Production of 15-F_{2t}-isoprostane as an assessment of oxidative stress in dairy cows at different stages of lactation

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ABSTRACT

Oxidative stress contributes to dysfunctional immune responses and predisposes dairy cattle to several metabolic and inflammatory-based diseases. Although the negative effects of oxidative stress on transition cattle are well established, biomarkers that accurately measure oxidative damage to cellular macromolecules are not well defined in veterinary medicine. Measuring 15-F_{2t}-isoprostane, a lipid peroxidation product, is the gold standard biomarker for quantifying oxidative stress in human medicine. The aim of our study was to determine whether changes in 15-F_{2t}-isoprostane concentrations in plasma and milk could accurately reflect changes in oxidant status during different stages of lactation. Using liquid chromatography–tandem mass spectrometry, 15-F_{2t}-isoprostane concentrations were quantified in milk and plasma of 12 multiparous Holstein-Friesian cows that were assigned to 3 different sampling periods, including the periparturient period (1–2 d in milk; *n* = 4), mid lactation (80–84 d in milk; *n* = 4), and late lactation (183–215 d in milk; *n* = 4). Blood samples also were analyzed for indicators of oxidant status, inflammation, and negative energy balance. Our data revealed that 15-F_{2t}-isoprostane concentrations changed at different stages of lactation and coincided with changes in other gauges of oxidant status in both plasma and milk. Interestingly, milk 15-F_{2t}-isoprostane concentrations and other indices of oxidant status did not follow the same trends as plasma values at each stage of lactation. Indeed, during the periparturient period, systemic 15-F_{2t}-isoprostane increased significantly accompanied by an increase in the systemic oxidant status index. Milk 15-F_{2t}-isoprostane was significantly decreased during the periparturient period compared with other lactation stages in conjunction with a milk oxidant status index that trended lower during this period. The results from this study

indicate that changes in 15-F_{2t}-isoprostane concentrations in both milk and plasma may be strong indicators of an alteration in redox status both systemically and within the mammary gland.

Key words: isoprostane, oxylipid, oxidative stress, reactive oxygen species

INTRODUCTION

Drastic shifts in feed consumption and energy demands before and after calving pointedly alter the metabolic environment of the modern dairy cow, setting the stage for predispositions to health disorders during the periparturient period (Sordillo and Mavangira, 2014). For example, mastitis may occur at any time during a production cycle but presents with a significantly higher incidence and severity during the periparturient period (Hogeveen et al., 2011). Several other infectious and metabolic disorders such as metritis, ketosis, and milk fever occur most frequently around the time of calving and early lactation (Pinedo et al., 2010). In dairy cattle, the underlying cause of increased incidence of disease during the periparturient period is metabolic stress. Metabolic stress ultimately compromises the immune status of the animal through a triad of risk factors including altered nutrient metabolism, dysfunctional inflammation, and oxidative stress (Sordillo and Mavangira, 2014). Altered nutrient metabolism can be triggered around the time of calving as the demand for energy increases due to fetal growth and the onset of lactation resulting in negative energy balance. Increased lipid mobilization as a consequence of negative energy balance may increase inflammatory responses and the generation of reactive oxygen species (ROS; Sordillo and Raphael, 2013). An imbalance between the production of ROS coupled with the decreased intake of dietary antioxidants due to decreased overall feed intake can lead to a pro-oxidant shift in the redox balance (Sordillo and Aitken, 2009). When excessive, ROS can damage cellular macromolecules such as lipids, protein, and DNA. The severity of oxidative stress can be determined by measuring any macromolecule damage, but lipid peroxidation events are often

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used to assess oxidative stress in dairy cattle (Castillo et al., 2005; Celi, 2011).

