Overstocking dairy cows during the dry period affects dehydroepiandrosterone and cortisol secretion

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Overstocking dairy cows during the dry period affects dehydroepiandrosterone and cortisol secretion. *Fustini et al.* Stressful situations trigger a number of changes such as the secretion of cortisol and dehydroepiandrosterone (DHEA) from the adrenal cortex, in response to adrenocorticotropic hormone. We investigated whether overstocking during the dry period affects DHEA and cortisol secretion and behavior in Holstein Friesian cows. Overstocking significantly increased DHEA concentration compared to control group ten days before calving and five days following a significant increase in plasma cortisol. Moreover, overstocking group showed a higher activities, as measured by counting the steps per hour, thus indicating the increased need of movement in the pen.

Overstocking dairy cows during the dry period affects dehydroepiandrosterone and cortisol secretion

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ABSTRACT

Stressful situations trigger a number of changes such as the secretion of cortisol (C) and dehydroepiandrosterone (DHEA) from the adrenal cortex, in response to adrenocorticotropic hormone (ACTH). The aim of this study was to verify whether overstocking during the dry period affects DHEA and C secretion and behavior in Holstein Friesian cows. Twenty-eight cows were randomly divided into two groups (14 animals each), balanced for number of lactations, BCS (body condition score) and expected date of calving. Cows in the far-off phase of the dry period (from 60 to 21 days before the expected calving date) were housed together in a bedded-pack. Then, animals from 21±3 days to the expected calving until calving were housed in pens with the same size but under different crowding conditions due to the introduction into the pen of heifers (interference animals). Control condition (CTR) had 2 animals per pen with 12.0 m^2 each, while the overstocked condition (OS) had three interference animals in the same pen with 4,8 m^2 for each animal. On days -30±3, -21±3, - 15±3, -10±3, -5±3 before and 10, 20, 30 after calving blood samples were collected from each cow for the determination of plasma DHEA and C concentrations by RIA. Rumination time, activity (steps/h), lying time (min) and lying bouts were also individually daily recorded daily. In both groups, there was an increase in DHEA before calving and after parturition the concentration declined rapidly. Overstocking significantly increased DHEA concentration compared to CTR group at day -10 (1.79±0.09 vs 1.24±0.14 pmol/ml) while an increase of C was observed at day -15 (3.64±0.52 vs 1.64±0.46 ng/ml). However, nNo relationship was found between DHEA and C. OS group showed significantly higher activity (step/hour), compared with CTR group. Daily lying bouts tended to be higher for OS group compared with CTR group in the first week of treatment. The overall results of this study show that overstocking during the dry period is associated with changes in DHEA

and C. Additional researches are required to determine whether these hormonal changes are effective in affecting the subsequent behaviour performance.

Key words: dairy cattle, cortisol, dehydroepiandrosterone, overstocking, dry period

INTRODUCTION

Stressful situations trigger a number of changes such as activation of the sympathetic nervous system and hypothalamic-pituitary-adrenal axis. As a consequence, the adrenal cortex, in response to adrenocorticotropic hormone (ACTH), starts to secrete both cortisol and dehydroepiandrosterone (DHEA). Cortisol and DHEA are produced in different sections of the adrenal cortex; the zona fasciculata secretes cortisol while the zona reticularis secretes DHEA and its sulfated metabolite dehydroepiandrosterone sulfate (DHEA-S) (Nguyen and Conley, 2008). In female primates, DHEA and DHEA-S are also produced in the ovary (Sirinathsinghji and Mills 1983) and in primates and rodents DHEA is produced within the central nervous system and in peripheral nerves (Baulieu, 1998).

Cortisol stimulates the mobilization of the energy needed to overcoming stressors; DHEA and DHEA-S are androgen precursors that have been shown to exert antioxidative and anti-inflammatory effects (Kalimi et al., 1994; Maninger et al., 2009) and to play a protective and regenerative role (Theorell, 2009; Mainger et al., 2009).

