period by experimental day is presented in Figure 1.

Plasma NEFA and glucose concentrations were greater during the overstocked period compared with the control period [ $0.11$  vs.  $0.09 \pm 0.006$  mEq/L ( $P = 0.002$ ) and  $65.3$  vs.  $64.2 \pm 1.1$  mg/dL ( $P = 0.05$ ), respectively], and overall 11,17-DOA concentration tended to be higher during the overstocked period compared with the control period ( $891$  vs.  $792 \pm 86$  ng/g of fecal DM;  $P = 0.10$ ). The insulin concentration of

cows did not differ between the overstocked and control periods ( $29.0$  vs.  $31.2 \pm 2.1$   $\mu$ IU/mL, respectively;  $P = 0.20$ ).

Glucose clearance from circulation was slower during the overstocked treatment as evidenced by a longer time to reach half-maximal glucose concentration and to reach basal glucose levels, a greater +AUC throughout the 180 min of sampling, and a tendency for a lower glucose CR between 0 and 60 min of the GTT (Table 1; Figure 2A;  $P \leq 0.05$ ). We observed an attenuated insulin response to the GTT during the overstocked treatment period compared with the control period; this was evidenced by lower peak insulin concentration following glucose administration, a lower +AUC value for the 240-min sampling period, and a lower insulin clearance rate between 15 and 75 min of the ACTH challenge (Table 1; Figure 2B;  $P \leq 0.02$ ). The rate of NEFA decline from circulation was slower during the first 30 min of the GTT during the overstocked treatment ( $P = 0.04$ ) and we observed a tendency ( $P = 0.09$ ) for NEFA concentration to decrease lower (lower nadir) following glucose infusion during the control treatment (Table 1; Figure 2C).

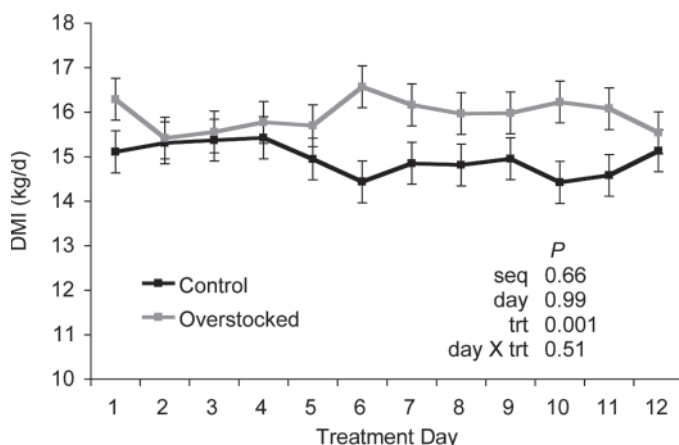
During the ACTH challenge, cows did not differ in basal cortisol ( $11.2$  vs.  $11.2 \pm 2.7$  nmol/L;  $P = 0.97$ ), peak cortisol ( $176.2$  vs.  $177.7 \pm 8.8$  nmol/L;  $P = 0.62$ ), CR<sub>60-180</sub> ( $1.30$  vs.  $1.36 \pm 0.06\%$ /min;  $P = 0.13$ ), +AUC<sub>240</sub> ( $18,824$  vs.  $18,467 \pm 859$  nmol/L  $\times$  240 min;  $P = 0.48$ ), or -AUC<sub>240</sub> ( $-52$  vs.  $-64 \pm 43$  nmol/L  $\times$  240 min;  $P = 0.80$ ) between the control and overstocked periods, respectively (Figure 3).

### Multiparous Cows Only

During the overstocked period, multiparous cows spent a smaller proportion of their total daily feeding time at the FB during the 3-h period following fresh feed delivery ( $25$  vs.  $29 \pm 2\%$ ;  $P = 0.05$ ). Average daily feeding time during the control and overstocked periods ( $263$  vs.  $265 \pm 20$  min/d) and the time to approach the FB following fresh feed delivery ( $40$  vs.  $59 \pm 15$  min) was not different in multiparous cows ( $P = 0.85$  and  $P = 0.20$ , respectively).

Average glucose, insulin, and 11,17-DOA concentrations were not different among multiparous cows during the overstocked or control treatment periods. A treatment by day interaction for plasma NEFA ( $P = 0.05$ ) indicated that multiparous cows had greater NEFA concentrations during the overstocked period relative to the control period, but only on d 5 of the treatment period (Figure 4).

During the GTT, multiparous cows had a slower insulin clearance rate during the overstocked period



**Figure 1.** Least squares means ( $\pm$ SE) DMI of 4 groups (expressed on a per cow basis) exposed to both a control and an overstocked stocking density treatment.