An alteration in the redox balance toward a greater pro-oxidant redox status can compromise dairy cattle immune competence by influencing the production of oxylipids, which are potent lipid mediators produced from oxidized PUFA that can control essentially every aspect of the inflammatory response (Sordillo, 2018). The role of oxylipids in regulating inflammation is convoluted because they may produce both pro- and anti-inflammatory effects (Mancuso et al., 1998; Cheng et al., 2008). In periparturient cows, for example, oxylipids can significantly affect the susceptibility and duration of diseases such as mastitis by overrecruiting leukocytes to the site of infection and increasing damage to the mammary gland (Mavangira et al., 2015; Sordillo, 2018). Oxylipids are generally produced enzymatically but can be nonenzymatically formed through direct oxidation of lipids by ROS. Because lipids are the most sensitive macromolecule to oxidative damage, nonenzymatic production yields quantifiable oxylipids, including the isoprostanes, that can be used as specific and sensitive markers for oxidative stress. Specifically, the 15-F_{2t}-isoprostane, also referred to as 8-isoprostane, has emerged in research as the gold standard for the measurement of oxidative stress in human and veterinary medicine (Niki, 2014; Mavangira et al., 2016).

The aim of this study was to quantify 15-F_{2t}-isoprostane in milk as a direct measure of oxidative stress in the mammary gland. Because the mammary gland is physiologically isolated to a certain extent from the systemic circulation by the blood–milk barrier, there is the potential that the oxidative environment of the mammary gland does not mimic that of the systemic circulation. Accurately quantifying the local oxidative environment of the mammary gland provides a new resource to redox balance research, allowing for direct quantification of oxidative damage rather than extrapolation from the systemic circulation.

MATERIALS AND METHODS

Chemicals

Acetonitrile, methanol, and formic acid of liquid chromatography–MS grade were purchased from Sigma-Aldrich (St. Louis, MO). Deuterated and nondeuterated oxylipid standards were purchased from Cayman Chemical (Ann Arbor, MI). Butylated hydroxy toluene was purchased from Acros (Waltham, MA), 156 EDTA and triphenylphosphine were purchased from Sigma-Aldrich, and indomethacin was purchased from Cayman Chemical.

Animals

This study was approved by the Michigan State University Institutional Animal Care and Use Committee (reference number 03/14-059-00), and cows were enrolled with client consent. The study was conducted at a commercial dairy operation in Michigan with an average of 3,300 lactating cows with an approximate rolling herd-average milk production of 12,250 kg. Cows were housed in freestall barns and grouped per lactation number, DIM, and milk yield. Cows were milked 2 times daily. Two diets, one for periparturient cows and a second for mid-lactation and late-lactation cows (Table 1), were formulated to meet the energy requirements based on production, and feed was delivered 2 times per day as a TMR.

Study Design

Twelve multiparous dairy cows of at least third lactation with an average age of 5.2 yr were enrolled and divided evenly according to their current stage of lactation into periparturient (**PP**; *n* = 4), mid-lactation (**ML**; *n* = 4), and late-lactation (**LL**; *n* = 4) groups. All animals in the periparturient group were 1 to 2

Table 1. Nutrient composition (% of DM unless noted) of the diets for the experimental animals

Item	Group ¹	
	PP ²	ML and LL ³
DM	46.14	47.64
Forage DM	47.00	40.87
CP	17.09	17.23
MP	12.48	11.97
ME, Mcal/kg	3.05	2.98
NE _L , Mcal/kg	1.81	1.79
ADF	17.51	16.23
NDF	27.48	29.56
Forage ADF	12.44	10.92
Forage NDF	17.19	17.46
NFC	44.33	42.36
Starch (nonsugar)	28.55	29.85
Fat	4.70	4.34

¹PP = periparturient; ML = mid lactation; LL = late lactation.

²Additions to base ration include the following. Vitamins (IU/kg): vitamin A, 8,328; vitamin D, 1,456; vitamin E, 35. Minerals (% of DM): Ca, 0.80; P, 0.37; K, 1.32; Mg, 0.29; S, 0.25; Na, 0.34; Cl, 0.48; salt, 0.47. Ca:P ratio = 2.16. Trace minerals (mg/kg): Zn, 61.97; Fe, 3.66; Cu, 14.08; Mn, 46.20; Co, 0.79; I, 0.79; Se, 0.28. Dietary cation anion balance salts = 19.48 mEq/100 g; Rumensin (Elanco, Greenfield, IN), 14.48 g/Mg.

