Relationship of concentrations of cortisol in hair with health, biomarkers in blood, and reproductive status in dairy cows

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ABSTRACT

Hair cortisol has been used to measure chronic stress in dairy cows as it offers the advantage of being noninvasive, fast, and able to indicate levels of cortisol over long periods. The aim of this study was to determine the associations between hair cortisol with clinical disorders, reproductive status, and the development of subclinical endometritis in dairy cows. Furthermore, we aimed to determine the association between hair cortisol concentrations and blood markers associated with metabolic status and acute inflammation. In experiment 1, cows ($n = 64$) were hair sampled every 3 wk from the tail switch beginning at calving (d 0) until d 126 for cortisol analysis; blood samples were collected every 3 wk from d 0 until 42 for β -hydroxybutyrate and glucose analysis. In experiment 2, cows ($n = 54$) were chosen retrospectively by diagnosis of subclinical endometritis (END), subclinical endometritis and at least 1 clinical disease (END+CLIN), or as healthy (control) using a cytobrush and ultrasonography at 30 ± 3 d in milk. At the same time, animals were hair sampled for cortisol analysis and blood sampled for haptoglobin and ceruloplasmin analysis. Health records were recorded throughout both experimental periods. Animals with clinical disease presented higher cortisol concentrations than clinically healthy animals in experiment 1 [geometric mean (95% confidence interval); 8.8 (7.8, 9.9) vs. 10.7 (9.6, 12.0) pg/mg]; however, animals diagnosed with subclinical endometritis in experiment 2 did not differ in hair cortisol concentrations [11.7 (9.8, 14.0), 12.2 (9.3, 15.9), 10.5 (8.1, 13.6) pg/mg for control, END, and END+CLIN, respectively]. In experiment 1, an effect of sample day was noted, where d 21 had higher cortisol concentrations than d 42, 84, and 126, but not from d 0 for both parities. Within both experiments, a parity effect was present where

multiparous animals consistently had higher cortisol concentrations than primiparous animals. Multiparous cows that became pregnant by 100 d postpartum had lower concentrations of hair cortisol at d 42 and 84 in milk. Lastly, other biomarkers associated with metabolic status and acute inflammation, such as glucose, β -hydroxybutyrate, haptoglobin, and ceruloplasmin, were not strongly correlated with measurements of cortisol in hair. Overall, hair cortisol measurements appear to be associated with clinical disorders and have a direct association with pregnancy status; however, concentrations of hair cortisol may not be suited to differentiate situations of stress with lower magnitudes, such as the development of subclinical disease.

Key words: chronic stress, disease, hair cortisol, dairy cattle

INTRODUCTION

Chronic stress, characterized as a sustained stress response or the excessive secretion of stress mediators, has been shown to impair the immune response and reproductive function in animals (Matteri et al., 2000; Chrousos, 2009). In cattle, several reports have shown greater concentrations of cortisol in plasma in different scenarios involving clinical diseases and inflammatory responses. Comin et al. (2013) reported greater concentrations of cortisol in hair from clinically compromised (e.g., laminitis, metritis, or mastitis) cows compared with those with an absence of disease. Additionally, Lavon et al. (2008) observed that animals administered *Escherichia coli* endotoxin showed an increase in cortisol in plasma, suggesting that an inflammatory response induces increases in cortisol. Some researchers have reported that an inflammatory infection caused by *E. coli* endotoxins administered into the uterine lumen has negative effects on reproduction, which suggests this may be due to the release of cortisol as a result of the inflammation (Lopez-Diaz and Bosu, 1992; Huszenicza et al., 2004). However, it is unclear if subclinical states of common disorders in dairy cows,

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such as subclinical endometritis and mastitis, trigger the same corticotropin-releasing hormone responses from the hypothalamus or if these responses can be measured using hair samples. In addition, increased concentrations of cortisol are known to cause immunosuppressive effects in dairy cattle and swine (Hopster et al., 1998; Tuchscherer et al., 2004) and are linked to decreases in antibody concentrations and reductions in phagocytic functions in monocytes and macrophages (Roth, 1985).

Chronic and acute stress can trigger disruptions in reproductive function. Chronic lameness in cattle, for example, is linked to disruptions in the pulsatile pattern of GnRH and consequent impairment of estrous behavior expression (Walker et al., 2008). Acute stress, characterized as short-term and normally measured through the blood, saliva, and feces, has detrimental effects on ovarian cyclicity by disrupting the normal release of hormones from the hypothalamic-pituitary-gonadal axis that controls reproduction (Dobson et al., 2003; Smith et al., 2003). Stressors, such as isolation, transport, and restraint (acute causes), have been found to interfere with the endocrine events preceding ovulation and, thus, result in ovulation failure (Moberg, 2000).

Most of the literature on dairy cattle concerning cortisol measurements has been done in plasma (Forslund et al., 2010), saliva (Negrão et al., 2004), or fecal samples (Huzzey et al., 2011). Alternatively, the measurement of cortisol in hair has recently received attention and has the advantage of being a noninvasive procedure (Comin et al., 2013; Burnett et al., 2014); however, no research has demonstrated the dynamic changes on how disease events (clinical and subclinical) and reproductive status are associated with concentrations of cortisol in hair over time. In addition, the relationship between cortisol in hair, energy metabolites, and acute-phase proteins is currently unclear and can provide valuable information about the validity of hair cortisol tests.