**Table 1.** Effect of stocking density treatment (control vs. overstocked) on the glucose, insulin, and NEFA responses to an intravenous glucose tolerance test (GTT) for 4 groups of cows (4 primiparous cows and 6 multiparous cows per group)

Item <sup>1</sup>	All cows (n = 10 per group)					
	Control	Overstocked	SEM	Difference <sup>2</sup>	SE Difference	P-value
Glucose (mg/dL)						
Basal	74.2	74.8	0.9	-0.6	0.6	0.40
CR <sub>0-60</sub> (%/min)	1.93	1.78	0.11	0.15	0.05	0.12
T <sub>1/2</sub> (min)	37.0	40.3	2.2	-3.3	0.7	0.04
T <sub>basal</sub> (min)	51.5	55.1	2.9	-3.6	0.8	0.05
+AUC180	2,657	2,882	165	-225	61	0.03
-AUC180	-665	-513	113	-152	81	0.20
Insulin (μIU/mL)						
Basal	21.0	22.9	1.6	-1.9	0.6	0.08
Peak	259.8	199.1	38.6	60.8	9.1	0.02
CR <sub>15-75</sub> (%/min)	4.3	3.6	0.4	0.7	0.1	0.02
+AUC180	6,692	5,258	1,104	1,434	130	0.008
-AUC180	-592	-609	83	17	53	0.78
NEFA (mEq/L)						
Basal	0.17	0.17	0.01	0.001	0.013	0.96
Nadir	0.07	0.08	0.004	-0.008	0.003	0.09
Slope <sub>0-30</sub> (%/min)	1.93	1.42	0.15	0.51	0.15	0.04
+AUC180	2,907	2,090	703	817	994	0.45
-AUC180	-7,684	-7,285	1,285	-398	1,694	0.83

<sup>1</sup>Basal = mean analyte concentration at  $t = -15$  and  $-5$  min of GTT; peak = highest insulin concentration; nadir = lowest NEFA concentration; CR<sub>t1-t2</sub> = clearance rate between t1 and t2 GTT; slope<sub>0-30</sub> = rate of NEFA decline from circulation during the first 30 min of GTT; T<sub>1/2</sub> = time to reach half maximal glucose concentration; AUC180 = area under the curve during the 180 min of the GTT (+AUC refers to AUC for sampled analyte concentrations above basal and -AUC refers to AUC for sampled analyte concentrations below basal).

<sup>2</sup>Difference between the treatment LSMEANS.

compared with the control period (CR<sub>15-75</sub>: 3.3 vs. 3.8 ± 0.3%/min;  $P = 0.02$ ). All other measured responses during the GTT were found to be either trends or not significant (Table 2). During the ACTH challenge, multiparous cows did not differ in basal cortisol (12.1 vs. 11.7 ± 3.8 nmol/L;  $P = 0.81$ ), peak cortisol (186.5 vs. 186.8 ± 9.5 nmol/L;  $P = 0.93$ ), CR<sub>60-180</sub> (1.28 vs. 1.35 ± 0.07%/min;  $P = 0.17$ ), +AUC<sub>240</sub> (20,051 vs. 19,431 ± 757 nmol/L × 240 min;  $P = 0.44$ ), or -AUC<sub>240</sub> (-51 vs. -86 ± 57 nmol/L × 240 min;  $P = 0.57$ ) between the control and overstocked periods, respectively.