³Additions to base ration include the following. Vitamins (IU/kg): vitamin A, 8,369; vitamin D, 1,455; vitamin E, 33. Minerals (% of DM): Ca, 1.04; P, 0.48; K, 1.10; Mg, 0.34; S, 0.26; Na, 0.56; Cl, 0.32; salt, 0.35. Ca:P ratio = 2.17. Trace minerals (mg/kg): Zn, 51.8; Fe, 7.6; Cu, 11.5; Mn, 37.0; Co, 0.6; I, 0.6; Se, 0.37. Dietary Cation Anion Balance salts = 27.6 mEq/100 g; Rumensin, 9.5 g/ton.

DIM. The mid-lactation animals were between 80 and 84 DIM. The late-lactation animals were 183 to 215 DIM. Negative milk bacterial cultures and an absence of overt clinical signs at the time of collection as well as SCC of <250,000 cells/mL on the most recent Dairy Herd Improvement Association test day were required for inclusion in each group. Bacterial milk cultures were performed on blood agar and selective medium for coliform bacteria according to the National Mastitis Council guidelines (Hogan et al., 1999). Briefly, individual mammary gland quarter milk (10 μ L) was streaked onto sheep blood agar and selective medium using a sterile loop inoculator. Cultures were incubated at 37°C, read at 24 and 48 h, and considered positive for bacterial growth based on the presence of 3 or more colonies on blood or selective medium.

Sample Collection and Analyses

Blood samples were collected via coccygeal venipuncture into serum-separator or 156 EDTA evacuated tubes and placed on ice with added antioxidant reducing agent (**AOR**) within 15 min of collection to be transported to the laboratory. Milk samples were aseptically collected before morning milking and flash frozen within 15 min of collection for transportation to the laboratory. Urine samples were collected via free catch in plain 15-mL conical tubes and flash frozen within 15 min of collection for transportation to the laboratory. At the laboratory, blood tubes were centrifuged at $2,000 \times g$ for 18 min at 4°C and plasma and serum were harvested, aliquoted, and snap frozen in liquid nitrogen within 2 h of initial collection for subsequent storage at -80°C and analyses within 2 mo. Plasma, urine, and milk 15-F_{2t}-isoprostane quantification samples were mixed with an AOR at 4 μ L/mL as described previously (Mavangira et al., 2015). Reactive metabolites including both reactive oxygen and nitrogen species (**RONS**) were analyzed with plasma and whole milk not containing AOR using the Cell Biolabs (San Diego, CA) OxiSelect in vitro ROS/RNS assay, which involves sample reaction with dichlorodihydrofluorescein DiOxyQ and fluorometric quantification as previously reported (Mavangira et al., 2016). Plasma and whole milk without added AOR were also used to determine antioxidant potential (**AOP**) as described previously (Re et al., 1999). In short, AOP was quantified by sample reaction with 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) solution. The ability of the sample to reduce 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) was standardized to Trolox used in a standard curve. Serum amyloid A and haptoglobin were measured in serum samples using the commercial assays from Tridel Development Ltd. (Maynooth, Ireland).

Plasma albumin, cholesterol, and nonesterified fatty acids (**NEFA**) were analyzed at the Michigan State University Veterinary Diagnostic Laboratory (Lansing, MI). The oxidant status index (**OSi**) was created by dividing the RONS value by AOP (Abuelo et al., 2013).

Quantification of 15-F_{2t}-Isoprostane

15-F_{2t}-isoprostanes were determined using liquid chromatography–tandem MS. Plasma (2 mL) and milk (4 mL) samples were mixed with an AOR mixture to prevent degradation of preformed oxylipids and prevent ex vivo lipid peroxidation as described previously (O'Donnell et al., 2009). The AOR mixture consisted of 50% methanol, 25% ethanol, and 25% water with 0.9 mM butylated hydroxy toluene, 0.54 mM EDTA, 3.2 mM triphenylphosphine, and 5.6 mM indomethacin. Samples were flash frozen in liquid nitrogen and stored at -80°C until analyses. A mixture of internal standards containing 5(S)-HETE-*d*₈, 15(S)-HETE-*d*₈, 8(9)-EET-*d*₁₁, PGE2-*d*₉, 8,9-DHET-*d*₁₁, AA-*d*₈, 2-AG-*d*₈, and AEA-*d*₈ was prepared to final concentrations of 0.25, 0.25, 0.5, 0.5, 0.25, 50, 2, and 0.25 μ M, respectively. The internal pure standards mixture was added to each sample and used to generate a 6-point standard curve ranging from 500 to 0.001 μ M in concentration of unlabeled fatty acid and oxylipid standards.