The aim of our study was to determine the associations between hair cortisol with clinical disorders, reproductive status, and the development of subclinical endometritis in dairy cows. Furthermore, we aimed to determine the association between cortisol in hair with energy metabolites in blood (glucose and BHBA) and acute-phase proteins in plasma (ceruloplasmin and haptoglobin). It was hypothesized that (a) the concentration of cortisol found in the hair of dairy cows has a negative relationship with the health and reproductive status of dairy cows, and (b) a positive association exists between concentrations of cortisol in hair and concentrations of BHBA, glucose, ceruloplasmin, and haptoglobin in blood during the transition period.

MATERIALS AND METHODS

This experiment was conducted between June 2011 and March 2012 at the University of British Columbia's Dairy Education and Research Centre (Agassiz, BC, Canada). All procedures were approved by the Animal Care Committee of the University of British Columbia. Animals used in this experiment were cared for as outlined by the guidelines provided by the Canadian Council for Animal Care (2009).

Animals and Housing

A total of 118 high-producing Holstein dairy cows were used in this study, which consisted of 2 experiments (experiment 1: $n = 64$; experiment 2: $n = 54$). Cows produced (mean $\pm$ SD) $12,236 \pm 2,219$ kg of milk (305-d mature-equivalent yield) and with BCS ranging from 1.75 to 3.25 at 30 ± 3 DIM. All animals were housed in the same naturally ventilated wooden-framed barn with a freestall design, equipped with deep sand-bedded stalls. Animals were milked twice daily at 0500 and 1500 h with automatic milking machines. Fresh TMR was delivered twice daily at approximately 0700 and 1600 h. The TMR was formulated following the NRC guidelines (NRC, 2001) to meet or exceed the requirements of a 620-kg Holstein cow producing 40 kg/d of 3.5% FCM; the animals had ad libitum access to both TMR and water.

During both experimental periods, various potential forms of stressors were recorded, including retained placenta, clinical hypocalcaemia, displaced abomasum, bouts of clinical mastitis and metritis, surgical procedures, milk yield (305-d mature-equivalent yield), and SCC. All animals were body condition scored at 30 ± 3 DIM on a 5-point scale from thin (1) to obese (5) as outlined by Edmonson et al. (1989). Animals were later classified as thin (BCS <2.75), average (BCS = 2.75), or moderate (BCS >2.75). All health and production information was collected by the dairy herd personnel with the assistance of the herd veterinarian, and confirmed and recorded by the project leader using the on-farm Dairy Comp 305 software (Valley Agricultural Software, Tulare, CA). Animals were considered healthy if they had an absence of clinical disease during the entire experimental period, or as clinically diseased if they were diagnosed with 1 or more clinical disease event.

Clinical mastitis was diagnosed by inspection for udder hardness, heat, and clots or discolored milk and with the use of the California Mastitis Test. Retained placenta was defined as the failure to expel the placenta within 24 h postpartum (LeBlanc, 2008). Clinical

metritis was defined as the presence of fetid watery red-brown uterine discharge, systemic signs of illness and a fever $>39.5^{\circ}\text{C}$ (Sheldon et al., 2006). Clinical milk fever was defined as a recumbent cow postpartum exhibiting anorexia and staggering, which responded appropriately to intravenously administered calcium. Animals were classified with clinical ketosis by having BHBA $\geq 1,400 \mu\text{mol/L}$ at wk 2 postpartum or later with signs of systemic illness (Walsh et al., 2007). Chronic lameness was defined as cows with obvious pain when walking and clinical symptoms of general distress (Hernandez et al., 2005). Individual animals were tested for SCC approximately every 35 ± 5 d by CanWest DHI (Guelph, ON, Canada); all milk samples were within 20 d of the sample day and were used to assess the mammary status of the animal.

Experiment 1

Sixty-four Holstein dairy cows were enrolled in experiment 1, including 20 primiparous and 44 multiparous cows. Each cow had hair sampled from the tail switch on the day of calving, and were resampled every 3 wk (21 ± 3 DIM) until 126 ± 3 DIM; however, only samples on 0, 21, 42, 84, and 126 DIM were subsequently analyzed for cortisol. Blood samples were collected at 0, 21, and 42 DIM and analyzed for glucose and BHBA. A visual schematic of the sampling schedule can be found in Figure 1. Analyses for glucose and BHBA were used to investigate possible correlations of these biomarkers and negative energy balance during the transition period with hair cortisol samples. Cows were submitted to the regular reproductive program of the dairy that consisted of a presynchronization with the administration of 2 injections of PGF_{2 α} 14 d apart, at 30 ± 3 and 44 ± 3 DIM, and AI performed upon spontaneous estrus from 50 until 100 DIM; if estrus was not detected by 100 DIM, cows were submitted to the Ovsynch protocol (Pursley et al., 1997). At the time of each presynchronization injection, animals were examined by per rectum palpation using ultrasonography for cyclicity, diameter of the inner layer of the cervix, asymmetry of the diameter of the endometrium between both uterine horns, and additionally for pregnancy diagnosis at 40 ± 7 d after AI; a more detailed description is included herein. Animals were classified as being cyclic if they had a presence of at least 1 corpus luteum at the time of either presynchronization injection. Estrous detection was carried out using an automated activity monitor (Heatime; SCR Engineering Ltd., Netanya, Israel). Data on the reproductive status, episodes of health disorders, and SCC test results were recorded for each animal. Milk test results for SSC were matched with

corresponding hair samples by DIM. Sampling methodologies are detailed below.