### Primiparous Cows Only

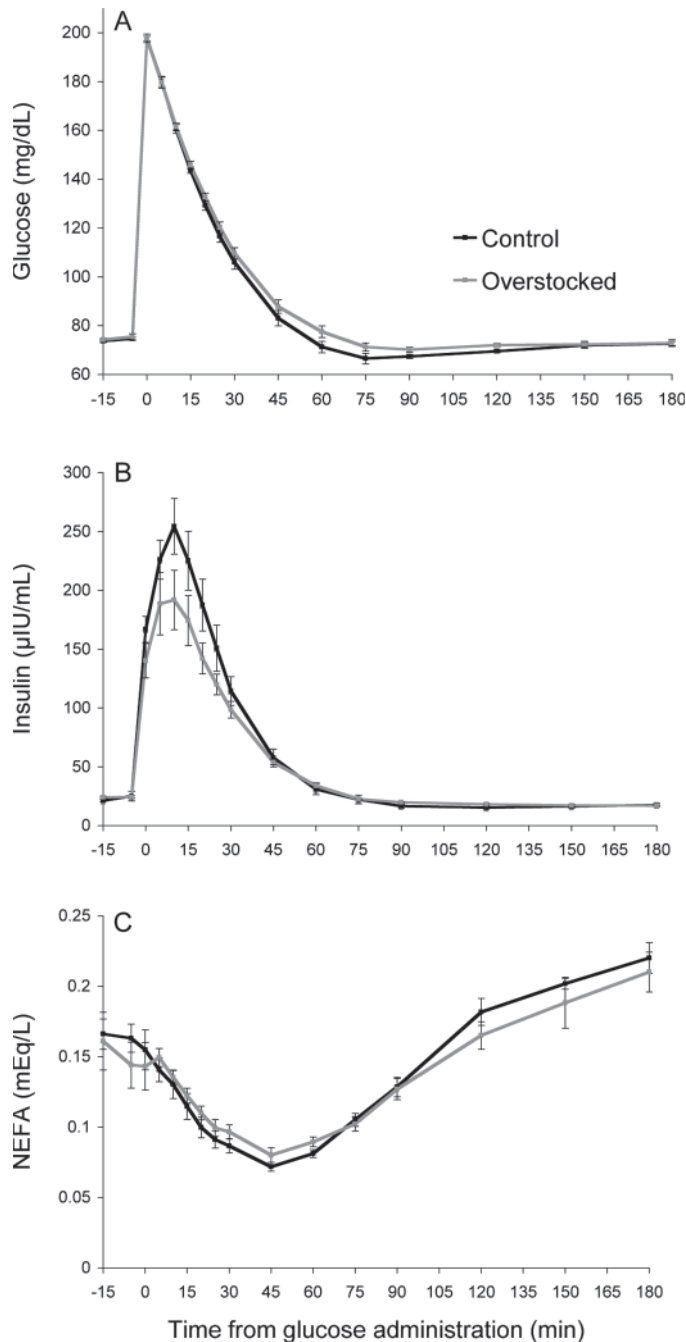
During the overstocked period, primiparous cows took longer to approach the FB following fresh feed delivery (81 vs. 32 ± 18 min;  $P = 0.04$ ) and spent a smaller proportion of their total daily feeding time at the FB during the 3 h following fresh feed delivery (18 vs. 27 ± 3%;  $P = 0.001$ ). Stocking density treatment did not affect the average daily feeding time of primiparous cows (208 vs. 208 ± 10 min/d for the control and overstocked periods, respectively;  $P = 0.97$ ).

Primiparous cows had greater NEFA, glucose, and 11,17-DOA concentrations during the overstocked period relative to the control period ( $P \leq 0.04$ ) but average daily plasma insulin was not affected by stocking density treatment (Figure 5).

During the GTT, primiparous cows had a lower rate of NEFA decline from circulation during the overstocked period relative to the control period (Slope<sub>30</sub>: 1.92 vs. 2.63 ± 0.17%/min, respectively;  $P = 0.02$ ). All other measured responses during the GTT were found to be either trends or not significant (Table 3). During the ACTH challenge, primiparous cows did not differ in basal cortisol (9.9 vs. 10.4 ± 2.2 nmol/L;  $P = 0.90$ ), peak cortisol (160.7 vs. 163.9 ± 8.8 nmol/L;  $P = 0.73$ ), CR<sub>60-180</sub> (1.31 vs. 1.36 ± 0.08%/min;  $P = 0.20$ ), +AUC<sub>240</sub> (16,983 vs. 17,021 ± 1,105 nmol/L × 240 min;  $P = 0.62$ ), or -AUC<sub>240</sub> (-53 vs. -31 ± 33 nmol/L × 240 min;  $P = 0.67$ ) between the control and overstocked periods, respectively.

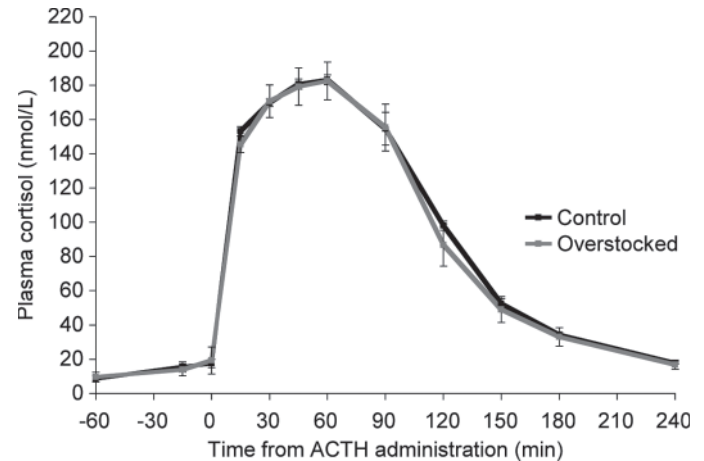
## DISCUSSION

The results of this study suggest that overstocking may alter physiological parameters associated with energy metabolism. Although overall DMI increased during the overstocked treatment period compared with the control period, overall NEFA, averaged across the entire treatment period, was also higher during the overstocked period. In dairy cattle, elevated concentrations in plasma NEFA are typically observed when intake cannot support energy requirements, thus requiring the mobilization of NEFA from adipose tissue to support energy demand (Bauman and Currie, 1980).



**Figure 2.** Effects of overcrowding on (A) glucose, (B) insulin, and (C) NEFA (arithmetic mean  $\pm$  SE) response to an intravenous glucose tolerance test. Data presented are the average of 4 groups ( $n = 10$  cows per group).