In humans, an acute psychosocial stress induces a DHEA and DHEA-S increase (Izawa et al., 2008; Lennartsson et al., 2012) while long-term psychosocial stress negatively affects both steroids levels (Izawa et al., 2012; Lennartsson et al., 2013). Elevated levels of DHEA and DHEA-S in response to the stressor have been found in both men and women, along with significantly increased ACTH, cortisol, heart rate and blood pressure.

In cows, circulating DHEA-S is significantly lower than DHEA, and DHEA release is very different among individuals (Feher et al. 1977, Marinelli et al., 2007). In cows as in most non-primate mammals DHEA could be considered an index of the P450c17 enzyme activity and the most important circulating precursor of ectopic androgens and estrogens synthesis; on the contrary, DHEA-S contribution as an androgen reservoir is rather limited (Marinelli et al., 2007).

Increased stocking density is a common practice among dairy producers; the behavioral consequences of this practice are well documented while the physiological ones have still not been thoroughly investigated.

Fregonesi et al. (2007a) observed in dairy cows a linear reduction in lying time as freestall stocking density increased while Huzzey et al. (2006) observed a linear reduction in feeding time as stocking density at the feed bunk ??? was increased.

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Moreover, increased aggressive displacements are often observed at the overstocked feed bunk or freestalls (Huzzey et al., 2006; Fregonesi et al., 2007b); these competitive environments can make it difficult for some cows to gain access to feed.

As for the physiological consequences of overstocking, previous works have shown that cows regrouped into a high stocking density group (Friend et al., 1977) or subjected to overcrowding in the resting area (Friend et al., 1979) present a higher cortisol response to ACTH challenge compared with cows that are not regrouped or overcrowded, respectively.

In contrast to cortisol, DHEA and DHEA-S have received little attention within the stress research area of domestic animals and no studies have investigated so far the effect of overcrowding on DHEA secretion.

Therefore, the aim of this study was to verify whether overstocking during the dry period affects DHEA and cortisol (C) secretion and behavior in Holstein Friesian cows.

Commento [MB1]: Io credo che occorrerebbe mettere un fine legato alla eventuale correlazione con altri elementi di stress quali il comportamento: cioè capire se DHEA e/o cortisplo possono essere da soli marcatori di stress. In questo caso il solo rilievo di modifiche ormonali non è sufficiente. Mi sembr un elemento di discussione da sottolineare

MATERIALS AND METHODS

Animals, housing and diet

Twenty-eight Holstein dairy cows were enrolled in this experiment. All animals were housed at the farm of the University of Bologna (Ozzano Emilia, Italy) and used according to

EEC animal care guidelines. The experimental procedures had been approved by the Ethical Committee of Bologna University.

Animals were randomly divided into two groups (14 animals each), balanced for number of lactations, BCS (body condition score) and expected date of calving.. Cows in the far-off phase of the dry period (from 60 to 21 days before the expected calving date) were housed together in a bedded-pack and received water and grass hay ad libitum. From 21±3 days until calving animals were housed in two bedded-pack groups where they had ad libitum access to water and were fed daily using total mixed ration. After calving cows were housed together in a bedded pack area for the first 2 weeks of lactation and then moved to a free-stall pen for the rest of lactation. The total mixed rations (TMR) were fed approximately at 7 am for lactating cows and 9 am for dry cows. TMR samples were collected weekly throughout the study and analyzed for the chemical composition according to the following methods: dry matter (DM) was determined gravimetrically drying the sample at 103°C to a constant weight, crude protein (CP), neutral detergent fibre (aNDFom), and acid detergent fiber (ADF) were determined according to Mertens (2002), and AOAC 973.18, respectively. Starch was determined according to AOAC official method (AOAC 996.11) and ether extract according to AOAC 920.390020. Diet composition and analysis for both dry period and lactation are shown in Table 1.

Experimental design, blood sampling and hormone assays

Animals from 21 days to the expected calving until calving were housed in pens with the same size (24,0 m^2 in total with 15,5 m^2 of resting area and 8,5 m^2 of feeding area) but in different crowding conditions due to the introduction into the pen of heifers (interference animals) having a body weight of 500-550 Kg. In particular, control condition (CTR) had 2 animals per pen with 12.0 m^2 each, while the overstocked condition (OS) had three interference animals in the same pen with 4,8 m^2 for each animal. Bunk space is 3.3 m long and design with a neck rail allowing a space of 1.65 for control animal and 0.66 for OS animal. Resting area is a deep-bedded pack with straw added twice a day.