Experiment 2

A different set of 54 Holstein dairy cows was used for experiment 2, consisting of 24 primiparous and 30 multiparous cows. Animals were chosen retrospectively by parity and divided into 3 groups according to their health status: diagnosed with subclinical endometritis and an absence of clinical disease (**END**), subclinical endometritis and at least 1 clinical disease (**END+CLIN**), or healthy with an absence of subclinical endometritis and clinical disease (control). All animals were enrolled at 30 ± 3 DIM. Samples of hair and blood were collected and a uterine health diagnosis was obtained from each cow using a cytobrush and ultrasonography (Figure 1). Subclinical endometritis was positively diagnosed when the proportion of neutrophils on the uterine smear was $>18\%$ in relation to the total number of cells (Kasimanickam et al., 2004); all animals in the END and END+CLIN groups were also diagnosed with some uterine luminal fluid (echogenic or nonechogenic fluid) by the ultrasound examination. Uterine smears with $\leq 18\%$ of neutrophils and absence of echogenic fluid in the lumen of the uterus were classified as healthy and placed in the control group; all animals in the control group had an absence of clinical disease. Blood samples were analyzed for acute-phase proteins, haptoglobin, and ceruloplasmin and hair was analyzed for cortisol. The SSC milk test closest to 30 ± 3 DIM postpartum was used to account for the occurrence of any subclinical states of mastitis early in lactation. Sampling methodologies are detailed herein.

Blood Sampling and Analysis

All blood samples were drawn by puncture of the median coccygeal vein or artery utilizing untreated Vacutainer tubes (Becton Dickinson Vacutainer Systems, Rutherford, NJ) for experiment 1 and K2 EDTA Vacutainer tubes for experiment 2. For experiment 1, whole blood was immediately analyzed for glucose (Precision Xtra blood glucose kit; Abbott Diabetes Care, Alameda, CA) and BHBA (Precision Xtra blood ketone kit; Abbott Diabetes Care) concentrations using the procedures described by Iwersen et al. (2009). For experiment 2, analyses for haptoglobin and ceruloplasmin were performed using the commercially available PHASE Haptoglobin kit (Tridelta Development Ltd., TP801, Maynooth, Ireland) and using a method outlined by Demetriou et al. (1974) determined by the rate of *p*-phenylenediamine oxidation, respectively.

Figure 1. Schematic of the experimental and sampling design of experiment 1 and 2. Hair samples were collected every 21 d, but were only analyzed for days indicated with □ for experiment 1. Hair was only sampled and analyzed on d 30 for experiment 2. Blood and cytology samples were collected and analyzed for the days indicated with ◆ and ★, respectively. Calving is indicated on the schematic as d 0 for both experiments.

Hair Sampling and Analysis

All hair samples collected for our study were harvested from the tail switch, as described by Burnett et al. (2014). For the first hair collection in either experiment, the hair was cut as close to the skin as possible using surgical scissors and the 3 cm closest to the skin was kept for analysis. For experiment 1, all subsequent hair samples were harvested from the same region and the total regrown hair was collected and used for analysis; only samples at 0, 21, 42, 84, and 126 DIM were analyzed. For experiment 2, only 1 sample was collected at 30 ± 3 DIM. Once the hair was collected, it was stored at room temperature in dry, nontransparent envelopes in a dark room until analysis. Hair samples were cleaned, dried, and ground according to

the procedures outlined in Burnett et al. (2014). Grinding was performed on 250 mg of sample in a 10-mL stainless steel milling cup with a 12-mm stainless steel ball in a Retsch Mixer Mill MM400 ball mill (Retsch, Haan, Germany) for 5 min at a frequency of 30 reps/s. Ground hair samples were stored in the dark in glass jars at room temperature.

Extraction of hair cortisol was performed using methanol following the procedures of Burnett et al. (2014), which were adapted from Koren et al. (2002). Hair cortisol concentrations were determined using a commercially available assay kit designed for salivary cortisol (Salimetrics Expanded Range, High Sensitivity 1-E3002, State College, PA), as previously performed by Davenport et al. (2006) and Bennett and Hayssen (2010). The sensitivity of the cortisol kit was $0.01 \mu\text{g/}$

dL. Samples were analyzed for cortisol content following the instructions supplied by the manufacturer. Duplicate 25- μ L samples were aliquoted into wells, and absorbance of the samples was read at 450 nm using a microplate plate reader (BioRad xMark, Hercules, CA). Results were calculated using the microplate reader software using a 4 parameter logistic curve fit. Results were converted from microgram to deciliter to picogram to milligram. The average intra-assay CV between samples on the plates was 3.51%.

Uterine Cytology and Ultrasonography

Uterine health monitoring was performed by method of uterine cytology and ultrasound. Cytological examination of the endometrium was performed by way of a cytobrush (CooperSurgical Inc., Trumbull, CT). The vulva was cleaned of any debris and the cytobrush, threaded onto a steel rod and covered with an outer steel tube, was inserted into the vagina and through the cervix. With contact of the uterine wall, the cytobrush was extended from inside the steel tube and immediately rotated to collect a sample. The cytobrush was then retracted inside the steel tube before removal from the uterus as to not contaminate the sample. Once retracted, the cytobrush was rolled onto a glass microscope slide to make an endometrial smear. Smears were air-dried and stained using a Romanowsky stain (Diff-Quick, Fisher Diagnostics, Middletown, VA). The slides were examined to determine the percent neutrophils. To do so, 3 sections of 100 cells each were counted from each slide, and the total number of neutrophils and epithelial endometrial cells were tallied. Subclinical endometritis was defined when the proportion of neutrophils was $>18\%$ in relation to the total number of the cells (Kasimanickam et al., 2004; Sheldon et al., 2006).

