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Effects of parity and week after calving on the metabolic, redox, and immune status of dairy cows

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ABSTRACT

At the onset of lactation in dairy cows, inflammation and oxidative stress may occur and result in a risk of pathologies and lower milk yield. To propose an innovative management strategy for cows during this period, it is essential to better understand these physiological variations. Our objective was to evaluate the metabolic, redox, and immune status of 7 primiparous and 8 multiparous Holstein cows during late gestation and the first months of lactation. Blood samples were collected between 3 wk before calving until 12 wk postpartum. Milk samples were also collected, but only at the time points after calving. The metabolic (nonesterified fatty acids [NEFA], BHB, glucose, urea, calcium) and redox (reactive oxygen metabolites [ROM], oxidative stress index [OSI], glutathione peroxidase activity, vitamin E) statuses were analyzed in plasma or erythrocytes. The expression of genes related to antioxidant functions was determined in leukocytes collected from milk. For immune status, plasma cytokine levels and the production of reactive oxygen species (ROS) in classical and regulatory neutrophils were measured in 2 whole blood ex vivo challenges. The data were analyzed using a mixed model that included the fixed effects of parity and week and their interaction. Milk yield, plasma NEFA, and BHB in wk 2 and 4 after calving were higher in multiparous cows than in primiparous cows, whereas glucose and calcium tended to be lower. Plasma ROM and OSI levels in wk 8 were higher in multiparous than in primiparous cows. Multiparous cows also displayed higher glutathione peroxidase activity in erythrocytes, and antioxidant transcription factor and superoxide dismutase-1 expression levels in milk leukocytes. Moreover, multiparous cows had higher plasma concentrations of vitamin E but lower plasma levels of cytokines CXCL10, CCL2, IL1Rα, and IFNγ. Following ex vivo whole blood stimulation with *Escherichia coli*, lower IL1α and TNFα levels were measured in multiparous than in primiparous cows. Intracellular ROS production by neutrophils was lower in multiparous than in primiparous cows. These results thus indicated marked physiological changes in wk 8 compared with wk 2 and 4 of lactation. These differences in the physiological status of primiparous and multiparous cows offer interesting perspectives for potential dietary strategies to prevent pathologies which take account of parity and week relative to calving.

Key words: primiparous, antioxidant response, immunity, lactation onset

INTRODUCTION

The onset of lactation is a period when dairy cows are highly susceptible to several metabolic or infectious diseases (Fleischer et al., 2001). During the peripartum period, oxidative stress (Castillo et al., 2005), and inflammation (Bertoni et al., 2008) can occur and may explain, or at least be associated with, a higher incidence of several infectious diseases.

Oxidative stress corresponds to an increase in reactive oxygen metabolites (**ROM**) along with a fall in antioxidant capacities (Sies, 1985). Increased ROM production is due to the increase in energy metabolism required to support lactation and can be considered as a consequence of the homeorhetic adaptation of cows (Bradford and Swartz, 2020). Oxidative stress can affect several cell functions by targeting ROM to DNA, RNA, proteins, or lipid membranes (Sordillo and Aitken, 2009). Higher ROM plasma levels and increased concentrations of nonesterified free fatty acids (**NEFA**) may result in an increase in lipid peroxidation (Bradford et al., 2015). It has also been shown that an intake of antioxidants can

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improve the immune response by reducing free radicals (Sordillo and Aitken, 2009).

In contrast, it has been observed in dairy cows that inflammation can occur and immune status be impaired during the peripartum period (Trevisi and Minuti, 2018). Immune status may be dysregulated and more specifically, reduced neutrophil functional capacity has been associated with the metabolic status of dairy cows during this period (LeBlanc, 2020). Impaired lipid metabolism, increased circulating concentrations of NEFA and BHB and oxidative stress are all associated with systemic inflammation (Sordillo and Aitken, 2009; Ingvartsen and Moyes, 2013; Bradford et al., 2015). A recent review challenged the dogma that the mobilization of body reserves is the cause of an altered inflammatory profile and instead suggested that the former is a consequence of the latter (Horst et al., 2021).

Such links between metabolic, redox, and immune status still need to be clarified because oxidative stress and inflammation may constitute a risk of physiological disturbances and diseases that induce tissue damage.

Multiparous and primiparous cows differ in their milk production levels and metabolic profiles, with multiparous cows mobilizing more body reserves. Parity is thus an interesting factor to consider when examining the relationships between metabolic, redox, and immune status in dairy cows. Moreover, the parity influences cow health. Multiparous cows are more likely to suffer from mastitis, ketosis, or displacement of the abomasum, whereas primiparous animals have a greater risk of developing metritis (Markusfeld, 1987; Lean et al., 2023).

Study findings on differences in parity-dependent redox status are currently inconsistent. Some studies have shown a higher production of ROM in primiparous or second lactation cows than in those with a higher lactation rank (Abuelo et al., 2013; Urh et al., 2019), and others indicated either higher (Elischer et al., 2015) or lower antioxidant potential (Omidi et al., 2017) in primiparous than in multiparous cows. In contrast, other studies observed a link between metabolic status and immune status without considering redox status. For example, one study showed that the inflammatory profile after calving was similar between the first and second lactations (Cattaneo et al., 2023), while another indicated that the immune response measured by the oxidative burst activity of neutrophils was more impaired in multiparous than in primiparous cows (Moya et al., 2008).

Our objective was to investigate the links between metabolic, redox, and immune status by exploring their variability as a function of parity and week of lactation. Our experiment was performed between 3 wk before and 12 wk after calving, where these statuses vary the most according to the literature (Castillo et al., 2005; Trevisi and Minuti, 2018; Minuti et al., 2020). Status was analyzed at the systemic and local levels in the mammary gland. Our hypothesis was that a multiparous cow has a lower redox status and altered immune functions, and hence, higher inflammation and oxidative stress due to a higher metabolic solicitation than primiparous cows.

MATERIALS AND METHODS

Animals, Experimental Design, and Animal Housing

This experiment was conducted at the INRAE Experimental Farm (IE PL, INRAE, Dairy Nutrition and Physiology, Le Rheu, Brittany, France accreditation for animal housing no. C-35–275–23") between August 23 and December 17, 2021. The experiment was approved by the Rennes Ethics Committee on Animal Experimentation and the French Ministry for Higher Education, Research and Innovation (APAFIS project number #31835–2021053017243978_v) and performed in compliance with all applicable provisions established by European Directive 2010/63/UE. Fifteen Holstein dairy cows, 7 primiparous (first lactation) and 8 multiparous (4 cows in second lactation, 2 in third lactation, one in fourth lactation, and one in fifth lactation), were monitored between 3 wk before and 12 wk after calving. We considered that 2 groups of 7 cows, one for each parity group, was sufficient to reveal a significant difference in milk production between the parity groups considering an effect size of 2, with a power of 0.95 and a type I error of 0.05 (estimated sample size = 7 cows [GPower, Universität Kiel, Germany]). The effect size was based on an expected difference between parities of at least 9 kg/d (Leclerc, 2008), and a standard deviation within the population at a given stage of lactation of 4.5 kg/d. The cows were assigned in 4 blocks depending on their predicted calving date and were selected in such a way that the proportion of primiparous and multiparous cows remained comparable between blocks. Within each block, the maximum interval between calving dates was 7 d. In our trial, to maintain a maximum interval of 7 d between calving dates within each block, the cows were switched between blocks according to their actual calving date. Finally, the first block included one multiparous and 3 primiparous cows, the second had 3 multiparous and 2 primiparous cows, the third had 3 multiparous and one primiparous cow, and the last one had multiparous and one primiparous cow.

The cows were housed in a freebarn stall with free access to feed and water with controlled individual intake, except for the 1 to 2 d around calving when they were kept separately. The cows were fed a TMR based on corn silage (Supplemental Table S1, see Notes; Inra, 2018). The ration was distributed twice daily in 2 equal-sized meals at 0830 and 1700 h. Feed distribution and feed

refusals were measured to calculate the DMI. The dairy cows were milked twice daily at 0700 and 1630 h. Milk yield was recorded individually at each milking.

Health events were monitored daily and any cows with health issues at calving (stillborn calf, abortion) or lameness problems requiring isolation from the herd were excluded. Body condition score was evaluated at around wk −2, 2, 8, and 12 for each cow.

Milk Samples and Milk Composition Determinations

Twice a week, milk samples were collected to determine their fat and protein composition and SCC using an infrared method (Mylab, Châteaugiron, France). The SCS was then calculated. Additional milk samples (50 mL) were collected in wk 4, 8, and 12 from the entire morning milking to determine the lactose content using an infrared method (Mylab). At the same time points, a 1.8-L milk sample was collected for the isolation of mammary epithelial cells (**MEC**) and leukocytes, as well as a 5-mL sample to determine sodium and potassium concentrations as markers of mammary epithelium integrity.

