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Measurement of fecal glucocorticoid metabolites and evaluation of udder characteristics to estimate stress after sudden dry-off in dairy cows with different milk yields

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ABSTRACT

Sudden dry-off is an established management practice in the dairy industry. But milk yield has been increasing continuously during the last decades. There is no information whether the dry-off procedure, which often results in swollen and firm udders, causes stress, particularly in high-producing dairy cows. Therefore, we evaluated the effect of a sudden dry-off on extramammary udder pressure and the concentration of fecal glucocorticoid metabolites (i.e., 11,17-dioxoandrostane, 11,17-DOA) as an indirect stress parameter. Measurements were carried out within the last week before dry-off and until 9 d after dry-off considering 3 groups of milk yield (i.e., low: <15 kg/d, medium: 15–20 kg/d, and high: >20 kg/d). Udder pressure increased in all yield groups after dry-off, peaked at d 2 after dry-off and decreased afterwards. Pressures were highest in high-yielding cows and lowest in low-yielding cows. But only in high-yielding cows was udder pressure after dry-off higher than before dry-off. Baseline 11,17-DOA concentrations depended on milk yield. They were highest in low-yielding (121.7 ± 33.3 ng/g) and lowest in high-yielding cows (71.1 ± 30.0 ng/g). After dry-off, 11,17-DOA increased in all yield groups and peaked at d 3. Whereas in medium- and high-yielding cows 11,17-DOA levels differed significantly from their respective baseline during the whole 9-d measuring period, low-yielding cows showed elevated 11,17-DOA levels only on d 3 after dry-off. However, especially the increase in 11,17-DOA after dry-off between the 3 yield groups was considerably different. Mean 11,17-DOA increase from baseline to d 3 was highest in high-yielding cows (129.1%) and considerably lower in low-yielding cows (40.1%). The highest fecal 11,17-DOA concentrations were measured on d 3 after dry-off, indicating that the stress was most intense on d 2, which is due to

an 18-h time lag; at about the same time, udder pressure peaked. Our results showed a negligible effect of a sudden dry-off on low-yielding cows. High-yielding cows, however, faced high extramammary pressures and increased glucocorticoid production. Considering animal welfare aspects, a review of the current dry-off strategies might be warranted.

Key words: dry-off, fecal glucocorticoid, stress, udder pressure

INTRODUCTION

Animal welfare in farm animals has become a major public concern (von Keyserlingk et al., 2009). Recently, numerous studies were conducted to assess potential stressors in cows, such as the transition period and its effects on postpartum health (Huzzey et al., 2011), different lactation stages (Fukasawa et al., 2008), weaning and separation of calf and dam (Loberg et al., 2008), and vaginal examinations (Pilz et al., 2012).

Although a sudden dry-off is a common management practice on commercial dairy farms (Dingwell et al., 2001), there is a dearth of science-based information on the question whether the dry-off procedure causes stress, particularly in high-producing dairy cows. Recently, studies analyzed the effect of different feeding strategies during dry-off on metabolic parameters (Odensten et al., 2005) and the influence of herd management strategies during the dry period on the prevalence of IMI (Green et al., 2007). Only one study, however, addressed animal welfare during dry-off (Tucker et al., 2009). The authors investigated the effect of drying-off dairy cows on their lying behavior, time budget, and udder characteristics (e.g., udder firmness). That study focused on the comparison of 2 different dry-off strategies (feed restriction vs. reduced milking frequency) in late-lactating cows. Consequently, milk yield prior to dry-off was low (9.6 ± 2.9 kg of milk/d). In many areas throughout North America and in most European countries, however, farmers follow common recommendations featuring an abrupt cessation of milking at the

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end of lactation (Newman et al., 2010). Without feed restriction (Tucker et al., 2009) or intermittent cessation of milking (Odensten et al., 2005), milk yield at the last day of milking is quite a bit higher. A decrease in milk production during the last week before dry-off by 22 to 47% and 3.7 to 10.4% was demonstrated in cows with intermittent and abrupt cessation, respectively (Dingwell et al., 2001). Dingwell et al. (2001) examined Ontario DHI records and discovered an average milk yield at the time of dry-off of 16.6 kg per day. Furthermore, it was demonstrated that about 20% of the cows had a daily milk yield exceeding 22 kg at the time of dry-off. In the 1990s, even peak lactation rarely exceeded 25 kg per day (Schutz et al., 1990). This increase in milk yield during the last 20 yr warrants recognition, especially because management procedures hardly changed.

Frequently, dairy farmers have reported increased vocalization, reduced feed intake, and prolonged standing times after dry-off in addition to apparent udder swelling (S. Bertulat, unpublished data). Such behavioral changes indicate elevated stress levels and can be signs of discomfort and pain (Anil et al., 2002; Rutherford, 2002). Similar behavior during the dry-off period in cows with restricted feed rations was described by Valizaheh et al. (2008). Those authors associated increased vocalization with the experience of distress during the dry-off procedure. Due to their study design, however, hunger was the suspected reason.

Nevertheless, considering this evidence, we hypothesized that a relationship exists between dry-off and elevated stress. The pressure within the udder after dry-off, caused by slowly decreasing milk secretion and cessation of milking, might cause discomfort and consequently create stress accompanied by behavioral changes. This assumption is substantiated by findings in women that suffer regularly from breast soreness and pain after a short weaning duration (Neighbors et al., 2003).

Unfortunately, an objective quantification of pain in animals is currently not possible (Anil et al., 2002; Rutherford, 2002). But estimating stress as a result of pain or discomfort by measuring cortisol and cortisol metabolites is an established method (Anil et al., 2002; Rutherford, 2002).

Blood is the most common sample material to measure cortisol (Echternkamp, 1984) and the analytical method is well proven (Neher, 1958). For the determination of low and chronic stress levels, the use of blood samples, however, has certain limits (Cook et al., 2000). Cortisol concentration increases in the blood immediately after stress exposure. Therefore, mere handling, restraining cows, and puncturing the blood vessel cause

an increase in blood cortisol (Echternkamp, 1984; Hopper et al., 1999) and confound the resulting cortisol concentrations.

Because limitations of blood cortisol measurements have become evident for several research topics, various alternatives have been investigated. Analytical methods using feces (Morrow et al., 2002), saliva (Negrão et al., 2004), milk (Fukasawa et al., 2008), hair (Comin et al., 2011), and urine (Pompa et al., 2011) have been shown to be advantageous for effectively measuring glucocorticoid metabolites as equivalents of stress. For our study, the determination of cortisol in the feces seemed to be most promising. Fecal samples can be obtained without stressful restraining and manipulating the cow and measuring fecal cortisol metabolites offers a feedback-free method that has proven to be useful for the evaluation of chronic stress (Möstl and Palme, 2002). A direct relationship between fecal glucocorticoid metabolites, blood cortisol, and adrenal activity has been demonstrated (Morrow et al., 2002).

The most important glucocorticoid metabolites measured in cow feces are 11,17-dioxoandrostanones (**11,17-DOA**; Palme and Möstl, 1997; Palme et al., 1999; Möstl et al., 2002). 11,17-Dioxoandrostanone is measured utilizing an enzyme immune assay developed by Palme and Möstl (1997) and validated, for example, by Morrow et al. (2002). Several studies (e.g., Palme et al., 2000; Morrow et al., 2002; Palme, 2005) described a time lag of 8 to 16 h between an increase in blood cortisol concentration coinciding with the stressor and an elevated concentration of fecal 11,17-DOA.