After the uterine sample collection, ultrasonography was performed on the uterus via rectal palpation using an ultrasonographic machine (Aloka SSD-500, Aloka Co Ltd., Wallingford, CT) equipped with a 7.5-MHz linear transducer. The following uterine health measures that have previously been demonstrated to be associated with subclinical endometritis (Cerri et al., 2012) were measured: diameter of the inner layer of the cervix (small < 18 mm $\leq$ large), asymmetry of the diameter of the endometrium between both uterine horns (moderate < 3 mm $\leq$ high), and lumen content (0 = no fluid, 1 = clear fluid, 2 = fluid with intermediary echogenicity, and 3 = fluid highly echogenic). In addition, cyclicity was assessed by ultrasonography of each ovary by recording the presence of at least 1 corpus luteum at either 30 ± 3 or 44 ± 3 DIM.

Statistical Analyses

All analyses for this experiment were performed using SAS (version 9.4; SAS Institute Inc., Cary, NC) with cow as the experimental unit. Prior to all analyses data were checked for normality using the UNIVARIATE procedure and probability distribution plots; any variables deemed not normal were transformed to fit normality and subsequently back-transformed for geometric means. The sample size was calculated using a single mean-sampling test (MedCalc 11, MedCalc Software bvba, Ostend, Belgium). The power analysis was based on a value of $\alpha = 0.05$ and $\beta = 0.20$. A conservative estimate was based on a previous study (Burnett et al., 2014) and pilot data from our laboratory. A mean value of 10 pg/mg with a null hypothesis value of 8.5 pg/mg and a standard deviation of 3 pg/mg yielded a minimum sample size of 18.

For experiment 1, hair cortisol and BHBA and glucose concentrations in whole blood were used as dependent variables. Parity, BCS, milk production, SCC, cervical diameter, horn asymmetry, pregnancy status, and disease incidence during the experimental period were evaluated. For experiment 2, hair cortisol, ceruloplasmin, and haptoglobin concentrations were used as dependent variables. The effects of group, parity, BCS, milk production, SCC, cervical diameter, horn asymmetry, and disease incidence before 30 DIM were used as independent variables. Continuous variables were analyzed using the MIXED procedure of SAS. For experiment 1, the model specified experimental day as the repeated measure and cow as the subject; an autoregressive covariance structure was used when more than 2 experimental days were included. Independent variables were first assessed by univariable analysis and eliminated from the final model once found insignificant. Pearson correlations were calculated using the CORR procedure and frequency statistics were calculated using the FREQ procedure.

RESULTS

Experiment 1

The mean hair cortisol concentration for the entire experimental period was 9.8 ± 3.7 pg/mg (mean $\pm$ SD) for $n = 64$ cows. For both primiparous and multiparous cows, DIM had an effect on hair cortisol: d 21 had greater concentrations of cortisol in hair than d 42, 84, and 126 (Figure 2; $P < 0.001$). The same effect was also observed when using only clinically healthy animals in the analysis ($P < 0.001$). Furthermore, when using only clinically healthy animals, parity significantly affected

hair cortisol concentrations, where multiparous animals had greater hair cortisol concentrations than primiparous [geometric mean (95% CI); 9.2 (8.2, 10.4) vs. 7.8 (7.0, 8.6) pg/mg; $P = 0.03$]. However, hair cortisol at d 0 (calving) did not differ between multiparous and primiparous cows ($P = 0.78$) or by the development of clinical disease within the experimental period ($P = 0.54$); as it is suggested that hair requires approximately 2 wk to grow from the follicle to the skin surface (Burnett et al., 2014), d 0 samples represent circulating cortisol concentrations from before calving. Multiparous cows with moderate and average BCS had greater concentrations of cortisol in hair than Thin cows at d 0 [12.0 (10.2, 14.1), 11.0 (9.1, 13.3), 8.7 (7.4, 10.3) pg/mg for moderate, average, and thin cows, respectively; $P = 0.03$]; BCS did not affect hair cortisol concentrations of primiparous cows at d 0 ($P = 0.66$). Somatic cell count did not have an effect on hair cortisol concentration for either parity ($P = 0.44$).

For multiparous cows, clinical disease during the experimental period had a significant effect on cortisol concentrations in hair. Clinically affected multiparous cows had greater cortisol concentrations in hair than those with an absence of disease, specifically animals that were afflicted with more than 1 disease event (Figure 3; $P = 0.02$). The frequency of disease events within the experimental period affected hair cortisol concentrations, as multiparous cows with 2 or more disease events had elevated hair cortisol concentrations in comparison to healthy cows (Figure 3; $P = 0.03$); from this trial we were unable to determine if clinical disease had an effect on primiparous cows due to the low incidence of disease in these animals (5.0%; 1/20). Furthermore, animals with clinical disease tended to

have higher hair cortisol at d 21 ($P = 0.05$), 84 ($P = 0.05$), and 126 ($P = 0.07$) DIM (Figure 4). Throughout the experimental period, 39.1% (24/64) of the overall animals were diagnosed with at least 1 clinical disease. The following types of clinical diseases were diagnosed during the experimental period: mastitis ($n = 19$), clinical metritis ($n = 7$), displaced abomasum ($n = 3$), retained placenta ($n = 4$), milk fever ($n = 5$), clinical ketosis ($n = 1$), and chronic lameness ($n = 2$). These clinical diseases were distributed among animals as follows: 13 animals were diagnosed with multiple clinical diseases, 7 were diagnosed with only mastitis (1 or more bouts per animal), and 5 were diagnosed with only 1 clinical disease other than mastitis.