The results of this study suggest that factors other than intake might regulate NEFA balance in the dairy cow during periods of overstocking; for example, changes in the concentrations of circulating hormones (e.g., insulin or glucocorticoids) that are important regulators of lipolysis and lipogenesis or changes to the sensitivity or

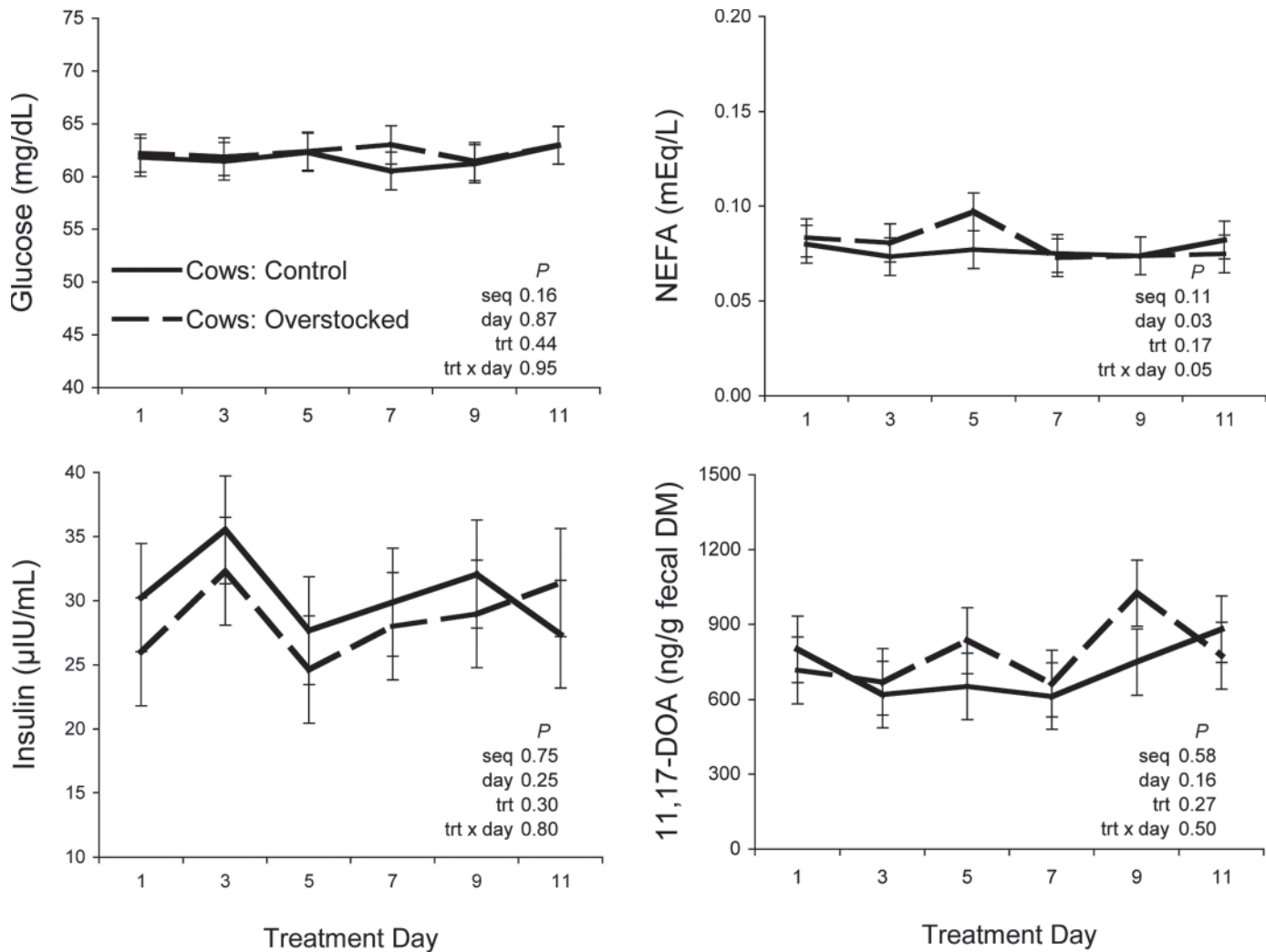


**Figure 3.** Effects of overcrowding on plasma cortisol (arithmetic mean  $\pm$  SE) response to an intravenous ACTH challenge. Data presented are the average of 4 groups ( $n = 10$  cows per group).

responsiveness of tissues to these hormones may alter plasma NEFA concentrations (Bauman and Currie, 1980; Andrews and Walker, 1999).