On days -30±3, -21±3, - 15±3, -10±3, -5±3 before and 10, 20, 30 after calving blood samples were collected from each cow before the morning feeding from the jugular vein into heparinized tubes for the determination of plasma DHEA and C concentrations.

After collection, blood samples were placed immediately on ice and centrifuged at 1200 x *g* for 20 min at 4°C. Plasma was harvested and stored at -20°C until steroids were measured. Plasma cortisol concentration was determined using a validated RIA as previously described (Tamanini et al 1983). The sensitivity of the assay was 4.3 pg/tube, and the intra- and inter-assay coefficients of variation were 5.4% and 8.6%, respectively. Cortisol plasma levels were expressed as ng/mL.

Plasma DHEA was measured by a microtiter RIA method previously described (Gabai et al., 2004), using a commercial anti-DHEA-7-carboxymethyloxime-BSA (Biogenesis, Poole, UK) that showed the following cross-reactions: DHEA 100%, 5α-androstane-3ß, 17 ß -diol 6.3%, androstenedione 1.3%, testosterone 0.1%, other related compounds less than 0.05%. The antiserum was used at a working dilution of 1:20,000. The tracer was [1,3,6,7 3H]DHEA (Perkin-Elmer Life Sciences; specific activity: 71 Ci/mmol; 30 pg/well). The standard curve was made by serially diluting (1.56–200 pg/well) a solution of DHEA (Sigma, Milan, Italy). The detection limit of the assay was 1.56 pg/well (software Riasmart; Perkin-Elmer Life Sciences). The results of the intra- and inter-assay precision test, expressed as coefficients of variation (CV), were 3.7 and 7.2%, respectively.

Body condition score

At enrolment, three weeks before calving, at calving and at 5 weeks of lactation, all cows were scored for body condition (1=emaciated and 5=obese; 0.25-unit increments, as described by Edmonson et al. (1989) and locomotion (1=normal locomotion and 5 = severely lame; as described by Sprecher et al., 1997). Cows with locomotion score ≥3 were considered lame.

Behaviour Monitoring

Rumination time was recorded using the Hi-Tag rumination monitoring system (SCR Engineers Ltd., Netanya, Israel). This rumination sensor includes a microphone that detects the rumination sounds, a motion sensor, a microprocessor, a storage unit and a battery. The sensor is fixed on collar and placed on the left side of the cow's neck. To guarantee the correct position of the tag a counter weight is placed on the bottom of the collar. The data are sent to a PC via antenna. A software (Data Flow software, SCR Engineers Ltd.) analyses the rumination time as minutes of 2 hours with a resolution of 2 minutes (Schirmann et al., 2009), and calculates the rumination time of the last 24 hours.

The cows were also equipped with another sensor (Pedometer Plus; S.A.E. Afikim) that monitored 3 parameters: activity (steps/h), lying time (min), and lying bouts (switching between standing and lying; Higginson et al., 2009). The tag was fitted to the rear leg of each cow and the data were accumulated and transmitted to management software (AfiFarm; S.A.E. Afikim) each time the cows passed an antenna located in the milking parlor. Behavioral data were collected every day but for statistical analysis the data were averaged per week.

Clinical Examination and Definitions of Diseases

All cows were examined at 1, 3±1, 10±1 days in milk (DIM) for diagnosis of retained foetal membrane, metritis, and acute metritis. Retained foetal membrane was defined as retention of foetal membrane after 24 h postpartum. Metritis was defined as cows with watery, pink or brown, and fetid uterine discharge. Cows with symptoms of metritis, rectal temperature >39.5°C, or anorectic, or depressed were considered to have acute metritis

(LeBlanc, 2010). All cows were observed once daily for displacement of abomasum and twice daily for mastitis throughout their lactation.