Within the transition period, whole-blood concentrations of glucose had a positive effect on cortisol concentrations in hair for multiparous cows [$m = 0.11$ natural log (Ln) cortisol/glucose, m = slope; $P = 0.03$]; a tendency for a negative relationship between hair

Figure 2. Effect of DIM on concentrations of cortisol (geometric mean $\pm$ SEM) extracted from hair samples collected at 0, 21, 42, 84, and 126 DIM from all cows in experiment 1. Days in milk significantly affected concentrations of hair cortisol ($P < 0.001$); hair samples collected at 21 DIM had greater concentrations of cortisol compared with postpartum collections but were not different from collection at calving (0 DIM).

Figure 3. Effect of disease on concentrations of cortisol (geometric mean $\pm$ SEM) extracted from hair samples from multiparous animals in experiment 1. Disease significantly increased concentrations of hair cortisol (panel A; $P = 0.02$). Animals with more than 1 disease bout were found to have more hair cortisol than healthy animals, but not from animals with only 1 disease bout (panel B; $P = 0.03$). Different letters (a,b) indicate significant differences ($P < 0.05$).

Figure 4. Hair cortisol concentrations (geometric mean $\pm$ SEM) of multiparous cows that were diagnosed as clinically diseased or clinically healthy by 126 DIM. Healthy animals had less hair cortisol at 21 (* $P = 0.05$), 84 (* $P = 0.05$), and 126 ($\dagger P = 0.07$) DIM than animals that were diagnosed with clinical disease within the experimental period in experiment 1.

cortisol and BHBA was noted ($m = -0.12 \text{ Ln cortisol/Ln BHBA}$; $P = 0.11$). For primiparous cows, glucose concentration had a positive relationship with cortisol concentrations in hair ($m = 0.09 \text{ Ln cortisol/glucose}$; $P = 0.04$); BHBA did not have an effect on hair cortisol concentrations in primiparous cows ($P = 0.18$). Correlations between hair cortisol, BHBA, and glucose at 21 DIM are shown in Figure 5. Although hair cortisol and blood glucose tended to be correlated, only 7% of the variation in hair cortisol was explained by blood glucose ($R^2 = 0.07$; $P = 0.07$).

Glucose concentrations of multiparous cows were affected by disease, where clinically diseased animals had higher concentrations than healthy animals [8.8 (7.8, 10.0) vs. 10.7 (9.4, 12.1) mg/dL]. The BHBA concentrations did not differ between clinically diseased or healthy cows. Neither cervix diameter ($P = 0.86$) nor asymmetry of uterine horns ($P = 0.20$) affected hair cortisol concentrations found within multiparous cows. However, primiparous cows with a cervix diameter classified as large were found to have greater hair cortisol concentrations within the transition period than those classified as small [9.3 (8.1, 10.6) vs. 7.5 (6.7, 8.4) pg/mg for large and small cervix diameter, respectively; $P = 0.02$).

Hair cortisol concentrations of multiparous cows that were pregnant at 100 DIM were lower at d 42 ($P = 0.04$) and 84 ($P = 0.03$), but not at d 0, 21, or 126 (Figure 6). These effects were not observed in primiparous cows that were pregnant at 100 DIM at any of the sampling periods analyzed (Figure 6). The proportion of multiparous cows that were clinically diseased or healthy was different between cows that were diagnosed

pregnant or nonpregnant at 100 DIM, where 20.0% of pregnant and 71.4% of nonpregnant multiparous cows were diagnosed with clinical disease ($P < 0.01$). No difference was observed in the proportion of pregnant and nonpregnant primiparous cows that were diagnosed with clinical disease (0.0 and 10.0% for pregnant and nonpregnant cows, respectively; $P = 0.33$). At the end of the presynchronization, 75.0% of all cows were classified as being cyclic (55.0% of primiparous and 84.1% of multiparous). Hair cortisol concentrations were not different for animals that were cyclic compared with those that did not cycle for either parity. There was no difference in pregnancy status at 100 DIM between primiparous and multiparous cows, where 47.4% of primiparous and 34.9% of multiparous cows were diagnosed as pregnant by 100 DIM ($P = 0.35$).