Sample Processing

Frozen plasma and whole milk samples were thawed on ice and processed as follows: 4 mL of milk was combined with 9.15 mL of 4°C acetonitrile, and 2 mL of plasma was combined with 5 mL of 4°C methanol and 2 μ L of 88% liquid chromatography MS-grade formic acid. For urine, 4 mL of sample was initially mixed with 24 μ L of formic acid. To each sample mixture, 15 μ L of internal standard cocktail was added, and the sample was vortexed for 2 min, incubated at room temperature for 15 min, and centrifuged at $4,816 \times g$ for 20 min at 4°C. For plasma and urine, the supernatant was diluted with 95 mL of HPLC water and 95 μ L of formic acid, and supernatant in milk samples was diluted with 170 mL of HPLC-grade water and 170 μ L of formic acid.

Solid Phase Extraction

Solid phase extraction was performed for each sample with Oasis HLB 12cc (500 mg) LP extraction columns (Waters, Medford, MA). Columns were conditioned with 6 mL of methanol followed by 6 mL of HPLC water. Extracts were loaded onto the columns, which were then washed with 6 mL of 20% methanol and then dried under full vacuum for 15 min. Analytes were

eluted with 6 mL of methanol and acetonitrile (50:50 vol/vol). The volatile solvents were removed under vacuum using a Savant SpeedVac Concentrator (ThermoFisher Scientific, Asheville, NC). The residues were reconstituted in 150 μ L of methanol, mixed at a 1.4:1 ratio with HPLC water, and stored in glass chromatography vials with glass inserts at -20°C until analysis. The curve for 15-F_{2t}-isoprostane was a 5-fold dilution ranging from 100 to 0.001 μM . The deuterated internal standard concentrations mentioned above were added to each point on the curves. The linear curves generated produced coefficient of determination (R^2) values of 0.99 with percentage deviations of less than 100%.

Liquid Chromatography–Tandem MS Analyses

Details of liquid chromatography–tandem MS procedures are described in Mavangira et al. (2015). Briefly, the quantification of 15-F_{2t}-isoprostane was accomplished on a Waters Acquity UPLC coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer using multiple reaction monitoring. Chromatography separation was performed with an Ascentis Express C18 HPLC column (10 cm $\times$ 2.1 mm, 2.7 μm ; Supelco, Bellefonte, PA) held at 50°C . The autosampler was held at 10°C . Mobile phase A was water with 0.1% formic acid, and mobile phase B was acetonitrile. Flow rate was fixed at 0.3 mL/min. Liquid chromatography separation took 15 min with linear gradient steps programmed as follows (A/B ratio): time 0 to 0.5 min (99/1), to (60/40) at 2.0 min; to (20/80) at 8.0 min; to (1/99) at 9.0 min; 0.5 min held at (1/99) until min 13.0; then return to (99/1) at 13.01 min, and held at this condition until 15.0 min. 15-F₁₂-isoprostane was detected using electrospray ionization in negative-ion mode. Cone voltages and collision voltages were optimized using Waters QuanOptimize software. All samples were processed, extracted, measured, and analyzed as a single batch, minimizing intra-assay variation; however, a specific intra-assay variation coefficient is not available. Because a single batch of samples was assayed, an inter-assay variation coefficient does not apply.

Statistical Analyses

Statistical comparisons were performed using the MIXED procedure in SAS (SAS Institute Inc., Cary, NC). Data were checked for normality in SAS and analyzed by 1-way ANOVA with Tukey's adjustment for multiple comparisons. Data failing to satisfy the Gaussian (normal) distribution were transformed (log or square root) and rechecked for normality before comparisons were performed. For presentation (tables or figures), transformed data were back transformed to

the observed scale and reported as the geometric means. Where data satisfied the Gaussian distribution requirements but with unequal variances, the Kenward-Roger adjustment for multiple comparisons was performed (this was done only for Haptoglobin data). Pearson correlation coefficients were determined both within specific lactation groups and across all sample periods for all data in which both milk and plasma samples were available for correlation within specific lactations and across all sample periods. All comparisons were considered different at $\alpha \leq 0.05$.