Blood Samples

Blood samples were collected after milking from the coccygeal or jugular veins, depending on the type of analysis, at wk −3, 4, 8, and 12. At wk −2 and 2, additional blood samples were collected for mineral and vitamin measurements only.

Blood from the coccygeal vein was collected in five 9-mL tubes. Three of these 9-mL tubes contained lithium heparin as an anticoagulant (Vacutest, Kima srl, Arzergrande, Italy) and were used to analyze the metabolic, redox balance, and inflammation markers detailed in the section on biochemical analysis. Two 9-mL tubes containing K_2 EDTA as an anticoagulant (Vacutest) were used for blood collected from the coccygeal vein to determine vitamin E concentrations. The plasma was separated by centrifugation at 1,200 × *g* for 10 min at 4°C and then stored at −20°C until subsequent analysis.

Some blood from the jugular vein was placed in 2 lithium heparin monovettes (S-Monovette, Sarstedt Marnay, France) preloaded with either heat-killed (**HK**) *Escherichia coli* or a control medium as previously described (Lesueur et al., 2022). An additional two 9-mL tubes were collected, one containing lithium heparin as an anticoagulant (Vacutest) used to determine the antioxidant enzymatic activity GSH-Px and the second containing K_2 EDTA as an anticoagulant (Vacutest) to determine the blood neutrophil phenotype and measure ROS production. The jugular blood in the 2 monovettes was used to perform an ex vivo challenge in the absence or presence of HK *E. coli* and to measure cytokine levels.

Cytokine and Chemokine Measurements With or Without HK E. coli Stimulation

The blood samples in the two 1-mL monovettes—one a control without bacteria and one containing HK *E. coli* (final concentration of 10^7 *E. coli* P4 bacteria)—were incubated and treated as previously described (Lesueur et al., 2022). Fifteen cytokines/chemokines were measured in the plasma using Luminex technology and the 15-plex kit from Merck-Millipore (SPRCUS617, Milliplex xMAP, Merck-Millipore, France) according to Lesueur et al. (2022). The results were supplied as median fluorescence intensities (**MFI**) recorded on a MAGPIX system (Luminex) and transformed into a decimal logarithm.

Hematological Profile and Flow Cytometry Analysis

The hematological profile was analyzed by the Labocea laboratory (Fougères, France) for determination of the white blood cell count (10^9/L) with a hematological counter and the percentage of lymphocytes, monocytes, polynuclear neutrophils, and polynuclear eosinophils by microscopic reading after May–Grünwald Giemsa staining. The percentages of each cell type were multiplied by the total concentration of white blood cells, to obtain their concentration.

Neutrophils were purified from blood after centrifugation at 1,000 × *g*, for 15 min at 20°C to recover the red pellet fraction containing the neutrophils. Then erythrocytes were lysed by adding 4 volumes of ammonium-chloride-potassium buffer (Gibco) per 1 volume of blood. The neutrophils were then washed twice with PBS, numerated with a TC20 Automated Cell Counter (Biorad, Hercules, CA), resuspended in PBS with 10% horse serum, 2 m*M* EDTA at a concentration of 10^7 cells/mL and 2×10^6 cells/wells were plated in a 96-well plate. Neutrophils were labeled with an anti-G1 IgM primary antibody (CH138A IgM, 1/500 [vol/vol], Kingfisher), and then with an anti-IgM secondary antibody coupled to Alexa Fluor 647 (goat anti-mouse IgM Alexa Fluor 647, 1/200 [vol/vol] Invitrogen). According to Rambault et al. (2021), regulatory neutrophils were labeled with an anti-class II major histocompatibility complex (**MHCII**) IgG1 primary antibody (CAT82A IgG_1, 1/500 [vol/vol], Kingfisher) and then with an anti-IgG1 secondary antibody coupled to Alexa Fluor 488 (goat anti-mouse IgG1 Alexa Fluor 488, 1/200 (vol/vol) Invitrogen). The ROS produced by neutrophils were quantified using CellROX Orange Flow Cytometry Assay Kits (CD10493, Invitrogen) according to the manufacturer's instructions. To induce ROS production, neutrophils were incubated

for 1 h at 37°C with or without 400 µ*M* tert-butyl hydroperoxide (**TBHP**) in a 96-well black microplate and then stained for 30 min with 100 n*M* CellROX orange reagent. A viability marker was added to differentiate dead cells after TBHP stimulation (Fixable Viability Dye Efluor 780, 1/10,000 [vol/vol], Invitrogen). Isotype controls of mouse IgG1 and IgM (Invitrogen) were used and served to discriminate nonspecific background during the analysis. A total of 120,000 events were acquired using a MACSQuant Analyzer 10 cytometer (Miltenyi Biotec), and after compensation, the results were analyzed with Kaluza software 114 (analysis version 2.1, 2009–2021 Beckman Coulter, Inc.; Rambault et al., 2021). Neutrophil subset percentages were measured in the blood by flow cytometry according to the gating strategy described in the Supplemental Figure S1 (see Notes).

MEC Count, Milk Leukocyte Gene Expression, and Milk Progesterone

To determine the count and exfoliation rate of MEC in milk, and gene expression in leukocytes, milk cells were prepared from 1.8 kg of fresh milk samples collected at morning milking. The milk was distributed into 8 tubes and cells were collected by centrifugation at 1,500 × *g* for 15 min at 4°C. The cell pellets were resuspended in 3 mL of PBS, pooled together, and the volume was adjusted to 225 mL of PBS before centrifugation at 1,000 × *g* for 10 min at 4°C. The cell pellet was resuspended in 3 mL of PBS then filtered through a 200-µm filter, and the volume was adjusted to 50 mL of PBS before centrifugation at 1,000 × *g* for 10 min at 4°C. The cell pellet was finally resuspended in 1 mL of PBS 1% BSA (m/v) (Sigma A7888, Darmstadt, Germany) before incubation with magnetic beads. The MEC and leukocytes were prepared using an immunomagnetic separation technique (Herve et al., 2017). After incubation with magnetic beads (Pan Mouse IgG, Dynal Biotech, Invitrogen) bound to an antibody specific for MEC directed against cytokeratins (clone 34βE12; Dako, Trappes, France), the samples were placed in a magnetic particle concentrator (MPC-S; Dynal Biotech, Invitrogen). After a few seconds on the magnetic rack, 2 phases could be distinguished: the supernatant containing milk leukocytes, and the cell fraction containing MEC bound to the magnetic beads. The supernatant containing the leukocytes was collected in a new tube and stored in 1 mL of TRIzol (Invitrogen Life Technologies, Carlsbad, CA) at −80°C until total RNA extraction. The cell count of the purified MEC suspension was analyzed using a Vi-Cell XR analyzer (Beckman Coulter, Brea, CA). The rate of MEC exfoliation was defined as the number of MEC exfoliated into milk per day and calculated by multiplying the MEC concentration measured during the morning milking by the daily milk yield.

After defrosting at room temperature, the milk leukocyte samples were crushed and homogenized in 1 mL of TRIzol and ground mechanically with 2.8-mm metal beads (P000925-LYSK0-A.0, Ozyme, Saint-Cyr-l'École, France) using a Precellys ball mill (DQ2368, Bertin, Montigny-le-Bretonneux, France), followed by extraction using an RNeasy Mini kit (RNeasy Mini Kit QIAGEN, Hilden, Germany; Boutinaud et al., 2008). The amounts of total RNA extracted from milk leukocyte samples were determined using a DeNovix DS-11 Spectrophotometer (DeNovix Inc., Wilmington, DE). Total RNA quality was assessed with a Bioanalyzer (Agilent Technologies, Massy, France). Complementary DNA was generated from total RNA using a SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Gene mRNA levels were measured by quantitative PCR using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA; Herve et al., 2019). The primers used for real-time PCR were as follows: *RPS5*, 40S ribosomal protein S5, (NM_001015531.1, forward primer 5′-GGAACATCAAGACCATTGCCG-3′, reverse primer 5′-GCGTAGGAATTGGAG GAGCC-3′); *PPIA*, cyclophilin A (NM_178320.2, forward primer 5′–GGATTTATGTGCCAGGGTGGTGA-3′, reverse primer 5′-CAAGATGCCAGGACCTGTATG-3′); *NFE2L2*, nuclear factor, erythroid 2 like 2, (NM_001011678.2, forward primer 5′ - AGGACATGGATTTGATTGAC - 3′, reverse primer 5′–TACCTGGGAGATGTTGGCA-3′); *SOD1*, superoxide dismutase 1, (NM_174615.2, forward primer 5′-TGTTGCCATCGTGGATATTGTAG-3′, reverse primer 5′-CCCAAGTCATCTGGTTTTTCATG-3′); *CXCL8*, C-X-C motif ligand 8 or IL8 (NM_173925.2, forward primer 5′-TGGGCCACACTGTGAAAA-3′, reverse primer 5′-GTGGCTGGAGTGGTTATTAG-3′); *IL6*, interleukin 6 (NM_173923.2, forward primer 5′-TGCTGGTCTTCTGGAGTA-3′, reverse primer 5′-GTGGCTGGAGTGGTTATTAG-3′) (Souza et al., 2018). The genes studied in milk leukocytes were expressed relative to the geometric mean of 2 reference genes (*PPIA* and *RPS5*) using a semi-absolute method, as previously reported (Boutinaud et al., 2004).