Experiment 2

The mean cortisol concentration was 12.6 ± 5.6 pg/mg (mean $\pm$ SD) for $n = 54$ cows. No effect of group was found for either parity (Figure 7; $P = 0.76$). As hair samples represent circulating cortisol concentrations from approximately 2 wk prior, we can conclude that animals which developed subclinical endometritis at 30 ± 3 DIM did not have a difference in hair cortisol concentration before diagnosis. Comparative statistics by group have been summarized in Table 1. When using only animals with an absence of clinical disease an effect of parity was seen, where multiparous cows had greater concentrations of cortisol in hair than primiparous [12.9 (10.4, 15.9) vs. 9.0 (7.4, 10.8) pg/mg; $P = 0.01$]. A disproportionate number of animals

Figure 5. Scatter plots representing Pearson correlations between hair cortisol and blood BHBA ($r = 0.10$; $P = 0.46$), hair cortisol and blood glucose ($r = 0.26$; $P = 0.07$), and glucose and BHBA ($r = -0.26$; $P = 0.07$) concentrations at 21 DIM in experiment 1.

were clinically ill by 30 DIM between parity; 12.5% of the primiparous animals were diagnosed with disease compared with 36.7% of multiparous animals ($P = 0.04$). For multiparous cows, an effect of BCS on concentrations of cortisol in hair was found, with moderate having greater cortisol than both average and thin [21.9 (14.6, 32.8), 11.7 (9.3, 14.6), and 12.9 (10.3, 16.1) pg/mg of cortisol in hair for moderate, average, and thin BCS, respectively; $P = 0.03$]. No effects of BCS were found for primiparous cows ($P = 0.56$). Additionally, milk yield and hair cortisol tended to have a negative relationship ($P = 0.02$), where greater milk yield was associated with lower hair cortisol. Average SCC from the first 2 milk tests postpartum did not have an effect on hair cortisol concentrations ($P = 0.73$). Group affected milk yield, where animals with an absence

Figure 6. Hair cortisol concentrations (geometric mean $\pm$ SEM) of multiparous (panel A) and primiparous cows (panel B) that were diagnosed nonpregnant or pregnant by 100 DIM. Within each panel, the asterisk (*) denotes difference between hair cortisol concentrations ($P < 0.05$).

of clinical disease produced more milk than clinically diseased cows [12,821 (11,915, 13,727), 12,753 (11,284, 14,223), 9,967 (8,812, 11,123) kg of milk for control, END, and END+CLIN groups respectively; $P = 0.001$]. Cyclicity, cervix diameter, and asymmetry of uterine horns had no effect on hair cortisol found within either parity.

A tendency for haptoglobin concentrations to be affected by group was noted, where the control and END groups had lower haptoglobin concentrations than the END+CLIN group (Table 1; $P = 0.07$). Ceruloplasmin concentrations were not affected by group (Table 1; $P = 0.27$). However, there was a tendency for BCS to affect ceruloplasmin concentrations. Animals with a moderate BCS had greater ceruloplasmin concentrations than both average and thin animals [11.09 (8.84, 13.34), 8.79 (7.47, 10.11), and 7.90 (6.30, 9.50) mg/dL for moderate, average, and thin BCS, respectively; P

Table 1. Comparative statistics [geometric mean (95% CI)] for the control, subclinical endometritis (END), and END plus at least 1 clinical disease (END+CLIN) groups of experiment 2¹

Item	Control	END	END+CLIN	P-value
Neutrophils (%)	4.2 (2.4, 6.5) ^a	37.9 (29.9, 48.1) ^b	73.1 (61.8, 85.3) ^c	<0.01
Milk production (kg)	12,821 (11,915, 13,727) ^a	12,753 (11,284, 14,223) ^a	9,967 (8,812, 11,123) ^b	<0.01
SCC (1,000 cells/mL)	65 (40, 106) ^a	70 (33, 151) ^a	63 (31, 128) ^a	0.98
Cortisol (pg/mg)	11.7 (9.8, 14.0) ^a	12.2 (9.3, 15.9) ^a	10.5 (8.1, 13.6) ^a	0.76
Haptoglobin (mg/mL)	0.62 (0.54, 0.71) ^a	0.64 (0.53, 0.76) ^a	0.80 (0.68, 0.92) ^b	0.07
Ceruloplasmin (mg/dL)	8.70 (7.32, 10.09) ^a	8.63 (6.50, 10.75) ^a	10.45 (8.78, 12.12) ^a	0.27

^{a-c}Geometric means with different superscripts differ within the row.

¹Milk production is represented as the 305-d mature-equivalent yield, concentrations of cortisol were measured from hair samples, whereas concentrations of haptoglobin and ceruloplasmin were measured in plasma samples.

= 0.07). Interestingly, if group is replaced by percent neutrophils in the model, both haptoglobin ($P = 0.02$) and ceruloplasmin ($P = 0.02$) are positively affected by percent neutrophils; however, hair cortisol concentration is not affected by percent neutrophils ($P = 0.41$).

No correlation was found between concentrations of cortisol in hair and haptoglobin or ceruloplasmin concentration in plasma (Table 2). A strong correlation between haptoglobin and ceruloplasmin concentrations was found ($P < 0.001$; Table 2). All correlations found between hair cortisol, plasma haptoglobin, plasma ceruloplasmin, neutrophils, milk yield, SCC, and cervix diameter are summarized in Table 2.

DISCUSSION

The aim of our study was to investigate the association of concentrations of cortisol in hair with known stressors (clinical and subclinical disease), pregnancy status, and with concentrations of other metabolic and inflammatory markers in blood (BHBA, glucose, ceruloplasmin, and haptoglobin). In addition, other

variables, such as DIM, milk yield, parity, BCS, cervix diameter, and asymmetry of the uterine horns, were evaluated. As initially hypothesized, cortisol concentrations in hair can be used to detect significant differences when comparing healthy with clinically diseased lactating cows and can demonstrate that multiparous cows pregnant by 100 DIM have lower levels of cortisol in hair at 42 and 84 DIM compared with nonpregnant ones. However, we were unable to find any associations between cortisol in hair and some important biomarkers found in blood. Furthermore, when the development of a subclinical form of disease that is common to dairy cows (endometritis) was evaluated, the concentrations of cortisol in hair were unaffected.