RESULTS

Indices of inflammation and liver function included measuring haptoglobin, serum amyloid A, and plasma albumin, whereas altered nutrient metabolism was quantified through NEFA and cholesterol concentrations (Table 2). Abundance of RONS was measured fluorometrically. In plasma, RONS levels were higher in PP and significantly lower ($P < 0.01$) in ML and LL ($P < 0.05$; Figure 1). In addition to RONS, the AOP was quantified by the ability of the sample to reduce 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) against a standard of Trolox (Figure 1). In plasma, AOP was lower in the PP period compared with other lactation periods but only significantly lower when compared with late lactation ($P < 0.01$). High levels of RONS and a low AOP during the PP created a significantly ($P < 0.01$) higher OSi during the PP period compared with ML and LL (Figure 1). These measurements allude to a systemic pro-oxidant environment in the cow during the PP compared with other stages, as would be expected in animals facing a negative energy balance.

In addition to measuring RONS, AOP, and the OSi in plasma, these values can be obtained in milk as has been shown previously (Albenzio et al., 2016; Mavangira et al., 2016). Rather than providing systemic values, milk provides a measure of oxidant status locally in the mammary gland. Results obtained from milk were overall inconsistent with those found systemically. Although a significant correlation ($P < 0.001$) between plasma and milk RONS was found across all lactation groups ($r = 0.95$), AOP levels in milk did not correlate with plasma AOP at any lactation stage. Specifically, AOP values in milk were significantly ($P < 0.01$) higher during the PP stage compared with the ML and LL stages (Figure 1). This combination of increased RONS in the presence of high AOP suggests that although an abundance of RONS exists, a likely compensatory response of local antioxidant mechanisms has been triggered to combat the pro-oxidant load and maintain a stable redox environment. When the OSi is applied,

Figure 1. Plasma and milk concentrations (mean $\pm$ SEM) of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in relative fluorescence units (RFU) per microliter (A), antioxidant potential in Trolox equivalents per microliter (B), and oxidant status index, the ratio of ROS and RNS to antioxidant potential (AOP; C) were inconsistently higher or lower between the 2 fluids in cows from the periparturient (PP), mid-lactation (ML), and late-lactation (LL) stages, ultimately revealing opposite trends in the oxidant status index. Bars with different letters (a,b) differ ($P < 0.05$) within each individual sample type of a single graph unless otherwise noted by a specific P -value.

it reveals a relatively low index value during the PP stage compared with the ML and LL stages, albeit not significant, concurring with this assessment.

Although these observations offer valuable insights, quantifying RONS or AOP only indirectly determines redox status. Measurement of isoprostanes, specifically 15-F_{2t}-isoprostane, has become the gold standard of direct oxidative stress quantification. When measured across lactation in plasma, 15-F_{2t}-isoprostane concentrations were significantly higher during the PP and LL stages compared with the ML stage ($P < 0.05$; Figure 2). Interestingly, in milk, 15-F_{2t}-isoprostane concentrations in the PP stage were significantly lower than those measured in the ML or LL stages ($P < 0.05$; Figure 2). A correlation ($P < 0.05$) between milk and plasma 15-F_{2t}-isoprostane concentrations was noted only during the ML stage ($r = 0.98$). 15-F_{2t}-isoprostanes were additionally quantified in urine across lactation (presented as mean $\pm$ SE mg/mg of creatinine). In urine, the PP stage (5.49 ± 3.02 mg/mg of creatinine) and LL stage (29.02 ± 15.03 mg/mg of creatinine) were found to have significantly higher concentrations than

the ML stage (0.25 ± 0.21 mg/mg of creatinine; $P < 0.05$). Although these trends are similar to those seen in plasma, no correlation was found between plasma and urine.