Increased NEFA concentration during the weeks around calving (e.g.,  $\geq 0.3$  mEq/L during the 2-wk period before calving) has been associated with an increased risk of disease, reduced milk yield, and compromised reproductive performance (Ospina et al., 2010a,b); however, this study was conducted during the early dry period (before 3 wk prepartum) and so it is unclear whether the increased NEFA concentrations observed during overstocked period could contribute to an increased risk for health or production complications after calving. Further, the NEFA concentrations observed in the present study during both treatment periods were low (0.9 to 0.11 mEq/L) compared with the prepartum NEFA concentration thresholds that Ospina et al. (2010a,b) identified as being predictive of postpartum health and performance outcomes (e.g.,  $\geq 0.3$  mEq/L); therefore, the observed increase in NEFA during the overstocked period may not be of biological significance. Future research will need to investigate whether changes in NEFA concentrations during the early dry period could have downstream consequences on health and performance.

The results of the GTT provided additional evidence that overstocking is associated with changes in energy metabolism. Overall glucose AUC estimates, time to half-maximal glucose concentration, and time to basal glucose concentration were increased during the overstocked treatment period but these differences were small and may not be of biological significance. For example, the time to reach basal glucose concentration following the GTT differed by only 4 min between stock-



**Figure 4.** Least squares means ( $\pm$ SE) plasma glucose, NEFA, insulin, and 11,17-dioxoandrostane (11,17-DOA) of multiparous cows in 4 groups during control (solid line) and overstocked (dashed line) stocking density treatments.

ing density treatments. On the other hand, stocking density treatment had a much greater effect on insulin response to glucose during the GTT. During the GTT, overall peak insulin secretion during the overstocked treatment was 61  $\mu$ IU/L lower than the peak insulin secretion during the control treatment (199 vs. 260  $\mu$ IU/L), a concentration difference 3 times that of basal insulin concentrations. Based on the insulin response, the glucose response curves to the GTT could be interpreted in 2 ways. First, decreased insulin secretion from the pancreas might explain the slightly reduced glucose clearance as there would be less endocrine signaling to upregulate glucose transporters for cellular glucose uptake; alternatively, the overstocking treatment may have had an insulin-sensitizing effect, because less insulin was required to produce similar glucose clearance rates (Leney and Tavaré, 2009).

The physiological responses to overstocking appear to have commonalities with the responses observed during compromised nutritional status. For example, previous work has shown that plane of nutrition can influence insulin secretion. Hove (1978) reported an attenuated insulin response following a GTT in ketonemic cows, whereas Holtenius et al. (2003) found that cattle that were fed below their metabolizable energy requirements had lower glucose-induced insulin secretion from the pancreas. Despite these similarities, overall group intake was greater during the overstocked period; however, group DMI in response to overcrowding should be interpreted with caution because it can mask individual differences in intake. It is likely that not all cows within a group have the same level of success at competing for access to the FB to achieve higher intake during overstocking. Future work will need to test this hypothesis



**Table 2.** Effect of stocking density treatment (control vs. overstocked) on the glucose, insulin, and NEFA responses to an intravenous glucose tolerance test (GTT) for 4 groups of multiparous cows

Item <sup>1</sup>	Multiparous cows (n = 6 per group)					
	Control	Overstocked	SEM	Difference <sup>2</sup>	SE Difference	P-value
Glucose (mg/dL)						
Basal	72.8	73.3	0.8	-0.5	0.4	0.37
CR <sub>0-60</sub> (%/min)	1.86	1.77	0.09	0.09	0.05	0.24
T <sub>1/2</sub> (min)	38.2	40.6	1.9	-2.4	0.6	0.06
T <sub>basal</sub> (min)	52.8	55.0	0.9	-2.2	1.0	0.12
+AUC180	2,656	2,777	151	-121	51	0.10
-AUC180	-643	-591	84	-52	29	0.22
Insulin (μIU/mL)						
Basal	19.6	20.3	2.3	-0.7	1.3	0.61
Peak	203.1	149.3	38.3	53.8	13.2	0.06
CR <sub>15-75</sub> (%/min)	3.8	3.3	0.3	0.6	0.1	0.02
+AUC180	5,354	4,067	1,116	1,287	564	0.15
-AUC180	-590	-544	148	-46	145	0.78
NEFA (mEq/L)						
Basal	0.12	0.12	0.01	0.01	0.02	0.73
Nadir	0.06	0.06	0.002	-0.002	0.002	0.45
Slope <sub>0-30</sub> (%/min)	1.46	1.08	0.19	0.38	0.27	0.21
+AUC180	2,215	1,578	624	637	867	0.50
-AUC180	-4,696	-3,997	1,707	-699	2,165	0.76

<sup>1</sup>Basal = mean analyte concentration at  $t = -15$  and  $-5$  min of GTT; peak = highest insulin concentration; nadir = lowest NEFA concentration; CR<sub>t1-t2</sub> = clearance rate between t1 and t2 GTT; slope<sub>0-30</sub> = rate of NEFA decline from circulation during the first 30 min of GTT; T<sub>1/2</sub> = time to reach half maximal glucose concentration; AUC180 = area under the curve during the 180 min of the GTT (+AUC refers to AUC for sampled analyte concentrations above basal and -AUC refers to AUC for sampled analyte concentrations below basal).