Hair cortisol has been described in the literature as a chronic stress marker in dairy cattle (Comin et al., 2013). The choice for using cortisol in hair, a relatively new biomarker in humans and domestic species (Meyer and Novak, 2012), as the target to measure chronic hypothalamic-pituitary-adrenal (HPA) axis activation relies in the sampling advantages associated with the long-term interpretation of the results. However, the literature on dairy cows lacks information on the validity of this test in relation to common stressors and factors (e.g., environmental, clinical and subclinical disorders, pregnancy status, and so on) and its correlation with other important biomarkers. The results from our study demonstrate that clinical disease early in lactation significantly altered the concentrations of cortisol in dairy cattle hair; however, experiment 2 lacked a difference in hair cortisol concentrations between animals that developed subclinical endometritis and those that did not. Our results are consistent with those of Comin et al. (2013), who reported that animals that were clinically compromised with mastitis, lameness, clinical metritis, or multiple illnesses had greater hair cortisol concentrations than clinically healthy animals.

Much of the literature relating cortisol to health and reproduction has used plasma as the main source of tissue for a biomarker of stress. Our findings are further supported by studies demonstrating increased plasma

Figure 7. Effect of group on concentrations of cortisol (geometric mean $\pm$ SEM) extracted from hair samples in experiment 2. Subclinical endometritis did not significantly increase concentrations of hair cortisol ($P = 0.76$).

Table 2. Pearson correlation coefficients (*P*-values in parentheses) found between hair cortisol, plasma haptoglobin, plasma ceruloplasmin, percent neutrophils,¹ milk yield,² SCC, and cervix endometrium diameter in Holstein dairy cows (experiment 2)

Item	Cortisol (pg/mg)	Haptoglobin (mg/mL)	Ceruloplasmin (mg/dL)	Neutrophils (%)	Milk yield (kg)	Cervix diameter (mm)	SCC (cells/mL)
Cortisol (pg/mg)	1.00	−0.13 (0.36)					
Haptoglobin (mg/mL)	−0.13 (0.36)	1.00					
Ceruloplasmin (mg/dL)	0.04 (0.78)	0.71 (<0.01)	1.00				
Neutrophils (%)	0.06 (0.66)	0.42 (<0.01)	0.33 (0.02)	1.00			
Milk yield (kg)	−0.27 (0.04)	−0.40 (<0.01)	−0.30 (0.03)	−0.35 (0.01)	1.00		
Cervix diameter (mm)	0.03 (0.84)	0.39 (<0.01)	0.24 (0.09)	0.54 (<0.01)	−0.15 (0.27)	1.00	
SCC (cells/mL)	0.005 (0.97)	0.43 (<0.01)	0.39 (<0.01)	0.23 (0.10)	−0.43 (<0.01)	0.36 (<0.01)	1.00

¹Percent neutrophils were measured as the percent neutrophil cells out of total neutrophil and epithelial cells found from a uterine cytology smear.

²Milk yield was measured as the 305-d mature-equivalent yield.

cortisol concentrations in animals with naturally occurring mastitis (Huszenicza et al., 2004), retained placenta (Peter and Bosu, 1987; Kaczmarski et al., 2006), hypocalcaemia (Horst and Jorgensen, 1982), and parturient paresis (Horst and Jorgensen, 1982; Forslund et al., 2010), but not in animals with puerperal metritis (Kaczmarski et al., 2006). On the contrary, Forslund et al. (2010) reported no evidence to support increased serum cortisol concentration as a result of clinical metritis, suggesting that specific illnesses may elicit different HPA responses. The use of hair cortisol concentrations as a biomarker for health status appears to be ideal for chronic health conditions found in cows, as it removes the short temporal changes that may affect cortisol concentrations found in plasma analysis (e.g., circadian rhythms, specific time of start and end of the clinical disease, degree of HPA response, and so on).

The diagnosis of subclinical endometritis does not describe the severity of the inflammation over time. Although animals with clinical metritis are more likely to be diagnosed with subclinical endometritis (Rutigliano et al., 2008; Galvão et al., 2010), not every animal diagnosed with subclinical endometritis has had clinical metritis. In experiment 2 we did not find an increase in hair cortisol, plasma haptoglobin, or plasma ceruloplasmin concentration in cows diagnosed with only subclinical endometritis. Furthermore, we found limited evidence that uterine health factors that are correlated with subclinical endometritis (cervix diameter and endometrial asymmetry between both uterine horns) affected hair cortisol concentrations. Although there is disagreement in the literature with respect to HPA axis activation caused by subclinical and clinical uterine infection, some reports suggest that no HPA axis activation exists, with no significant increase in plasma cortisol concentrations in animals with metritis (Forslund et al., 2010; Galvão et al., 2010). Kaczmarski et al. (2006) also reported that animals with pu-

erperal metritis did not have increased plasma cortisol concentrations. The current paper is, to our knowledge, the first publication to demonstrate that concentrations of cortisol in hair are not different between cows with or without the development of subclinical endometritis. Future research is required to determine if hair cortisol concentrations differ at the time of subclinical endometritis diagnosis by determining and accounting for the time delay present due to the time required for the hair segment to grow out from beneath the skin.