Mammary epithelium integrity was estimated by measuring the Na^+:K^+ ratio. Milk minerals were measured in whole milk using the ICP-OES method (5110 Agilent Technology, Les Ulis, France), as previously reported (Herve et al., 2023).

To measure milk progesterone concentrations, 2-mL milk samples were collected twice weekly from wk 3 to 12 of lactation. These milk samples were stored at −20°C until progesterone determinations using commercial ELISA kits (Milk Progesterone ELISA, Ridgeway

Science Ltd., Lydney, UK). Progesterone concentrations were used to calculate the percentage of cows in the luteal phase ([progesterone] > 3 ng/mL) and follicular phase ([progesterone] < 3 ng/mL).

Biochemical Analysis: Plasma Metabolites, Haptoglobin, and Redox Status Determination

Plasma glucose, urea, NEFA, and haptoglobin concentrations, ROM, biological antioxidant potential (**BAP**), and ferric reducing antioxidant power (**FRAP**) antioxidant activities were measured using a multiparameter analyzer (Kone Instrument Corp., Espoo, Finland). Glucose was measured with the Glucose GOD-POD commercial kit (Thermo Fisher Scientific, Waltham, MA), urea with a urea commercial kit (Thermo Fisher Scientific), and NEFA with the NEFA-HR commercial kit (Fujifilm WAKO, Mountain View, CA). Plasma haptoglobin determinations were based on measurement of the peroxidase activity of hemoglobin at 37°C by absorption at 600 nm, with the phase Haptoglobin Assay T801 commercial kit (Tridelta Ltd., Maynooth, Ireland). Plasma BAP was measured with commercial BAP Test kit (Diacron Labs S.r.l, Italy). Plasma FRAP determination was based on reducing the TPTZ-Fe^{3+} complex (2,4,6-Tris(2-pyridyl)-s-triazine, for the spectrophotometric determination of Fe, > = 98%; Sigma-Aldrich) to TPTZ-Fe^{2+} at 37°C by absorption at 593 nm (Benzie and Strain, 1996). Plasma ROM was measured using the Fenton reaction at 37°C by absorption at 505 nm, with the dROM Test kit (Diacron Labs S.r.l, Italy). After antioxidant measurement, an oxidative stress index (**OSI**) was calculated as the ratio of ROM on FRAP ($\mathbf{OSI_{FRAP}}$) and as the ratio of ROM on BAP ($\mathbf{OSI_{BAP}}$; Abuelo et al., 2013).

Activity of the glutathione peroxidase (**GSH-Px**) enzyme was measured in plasma and erythrocytes, which were isolated and then stored at −80°C until subsequent analysis (Bernabucci et al., 2005). The measurement of GSH-Px activity in plasma and erythrocyte lysates was based on the amount of NADPH formed by an enzymatic reaction. A solution of 20 m*M* glutathione (**GSH**) and 2 m*M* NADPH formed a GSH-$NADPH_2$ complex; the addition of 3 m*M* H_2O_2 then released NADPH at pH 7.0 measured at 37°C by absorbance at 340 nm after 300 s. This protocol was adapted according to a GSH-Px assay protocol used in pigs (Lebret et al., 2018). These data were transformed into a decimal logarithm.

Plasma calcium concentrations were obtained by ICP-OES (5110 Agilent Technology), with nitric acid (2% vol/vol), the addition of Triton X-100 (0.01% vol/vol), and ultrapure water.

α-Tocopherol was quantified in duplicates from plasma (Chauveau-Duriot et al., 2010). δ-Tocopherol was used as an internal standard to consider potential variations in purification efficiency. Briefly, compounds were purified from plasma through 2 successive hexane extractions after ethyl alcohol deproteinization. The extracts were injected into an Acquity UPLC system (Waters, Saint-Quentin-en-Yvelines, France) using a 150 × 2.1 mm, HSS T3, 1.8-µm column for separation, followed by detection with a photo diode array detector at 295 nm. Quantification was performed relative to the standard curves of these 2 compounds.

Statistical Analysis

All data were analyzed using the lmerTest-package (version 3.1–3, 2020) for a linear mixed-effects models procedure on R Studio (version 1.3.1093, 2009–2020). According to the following statistical model, a type III ANOVA test was used:

$$Y_{ijkl} = \mu + \text{parity}_i + \text{week}_j + (\text{parity} \times \text{week})_{ij} + \text{cow}_k + \text{block}_l + E_{ijkl}.$$

Y_{ijkl} was the analyzed variable; parity_i was the parity (i.e., primiparous vs. multiparous [fixed effect, 1 df]); week_j was the sampling week relative to calving date (fixed effect df depending on the sampling numbers for each analyzed variable); block_l was included as a fixed effect (3 df); cow_k as a random effect; and E_{ijkl} was the residual error. The means and SEM were used to draw the graph, and the contrasts were calculated. Post hoc 2-by-2 comparisons between modalities of parity, weeks, or parity and week interaction were also calculated according to Satterthwaite's method. The threshold of significance were set at $P < 0.05$, and trends were noted at $P \leq 0.10$. Student's *t*-test was used just once when the multiparametric test failed to indicate an interaction between parity and week to show a difference between multiparous and primiparous cows at just one point in the kinetics.

Principal component analysis (**PCA**) based on a correlation matrix was carried out using the R studio FactoMineR package (version 2.4, 2020). Main and nonredundant variables were considered relative to milk yield, milk progesterone, metabolic (NEFA, BHB, glucose, urea, calcium), redox (ROM, BAP, FRAP, vitamin E, plasma, and erythrocytes GSH-Px activity, *NFE2L2* and *SOD1* mRNA) and immune profiles (haptoglobin, white blood cells, cytokine total mean without or with HK *E. coli*, ROS production by classical or regulatory neutrophils with or without TBHP). The management of missing data was necessary to draw this PCA and was ensured by imputation using a 2-dimensional PCA model and a good compromise. Parity, blocks, and weeks of lactation were selected as additional qualitative variables.

Some values were deleted (one value for BAP, one value for haptoglobin, 8 for vitamin E, and one leukocyte RNA) because the assays gave values out of range, or, for RNAs, because of the lack of detection of 18S and 28S ribosomal subunits indicating poor RNA quality.

RESULTS

Milk Yield, DMI, Plasma Metabolites, and Milk Composition

As expected, milk yield and DMI were influenced by both weeks and parity. Between wk 1 and 12 relative to calving, the milk yield progressively increased ($P < 0.001$) and multiparous dairy cows produced more milk than primiparous cows ($P = 0.01$). The mean milk yield between wk 1 and 12 was 34.9 kg/d ± 1.48 versus 25.2 kg/d ± 1.56 in multiparous and primiparous cows, respectively (Figure 1A). Dry matter intake increased until wk 8 of lactation ($P < 0.001$) and was higher in multiparous than primiparous cows ($P < 0.001$, Supplemental Table S2, see Notes). Plasma NEFA concentrations in all cows were higher in wk 2 and 4 after calving compared with other weeks ($P < 0.001$), and plasma BHB was higher between 2 and 12 wk after calving compared with wk −3 ($P < 0.001$). Plasma NEFA and BHB were higher in multiparous than in primiparous cows in wk 4 after calving ($P = 0.01$ and 0.02, respectively; Figure 1C and 1B). Plasma urea fell from wk 2 to 8 ($P < 0.001$) and was higher in multiparous than in primiparous cows ($P = 0.01$, Figure 1D). Plasma glucose concentrations decreased more in wk 2 and 4 after calving than during other weeks ($P < 0.001$), but multiparous cows displayed lower plasma glucose than primiparous cows ($P = 0.01$), particularly in wk −3, 2, and 4 ($P = 0.02$; Figure 1E). Plasma calcium concentrations fell on d 1 after calving compared with other sampling points (day effect, $P = 0.01$). Furthermore, multiparous cows tended to have less calcium in their plasma than primiparous cows ($P = 0.07$, Figure 1F). No differences in the SCS and lactose, protein, and fat concentrations in milk were observed between primiparous and multiparous cows ($P > 0.10$). The milk fat:protein ratio tended to be higher in multiparous than in primiparous cows ($P = 0.06$, Supplemental Table S2). In all cows, the BCS fell after 2 wk of lactation ($P < 0.001$). The BCS tended to be lower in multiparous cows than in primiparous cows ($P = 0.09$). In wk 4, 50% of multiparous cows and 71% of primiparous cows were in the luteal phase; in wk 8 these percentages were 62.5% and 100% and in wk 12 they were 87.5% and 57% in multiparous and primiparous cows, respectively. At a 0.95 level of confidence, the proportions of cows in the luteal phase indicated a difference between primiparous cows and multiparous cows, most of the primiparous cows being in the luteal phase in wk 8 of lactation (critical CI: 2.19 > 1.96; Supplemental Figure S2, see Notes).