Production parameters

After calving, cows were milked twice daily at 07.30 and 19.30 h and individual yield of milk (AfiFlo milk meters, S.A.E. Afikim), concentrations of fat, true protein, and lactose (AfiLab on-line real-time milk analyzer, S.A.E. Afikim) were recorded by the Afikim milking system. The AfiLab system is calibrated once monthly with data on milk composition from 90 cows analyzed by the ARAER Laboratoty (Modena, IT). Concentrations of milk components from each milking were used to calculate the daily yields of fat, protein, and lactose after adjusting for milk production during each milking. The ECM yield (energy connect milk) was calculated as [(0.327 × milk yield) + (12.95 × fat yield) + (7.2 × protein yield)] (Orth, 1992). Daily values were averaged into weekly means for statistical analyses.

Statistical Analysis

The experiment had a randomized switch-back design with pen as the experimental unit. Seven replicates were used, six of them had a nulliparous and a parous cows together and one replicate had only parous cows. All statistical analysis were conducted using SAS version 9.2 (SAS/STAT, SAS Institute Inc., Cary, NC). Data were tested for non-normality by the Shapiro test. Binomial dependent variables were analyzed by logistic regression using GLIMMIX procedure with a binary distribution. Continuous data were analyzed by ANOVA for repeated measures using the MIXED procedure. The structure of covariance (autoregressive, unstructured, or compound symmetry) was chosen according to the Bayesian Akaike information criteria. In all models, treatment (OS vs Control), replicate (1 to 7), and parity (nulliparous vs parous) were included as fixed effect. For analysis of repeated measurements variables, time and the interaction between treatment and time were included in the model as fixed effect. Only the independent variables with $P < 0.10$ were retained in the model. Cortisol data were handled by log transformation to match normality.

RESULTS

At enrollment days of gestation (CTR = 258.8 ± 5.3 d, OS = 257.7 ± 4.7 d; P = 0.35), lactation number (CTR = 1.41 ± 1.33 lactation, OS = 1.29 ± 1.27 lactation; P = 0.62) and BCS (CTR = 3.64 ± 0.35 kg, OS = 3.52 ± 0.34 kg; P = 0.26) were not different among treatments. Among cows, treatment did not differ regarding previous lactation 305-d mature equivalent milk yield (CTR = 10,252 ± 231.1 kg, OS = 10,038 ± 191.7 kg; P = 0.39). Upon calving, gestation length was not different among treatments (CTR = 279.9 ± 5.0 d OS = 278.7 ± 4.2 d; P = 0.32). Days dry tended (P = 0.10) to be shorter for OS cows compared with CTR cows (CTR = 55.6 ± 12.6 d, OS = 48.6 ± 3.0 d). Calves weight was not different (P = 0.46) among treatments (CTR = 41.5 ± 3.7 d, OS = 41.7 ± 4.3 d). No animals carried twins. Incidence of peripartum diseases was not different between CTR and OS treatments. No animals had displaced abomasum and mastitis in the first 5 weeks after calving. One cow had metritis in the OS group while no cows in CTR group. Body condition score and lameness score were not affected by treatment.

DHEA and Cortisol concentrations

In both groups, there was an increase in DHEA before calving and after parturition the concentration declined rapidly. Overstocking significantly ($P<0.05$) increased DHEA concentration compared to CTR group at day -10 (1.79±0.09 vs 1.24±0.14 pmol/ml) while an increase of C was observed ($P<0.05$) at day -15 (3.64±0.52 vs 1.64±0.46 ng/ml) (Figure 1). No relationship was found between DHEA and C.

Monitoring Behavior

Rumination time

There were no differences between treatments regarding rumination time (total minutes of rumination/day) (Table 2).

Activity behavior

OS group showed significantly higher activity (step/hour), compared with CTR group, as reported in Table 3.

Lying behavior

Total minutes of lying time per day was not different among OS and CTR groups (Tab. 4). Daily lying bouts tended to be higher for OS group compared with CTR group in the first week of treatment. In the following weeks before calving, no difference was recorded between groups.

DISCUSSION

To our knowledge, this is the first study that demonstrates the difference in time-course variation of DHEA and cortisol secretion in response to overstocking during the dry period in Holstein Friesian cows. In both groups, an increase in DHEA was observed before calving, which tended to be more evident in the overstocked group, although the difference between groups was significant only at -10 days. Then, DHEA concentrations rapidly declined after parturition.