The overall objective of this study was to evaluate the stress caused by drying-off dairy cows. Specifically, we set out (1) to quantify the changes of fecal 11,17-DOA concentration and udder pressure after a sudden dry-off, (2) to determine the effect of milk yield prior to dry-off on the fecal 11,17-DOA concentration and udder pressure, and (3) to evaluate the relationship between udder pressure and fecal 11,17-DOA concentration in the early dry period.

MATERIALS AND METHODS

Cows, Housing, and Feeding

This study was carried out on a commercial dairy farm in Brandenburg, Germany from April 2011 to August 2011. A total of 80 healthy, late-lactating (343 ± 39 DIM; mean \pm SD) and pregnant (49 ± 18 d before calving) Holstein-Friesian dairy cows were included in the experiment. All cows were managed according to the guidelines set by the International Cooperation on Harmonization of Technical Requirements for Regis-

tration of Veterinary Medicinal Products (Hellmann and Radeloff, 2000). The experimental procedures reported herein were conducted with the approval of the Institutional Animal Care and Use Committee. Cows were housed in a straw-bedded freestall barn and fed a roughage mix delivered twice per day at 0830 and 1700 h. Late-lactating cows received (on a DM basis) 54.3% corn silage, 25.4% haylage, 16.3% distillers grains, 0.9% corn, 0.8% soy, 2.0% rapeseed, and 0.3% basic mineral mix. Dry cows received (on a DM basis) 64.7% haylage, 32.8% corn silage, 1.7% hay, 0.3% corn, and 0.5% mineral mix. Concentrate was available for each lactating cow individually via an automatic feeder (35% wheat, 35% rye, 24% rapeseed extract, 5% soy, 1% oil, on a DM basis). All cows had access to fresh water in their pen.

Lactating cows were milked twice daily in a 2 × 8 Herringbone milking parlor (Alpro System; DeLaval, Tumba, Sweden) from 0600 to 0900 h and 1600 to 1900 h. Cows were dried off once per week based on their estimated calving date (7 wk before calving) or daily milk yield (<5 kg milk per day). All cows remained in the late-lactation pen until their last milking, received the same diet, and were milked twice daily. On the day of dry-off after the evening milking, cows scheduled for dry-off were treated with 150 mg of cefquinome (Cobactan DC; Intervet Deutschland GmbH, Unterschleißheim, Germany) administered into the teat canal and were transferred to the dry cow pen.

General Health Status and Milk Yield

Cows were enrolled 7 days before dry-off (54.7 ± 6.9 d to calculated calving date) and followed up for 9 d after drying off (Figure 1). A general examination was performed, including heart and breathing rate, rectal temperature, and rumination. Lameness was scored on a 5-point scale according to Sprecher et al. (1997). Udder quarters were palpated to diagnose pathological conditions (warmness, swelling, nodules, and changes in udder firmness). Additionally, the milk was visually examined on a dark surface. Examinations were repeated 1 d prior to dry-off and a California mastitis test was performed. Cows with signs indicative of mastitis, udder or teat lesions, alterations of the udder tissue, or cows with less than 4 functional quarters were excluded. Cows suffering from infectious or metabolic disease or lameness (i.e., lameness score >3) were also excluded. General and udder examinations were repeated 9 d after dry-off, when the cow completed the study. Cows were retrospectively withdrawn if any of the signs mentioned above were observed.

Cows were assigned to 1 of 3 groups according to their average milk yield during the last 7 d before dry-off. High-yielding cows ($n = 25$) produced more than 20 kg of milk per day, medium-yielding cows ($n = 29$) 15 to 20 kg per day, and low-yielding cows ($n = 26$) less than 15 kg. Milk yield per cow per milking was recorded by a milk meter integrated into the milking parlor and documented.

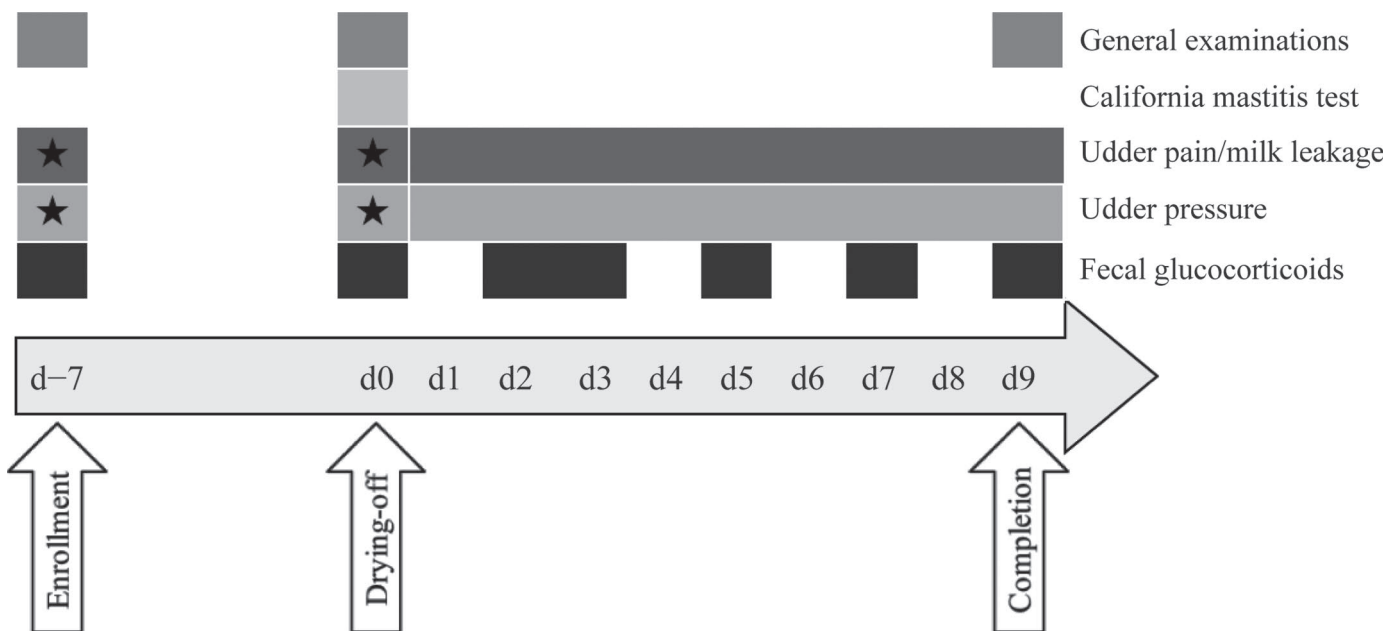


Figure 1. Schematic representation of examinations and sample collection. General examinations included clinical and udder examination and locomotion scoring (Sprecher et al., 1997). ★ = before and after milking.

Relevant cow data (i.e., age, parity, and DIM) were downloaded from the on-farm computer system. Test-day information (i.e., SCC, fat percentage, protein percentage, lactose percentage, and milligrams of urea per liter) was provided by the local DHIA (Landeskontrollverband Brandenburg e.V., Waldsiefersdorf, Germany). Cows were reviewed for all relevant events (e.g., disease, culling, and euthanasia) up to 21 d after calving. Hourly ambient temperature (AT) and relative humidity (RH) data were downloaded from the local weather station 15 km from the farm. The temperature-humidity index (THI) was calculated according to the equation reported by Kendall et al. (2008): $THI = (1.8 \times AT + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times AT - 26)]$. Hourly values were averaged for each trial day individually.