Plasma and Erythrocyte Markers of Oxidative Status

Plasma ROM concentrations were higher in wk 2 and 4 than in wk −3 and 8 of lactation ($P < 0.001$, Figure 2A). Plasma ROM also tended to be higher in multiparous cows ($P = 0.09$), especially at wk 8 after calving (parity × week interaction, $P = 0.03$). Plasma FRAP tended to decrease between wk −3, 4, and 8 and to increase in wk 12 ($P = 0.06$). It was unaffected by parity ($P = 0.91$, Figure 2B). Plasma BAP was higher at wk 12 of lactation than in other weeks ($P = 0.01$), and was also unaffected by parity ($P = 0.15$, Figure 2C). Plasma OSI_{FRAP} was higher in wk 2 and 4 than between wk −3 and 8 of lactation ($P < 0.001$). In wk 8, plasma OSI_{FRAP} was higher in multiparous than in primiparous cows (parity × week interaction, $P = 0.07$, Figure 2D). Plasma OSI_{BAP} was higher in wk 2 and 4 than between wk −3 and 8 of lactation ($P < 0.001$). As for plasma OSI_{FRAP}, in wk 8, OSI_{BAP} was higher in multiparous cows than in primiparous cows (parity × week interaction, $P = 0.01$, Figure 2E). Plasma GSH-Px activity in plasma was higher in wk 2 than in wk 8 of lactation ($P = 0.02$) but was not affected by parity ($P = 0.73$, Figure 2F). In wk −3 and 4, erythrocyte GSH-Px activity was higher in multiparous cows than in primiparous cows (parity × week interaction, $P = 0.01$; Figure 2G). Plasma vitamin E (α-tocopherol) concentrations fell between wk −3 and 2, and then increased after wk 4 until wk 12 of lactation ($P < 0.001$, Figure 2H). Plasma α-tocopherol was higher in multiparous cows than in primiparous cows ($P < 0.01$).

Counting and Measuring the Immune Capacities of Immune Cells

Plasma haptoglobin was unaffected by either week, parity, or their interaction ($P > 0.10$, Figure 3A). The white blood cell count rose sharply between wk −3 and 2 and then fell at wk 4 in multiparous cows, whereas it only slightly decreased between wk 4 and 8 in primiparous cows (parity × week interaction, $P < 0.05$, Figure 3B). Lymphocyte levels fell between wk 2 and 4 ($P < 0.001$, Figure 3C) and were unaffected by either parity or the parity × week interaction. To determine if neutrophils were elevated in wk 2, a Student's *t*-test was applied because the parity × week interaction *P*-value was 0.11. The blood neutrophil counts was higher in multiparous cows in wk 2 than in primiparous cows ($P < 0.05$, Figure 3E). Blood eosinophil counts were higher in multiparous cows ($P = 0.03$, Figure 3F), whereas multiparous cows tended to have fewer monocytes before calving than

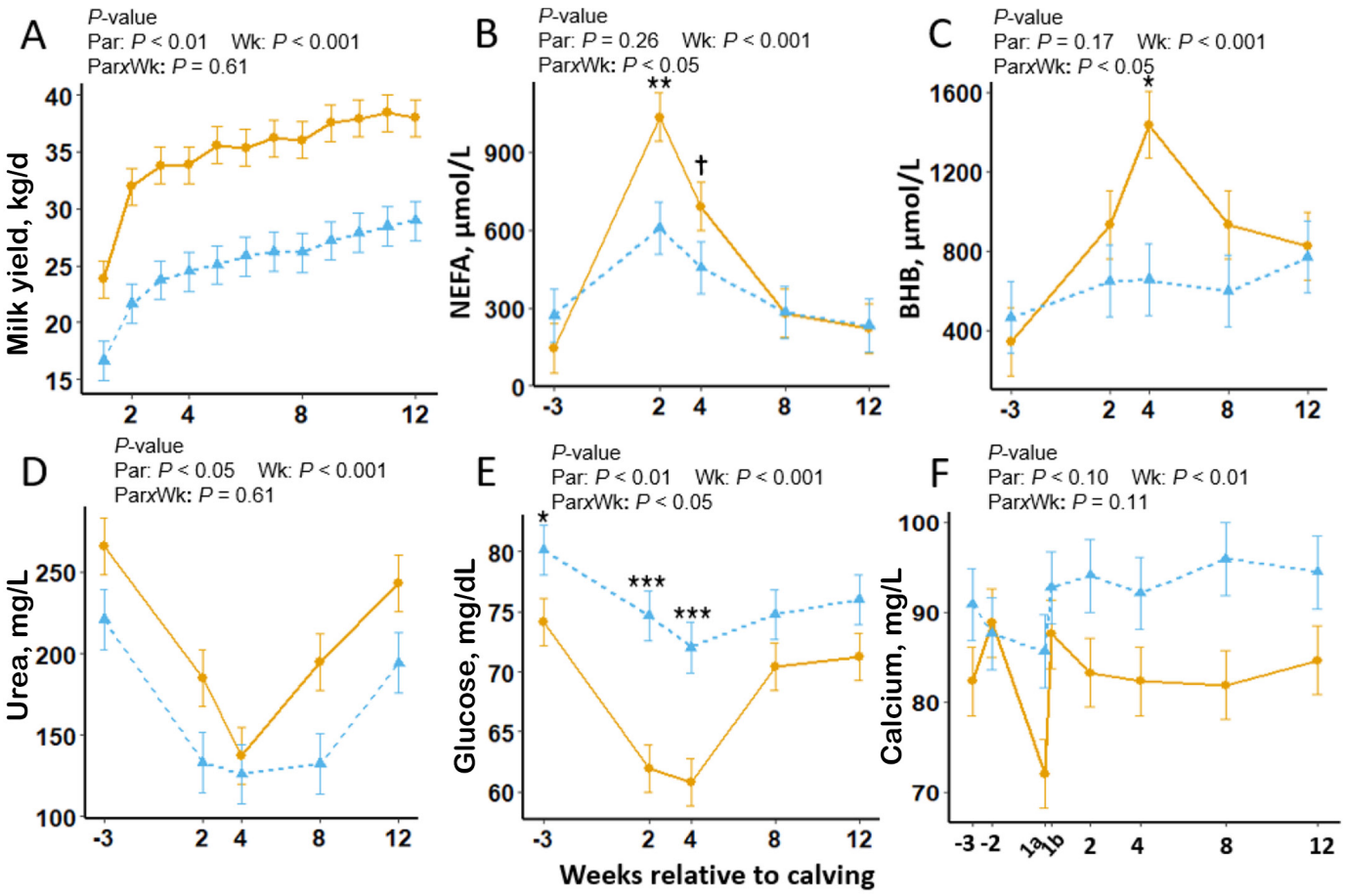

Figure 1. Milk production and plasma biomarkers of metabolic status measured in primiparous (n = 7) and multiparous (n = 8) dairy cows between wk −3 and 12 relative to calving. Milk yield (kg/d; A) from 1 to 12 wk relative to calving. Metabolic biomarkers in plasma, such as nonesterified fatty acids (NEFA; µmol/L; B), BHB (µmol/L; C), urea (mg/L; D), and glucose (mg/dL; E) from −3 to 12 wk relative to calving. Plasma calcium (mg/L; F) from −3 to 12 wk relative to calving, where 1a corresponds to 1 d after calving and 1b corresponds to 3 d after calving. Primiparous cows are represented by the dotted line (▲) and multiparous by the solid line (●). Adjusted means and SEM values are represented as a function of the week relative to calving calculated according to a mixed model. Significant differences between parity (Par) at each week (Wk) and their interaction (Par×Wk) are noted as ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$, and trends are noted at †$P \leq 0.10$. P-values are indicated above the graphs.

primiparous cows ($P = 0.06$, Figure 3D). Parity did not affect lymphocyte and monocyte counts after calving ($P > 0.10$, Figure 3C and 3D).

The percentage of classical neutrophils (corresponding to those not expressing the class II major histocompatibility complex and noted MHCII$^-$) was lower at wk −3 and 4 than at wk 8 and 12 ($P = 0.01$, Figure 4A) and was higher in multiparous cows than in primiparous cows ($P = 0.01$) regardless of week (parity × week interaction, $P = 0.63$). Consistent with this, inverse variations were observed for the percentage of regulatory neutrophils expressing the class II major histocompatibility complex and noted MHCII$^+$ ($P = 0.02$, Figure 4D).