DISCUSSION

Oxidative stress in dairy cattle can cause increased disease susceptibility, longer durations of clinical disease, and slower return to peak production (Bertoni et al., 2008; Jozwik et al., 2012; Sordillo and Raphael, 2013). Although a plethora of studies have outlined potential causes and consequences of systemic oxidative stress in dairy cattle (Sordillo and Aitken, 2009), few have addressed the oxidative environment of the mammary gland. The detrimental effects of oxidative stress on immune defenses are due, in part, to the peroxidation of cellular lipids resulting in the generation of several oxidized lipid metabolites, including the family of isoprostanes (Sordillo, 2018). Indeed, previous works in cattle have validated the use of measuring 15-F_{2t}-isoprostane in the plasma and milk of cows with

Table 2. Serum and plasma metabolites (mean $\pm$ SEM) from periparturient ($n = 4$), mid-lactation, ($n = 4$), and late-lactation ($n = 4$) cows

Metabolite	Periparturient		Mid lactation		Late lactation	
	Mean	SEM	Mean	SEM	Mean	SEM
Serum amyloid A, ¹ μ g/mL	135.71	33.91	91.54	33.91	28.56	33.91
Haptoglobin, ¹ mg/mL	1.378	0.051	1.064	0.010	0.003	0.009
Albumin, ² g/dL	2.98 ^b	0.05	3.03 ^b	0.06	3.57 ^a	0.06
Cholesterol, ² mg/dL	54.75 ^b	2.14	205.25 ^a	169.40	265.00 ^a	11.17
Nonesterified fatty acids, ² mEq/L	0.64 ^a	0.11	0.09 ^b	0.11	0.10 ^b	0.11

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).

¹Serum sample.

²Plasma sample.

Figure 2. Plasma and milk concentrations (mean $\pm$ SEM) of 15-F_{2t}-isoprostanes were elevated in plasma of periparturient (PP) cows, whereas milk 15-F_{2t}-isoprostanes were elevated in mid-lactation (ML) and late-lactation (LL) cows. Bars with different letters (a,b) differ ($P < 0.05$) within each individual sample type.

mastitis and during early lactation as an assessment of oxidative stress (Vernunft et al., 2014; Mavangira et al., 2016). A systemic pro-oxidant shift of the redox balance in dairy cattle is integral to the development of metabolic stress in the periparturient period. In this study, increased plasma 15-F_{2t}-isoprostane concentrations paralleled the higher plasma OSI, a more comprehensive measure of oxidative balance that takes into account both RONS and AOP (Abuelo et al., 2013). Additionally, 15-F_{2t}-isoprostane reflected biomarkers of inflammation (acute phase proteins) and altered nutrient metabolism (NEFA, cholesterol) characteristic of metabolic stress (Bertoni et al., 2008; Sordillo and Raphael, 2013; Sordillo and Mavangira, 2014). Thus, the findings of this study support the contention that plasma 15-F_{2t}-isoprostane concentrations are a valid way to assess oxidative stress at different stages of the lactation cycle.

During acute coliform mastitis, milk 15-F_{2t}-isoprostane concentrations were reported to resemble changes observed in plasma and urine (Mavangira et al., 2016). Similarly, changes in plasma and milk 15-F_{2t}-isoprostane concentrations have been shown to be correlated in healthy cows during the second month of lactation (Vernunft et al., 2014). Interestingly, it was during this ML stage in the current study that 15-F_{2t}-isoprostane concentrations were significantly correlated between milk and plasma. Whereas 15-F_{2t}-isoprostane concentrations in plasma and milk were correlated strictly during the ML period, values of RONS for plasma and

milk were correlated throughout all 3 lactation periods, even though milk RONS amounts were 100 times those found in plasma. Nevertheless, these values are consistent with those reported previously using the same assay methodology (Abuelo et al., 2016; Mavangira et al., 2016). The difference in RONS abundance is most likely due to the concentration of the metabolites in milk while they are relatively diluted throughout the body in plasma. In our study, the concentrations of 15-F_{2t}-isoprostane in milk and plasma did not consistently trend in similar directions across all lactation stages. Indeed, 15-F_{2t}-isoprostane concentrations were greatest in plasma but lowest in milk around the time of calving. Vernunft et al. (2014) also noted a comparable trend, reporting an increase in milk isoprostanes between d 38 and 71 postpartum, whereas plasma isoprostane concentrations decreased during this period.