Udder Pressure

Udder pressure was measured using a hand-held dynamometer (Penefel DFT 14; Agro Technologie, Forges-les-Eaux, France) equipped with a 15-mm measuring tip and a plastic plate (70 × 100 mm) 20 mm behind and parallel to the surface of the measuring tip, as previously validated by Bertulat et al. (2012). The unit was programmed to a threshold of 0.3 kg and to display mean and coefficient of variation of 5 consecutive measurements. Mean values with a coefficient of variation exceeding 10% were discarded and the measurement repeated. Seven investigators were trained in handling the dynamometer according to a standard operating procedure based on previous recommendations (Bertulat et al., 2012). The penetration depth and the measuring point were defined by the plate attached to the dynamometer and a point marked in the middle of the udder with an animal marker pen, respectively. Measurements confounded by movements of the cow were repeated. Pressure measurements were always conducted by 2 investigators in the middle of the left hind quarter. Both investigators used the same dynamometer and recorded the pressure values independently within 2 ± 1 min. A mean pressure value was calculated based on values from both investigators.

On the day of enrollment and the day before dry-off, measurements were conducted in the barn 1 ± 0.5 h before the evening milking and a second time in the milking parlor directly after milking. During the experiment, udder pressure in dry cows was measured once per day in the dry-off pen. After dry-off, all measurements were carried out at 1400 ± 1 h. In addition to the pressure measurement, udders were visually examined and palpated. Milk leakage (i.e., milk observed dripping from 1 teat or more), signs of IMI, and udder pain (i.e., avoidance behavior during palpation) were evaluated.

Fecal Glucocorticoids

Fecal samples from each cow were collected on the day of enrollment, the day of dry-off and day 2, 3, 5, 7, and 9 after dry-off. About 50 to 100 g of feces was obtained manually from the rectum immediately after measuring the udder pressure. Disposable gloves were changed after every cow. According to Palme (2005), 10 to 15 g (equates 8 to 12 mL) of feces from different locations on the glove were filled into fecal sample tubes [Wirtschaftsgenossenschaft deutscher Tierärzte eG (WDT), Garbsen, Germany]. Samples were stored on ice immediately and frozen at -26°C within 2 h after collection.

For the extraction of the fecal glucocorticoid metabolites, samples were thawed at room temperature, stirred, and 0.5 g of feces was dispersed in 5 mL of 80% methanol (Palme and Möstl, 1997). Subsequently, the dispersion was vortexed for 30 min and centrifuged at $3,750 \times g$ for 15 min (Palme et al., 1999). The supernatant was transferred into aliquots of 1.5 mL and stored at -18°C until further analysis. A group-specific enzyme immunoassay (i.e., 11-oxo-etiocholanolone enzyme immunoassay) was carried out to determine the 11,17-DOA concentration (Palme and Möstl, 1997; Palme et al., 1999; Morrow et al., 2002). All samples were analyzed in duplicate. Intraassay and interassay coefficients of variation were calculated. Concentrations are stated in nanograms of 11,17-DOA per gram of fresh feces.

Caused by the time lag between elevated stress level and increased 11,17-DOA concentration, 11,17-DOA concentrations are indicative of stress 12 to 18 h earlier. To avoid confusion between sampling and time of stress experienced, days of fecal sampling were designated with an "F" (e.g., d 2_F , 3_F , 5_F , 7_F , and 9_F).

Statistical Analysis

Data were entered into Excel spreadsheets (version 2010; Microsoft Corp., Redmond, WA) and statistical analyses were performed with IBM SPSS Statistics for Windows software (version 20.0; IBM Deutschland GmbH, Ehningen, Germany). Homogeneity of the proportion of parity (i.e., first, second, or third-or-higher lactation) and yield group (i.e., low, medium, or high yield) was evaluated with a χ^2 test. The normal distribution of the 11,17-DOA and pressure values was assessed by plotting and visually examining the data and calculating a quantile-quantile (Q-Q) plot.

To summarize 11,17-DOA and udder pressure values measured before dry-off, 3 baseline values were calculated individually for every cow. The 11,17-DOA baseline averaged 11,17-DOA concentrations measured

on d -7_F and 0_F . The first udder pressure baseline averaged values measured before milking on d -7 and 0 ; the second udder pressure baseline averaged pressure values measured after milking. To verify the validity of this approach, the association and difference between 11,17-DOA concentrations of d -7_F and 0_F and between pressure values of d -7 and 0 , each before and after milking, were investigated using Pearson's correlation and paired *t*-test. Pressure values before milking (mean difference \pm SD; 0.057 ± 0.34 kg, $P = 0.17$) and after milking (mean difference \pm SD; -0.002 ± 0.12 kg, $P = 0.87$) did not differ between d -7 and 0 . Also, 11,17-DOA concentrations between d -7_F and 0_F did not differ (mean difference \pm SD; -10.3 ± 52.1 ng/g, $P = 0.091$). Thus, an 11,17-DOA baseline, a before milking udder pressure baseline, and an after milking udder pressure baseline were calculated accordingly.

Further analyses were carried out applying a linear mixed-model ANOVA. All mixed-model ANOVA were built according to the model-building strategies described previously (Dohoo et al., 2009). In brief, in a first step, each parameter considered for the mixed model was separately analyzed in a univariate model, including the parameter as a fixed factor (i.e., ordinal parameter) or covariate (i.e., continuous parameter). Only parameters resulting in univariate models with $P \leq 0.2$ were included in the final mixed model. Furthermore, all independent parameters were tested with Spearman's correlation (i.e., ordinal parameter) or Pearson's correlation (i.e., continuous parameter) for collinearity. If 2 parameters showed a high, significant correlation ($r > 0.60$), only the one resulting in the univariate model with the smaller *P*-value was used in the final mixed model. This final model was built in a manual backward stepwise manner by removing parameters resulting in $P > 0.05$ until all remaining parameters showed a significant effect. The covariance structure was chosen based on the model with lowest Akaike information criterion value. Post hoc comparison was carried out applying the least significant difference test. The significance level was set at $P \leq 0.05$.

The effect of dry-off on udder pressure values was evaluated in a linear mixed-model ANOVA, considering days as the repeated measure. The effect of yield group as fixed factor and the random effect of cows within yield groups were included in this model. Moreover, the diagonal covariance structure was used. The effects of parity, DIM, age, SCC, and their potential interactions on udder pressure were tested accordingly. Due to an interaction ($P < 0.001$) between days and yield groups we evaluated the within yield group between days effect and the within days between yield groups effect.

The effect of several parameters on the occurrence of milk leakage after dry-off was evaluated in a binary

logistic regression model. A conditional backward stepwise manner was selected and significance levels of 0.1 and 0.05 were chosen to exclude and include terms, respectively. According to Peduzzi et al. (1996), we included a maximum of 7 parameters (i.e., yield groups, days after dry-off, udder pressure, parity, SCC, 11,17-DOA concentration, and DIM) and ensured at least 10 cases each with and without milk leakage to obtain reliable estimates. Odds ratios, 95% confidence intervals, and significance levels are reported.