Without TBHP stimulation, classical MHCII$^-$ neutrophils ($P = 0.09$) and regulatory MHCII$^+$ neutrophils ($P = 0.09$) tended to produce less ROS in multiparous cows than in primiparous cows (Figure 4B and 4E). With TBHP stimulation, regulatory MHCII$^+$ neutrophils from multiparous cows produced less ROS than MHCII$^+$ neutrophils from primiparous cows ($P = 0.05$), whereas classical MHCII$^-$ neutrophil ROS production was not affected by parity ($P = 0.42$, Figure 4C and 4F).

A panel of 15 cytokines in plasma was measured following the ex vivo challenge of blood cells with HK *E. coli*. In control blood samples (incubation in the absence of HK *E. coli*), the mean $\log_{10}$ (**MFI**) tended to be lower in multiparous cows than in primiparous cows ($P = 0.05$, Figure 5A). It was also specifically the case for the $\log_{10}$ (MFI) of CCL2, CXCL10, IFNγ, and IL17A ($P < 0.05$). After the ex vivo HK *E. coli* challenge, the mean $\log_{10}$ (MFI) for the 15 cytokines was higher in wk −3 and 8 than in wk 12 of lactation ($P = 0.01$). Between wk −3 and 8 of lactation after the HK *E. coli* challenge, the $\log_{10}$ (MFI) of several plasma cytokines (such as CCL3,

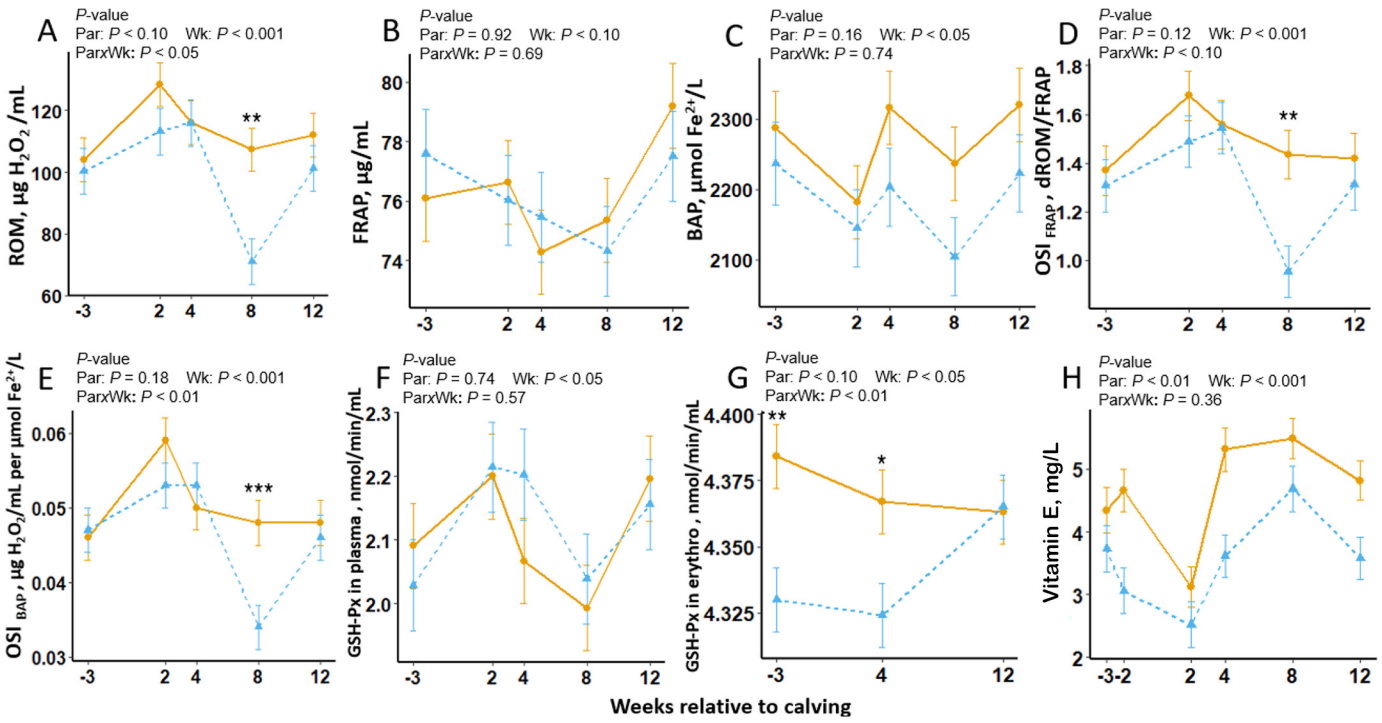

Figure 2. Plasma biomarkers of redox balance and enzymatic activity in plasma or erythrocytes in primiparous (n = 7) and multiparous (n = 8) dairy cows between wk −3 and 12 relative to calving. Reactive oxygen metabolite (ROM; µg H_2O_2/mL; A), ferric reducing antioxidant power (FRAP; µg/mL; B), biological antioxidant power (BAP; µmol Fe^{2+}/L; C), oxidative stress index (OSI_{FRAP}; D), and OSI_{BAP} (µg H_2O_2/mL per µmol Fe^{2+}/L; E) were measured in plasma. The antioxidant enzyme activity of glutathione peroxidase (GSH-Px) was determined in plasma (F) and erythrocytes (erythro, nmol/min/mL; G). Vitamin E (mg/L; H) was measured in plasma. Primiparous cows are represented by the dotted line (▲) and multiparous cows by the solid line (●). Adjusted mean and SEM values are represented as a function of the week relative to calving calculated according to a mixed model. Significant differences between parity (Par) at each week (Wk) and their interaction (Par×Wk) are noted as ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$. P-values are indicated above the graphs.

CXCL10, IL1α, and TNFα) was lower in multiparous cows than in primiparous cows ($P = 0.04$) (Figure 5B).

Expression of Genes Relative to Antioxidant and Inflammation Responses in Milk Leukocytes and Mammary Epithelium Integrity

The expression of 2 antioxidant genes, namely *NFE2L2* and *SOD1,* was higher at wk 4 than wk 12 ($P = 0.06$ and $P = 0.01$ for *NFE2L2* and *SOD1,* respectively, Table 1). *NFE2L2* expression was higher in multiparous cows than in primiparous cows ($P = 0.05$) whatever the week, whereas that of *SOD1* only tended to be higher in multiparous cows than in primiparous cows at wk 4 of lactation (parity × week interaction, $P = 0.06$). The expression of *CXCL8* tended to be higher in multiparous cows than in primiparous cows ($P = 0.09$), but *IL6* expression was unaffected by parity ($P = 0.15$, Table 1). Biomarkers of mammary epithelium integrity were high in wk 4 of lactation and then decreased at wk 8 and 12 after calving (SCC, SCS, MEC concentration, MEC exfoliation, Na^+:K^+ ratio in milk). However, plasma lactose concentrations did not vary over the weeks of our study. Moreover, parity did not affect any measured biomarkers of mammary epithelium integrity ($P > 0.10$, Supplemental Table S3, see Notes).

Integrative Approach Describing the Metabolic, Redox, and Immune Status of Dairy Cows as a Function of Parity and Lactation Week

The 2 main dimensions of the PCA analysis including blood and milk data explained half of all variability, with a percentage of inertia of 27.4% for the first dimension and 22.3% for the second dimension (Figure 6A). Dimension 1 was negatively influenced by calcium, plasma glucose, and neutrophil activity, with or without stimulation. By contrast, dimension 1 was positively affected by the levels of antioxidant, inflammatory gene expression in leukocytes, BHB, and NEFA. Dimension 1 highlighted a negative correlation between metabolic changes (NEFA, BHB) and the functional capacities of neutrophils (classical and regulator neutrophils ROS with or without TBHP; Supplemental Table S4, see Notes). Dimension 2 was influenced by redox biomarkers such as antioxidant capacities (FRAP, BAP, plasma GSH-Px enzyme activ-