Commento [MB2]: Poiché vi è solo una modifica di pattern ornonali, non sarebbe utile discutere qui il modello di stress utilizzato^ cioò discutere perché si è scelto di “stressarla” secondo quell’area per animale? Magari riportando che altri studi ritengono questo modello sufficiente per creare uno stress importante….

In primates and rodents, it is generally accepted that DHEA is secreted mainly by the adrenal cortex and the ovary (Baulieu, 1998), and peripheral tissues are able to metabolize this steroid into active androgens and estrogens (Labrie, 1991). In pregnant primates and horses, placenta can utilize circulating DHEA to synthesize estrogens (Strauss et al., 1996). In addition to their role as androgen and estrogen precursors, both DHEA and DHEA-S play an important protective and regenerative role (Theorell, 2009; Maninger et al., 2009). In humans, DHEA and DHEA-S levels significantly increase in response to acute psychological stress (Lennartsson et al., 2012) and it has been suggested that these steroids play a protective role during the stress reaction, antagonizing the effects of cortisol (Hechter et al., 1997; Morgan et al., 2004). The stress-induced DHEA and DHEA-S increase likely has behavioral and emotional effects. Studies on mice showed antidepressant, anxiolytic, anti-aggression, and memory-enhancing effects of DHEA-S (Melchior and Ritzmann, 1994). In the cow, Marinelli et al. (2007) suggested that the placenta is the most important source of DHEA, which utilizes mainly the Δ5 steroidogenic pathway to produce estrogen (Geiser & Conley 1998). Previous works (Gabai et al., 2004; Marinelli et al., 2007) indicate that the DHEA placental secretion increases in late pregnancy, probably depending upon the tissue mass (Geiser & Conley 1998), and suddenly decreases after parturition. Therefore, the DHEA increased observed in the OS group approximately five days following a significant increase in plasma cortisol was quite surprising. Indeed, adrenal DHEA has been reported being secreted synchronously with cortisol during night and day (Rosenfeld et al., 1971), and the delay in DHEA secretion in respect to cortisol was unexpected. A possible explanation resides in the stimulating glucocorticoid effect on the placental CYP17 enzyme in the cow (Gross and Williams, 1988; Shenavai et al., 2012) that, in turn, could speed up the conversion of pregnenolone into DHEA.

Walking is associated to an increase in plasma cortisol concentrations (Coulon et al., 1998) and, likely, the OS cows, which displayed the greater number of steps per hour and thus were more active, experienced higher cortisol concentrations during the pre-partum period, likely resulting in the higher cortisol concentrations observed on day -15. The suitability of blood cortisol as a stress biomarker in livestock is in doubt because its variability and as blood sampling is an invasive technique that can cause the activation of the HPA (Mormede et al., 2007). Therefore, the intrinsic variability in plasma cortisol could have masked the greater HPA activation associated with OS and increased walking. Moreover, it is possible that the cows' HPA axis responded to increased walking during the first days of the OS treatment and then animals incurred in habituation. Indeed, Coulon et al. (1998) observed that cortisol concentrations were higher on days 1 and 8 in cows that walked in comparison with cows that remained at the barn, but the difference was not anymore evident after 20 days.

As glucocorticoids can alter placental steroidogenesis (Gross and Williams, 1988; Shenavai et al., 2012), it is possible that the modified endocrine milieu affects pregnancy length. However, in this experiment the increased plasma DHEA observed in OS cows was not associated with differences in pregnancy length, although days dry tended to be lower for OS animals.

Current recommendations for feed bunk space for prepartum freestall-housed dry cows is to provide a minimum of 0.76 m of linear bunk space per cow (Nordlund et al., 2006). In the present study, control cows had 1.2 m of bunk space per cow and OS cows had only 0.66 m of bunk space, which should provide adequate to limited bunk space. Reducing linear feeding space has been observed to increase competition at the feed bunk (Huzzey et al., 2006 Collings et. al., 2011). However the results of these studies, while showing more cow displacements from the feed bunk, the effect on DMI is little in some studies with mid-lactation cows (Collins et al., 2011) but greater in others that studied dry cows (Huzzey, 2013). In a study on lactating cows, it was observed a reduction in feeding time in multiparous cows (Proudfoot et al., 2009) and, in other studies, the competitively fed cows had fewer meals per day with a tendency of larger and longer meals (Olofsson, 1999; Hosseinkhani et al., 2008). Olofsson (1999) found that competition slightly increased the DMI of dairy cows, and this increase was driven by an increase in feeding rate. Based on