The effect of dry-off on 11,17-DOA values was evaluated in a linear mixed-model ANOVA, considering days as the repeated measure. The effect of yield group and day as fixed factor and the random effect of cows within yield groups were included in this model. The diagonal covariance structure was used. The between groups within day and the within group between days effect was evaluated in the same model. Furthermore, the following parameters were tested as factors (i.e., ordinal data) and covariates (i.e., continuous data): udder pressure of the previous day, yield group, age, parity, DIM, SCC before dry-off, 21-d survival rate (i.e., culling or remaining in the herd), BW, milk leakage, and mean daily THI. Visually examining the 11,17-DOA values, a clear difference existed between baselines and days after dry-off. Therefore, the model was rerun twice covering only baseline values and excluding the baseline, respectively. In the model assessing effects on baseline 11,17-DOA, udder pressure values measured before milking were used. Cows within yield groups were included as random effect and the scale identity covariance structure was used in both ANOVA. Post hoc comparison was carried out applying the least significant difference test.

Baseline values were assigned to 3 equal groups using the percentile function in SPSS to visualize the heterogeneity of yield groups among cows with low, medium, and high baseline values. The time lag between stressor and elevated 11,17-DOA concentration was verified by calculating Pearson's correlation for 11,17-DOA and udder pressure of the same day the fecal sample was obtained and for 11,17-DOA and udder pressure of the day before the sample was obtained.

For better assessment of the variations in 11,17-DOA concentrations, the changes in 11,17-DOA concentrations after dry-off were calculated relative to the individual baselines (i.e., **11,17-DOA_{rel}**). These were computed for each individual cow and day using the following formula:

$$11,17\text{-DOA}_{\text{rel}} = \frac{11,17\text{-DOA} - \text{baseline}}{\text{baseline}} \times 100.$$

The effect of dry-off on 11,17-DOA_{rel} values was evaluated in a linear mixed-model ANOVA, considering days as the repeated measure. The random effect of cows within yield groups was included in this model and the diagonal covariance structure was used. The effects of days after dry-off, yield group, udder pressure, parity, DIM, and udder pain on 11,17-DOA_{rel} values were tested in this model. The model was rerun to assess the within days between groups effect.

Because 11,17-DOA concentrations for cows after dry-off were not available for a sample size calculation, a post hoc power analysis was performed using the G*Power program (version 3.1.3; University of Düsseldorf, Düsseldorf, Germany) to verify the level of the effect of drying-off on the 11,17-DOA concentration. A post hoc repeated-measures ANOVA between factor analyses model was applied to calculate the power of analysis (1- β) and the effect size (f), accepting a null hypothesis error of 0.05.

RESULTS

Eighty cows in first (n = 31), second (n = 26), and third-or-higher (n = 23) lactation met the inclusion criteria and were enrolled in the study. The distribution of parity was homogeneous between the 3 yield groups ($P = 0.21$). Four cows had to be excluded from further analyses due to group change in the dry period (n = 2) or due to mastitis (n = 2). A total of 551 fecal samples were collected and analyzed and 1,024 udder pressure measurements were carried out. The intra- and interassay coefficients of variation for the 11-oxo-etiocholanolone enzyme immunoassay were 10.1 and 14.5%, respectively.

The power of analysis for the repeated measurement of 11,17-DOA concentration before and after dry-off in 3 yield groups was 0.9996, with an effect size of $f = 0.318$. The power of analysis was within the limits set by Cohen (1988) and Prajapati et al. (2010) and the effect size of this study was acceptable (Cohen, 1988). The chance of error in accepting the null hypothesis was 0.04%.

The threshold for heat stress (i.e., $\text{THI} \geq 72$) established by Armstrong (1994) was not exceeded during the entire trial period. The mean THI for May, June, July, and August was 58.8 ± 4.6 , 63.6 ± 3.2 , 64.4 ± 3.1 , and 64.1 ± 2.9 , respectively. A significant difference in THI between the various months did not exist ($P = 0.097$).

Udder Pressure, Milk Leakage, and IMI

Udder pressure baseline values before and after milking averaged 0.72 ± 0.24 and 0.48 ± 0.10 kg for low-,

0.95 ± 0.25 and 0.56 ± 0.19 kg for medium-, and 1.01 ± 0.25 and 0.53 ± 0.104 kg for high-yielding cows, respectively. Mean pressure before milking differed between low- and medium- ($P = 0.001$) as well as between low- and high-yielding cows ($P < 0.001$). No difference existed between yield groups after milking ($P = 0.14$).

An overall effect of yield group ($P = 0.001$) and day ($P < 0.001$) on udder pressure could be evaluated in the linear mixed-model ANOVA. The post hoc comparison showed that udder pressure increased in all yield groups ($P < 0.001$) after dry-off and peaked on d 2 (Figure 2). But only in high-yielding cows was udder pressure after dry-off (i.e., d 2) higher than udder pressure measured in late lactation before milking ($P = 0.007$). After d 2, udder pressures declined in all 3 groups; however, baseline pressures measured in late-lactating cows after milking ($P < 0.05$) were not reached within 9 d. Considering the different yield groups, udder pressures after dry-off were highest in high-yielding cows. They differed between high- and low-yielding cows and between medium- and low-yielding cows for 9 (last sampling day) and 7 d after dry-off, respectively ($P < 0.05$). High-yielding cows had a higher udder pressure on d 3 and 4 ($P < 0.05$) compared with medium-yielding cows.

In addition to an effect of yield group and day on udder pressure, an interaction between day and yield group ($P = 0.003$) could be demonstrated. There was no effect, however, of parity ($P = 0.22$), DIM ($P = 0.076$), SCC ($P = 0.084$), or age ($P = 0.12$) on udder

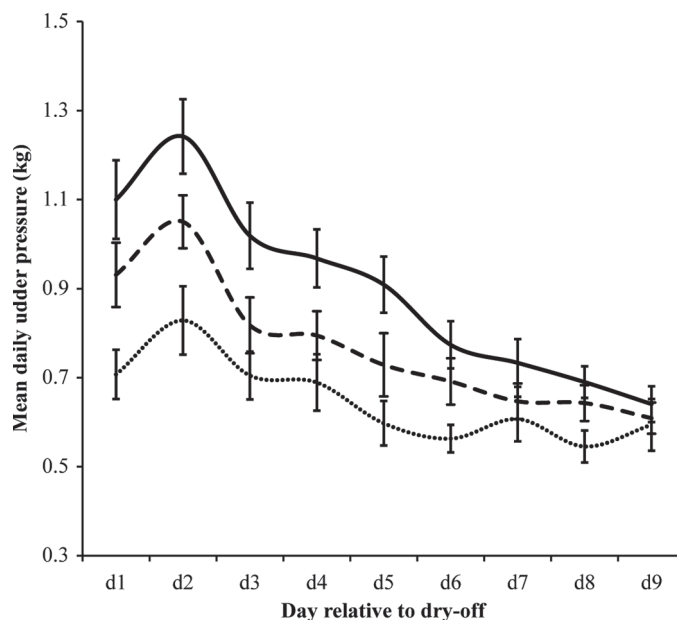


Figure 2. Udder pressure (mean \pm SE; kg) after dry-off considering low- (n = 25; <15 kg/d; dotted line), medium- (n = 27; 15–20 kg/d; dashed line), and high- (n = 24; >20 kg/d; solid line) yielding cows.

pressure after dry-off. The correlation coefficient between SCC and udder pressure after dry-off was -0.226 ($P = 0.042$).