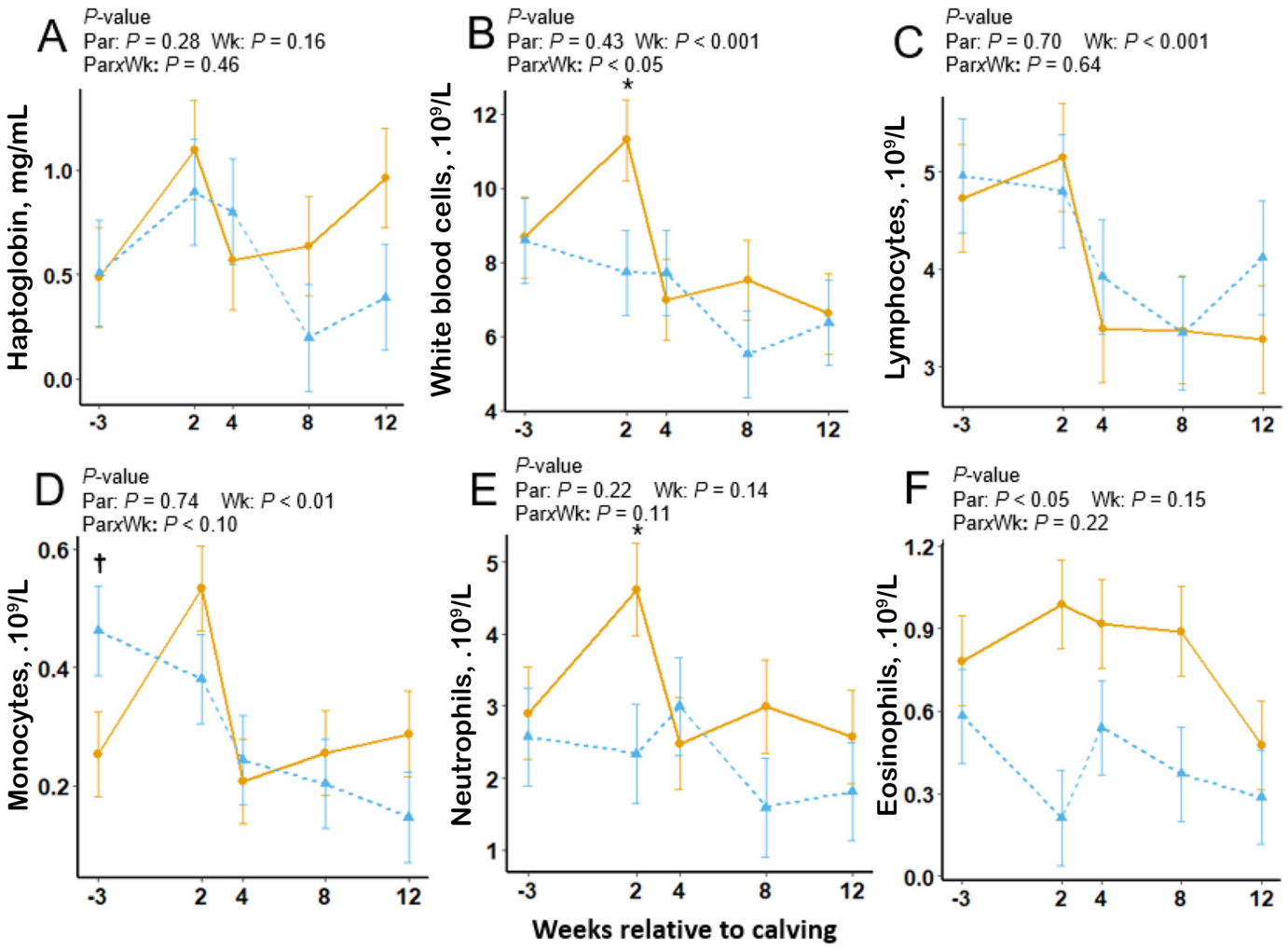

Figure 3. Measurement of health biomarkers in primiparous (n = 7) and multiparous (n = 8) dairy cows with haptoglobin concentrations in plasma and the leukocyte composition in whole blood from wk −3 to 12 relative to calving. Haptoglobin concentrations (mg/mL; A) and the white blood cell count (0.10^9/L; B), lymphocytes (C), monocytes (D), neutrophils (E), and eosinophils (F) measured between −3 and 12 wk relative to calving. Primiparous cows are represented by the dotted line (▲) and multiparous cows by the solid line (●). Adjusted mean and SEM values are represented as a function of the week relative to calving calculated according to a mixed model. Significant differences between parity (Par) at each week (Wk) and their interaction (Par×Wk) are noted as *$P < 0.05$, and trends are noted at †$P \leq 0.10$. P-values are indicated above the graphs. Student's t-test was applied to demonstrate a significant difference in the neutrophil count at wk 2. The Welch two-sample t-test were applied with the t-test function under Rstudio on data for multiparous and primiparous cows (t = 2.30, df = 8.28) with a P-value of 0.049. A sample estimate was the mean of multiparous cows (4.56) and the mean of primiparous cows (2.25).

ity) and ROM. Milk progesterone, white blood cells, and haptoglobin were projected in the same plane. Contrastingly, cytokine production, vitamin E (α-tocopherol), and erythrocyte GSH-Px enzyme activity were located at the other extremity of the axis of dimension 2 (Supplemental Table S4). By only taking account of qualitative variables of parity in primiparous and multiparous cows, the PCA showed that these 2 physiological profiles opposed each other according to dimensions 1 and 2 (Figure 6B). More specifically, the dispersion of qualitative variables (parity, weeks, and blocks) made it possible to visualize on dimension 2 the influence of wk 8 of lactation compared with that of wk 1 and 2, while the influence of weeks −3 and wk 4 was observed in dimension 1. Dimension 1 enabled the differentiation of parities, but dimension 2 showed that although parity influenced the projection of this axis, this was less marked than week of lactation (Figure 6C).

DISCUSSION

The objective of this study was to investigate the links between the metabolic, redox, and immune status in multiparous and primiparous dairy cows during the last

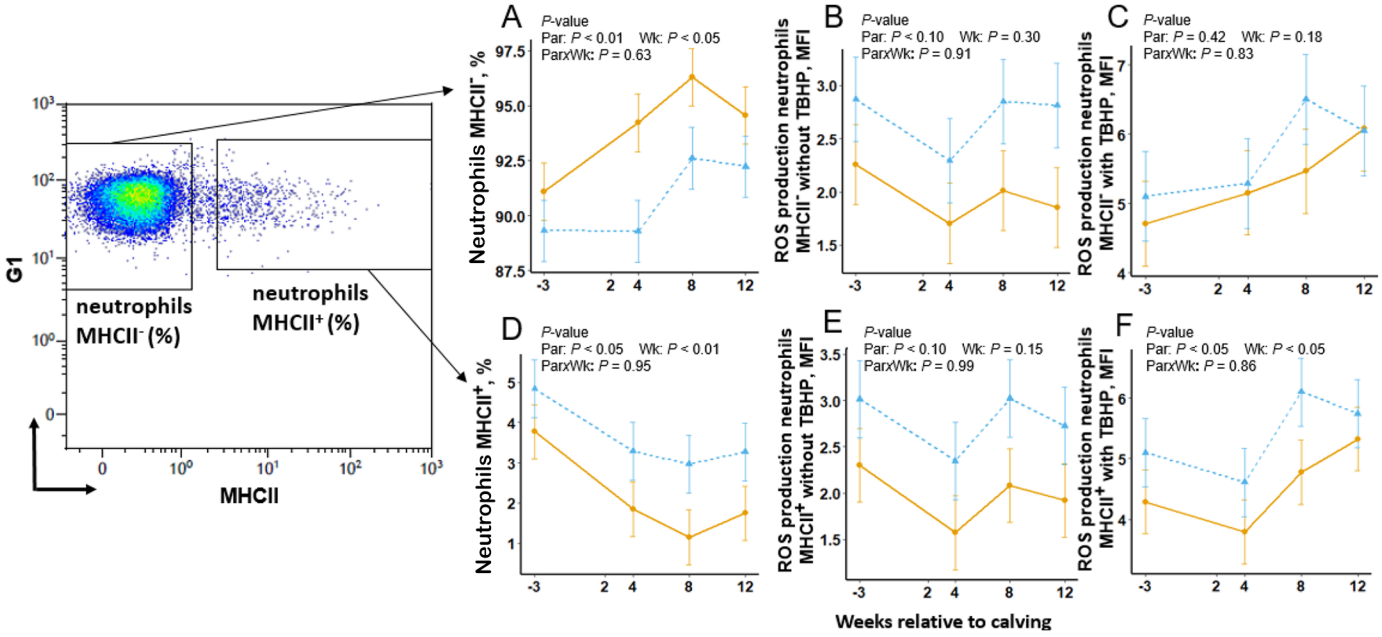

Figure 4. Flow cytometry identification of 2 types of neutrophils isolated from blood and measurement of their intracellular reactive oxygen species (ROS) production with or without ex vivo stimulation in primiparous (n = 7) and multiparous (n = 8) dairy cows between weeks −3 and 12 relative to calving. Percentage of neutrophils without class II major histocompatibility complex ($MHCII^-$; A) and class II major histocompatibility complex neutrophils ($MHCII^+$; D). Mean fluorescence intensity (MFI) measurement of intracellular ROS production by flow cytometry of $MHCII^-$ neutrophils without (B) or with (C) tert-butyl hydroperoxide (TBHP) stimulation. Measurement of intracellular ROS production of $MHCII^+$ neutrophils without (E) or with (F) TBHP stimulation. Primiparous cows are represented by the dotted line (▲) and multiparous cows by the solid line (●). Adjusted mean and SEM values are represented as a function of the week relative to calving calculated according to a mixed model. Significant differences between parity (Par), week (Wk) and their interaction (Par×Wk) are noted above the graphs.