Commento [MB3]: Rispetto al commento precedente qui vi è il riferimento che intendevo almeno per lo spazio di accesso alla mangiatoia these studies, it is not surprising to have little or no effect on DMI with the feed bunk restriction used in the current study.

Rumination times were not different in OS animals in the current analysis. This parameter can be a key indicator of DMI, therefore animal in both groups had similar rumen activities and more than likely similar intakes.

In some studies, lying time has been shown to be decreased with increased stocking density (Krawczel et al., 2012; Lobeck-Luchterhand et al., 2015); however, other studies using late lactation or dry cows showed no differences (Collings, 2011; Huzzey et al., 2012). It is consistent that dry cows with more available time throughout the day (Grant, 2001) would have sufficient hours available to allow for a normal number of lying hours. Lying time has a higher priority for cows than eating when these two behaviors are restricted (Munksgaard et al., 2005). This could explain why although the space was consistently lower in OS animal (3.3 m^2 of bedded area versus 7.8 m^2 for control animals), the resting time did not changed. The time budgets of prepartum cows tend to be interrupted less than lactating dairy cows, because the animals are not moved outside the pen for milking and do not have cycling activity with estrus behavior. Both groups, however, showed a daily lying time lower than the recommend 12 hours/day (Munksgaard et al., 2005). Comfort of the bedding surface could be an important factor in determining daily lying time (Fregonesi et al., 2007b). In a study with either 9 or 4.5 $m^2$of bedded area per cow there were no difference in lying time (Fregonesi and Leaver, 2002). Animals could better tolerate overcrowding when open pack area is present compared with stall barn, since they can lie down at the same time staying closer one to the other. Using free stall type bedding, lying time linearly decreased when stocking density increased from 100% to 150% (Fregonesi et al., 2007a). In same condition, Krawczel et al. (2012) reported lying time was reduced for stocking densities of 131 and 142% compared with 100 or 113%.

Commento [MB4]: Questa frase però io non la metterei, nel senso che potrebbe indurre il reviewer a criticare la scelta del modello vedendo che già altri hanno riportato lo stesso risultato. Magari sottolineerei le eventuali differenze con quei lavori che magari non prevedevano le analisi ormonali

Mean lying bouts tended to be higher in OS group the first week of overcrowding, indicating an adjustment period was occurring. Animals had a resting time that is more disrupted, considering that the daily lying time were divided in more bouts. After this first week, the behavior was similar in OS and control animals. Competition at the feed bunk generally increased standing time in multiparous transition cows (Proudfoot et al., 2009) and in midlactation cows (Olofsson, 1999; Huzzey et al., 2006). The importance of this is determined by the overall DMI of the animals. Excessive standing time is a risk factor for developing lameness conditions such as claw horn lesions (Greenough and Vermunt, 1991; Singh et al., 1993). Avoiding excessive standing is important throughout lactation, but in particular during transition when animals are subjected to many endocrine and metabolic changes (Goff and Horst, 1997).

In our study OS animals showed higher activity~~ies~~, measured by the number of steps per hour, that indicates the increased need of movement in the pen. This represents another indication of stress occurring in this phase. An increased number of animal displacements and animal movement would be expected with overcrowding and feed bunk restriction (Collings, 2011; Huzzey 2012) and the stress of this could be expected to alter parameters being measured in this study.

Energy corrected milk production were not different among treatments. Recent study (Silva et al., 2014) reports no difference in ~~-~~yield of ECM. It would be expected that the minimal differences in cow behavior and DMI as observed in this study, would not carry through to any differences in DMI or early lactation milk production~~ in these animals~~.

The overall results of this study show that overstocking during the dry period is associated with changes in DHEA and cortisol. However, a~~A~~dditional researches are required to determine whether these hormonal changes are effective in affecting ~~the subsequent~~ behavior ~~performance~~ or can affect the duration of the dry period.