Before dry-off, milk leakage was observed in 2 cows before milking; both were high yielding. After dry-off, 49 events of milk leakage in 27 different cows (33.8%) were recorded. Eight out of these 27 cows had milk leakage on more than 1 day after dry-off. The probability of the occurrence of milk leakage after dry-off was significantly associated with yield group ($P < 0.001$), parity ($P = 0.006$), and udder pressure ($P = 0.016$). Cows with a high udder pressure were more likely to show milk leakage than cows with low pressure values (odds ratio = 3.35; 95% CI = 1.26–8.93; $P = 0.016$). Additionally, animals in their third-or-higher lactation displayed 3.53-fold higher odds of having milk leakage than cows in first lactation (95% CI = 1.42–8.80; $P = 0.007$). No difference existed between cows in first and second lactation ($P = 0.73$). Furthermore, high-yielding cows were 5.07 times more likely to show milk leakage than low-yielding cows (95% CI = 1.83–14.04; $P = 0.002$; Figure 3). A difference between low- and medium-yielding cows was not significant ($P = 0.69$). The concentrations of 11,17-DOA ($P = 0.70$), DIM ($P = 0.99$), and SCC ($P = 0.43$) were not significantly associated with the likelihood of the occurrence of milk leakage. No difference was observed in the probability of milk leakage between d 1 and 9 after dry-off ($P = 0.66$).

Two cows developed clinical mastitis (i.e., firm, heated, and reddened quarter; abnormal milk with clots and pus) during the first 9 d after dry-off. Both cows were low yielding and did not show any signs of udder pain before the day of diagnosis (i.e., 4 and 6 d after

dry-off) and no milk leakage. However, these cows were not included in the analyses described above.

Fecal Cortisol Metabolites

11,17-Dioxoandrostande baseline concentrations ranged from 30.0 to 184.9 ng/g. These baseline concentrations were affected by yield group ($P < 0.001$) and udder pressure before milking ($P = 0.014$). A difference was observed between low- and medium- ($P = 0.003$), low- and high- ($P < 0.001$), and medium- and high-yielding cows ($P = 0.013$). Interestingly, most high-yielding cows had low and most low-yielding cows had high 11,17-DOA baseline concentrations (Figure 4). Age, parity, and SCC had no effect on 11,17-DOA baselines. They were excluded from the final model, because they resulted in univariate models with $P \geq 0.2$. Furthermore, no effect was observed of DIM on the baseline 11,17-DOA concentration ($P = 0.53$).

After dry-off 11,17-DOA concentrations up to 412.39 ng/g were measured. For all cows, concentrations of 11,17-DOA increased significantly from d 2_F to 3_F, peaked on d 3_F, and decreased again subsequently (Table 1). In high- and medium-yielding cows, 11,17-DOA increased from baseline to d 2_F ($P < 0.001$) and in medium-yielding cows, a second increase occurred from d 2_F to 3_F ($P = 0.004$). In both yield groups, 11,17-DOA concentrations decreased from d 3_F to 5_F ($P < 0.05$) and remained at an elevated concentration compared with the baseline until d 9_F ($P < 0.05$). Subsequently, no differences were found between d 5_F, 7_F, and 9_F ($P > 0.05$) in medium- and high-yielding cows, respectively. In low-yielding cows, however, only 11,17-DOA concentration on d 3_F differed from the baseline ($P = 0.005$). In this group, there was neither a difference between baseline 11,17-DOA concentrations and concentrations measured on d 2_F, 5_F, 7_F, and 9_F, nor between 11,17-DOA concentrations measured on any day after dry-off ($P > 0.05$).

Besides the effect of day ($P = 0.005$) on the 11,17-DOA concentration after dry-off, the concentration was furthermore affected by udder pressure ($P = 0.05$). However, considering only the days after dry-off, 11,17-DOA concentrations did not differ ($P = 0.83$) between the 3 yield groups. The average 11,17-DOA concentration after dry-off (d 2_F–9_F) was 143.27 ± 65.0 , 139.25 ± 70.1 , and 128.2 ± 77.5 ng/g for low-, medium-, and high-yielding cows, respectively. The univariate models for age, DIM, BW, and THI were again not significant and these parameters were excluded from the final model. Also, no effect was observed of parity ($P = 0.39$) or milk leakage ($P = 0.26$) on the 11,17-DOA concentration after dry-off.

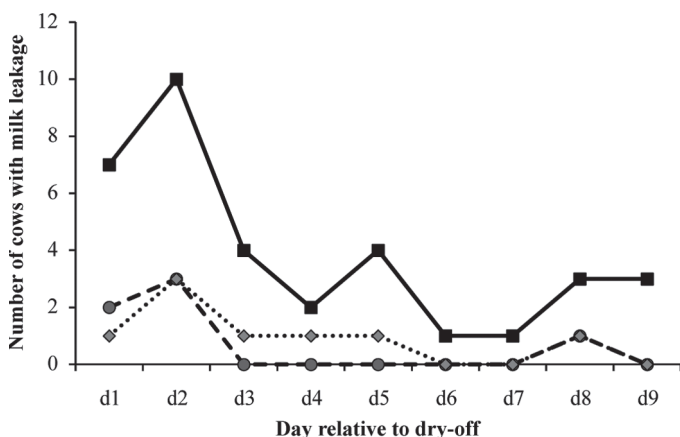


Figure 3. Number of cows with milk leakage after dry-off considering milk yield: low ($n = 25$; <15 kg/d; dotted line), medium ($n = 27$; 15 – 20 kg/d; dashed line), and high ($n = 24$; >20 kg/d; solid line).

Table 1. Mean daily 11,17-dioxoandrostande concentration (mean ± SE; ng/g) on different days of fecal sampling (designated with an F) before and after dry-off in 76 cows with varying milk yield

Day relative to dry-off	Yield group		
	Low (<15 kg)	Medium (15–20 kg)	High (>20 kg)
Baseline	121.7 ± 6.8	94.0 ± 6.3	71.1 ± 6.1
d 2 _F	132.8 ± 12.1	118.9 ± 10.9	129.8 ± 19.8
d 3 _F	163.2 ± 14.1	164.6 ± 16.1	136.6 ± 14.0
d 5 _F	131.9 ± 14.8	134.1 ± 13.0	113.3 ± 11.9
d 7 _F	140.1 ± 11.1	143.3 ± 13.7	125.2 ± 14.8
d 9 _F	148.4 ± 14.0	135.2 ± 11.6	135.9 ± 18.1

The 3 different yield groups showed diverging increases of 11,17-DOA concentrations after dry-off (11,17-DOA_{rel} values). Both the yield group ($P = 0.01$) and the experimental day ($P < 0.001$) had an effect on the change in 11,17-DOA concentration (Table 2). Although 11,17-DOA_{rel} values of low- and high-yielding cows differed ($P = 0.003$), no difference between low- and medium- ($P = 0.074$) and medium- and high-yielding cows ($P = 0.12$) was found. Within days, high-yielding cows had higher 11,17-DOA_{rel} values than low-yielding cows ($P < 0.02$) on all days after dry-off. Parity, DIM, and udder pain had no effect ($P > 0.05$) on 11,17-DOA_{rel} values.