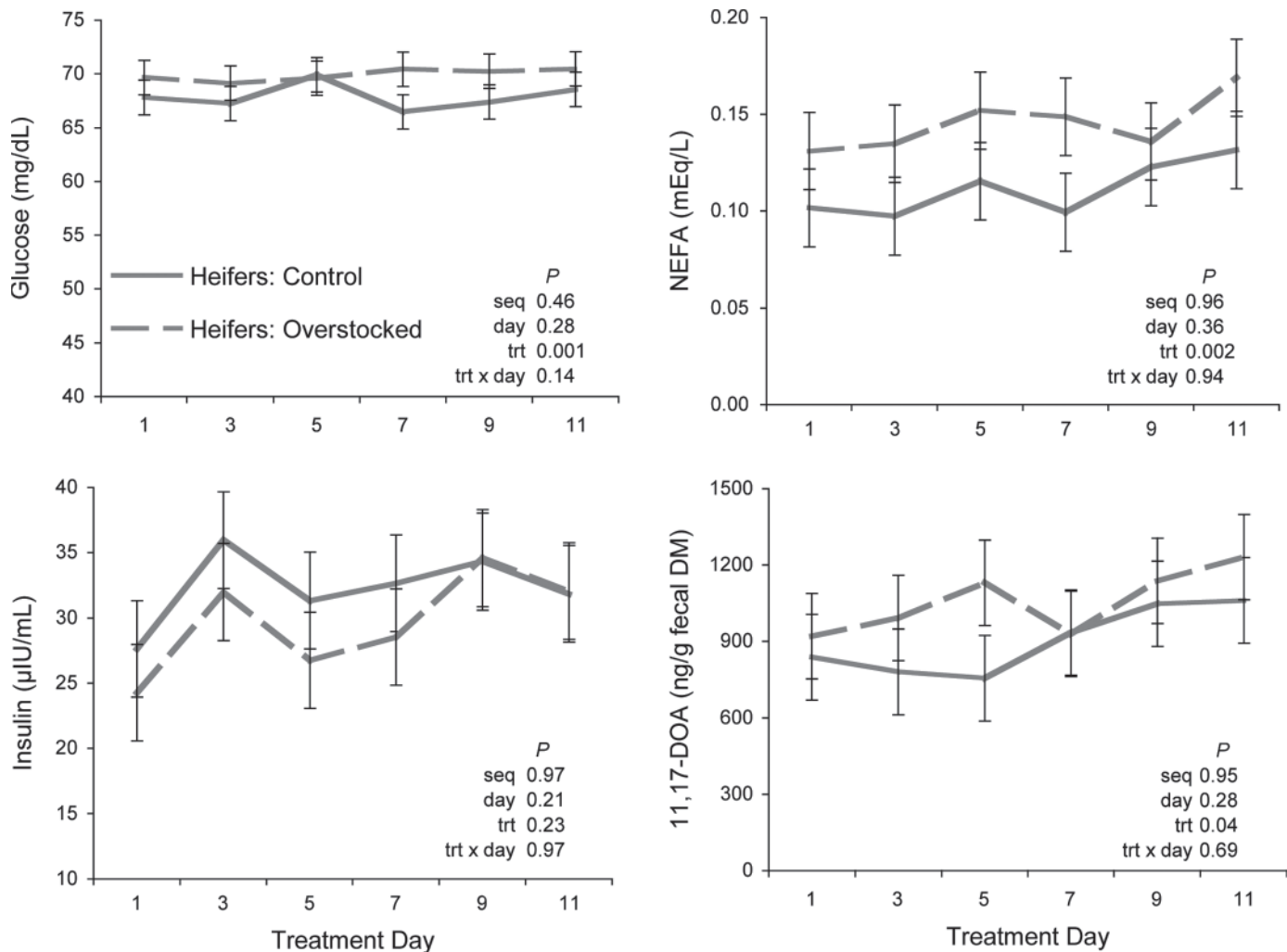
<sup>2</sup>Difference between the treatment LSMEANS.

by exploring how success at competitive interactions at the feed bunk during overstocking is related to feeding behavior and analytes associated with energy metabolism.