Commento [MB5]: Se si dice questo occorre indicare quali aspetti addizionali occorre includere che possano coprire eventuali dubbi su questo aspetto, se non vi sono allora io sottolineerei che gli aspetti ormonali non sono correlati a modifiche comportamentali.

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Table 1. Ingredients and chemical composition of the rations.

Composition	TMR Dry period	TMR Lactation
Ingredients (% of DM)		
Grass hay[1]	71.0	48.6
Corn ground fine	-	20.0
Sorghum grain meal	-	16.5
Soybean meal	-	7.9
Molasses	-	0.5
Concentrate mix [2]	29.0	-
Vitamins and minerals [3]	-	1.7
Chemical composition (% of DM)		
Crude protein	12.37	14.12
aNDFom	44.71	33.46
ADF	31.50	19.87
ADL	5.82	4.07
Starch	11.06	23.71
EE	3.28	3.48
Ash	5.60	6.71
NEl (Mcal/Kg of DM)	1.48	1.68

[1]Grass hay chemical composition on a dry matter basis was: 8.9% crude protein, 54% aNDFom, 39.9% ADF, 7.5% ADL, 8,8% ash.

[2] Concentrate mix: 48% corn meal, 20% soybean meal, 15% wheat bran, 10% beet pulp, 5% sunflowers meal, 2% mineral mix (4% Ca, 6% P, 4% Na, 10% Mg, 2000 mg/Kg of Zn, 1500 mg/Kg of Fe, 1000 mg/Kg of Mn, 175 mg/Kg of Cu, 150 mg/Kg I, 30 mg/Kg of Se, ,2000000 IU/Kg of vitamin A, 60000 IU/Kg vitamin D3, and 10000 mg/Kg of vitamin E).

[3] The lactating cows vitamins and minerals supplement contained 1,4% Ca, 8,3% P, 16 % Na, 5,5 % Mg, 4000 mg/Kg of Zn, 4000 mg/Kg of Mn, 400 mg/Kg of Cu, 400 mg/Kg I, 40 mg/Kg of Se, 20 mg/Kg of Co,1200000 IU/Kg of vitamin A, 200000 IU/Kg of vitamin D3, and 1000 mg/Kg of vitamin E.

Table 2. Mean ruminating period (total minutes/day) in response to treatment. The animals were overstocked (OS) for three weeks before calving.

Weeks before and after calving	Control	OS	SEM	P-value
-4	567.98	564.15	8.17	0.67
-3	561.98	542.28	8.97	0.21
-2	550.69	551.43	9.36	0.98
-1	525.10	512.30	12.85	0.58
1	489.24	478.29	11.86	0.59
2	590.91	608.39	9.97	0.28
3	557.39	572.89	11.01	0.07
4	554.53	576.96	10.88	0.31

Table 3. Mean activity (step/hour) in response to treatment.

Weeks before and after calving	Control	OS	SEM	P-value
-4	75.46	82.48	3.01	0.18
-3	75.04	109.20	4.72	<0.001
-2	73.52	109.41	4.60	<0.001
-1	79.73	113.15	5.26	<0.01
1	102.89	102.08	5.85	0.85
2	83.77	90.42	4.11	0.54
3	81.74	88.44	3.79	0.21
4	82.67	91.60	4.12	0.29

Table 4. Mean lying period (minutes/day) in response to treatment.

Weeks before and after calving	Control	OS	SEM	P-value
-4	659.1	672.5	10.94	0.55
-3	660.7	670.1	12.90	0.87
-2	672.2	659.9	19.96	0.54
-1	643.1	630.6	16.49	0.41
1	683.9	688.1	19.75	0.81
2	620.0	667.2	18.67	0.41
3	621.0	607.2	19.35	0.38
4	624.5	605.7	19.83	0.33

Table 5. Mean lying bouts in response to treatment.