Interestingly, udder pressure and 11,17-DOA concentrations showed a similar curve, but with a time lag of 1 d (Figure 5). The correlation coefficient between both parameters measured on the same day was 0.114 ($P = 0.027$). Considering the time lag and correlating 11,17-DOA values with the udder pressure measured on the previous day, the correlation coefficient increased slightly to 0.158 ($P < 0.001$).

Cows culled (i.e., slaughtered, euthanized, or died) within 21 d after calving ($n = 11$) due to metabolic disease or mastitis showed higher 11,17-DOA concentrations before (culled cows = 108.78 ± 41.4 ng/g; survived cows = 93.21 ± 37.1 ng/g; $P = 0.021$) and after

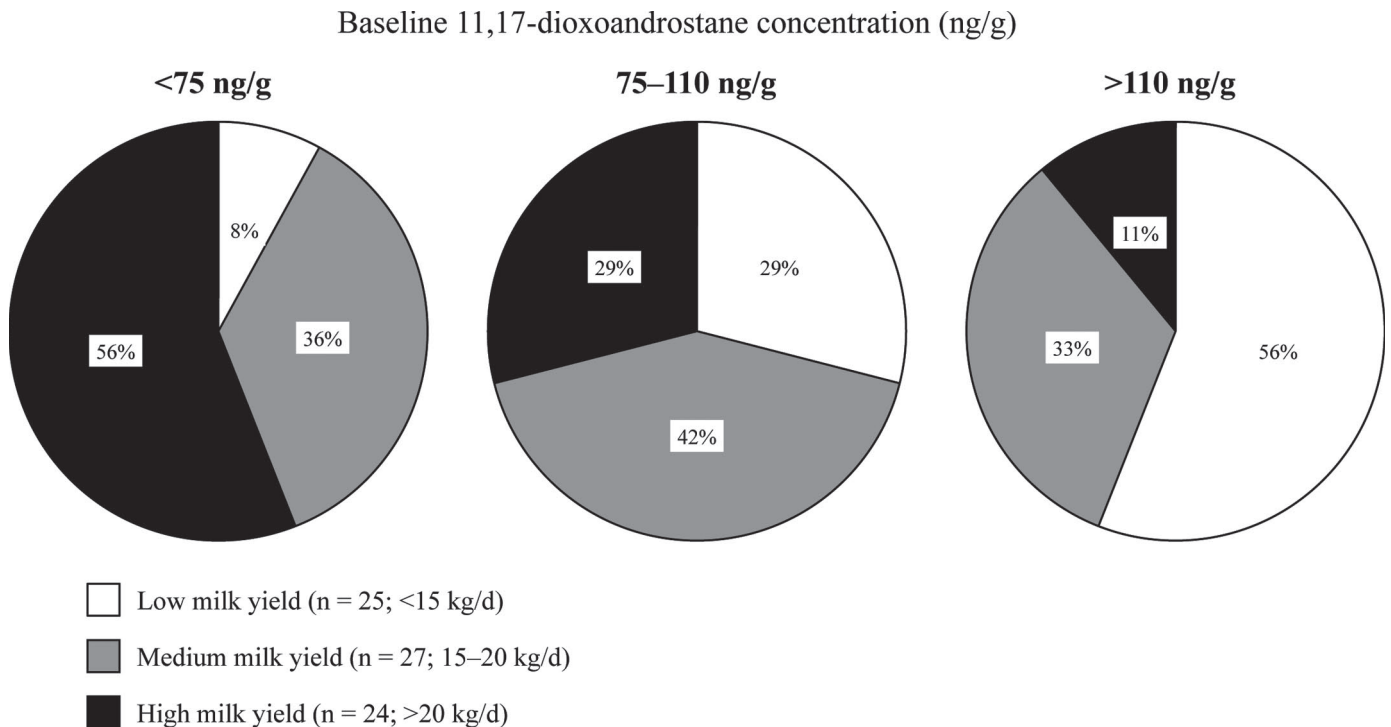


Figure 4. Distribution of daily milk yield in cows with different baseline 11,17-dioxoandrostande concentrations: low milk yield ($n = 25$; <15 kg/d), medium milk yield ($n = 27$; 15–20 kg/d), and high milk yield ($n = 24$; >20 kg/d).

Table 2. Percentage increase (mean \pm SE; %) in 11,17-dioxoandrostande (11,17-DOA) concentration in relation to the baseline in different yield groups and on different days of fecal sampling (designated with an F) after dry-off in 76 dairy cows¹

Day relative to dry-off	Yield group			<i>P</i> -value ²
	Low (<15 kg)	Medium (15–20 kg)	High (>20 kg)	
d 2 _F	10.6 \pm 8.3	33.9 \pm 11.3	117.7 \pm 41.4	0.008
d 3 _F	40.1 \pm 13.1	81.2 \pm 16.1	129.1 \pm 37.5	0.044
d 5 _F	14.7 \pm 12.5	55.5 \pm 15.1	83.9 \pm 29.6	0.060
d 7 _F	17.9 \pm 9.0	60.4 \pm 13.0	94.2 \pm 27.7	0.017
d 9 _F	24.0 \pm 10.4	48.5 \pm 9.3	128.8 \pm 44.1	0.015
<i>P</i> -value ³	0.060	<0.001	0.01	

¹The change in 11,17-DOA concentration after dry-off (11,17-DOA_{rel}) was calculated relative to the individual baselines as follows: $11,17\text{-DOA}_{\text{rel}} = \frac{11,17\text{-DOA} - \text{baseline}}{\text{baseline}} \times 100$.

²Within day between groups effect.

³Within group between days effect.

dry-off (culled cows = 162.76 \pm 83.4 ng/g; survived cows = 132.73 \pm 68.0 ng/g; *P* = 0.043) compared with cows remaining in the study.

DISCUSSION

Milk yield has been increasing continuously since the beginning of the 20th century (Lucy, 2001). Management practices implemented to dry-off dairy cows, however, have stayed the same except for the approval

of new drugs to decrease the risk of infection (e.g., antibiotic drugs and teat sealant; Berry and Hillerton, 2002; Godden et al., 2003). Considering the increased milk yield per cow, one might speculate that drying-off cows with considerable milk production could pose an animal welfare issue. To our knowledge, this is the first study evaluating the influence of milk yield on stress hormones directly after dry-off and correlating high milk yield in late-lactating cows with high extramammary udder pressure and elevated stress levels.