Although several trends exist between our results and those published previously with respect to oxidant status in plasma and milk, the absolute concentration of isoprostanes varies in the literature. Several reasons may explain the conflict between the findings in our study and results reported previously. The health status of a dairy cow can clearly affect lipid peroxidation, as indicated previously with the influence of coliform mastitis on isoprostane concentration in urine, milk, and plasma (Mavangira et al., 2016). All cows in the present study were clinically healthy, as was shown by haptoglobin, serum amyloid A, and plasma albumin concentrations residing within published reference limits at all stages of lactation. The significant differences noted in the PP compared with other stages of lactation are consistent with previous studies and represent a normal physiologic variation found during this time (Cozzi et al., 2011). Additionally, concentrations of NEFA and cholesterol were consistent with previously described values for the 3 different stages of lactation (Bell, 1995; Adewuyi et al., 2005; Contreras et al., 2010). A previous study in healthy cows also reported concomitant increases in both plasma and milk concentrations of 15-F_{2t}-isoprostane as related to ovulation (Vernunft et al., 2014). An important distinction between the previous and present study is the analytical methods used to determine 15-F_{2t}-isoprostane concentrations. Vernunft et al. (2014) used commercial ELISA-based assays that are known to be susceptible to cross-reactivity problems due to the structural similarities across the entire prostanoid series of molecules. As such, ELISA-based assays are considered only as semiquantitative estimations that often yield significantly higher 15-F_{2t}-isoprostane concentrations than corresponding chromatography and MS methods that were used in the present study (Klawitter et al., 2011; Sordillo, 2018). Thus, the methods used to measure isoprostanes should

be carefully considered when comparing studies in the scientific literature and interpreting the information. Additional differences, such as those noted between Contreras et al. (2017) and the presented results, may stem from differences in the basal redox status of cattle that live under different management conditions and are fed different rations (Hanschke et al., 2016; Khatti et al., 2017). Sampling time may also contribute to published differences because small changes in the physiological state of a cow may significantly affect isoprostane production. As such, it is difficult to directly compare absolute values of cattle in their dry period or shortly after the periparturient period with those of cattle at parturition or near peak lactation. The differences in 15-F_{2t}-isoprostane concentrations reported in the literature emphasize the need to establish more consistent sampling times and analytical techniques before this metabolite of lipid peroxidation can be used as a standardized biomarker of oxidative stress and disease susceptibility.

Although milk and plasma 15-F_{2t}-isoprostane concentrations did not follow the same trends across lactation stage, the lower milk concentration is consistent with the trends found in a recent study addressing shifting quantities of biosynthesized oxylipids across lactation (Kuhn et al., 2017). For example, the ratio of arachidonic acid–derived oxylipids in milk, including 9-hydroxyeicosatetraenoic acid (**HETE**) and 11-HETE to 15-HETE, was significantly lower during the PP stage compared with the ML and LL stages (Kuhn et al., 2017). Although these HETE are derived from the same fatty acid substrate, 9-HETE and 11-HETE are primarily nonenzymatically produced through oxidation by ROS, whereas 15-HETE is primarily enzymatically biosynthesized by the 15-lipoxygenase-1 enzyme (Kuhn et al., 2017). Expression of 15-lipoxygenase-1 is increased significantly in mammary tissue around the time of calving (Aitken et al., 2009); however, our data and others do not reflect a significant increase in 15-HETE during this period (Raphael et al., 2014; Kuhn et al., 2017). Rather, a decrease in nonenzymatically derived 9-HETE and 11-HETE was responsible for the change in the ratio of enzymatic and nonenzymatic pathways of oxidation (Kuhn et al., 2017). Lower milk concentrations of 9-HETE and 11-HETE during the PP stage may suggest that the pro-oxidant environment in the mammary gland was not sufficient to drive non-enzymatic oxylipid biosynthesis.

The potential role of negative redox balance in the mammary gland around calving and into early lactation was previously characterized (Hodgkinson et al., 2007; Aitken et al., 2009). Changes in antioxidant defenses (e.g., glutathione peroxidase) and measures of inflammation (including vascular cell adhesion molecule 1 or