Weeks before and after calving	Control	OS	SEM	P-value
-4	14.39	14.91	0.43	0.66
-3	14.19	16.12	0.52	0.09
-2	14.26	16.03	0.59	0.20
-1	15.11	16.55	0.69	0.32
1	16.59	17.71	0.50	0.42
2	13.72	14.67	0.54	0.27
3	13.50	13.63	0.49	0.30
4	12.66	12.23	0.65	0.42

Table 6. Mean ECM yield in response to treatment.

Weeks after calving	Control	OS	SEM	P-value
1	24.2	21.5	1.34	0.46
2	34.8	32.1	1.69	0.53
3	36.6	33.9	1.55	0.77
4	38.2	36.9	1.45	0.65

Figure captions

Figure 1. Plasma cortisol and DHEA concentrations before and after calving in CTR (●) and OS (■) group. The asterisk indicates a statistically significant difference between CTR and OS ($P < 0.05$) group. Values are mean ± SEM.

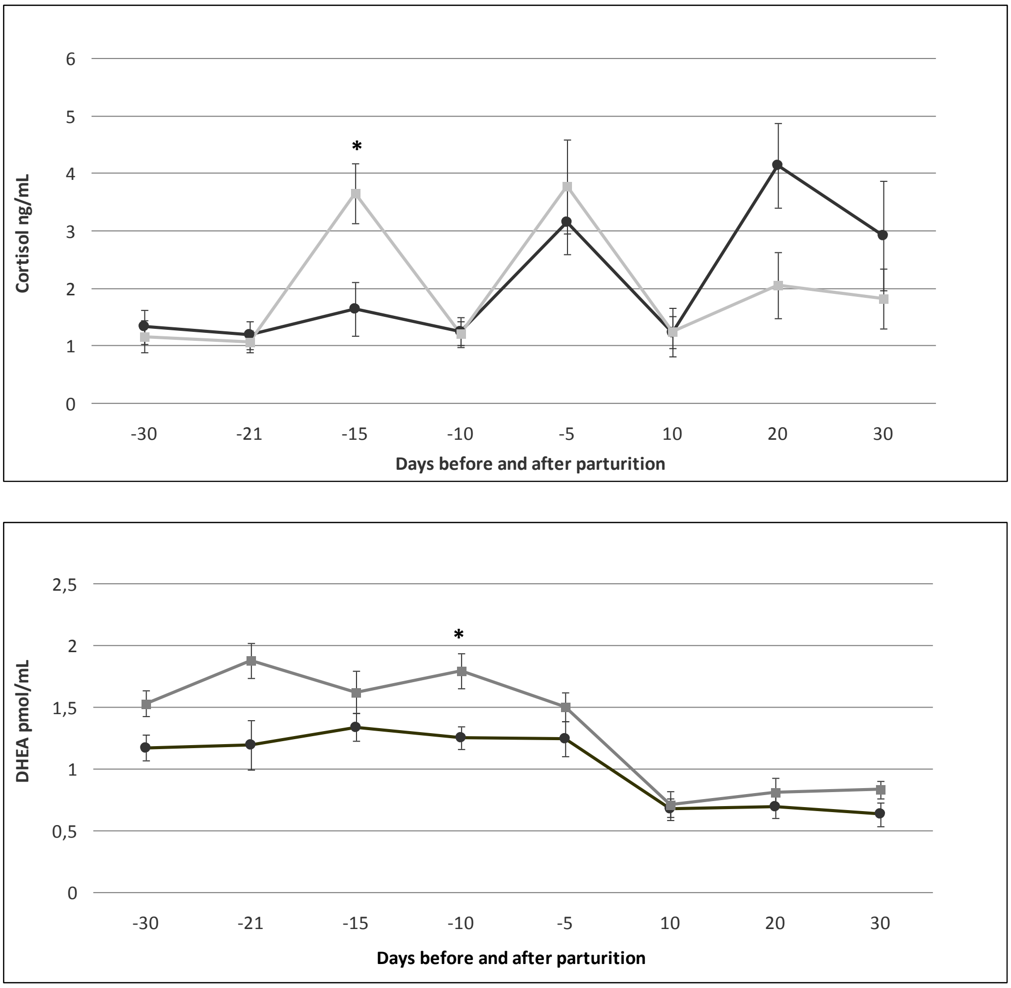

Figure 1. Fustini et al.