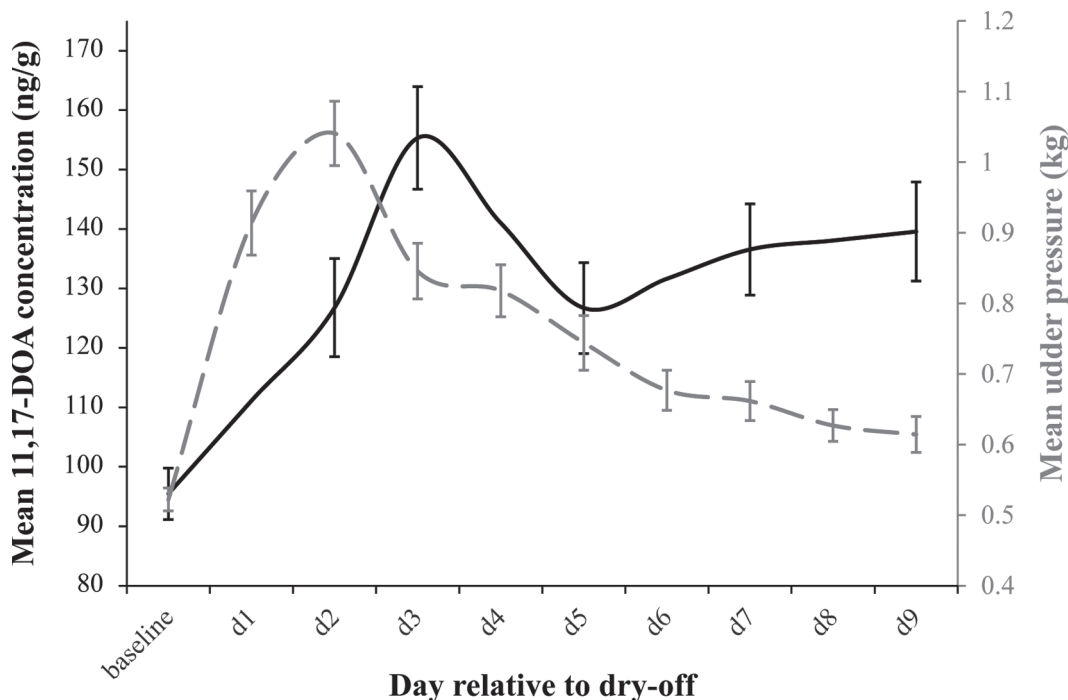


Figure 5. Mean (\pm SE) daily 11,17-dioxoandrostande concentration (11,17-DOA; solid line) and udder pressure (dashed line) after dry-off in 76 dairy cows.

Udder Pressure and Milk Leakage

Two udder pressure baselines (i.e., before and after milking) were established in late-lactating cows before dry-off. The udder pressure baseline evaluated after milking was lower in all yield groups compared with before milking. This confirms data recently reported by Bertulat et al. (2012), who demonstrated a similar decrease in udder pressure due to milking. In the current study, udder pressure before milking differed considerably between yield groups and high milk yield was associated with high pressure values before milking. This observation underlines that udder pressure depends on the milk volume in the udder. A similar relationship between high milk yield and high intramammary udder pressure in lactating cows was reported by Tucker et al. (1961) and Graf and Lawson (1968).

After milking, udder pressures were similar in all cows irrespective of their milk yield. While the intramammary udder pressure is solely determined by the amount of milk within the udder (Tucker et al. 1961), extramammary udder pressure is also influenced by the firmness of the udder tissue (Bertulat et al., 2012). As the intramammary udder pressure after milking is negligible because all milk has been withdrawn, the remaining extramammary pressure measured after milking corresponds with the firmness of the udder tissue. Our results indicated that the firmness of the udder tissue was similar in all cows regardless of their milk yield. This observation warrants further research on the diagnostics of udder ailments.

Data on udder pressure after dry-off are sparse. Overall, in our study, the development of udder pressure after dry-off with an initial increase, a peak within 2 d, and a subsequent decrease was similar to results published previously (Tucker et al., 2009). A direct comparison of udder pressure values measured in both studies is not possible, however, because of different measuring devices, resulting in values with varying units. Furthermore, Tucker et al. (2009) compared cows with different feed rations and milking frequencies. Consequently, the milk yield averaged 9.3 ± 1.0 kg/d, which is comparable only to our low-yielding cows.

Anecdotal evidence from the field suggests that especially high-producing cows show firm and swollen udders. To our knowledge, however, studies are not available describing a relationship between milk yield and extramammary udder pressure after dry-off. Our study showed that udder pressure after dry-off was highest in high-yielding cows and lowest in low-yielding cows. The correlation between milk yield and udder pressure on d 2 ($r = 0.411$; $P < 0.001$) was within the range described by Graf and Lawson (1968) for milk yield and intramammary udder pressure. A recent study (Tucker

et al., 2009) demonstrated a significantly lower udder pressure ($P \leq 0.012$) in cows with a lower (i.e., 8 kg of DM/d) compared with a higher feeding treatment (i.e., 16 kg of DM/d). The cows with 8 kg of DM/d also produced less milk ($P = 0.016$). The lower udder pressure and lower milk yield in cows with 8 kg of DM/d supports our results. The increase in udder pressure shown by Tucker et al. (2009) between pressure values measured for un milked udders before dry-off and 2 d after dry-off was similar to our findings in low- and medium-yielding cows (average increase of 12.8%). The differences between before and after dry-off, however, in high-yielding cows (i.e., >20 kg) were considerably higher. Therefore, we suspect that the higher milk secretion of high-yielding cows leads to a greater increase in udder pressures after dry-off.

In our study, udder pressure was measured once daily for 9 d after dry-off. For the whole period, pressure values in all 3 yield groups exceeded the baseline values after milking. This is in contrast to Tucker et al. (2009), who demonstrated that baseline pressure values were reached within 4 days after dry-off. Probably this discrepancy can be explained by the different milk yield of the cows enrolled (9.3 ± 1.0 vs. 17.6 ± 6.7 kg/d) and the different measuring methods. According to Hurley (1989), the total milk volume in udders decreased by 75% within 11 d after dry-off. Therefore, higher milk yield at the time of dry-off results in higher milk volume remaining in the udder after dry-off and a prolonged interval until complete resorption of the milk.

Our data did not show an influence of DIM, parity, and age on udder pressure. But similar to previous studies (Raubertas and Shook, 1982; Jones et al., 1984), an effect of DIM and SCC on milk yield was noted.

Milk leakage was diagnosed in 2 late lactating cows (2.5%) before dry-off, which confirms previous findings of 2% milk leakage before dry-off (Tucker et al., 2009). After dry-off, 31.6% of cows leaked milk within the first week after dry-off. The prevalence, however, varied between 56.0% in high-yielding and 15.4% in low-yielding cows. This yield-related prevalence confirms again the results of Tucker et al. (2009) who described up to 15% milk leakage after dry-off in cows with lower yield and up to 45% in higher-yielding dairy cows. Furthermore, our study indicated that a high extramammary udder pressure increased the risk of milk leakage. For lactating cows, a similar relationship between intramammary udder pressure and milk leakage has been demonstrated (Rovai et al., 2007). In contrast to Rovai et al. (2007), our data, however, also revealed a relationship between milk leakage after dry-off, parity, and udder pressure. A relationship between the decreasing integrity of the teat canal in higher-parity cows and enhanced risks for IMI was already demonstrated in a previous study (Ding-

well et al., 2004). We speculate that these conditions of the teat canal in older cows provoke milk leakage, too. It remains unclear why this effect was not observed in peak-lactation cows (Rovai et al., 2007). We presume that the udder pressure plays an important role; nonetheless, further studies are warranted to elucidate this association.