tumor necrosis factor α) were not significantly increased in mammary tissues during the periparturient period when compared with other physiological stages of the production cycle (Hodgkinson et al., 2007; Aitken et al., 2009). Although the periparturient period is associated with an increased production of systemic and milk ROS values, the mammary gland antioxidant defense mechanisms may be enhanced to combat local ROS accumulation and tissue damage. In support of this contention, previous research showed increased activity in the selenoproteins glutathione peroxidase-1 and glutathione peroxidase-4, which play key roles in the protection of cells against ROS-induced peroxidation of membrane phospholipids (Aitken et al., 2009; Sordillo and Aitken, 2009). Thus, the lower milk concentrations of 15-F_{2t}-isoprostane reported in the present study during the onset of lactation may be explained by increased AOP associated with mammary tissues during a time of increased metabolic demands. Whole milk was used in this study to include all considerations within milk that may affect redox status. Unpublished work in our laboratory evaluated the difference between centrifugally separated milk samples and whole milk samples, revealing that whole milk offered a higher AOP. Additionally, work by Albenzio et al. (2016) showed that whole milk contained higher levels of RONS than individual milk protein fractions. Indeed, different milk fractions have been shown to harbor specific antioxidant abilities (Chen et al., 2000; Castillo et al., 2013).

Systemic oxidative stress may still play a significant role in the onset of mastitis as well regardless of the local oxidant environment. At the onset of infection, when a robust yet measured inflammatory response is necessary to contain and eliminate foreign pathogens, oxidative stress dysregulates the control over this response. Reactive oxygen species increase the permeability of the endothelial barrier between the circulation and the mammary gland, allowing for increased extravasation of leukocytes (Boueiz and Hassoun, 2009). Additionally, neutrophils in an increased oxidant environment produce less superoxide dismutase than those supplemented with antioxidants, decreasing their killing abilities (Politis et al., 2004). In turn, this dysregulated response leads to the onset of infection, and the severity of mastitis during this period is heightened by a systemic shift toward a pro-oxidant redox balance. Without proper control of the inflammatory response, an overreaction to the initial stimulus can occur, causing further tissue damage from inflammatory cells as well as chronic inflammation (Aitken et al., 2011).

Current research in cattle is only beginning to uncover the abilities of local compartments, such as the urinary tract, ovaries, or mammary gland, to regulate their local oxidant environment independently from the

systemic circulation (De Bie et al., 2016). Although the focus of this study was on the mammary gland, data generated from urine 15-F_{2t}-isoprostane were in line with previous research in our laboratory, which has also shown no correlation between plasma and urine 15-F_{2t}-isoprostane (Mavangira et al., 2016). Both human and murine models have shown a direct correlation between 15-F_{2t}-isoprostane excretion in urine and plasma levels, making our findings noteworthy (Takahashi et al., 1992; Ware et al., 2011). The lack of a significant correlation between these fluids in cattle suggests that there may be another variable affecting the excretion of 15-F_{2t}-isoprostane in cattle, such as differential phospholipase activity (Stafforini et al., 2006).

Although not the first to quantify 15-F_{2t}-isoprostane in milk, this study validates the use of measuring 15-F_{2t}-isoprostane in milk to quantify oxidative stress in the mammary gland across different stages of the lactation cycle. The methodology used in the present study is arguably more accurate than ELISA-based assays (Klawitter et al., 2011), and we showed for the first time that milk 15-F_{2t}-isoprostane is not merely a reflection of plasma concentration but rather reflects lipid peroxidation events of the mammary gland. Additionally, this study supports previously reported data suggesting that the mammary gland may maintain its oxidative balance in the face of systemic oxidative stress during the periparturient period. The current study focused on comparing stages of lactation that represent drastically different physiological environments and, more specifically, significant differences in oxidant status; however, it is recognized that dramatic physiological changes still occur between each of the stages studied herein. Specifically focusing on changes that occur between calving and the immediate postpartum period may better characterize the oxidative changes occurring in the periparturient period. Due to the small sample size used, although significant differences were found in 15-F_{2t}-isoprostane concentration and other indicators of redox status both amid different lactation stages and between milk and plasma, a more robust study following a greater number of individuals across their entire lactation would be beneficial to sustain these conclusions. The deductions expressed in this paper are supported by the modest power of the post hoc analyses of 0.69 for plasma 15-F_{2t}-isoprostane concentrations; however, despite the low numbers, the analyses for milk 15-F_{2t}-isoprostane concentrations had a high power of 0.87. The ability of the mammary gland to regulate oxidant status independent of systemic measures may direct future studies and interventions toward the most effective strategies to prevent and control oxidative stress.

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