3 wk of gestation and the first 12 wk of lactation. The second aim was to understand the potential links between these statuses by observing differences in physiological profiles between primiparous and multiparous cows over the same period.

Metabolic Status that Differed Between Parities at Early Lactation with Multiparous Cows Mobilizing More of Their Body Reserves

In our study, milk production increased gradually during the first 12 wk of lactation, with multiparous cows producing on average 9.2 kg more milk than primiparous cows. Relative to this increased milk production, DMI was also higher in multiparous cows than in primiparous cows. This was expected and partly related to a higher capacity of ingestion for multiparous (Leclerc, 2008). As also observed by others, and compared with primiparous cows, multiparous cows had higher plasma concentrations of NEFA (on wk 2), BHB (on wk 4), and urea, which indicate a greater body reserve mobilization despite a similar fall of BCS. The BCS we recorded in this study were low compared with published recommendations (Roche et al., 2009) whatever the parity, likely due to the genetic particularity of this herd, or to measurement bias, or both (Gasselin et al., 2020). However, these low BCS were not atypical when compared with other dairy herds (Cattaneo et al., 2023). Multiparous cows had a tendency toward lower plasma calcium at d 1 and generally lower plasma glucose concentrations during the 12-wk period (Santos et al., 2004; Wathes et al., 2007; Yehia et al., 2020).

Yet despite their greater solicitation of energy metabolism, multiparous cows did not exceed the thresholds indicative of metabolic disease such as plasma NEFA >1,000 µmol/L, plasma BHB >1,400 µmol/L or plasma calcium <84 mg/L (Chapinal et al., 2012; McArt et al., 2013; LeBlanc, 2020). At the same time, the SCC in milk was lower than 200,000 cells/mL, indicating a lack of any mammary infection (Schukken et al., 2003).

According to the Indicators Chosen for our Study, Multiparous Cows Displayed Increased Redox Metabolism

Because of their higher milk production, greater oxidative stress was expected in multiparous than primiparous cows (Song et al., 2016). We therefore evaluated redox status as a function of parity in healthy cows. Oxidative stress results from an imbalance in the redox

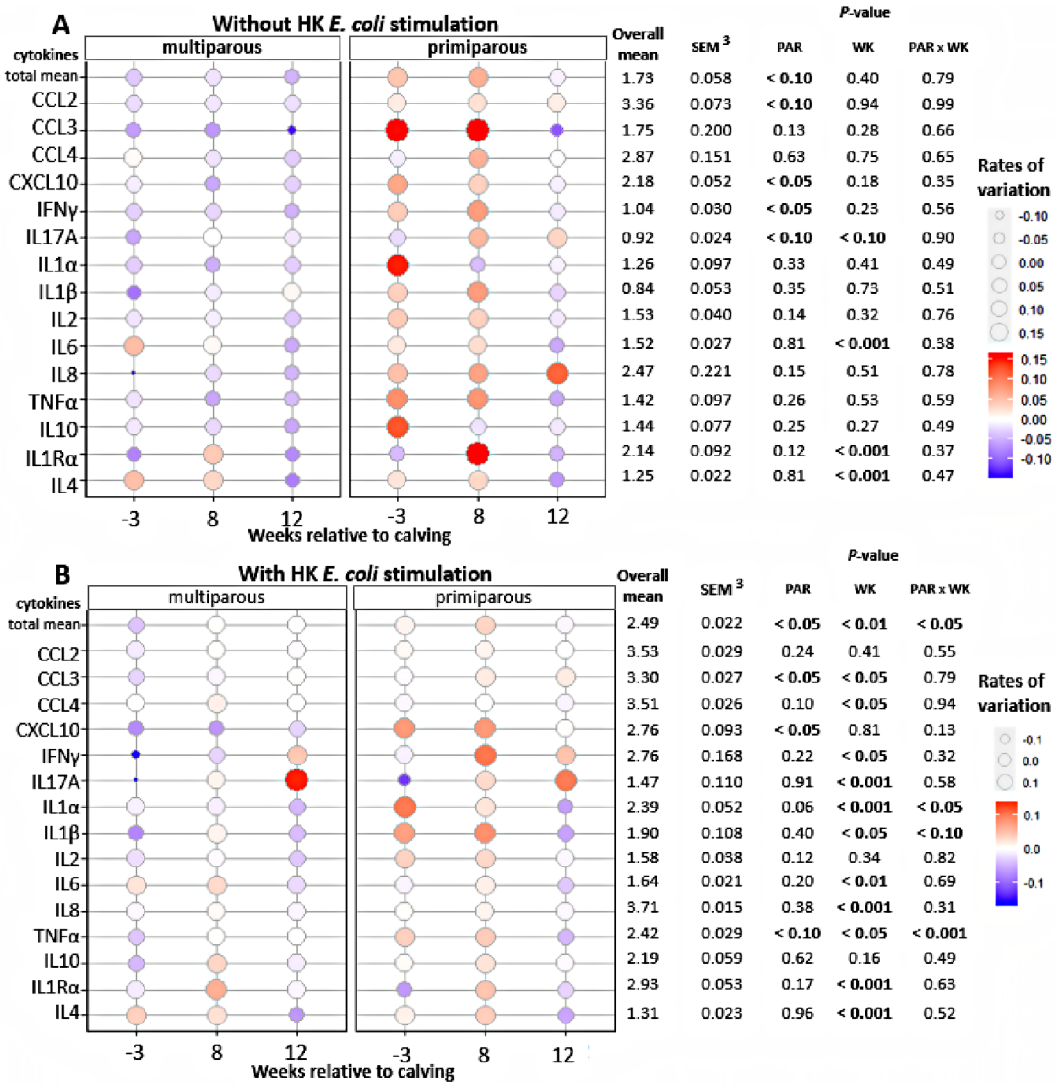

Figure 5. Measurement of 15 cytokines in plasma expressed in median fluorescence intensities (MFI) after an ex vivo heat-killed (HK) *Escherichia coli* challenge in primiparous (n = 7) and multiparous (n = 8) dairy cows between wk −3 and 12 relative to calving. Without HK *E. coli* stimulation (A) and with HK *E. coli* stimulation (B). Multiparous cows are represented on the left on the panel and primiparous cows on the right. The rates of variation for each cytokine were calculated to represent the difference between primiparous and multiparous cows as a function of the challenge and the week of lactation. The rates of variation are represented on the heat maps to observe the effect of parity according to the challenges with or without stimulation. The formula for the rate of variation was calculated as follows: Rate of variation = (MFI mean − overall mean)/overall mean. Probability: PAR = effect of parity, primiparous vs. multiparous; WK = week effect, wk 4, 8, and 12 relative to calving; PAR × WK = interaction between parity and week. The overall means and SEM were calculated for each cytokine. Bolding indicates *P*-values < 0.10.

status of an organism, typically due to an increase in the production of reactive oxygen species that exceeds the body's antioxidant defenses. For example, during oxidative stress, superoxide radicals, hydrogen peroxide, and hydroxyl radicals are elevated and are associated with low enzymatic (e.g., glutathione peroxidase) or nonenzymatic (e.g., vitamin E) antioxidant capacities (Miller et al., 1993). As also previously observed in the literature, the peripartum period was followed by an increase in ROM and variations in redox status related to metabolic status (Castillo et al., 2005; Invernizzi et al., 2019). In our study, both primiparous and multiparous cows experienced oxidative stress during the onset of lactation compared with the last 3 wk of gestation, with higher ROM and OSI values in wk 2 and 4, evaluated from FRAP or BAP (Benzie and Strain, 1996; Abuelo et al., 2013; Gong and Xiao, 2016; Hejel et al., 2021). The lack of difference between primiparous and multiparous cows for oxidative stress was previously reported during the first 4 wk of lactation (Yehia et al., 2020). However, we observed a later effect of parity: Plasma ROM and OSI were still high after 8 wk of lactation in

multiparous cows, whereas their levels fell in primiparous cows.

We further analyzed the redox status of the cows by determining GSH-Px activity in either plasma or erythrocytes. The further analysis of the redox status of the cows showed that probably in response to oxidative stress, plasma GSH-Px activity increased at the onset of lactation as previously suggested (Bernabucci et al., 2002). In line with a previous study, parity did not affect plasma GSH-Px activity (Schuermann et al., 2019), although we observed an effect of parity on GSH-Px activity in blood erythrocytes, with higher activity in multiparous cows than in primiparous cows at wk −3 and 4 relative to calving. This higher antioxidant capacity in multiparous cows was accompanied by higher plasma vitamin E (α-tocopherol) levels, which could have resulted from higher ingestion in multiparous cows that induced more vitamin E availability in these cows. Indeed, it has already been demonstrated that plasma α-tocopherol levels are quantitatively closely linked to intake in dairy cows (Calderón et al., 2007). Moreover, an injection of vitamin E and selenium was shown to induce an increase in GSH-Px activity in dairy heifers (Hoffman et al., 1978). According to the literature, the increased GSH-Px activity in erythrocytes in our study could have been due to the higher plasma levels of vitamin E, which is lipophilic and close to the cell membrane (Wang and Quinn, 1999; Atkinson et al., 2021). Vitamin E may therefore play a synergistic role with cell GSH-Px activity in reducing lipid peroxidation and H_2O_2 (Liao et al., 2022).