Glucocorticoids are also important moderators of energy metabolism; they increase the supply of glucose by promoting hepatic gluconeogenesis and decrease the utilization of glucose by cells elsewhere in the body, possibly by altering these cells' responsiveness to insulin (Andrews and Walker, 1999). These steroids can also directly inhibit insulin secretion from the pancreas (Lambillotte et al., 1997) and increase rates of lipolysis (Andrews and Walker, 1999). In the present study, no differences were observed in the plasma cortisol response to ACTH challenge between the 2 stocking density treatments. This was in contrast to the results of Friend et al. (1979), who reported a greater cortisol response to an ACTH challenge when cattle were exposed for 7 d to the same level of crowding in the lying stalls as used in the present study (1 stall/2 cows). Prolonged overstocking may be considered a chronically stressful situation, and the physiological responses to prolonged stressors are not necessarily constant over time (Mormède et al., 2007). In the present study, the ACTH challenge was administered after a 14-d treatment period and thus the lack of a cortisol response during the ACTH challenge could have reflected a physiological desensitization to stressors associated with crowd-

ing. Plasma cortisol response to ACTH challenge can also be influenced by ACTH dose level. As the dose of ACTH increases, maximum cortisol concentrations do not change but the cortisol response is prolonged over time (Lay et al., 1996); this response can make treatment differences more difficult to detect. The dose of ACTH used in the present study was adjusted for each cow's BW and was lower than the dose used by Friend et al. (1979); therefore, it is unlikely that the ACTH dose used in the current study was too high and masked treatment differences.

Concentrations of 11,17-DOA tended to be greater during the overstocking period, suggesting that overall daily cortisol secretion might have been higher during overstocking. Overstocking is characterized by increased social interactions (Huzzey et al., 2006; Fregonesi et al., 2007), many of which can be aggressive and thus potentially experienced by the animal as acute stressors capable of inducing a physiological stress response. In the present study, we observed a greater frequency of competitive displacements from the FB during the overstocked period relative to the control period (50 vs. 28 displacements/d) and evidence of increased feeding rate (no difference in feeding time despite greater DMI during overstocking). Even with no difference in the amount of cortisol secreted from the adrenal gland upon stimulation, higher average 11,17-DOA concentrations could have been achieved if the adrenal gland



**Figure 5.** Least squares means ( $\pm$ SE) plasma glucose, NEFA, insulin, and 11,17-dioxoandrostane (11,17-DOA) of primiparous cows in 4 groups during control (solid line) and overstocked (dashed line) stocking density treatments.

were stimulated to secrete cortisol more frequently (e.g., during periods when cows were engaged in competitive displacements at the FB). We found no effect of day or a treatment by day interaction on 11,17-DOA concentrations, suggesting that concentrations of 11,17-DOA were constant over time during each treatment period; this observation was not consistent with the hypothesis that a physiological adaptation or desensitization occurred at the level of the adrenal gland in response to the stress of overstocking. Although it is clear that cortisol has the capacity to affect energy metabolism through a variety of pathways, it is unclear whether the observed trends for greater 11,17-DOA concentration during overstocking reflect an increase in circulating cortisol sufficient to influence daily glucose and NEFA concentrations or the insulin response to the GTT. Although not measured in this study, other

moderators of the stress response such as epinephrine or norepinephrine may play a role in moderating these observed differences in energy metabolism (Malaisse et al., 1967).

In the present study, cows were overstocked both at the lying stalls and at the FB; overstocking of both resources likely influenced overall treatment responses. Previous work has shown that cows will sacrifice feeding time to gain additional resting time when access to both resources is limited (Metz, 1985). Behavior at the lying stalls was not measured in the current study; however, during the overstocked period, cows took longer to approach the FB following fresh feed delivery and spent a smaller proportion of their total daily feeding time within the 3-h period following fresh feed delivery. This observation may be evidence of some cows displaying a preference for resting during a

**Table 3.** Effect of stocking density treatment (control vs. overstocked) on the glucose, insulin, and NEFA response to an intravenous glucose tolerance test (GTT) for 4 groups of primiparous cows

Item <sup>1</sup>	Primiparous cows (n = 4 per group)					
	Control	Overstocked	SEM	Difference <sup>2</sup>	SE Difference	P-value
Glucose (mg/dL)						
Basal	76.3	77.2	1.0	-0.9	1.1	0.47
CR <sub>0-60</sub> (%/min)	2.03	1.80	0.15	0.23	0.10	0.14
T <sub>1/2</sub> (min)	35.2	39.8	2.7	-4.6	1.7	0.11
T <sub>basal</sub> (min)	49.5	55.2	3.6	-5.7	2.5	0.15
+AUC180	2,660	3,040	204	-380	172	0.11
-AUC180	-697	-396	165	-301	229	0.26
Insulin (μIU/mL)						
Basal	22.8	26.7	2.9	-3.9	3.1	0.28
Peak	339.8	269.1	48.8	70.7	27.2	0.12
CR <sub>15-75</sub> (%/min)	5.0	4.2	0.4	0.8	0.3	0.10
+AUC180	8,533	6,931	1,539	1,602	650	0.13
-AUC180	-585	-718	66	133	94	0.22
NEFA (mEq/L)						
Basal	0.24	0.25	0.03	-0.01	0.01	0.56
Nadir	0.08	0.10	0.01	-0.02	0.01	0.22
Slope <sub>0-30</sub> (%/min)	2.63	1.92	0.17	0.71	0.09	0.02
+AUC180	3,946	2,857	903	1,088	1,277	0.43
-AUC180	-12,166	-12,218	1,676	53	1,842	0.98