In agreement with the current study, Galvão et al. (2010) reported that animals with subclinical endometritis did not have significantly different haptoglobin concentrations compared with healthy animals between parturition and 42 DIM. On the contrary, Huzzey et al. (2009) reported that even animals with mild metritis had significantly higher haptoglobin concentrations than healthy animals; mild metritis was defined as either having a vaginal discharge score of 4 without fever, or a vaginal discharge of 2 or 3 with or without fever. Huzzey et al. (2009) reported that animals with metritis (both mild and severe) had increased serum haptoglobin concentrations until 12 d postpartum.

The detrimental effects of chronic stress on fertility were demonstrated in our study by an increase in days open found in multiparous animals that had higher hair cortisol concentrations at 42 and 84 DIM. The causes for delayed pregnancy may be due to the adverse effects stress and stress mediators have on the HPA axis, specifically on ovulation and embryonic loss; especially as samples at 42 and 84 DIM represent the critical time of first AI. Chronic stress has a negative effect on GnRH, resulting in a postponed LH surge (Dobson and Smith, 2000) and delayed ovulation times (Walker et al., 2008); during chronic stress, the effect can be so severe that the animal may enter anestrus (Dobson and Smith, 2000). Ovulation times are especially important in modern dairy farming systems where the

use of AI is relied upon. Delaying the LH surge also affects embryonic development by depriving the ovarian follicles gonadotropin support, resulting in reduced estradiol production and, thus, decreasing the quality and fertility of oocytes (von Borell et al., 2007).

Furthermore, increased cortisol concentrations found at 42 and 84 DIM may be indicative of increased disease during the transition period. As each sample represents a 3-wk period of hair growth before the sample date, it may be concluded that hair samples from 42 and 84 DIM represent circulating cortisol concentrations from very early in lactation. As animals that were nonpregnant at 100 DIM had a higher prevalence of clinical disease, in addition to increase cortisol concentrations, it may be symptomatic of transition diseases. Transition diseases, such as metritis and retained placenta, have been associated with reduced pregnancy rates and a longer calving to pregnancy interval (Dobson and Eslemont, 2002; LeBlanc, 2010).

The current experiment demonstrated an effect of parity on concentrations of cortisol in hair. In both of our experiments, multiparous animals had greater hair cortisol concentrations than primiparous animals. A relative bias was noted in our analysis, as multiparous cows had greater incidence of clinical disorders [experiment 1: 54.6% (24/44); experiment 2: 36.7% (11/30)] than primiparous cows [experiment 1: 5.0% (1/20); experiment 2: 12.5 (3/24)]; thus, only animals absent of clinical disease were used in the analysis for parity. Using milk cortisol concentrations, Fukasawa et al. (2008) observed no difference in concentrations of cortisol between primiparous and multiparous cows and found that milk samples had greater concentrations of cortisol in early lactation (7–90 DIM) than in mid and late lactation. When compared with other studies that used plasma samples, these results are conflicting. Galvão et al. (2010) reported that primiparous cows tended to have greater cortisol concentrations in plasma, but samples were collected only until 42 DIM. One report suggested that cortisol concentrations in plasma peak at parturition and return to baseline after 10 d postpartum (Forslund et al., 2010), whereas a different study reported increases in cortisol only last from 1 d before parturition until 3 d after (Goff et al., 1989). The different results observed in studies using plasma, milk, and saliva samples as the medium to measure HPA axis activation is another example of how different methodologies and experimental designs between studies play a vital role in the interpretation of results.

In our study, during the transition period, representing 3 wk before and after calving, multiparous animals that were clinically diseased had greater glucose concentrations than healthy multiparous animals, and hair

cortisol concentration had a significant positive relationship with plasma glucose. Galvão et al. (2010) also found a positive association between plasma cortisol and plasma glucose; but, similar to our study, this association only explained 7% of the variation. Previous research has shown that plasma glucose concentration decreases rapidly during the first week postpartum, but begins to recover during the second week postpartum (Vazquez-Añon et al., 1994; Galvão et al., 2010). The samples from our study were collected at 21 and 42 DIM, which could explain the absence of an effect of DIM.

Even though it has been reported that daily milk production and milk cortisol concentration are not well correlated (Fukasawa et al., 2008), in experiment 2 we found that cows with greater lactation milk yields had lower concentrations of cortisol in hair at 30 DIM. The relationship of cortisol with respect to the daily milk production on the sample day might be different from that with the whole lactation yield (Fukasawa et al., 2008). Another study reported that animals with greater milk production have decreased adrenocortisol reactivity, but suggested these differences may be more related to factors contributing to negative energy balance than milk production; overall, in that study, baseline levels of cortisol were similar between high- and low-producing animals (Beerda et al., 2004).

CONCLUSIONS

The measurement of cortisol concentrations in hair can detect significant differences when comparing healthy with clinically diseased lactating cows and can detect a difference in hair cortisol of multiparous cows that are pregnant by 100 DIM. We were unable to find any associations between cortisol in hair and some important markers found in blood. Furthermore, when subclinical endometritis was evaluated, concentrations of hair cortisol were unaffected before diagnosis. Hair cortisol measurements appear to be associated with clinical disorders and have a direct association with pregnancy status; however, concentrations of cortisol in hair may not be suited to differentiate situations with disorders of lower magnitude, such as subclinical endometritis.

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