Fecal Cortisol Metabolites

In order to verify a relationship between udder pressure and elevated stress levels we measured the concentration of 11,17-DOA in fecal samples before and after dry-off. First, baseline 11,17-DOA concentrations were established and compared between the varying yield groups. A clear relationship existed between average milk yield before dry-off and the baseline 11,17-DOA concentration. Interestingly, this relationship was negative, as low-yielding cows had high and high-yielding cows had low baseline 11,17-DOA concentrations. This observation contradicts results presented by Odensten et al. (2007), who showed similar blood cortisol concentrations in dairy cows with different yield classes (low = 5.0–11.4 kg/d; medium = 11.5–17.7 kg/d; high = 17.8–29.5 kg/d) before dry-off. Variations in the 11,17-DOA concentration could be caused by miscellaneous external triggers like transportation (Palme et al., 2000) or stressful handling (Saco et al., 2008). In our study, however, all cows were kept under identical conditions in the same pen. Clinical or subclinical diseases have been established by different authors as triggers for elevated stress levels (e.g., Peter and Bosu, 1987; Hockett et al., 2000). In this study, disease events are an unlikely reason for elevated 11,17-DOA concentrations because general health (i.e., body temperature, heart and breathing frequency, rumination, and BW) and udder health status were monitored multiple times throughout the study and cows with signs indicative of disease were withdrawn from analyses. Furthermore, an individual variability in the basal glucocorticoid concentration was already proven in cats (Graham and Brown, 1996) and is suspected also in cows (Palme et al., 2000; Morrow et al., 2002). A relationship between higher feed efficiency and, therefore, better performance in steers with higher baseline 11,17-DOA concentration was demonstrated by Montanholi et al. (2010). Our study, however, provides the first evidence that baseline 11,17-DOA concentrations in dairy cows could be yield related. A similar relationship between high milk yield and lower blood cortisol was demonstrated in dairy cows 30 and 90 d postpartum (Sartin et al., 1988). Those authors hypothesized that high milk yield may be correlated with a faster hormone metabolism and, thus, lower cortisol levels. Further evidence was provided by

Wiltbank et al. (2006), who evaluated a relationship between milk yield, elevated steroid metabolism, and as an extension elevated metabolic activity. Both papers related high milk yield to a faster metabolism, but were unable to substantiate this assumption and demand further research. As there is a lack of controlled studies investigating 11,17-DOA concentrations during peak and mid lactation, the reasons for those differences in baseline 11,17-DOA concentrations remain speculative. A relationship between high milk yield, faster metabolism, and lower 11,17-DOA concentrations could neither be validated nor rejected by our results.

Despite the fact that sudden dry-off is a common management practice, there is a dearth of information about the intensity of stress cows might suffer as a consequence of this procedure. The current study was able to demonstrate that fecal 11,17-DOA, an established indicator of stress, increased following dry-off. A similar increase in blood cortisol concentration after dry-off was reported previously (Odensten et al., 2007). In their study, however, stress levels were evaluated over a 4-wk period before and after last milking, including a 5-d dry-off regimen with prolonged milking intervals combined with a feed change (i.e., reduction in energy density) before the last milking. Regardless of the type of dry-off, both studies were able to demonstrate an effect of milk yield on stress levels after dry-off. In agreement with the results of the present study Odensten et al. (2007) showed an increase in blood cortisol concentration in high- (17.8–29.5 kg/d) and medium- (11.5–17.7 kg/d) yielding cows after dry-off. In contrast to the current study, however, no effect was evident in low-yielding (5.0–11.4 kg/d) cows. The latter might be due to different thresholds for the classification of the 3 yield classes. In our study, the threshold between low- and medium-yielding cows was 15 kg/d, whereas in the study cited above, the threshold between low and medium milk yield was set at 11.4 kg/d. Consequently, 10 out of 26 cows (i.e., 38.5%) classified in the low-yield group in our study would have been in the medium-yield group defined by Odensten et al. (2007).

The increases (11,17-DOA_{rel} values) between baseline 11,17-DOA concentrations and values measured after dry-off differed considerably between yield groups. Whereas high-yielding cows had the lowest 11,17-DOA concentrations before and the highest increase after dry-off, low-yielding cows had the highest baseline and only a slight increase. The measurement of stress hormones to estimate discomfort and pain is an established method (Anil et al., 2002; Rutherford, 2002). The significant increase of 11,17-DOA in high-yielding cows might indicate discomfort due to high udder pressure.

This assumption is substantiated by the similarity of udder pressure and 11,17-DOA profiles (Figure 5).

Both parameters peaked within a few days after dry-off and decreased subsequently. Levels of both pressure and 11,17-DOA were elevated until the end of the study period in medium- and high-yielding cows. As reported earlier for fecal 11,17-DOA determinations (Palme et al., 2000; Morrow et al., 2002; Palme, 2005), a time lag of 8 to 16 h between stress exposure and elevated 11,17-DOA concentrations existed. The highest 11,17-DOA concentrations on d 3_F indicate that the stress was most intense on d 2, on which the udder pressure peaked as well. Low-yielding cows with low pressure experienced elevated 11,17-DOA levels only on d 3_F after dry-off. A relationship between high intramammary pressures after dry-off and an increase in stress hormones has been assumed previously (Odensten et al., 2007). Our results confirm this hypothesis, although the correlation between pressure and 11,17-DOA was low ($r = 0.158$).

As our study was conducted on a commercial dairy farm, drying-off was accompanied by a group and ration change, which are common management practices in modern dairy farms (Bushe and Oliver, 1987; Dingwell et al., 2001; Tucker et al., 2009). These changes, however, might have influenced the 11,17-DOA concentrations. The concentration of 11,17-DOA increased in all yield groups after dry-off, but the increase was greatest in high-yielding cows. This difference cannot be explained by a group or ration change, because all cows were exposed to identical management procedures and had to adjust to the same changes irrespective of their yield group. Several studies evaluated the effect of regrouping on dairy cows (von Keyserlingk et al., 2008; Schirmann et al., 2011). Schirmann et al. (2011) showed that an effect of regrouping on the feeding, social, rumination, and lying behavior of dairy cows lasted only for 1 d after regrouping. In our study, however, stress levels peaked only 2 d after dry-off and remained elevated for at least 9 d in medium- and high-yielding cows, indicating that other factors than a group or ration change, presumably elevated udder pressure, were prevalent. Nevertheless, an effect of regrouping could neither be validated nor rejected. Especially in low-yielding cows, the group and ration change might have contributed to the increase in 11,17-DOA concentration.

In addition to indicating stress, elevated 11,17-DOA concentrations before calving can be a predictor for adverse events (Huzzey et al., 2011). Those authors described a relationship between elevated 11,17-DOA concentrations 3 to 2 wk before calving and the probability of culling within the first 30 DIM. A similar association could be demonstrated in our study. Cows culled within the first 21 d after calving had elevated 11,17-DOA concentrations before and after dry-off. Due to the long interval between elevated 11,17-DOA

concentration and event, this relationship should be interpreted carefully and further research is warranted to substantiate these findings. Several studies established that low milk yield in the previous lactation influenced treatment decisions and increased the risk of culling (Gröhn et al., 1998; Weigel et al. 2003; Norman et al., 2007). Considering the relationship between high 11,17-DOA baseline concentrations and low milk yield, it could be speculated that cows were not culled due to high 11,17-DOA but due to their low milk yield.

CONCLUSIONS

The results of the current study indicate that a reevaluation of the well-established dry-off procedures in dairy cows is warranted by demonstrating a relationship between a sudden dry-off and an increase in udder pressure and fecal stress hormones. High-yielding cows showed higher udder pressure and a greater increase in their stress levels after dry-off. The effect of a sudden dry-off on low-yielding cows was negligible. Further research should focus on long-term effects on stress and metabolism, particularly in high-yielding cows and subsequently assess animal health and performance parameters. Considering a reevaluation of current dry-off strategies, especially a reduction of milk yield before dry-off should be researched (e.g., by applying different dry-off strategies such as gradual feed restriction and cessation of milking).

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