<sup>1</sup>Basal = mean analyte concentration at  $t = -15$  and  $-5$  min of GTT; peak = highest insulin concentration; nadir = lowest NEFA concentration; CR<sub>t1-t2</sub> = clearance rate between t1 and t2 GTT; slope<sub>0-30</sub> = rate of NEFA decline from circulation during the first 30 min of GTT; T<sub>1/2</sub> = time to reach half maximal glucose concentration; AUC180 = area under the curve during the 180 min of the GTT (+AUC refers to AUC for sampled analyte concentrations above basal and -AUC refers to AUC for sampled analyte concentrations below basal).

<sup>2</sup>Difference between the treatment LSMEANS.

time that would otherwise be considered a peak feeding period; cows are highly motivated to eat during the period following fresh feed delivery (DeVries and von Keyserlingk, 2005). These altered feeding patterns during the overstocked period suggest that stocking pressure (cow numbers) at the FB during peak feeding time was lower than the treatment-defined stocking rate of 200%. If overstocking the lying stalls reduced stocking pressure (and thus competition level) at the FB, it is possible that the physiological response to overstocking will differ depending upon which resource is overstocked and the magnitude of the stocking rate. For example, although we observed a greater frequency of competitive displacements at the FB during the overstock treatment relative to the control treatment, this level of competition might have been greater had the stalls not also been overstocked, thus resulting in a different physiological profile for the cows in the group. These hypotheses require further investigation.

Stocking density treatment had few effects on the measured physiological and behavioral parameters of multiparous cows (e.g., Figure 4); this may be evidence of multiparous cows being effective in adapting to a competitive feeding and resting environment. The glucose, insulin, and NEFA responses of multiparous cows to the GTT were similar to the overall group responses (e.g., multiparous cows had lower peak insulin secretion

during the overstocked treatment relative to the control treatment) but almost all these associations were trends. This was likely due to fewer animals being used to generate group averages (6 vs. 10 cows per group), leading to more variation between groups and thus a higher type I error risk for the GTT parameters.

When considering the responses of only the primiparous cows in each of the 4 groups, overstocking was associated with higher glucose, NEFA, and 11,17-DOA concentrations relative to the control period (Figure 5). Primiparous cows also took longer to approach the FB following fresh feed delivery and had a smaller proportion of their total daily feeding time within the 3-h period following fresh feed delivery during the overstocked period compared with the control period. Glucose, insulin, and NEFA responses to the GTT were also similar to the overall group responses but were associated with a higher type I error risk, likely because of the smaller number of primiparous animals used to generate group means (4 primiparous cows/group).

This study was not designed to make direct comparisons between primiparous and multiparous cows housed together, because this was a pen study and the cows within a pen were not independent units of analysis. However, after evaluating group responses to overstocking based on summarized data from either primiparous cows only or multiparous cows only, the results suggest

that the physiological responses to overstocking may be influenced by parity. Because primiparous and multiparous cows were commingled in the present study, the exact manner by which parity may moderate physiological responses to overstocking is unclear. Heifers have been reported to spend less time feeding, have lower DMI, spend less time lying down, and be involved in more aggressive interactions when grouped with multiparous cows (Phillips and Rind, 2001); therefore it seems reasonable to speculate that when commingled in an overstocked environment primiparous cows may experience more adverse effects relative to multiparous cows. This hypothesis, however, requires further testing using experimental designs appropriate for comparing responses between parities. Future work in this area could begin by exploring whether overstocking alters aspects of physiology in cows housed within groups of the same parity.

## CONCLUSIONS

It is important to understand how overstocking influences dairy cow physiology in addition to behavior because this knowledge contributes to a better understanding of the ways in which overstocking can affect overall dairy cattle health and well-being. The overall results of this study show that overstocking during the dry period is associated with changes in physiology. These changes were related to energy metabolism and might be moderated by altered pancreatic insulin secretion or peripheral tissue responses to insulin. The role of cortisol in influencing these effects is still unclear. Additional research is required to determine whether these physiological changes are of a magnitude significant enough to affect subsequent health and performance. Future work in this area should also explore whether physiological changes vary depending on the magnitude of overstocking, whether these changes differ depending on the resource being overstocked (i.e., lying stalls vs. feed bunk), and the effects of parity on the behavioral and physiological responses to overstocking. Finally, understanding how individual behavioral strategies in response to overstocking correlate with physiological outcomes may help to identify management interventions that can minimize both the negative behavioral and physiological consequences of overstocking.

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