As well as being more strongly expressed in milk leukocytes during early lactation, *NFE2L2* and *SOD1* (2 genes involved in regulating the oxidative response) were also more strongly expressed in milk leukocytes from multiparous cows. This was consistent with the increased expression of genes encoding other antioxidant enzymes (GSH-Px and thioredoxin reductase) in the mammary tissue of cows during early lactation (Aitken et al., 2009). The literature assumes that in a cell, *NFE2L2* (an antioxidant gene transcription factor) may be activated by the increase in H_2O_2 to resolve the local oxidative stress related to high milk yield in multiparous cows (de Freitas Silva et al., 2018; Ayemele et al., 2021).

Overall, evaluation of the redox status indicates that compared with primiparous cows, multiparous cows had a higher production of ROM accompanied by higher antioxidant capacities: more vitamin E in the blood, higher antioxidant enzyme activity in erythrocytes, and higher antioxidant gene expression levels in milk leukocytes.

Transient Systemic Inflammation at the Onset of Lactation More Pronounced in Multiparous Cows than Primiparous Cows

The metabolic status of cows in early lactation may influence their immune status, as previously observed (Deng et al., 2021), and inflammation may also affect metabolic status (Horst et al., 2021). We first evaluated inflammatory status by determining plasma haptoglobin concentrations and differential cell counts in blood. In both multiparous and primiparous cows, plasma haptoglobin appeared to increase at wk 2 when compared with the last 3 wk of gestation. Inflammation was observed in almost all cows during the transition period (Horst et al., 2021). Parity did not affect haptoglobin in our study, which is consistent with other findings in the literature (Cattaneo et al., 2023). However, associated with the wk 2 increase in haptoglobin, a transient increase in white blood cells was only observed in multiparous cows and was attributed to a rise in neutrophil levels. The maximum of white blood and neutrophil cell counts in multiparous are close to the thresholds (12×10^9/L and $4 \times$

Table 1. Abundance of antioxidant mRNA (*NFE2L2* and *SOD1*) and immune function mRNA (*CXCL8* and *IL6*) determined by real-time quantitative RT-PCR in milk isolated leukocytes from wk 4 to 12 relative to calving in primiparous (n = 7) or multiparous (n = 8) dairy cows

	Primiparous				Multiparous				*P*-value[1]		
Item[2]	4	8	12	SEM	4	8	12	SEM	PAR	WK	PAR × WK
NFE2L2	6.15	6.17	5.39	0.342	7.14	6.38	6.29	0.322	<0.05	<0.10	0.41
SOD1	7.49[b]	7.72	7.41	0.171	8.09[a]	7.95	7.21	0.161	0.17	<0.05	<0.10
CXCL8	8.32	8.68	8.77	0.514	9.67	9.86	9.58	0.486	<0.10	0.71	0.73
IL6	5.87	5.73	5.69	0.356	6.91	6.20	5.92	0.336	0.15	0.13	0.37
Mean of reference genes[3]	5.16	4.93	4.96	0.190	4.60	4.61	4.99	0.179	0.13	0.54	0.28

[a,b]Mean values in the same row showing a difference ($P < 0.05$) or tendency ($P < 0.10$) regarding the interaction between parity and week relative to calving.

[1]Probability: PAR = effect of parity, primiparous vs. multiparous; WK = week effect, wk 4, 8, and 12 relative to calving; PAR × WK = interaction between parity and week.

[2]Milk samples were collected on wk 4, 8, and 12 after calving; then leukocytes from milk were prepared, and analyses of the gene expression of *NFE2L2* (nuclear factor erythroid-2-related factor 2), *SOD1* (superoxide dismutase 1 [Cu-Zn]), *CXCL8* (interleukin 8), and *IL6* (interleukin 6) were made, using *RPS5* (ribosomal protein S5) and *PPIA* (cyclophilin A) as reference genes.

[3]Decimal logarithm of the geometric mean of *RPS5* (ribosomal protein S5) and *PPIA* (cyclophilin A) genes.

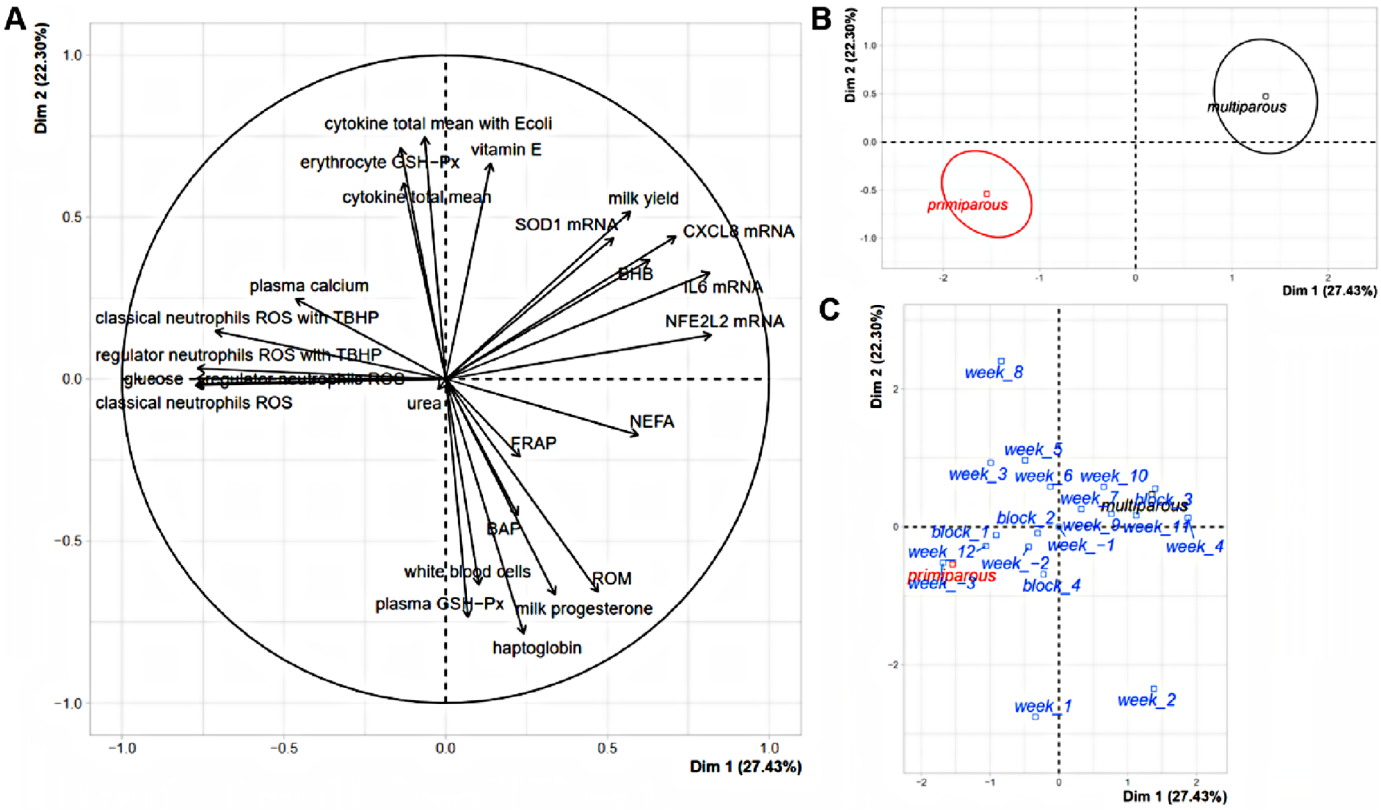

Figure 6. Principal component analysis (PCA) of metabolic status, redox, and immune balance parameters in dairy cows in early lactation according to 3 qualitative variables (week, parity, and block). (A) Representation of PCA variables of the first dimension (Dim 1; 27.43% inertia) and second dimension (Dim 2; 22.30% inertia). Blood and milk biomarkers were grouped on the PCA to demonstrate and affirm a link between the biomarkers of different statuses during a longitudinal experiment. (B) Representation of the parity qualitative variable only according to primiparous and multiparous dairy cows only. (C) Representation of the dispersion of qualitative variables influencing this PCA (week, parity, and block). NEFA = plasma nonesterified fatty acid; BHB = plasma β-hydroxybutyrate; *NFE2L2* mRNA = nuclear factor erythroid-2-related factor 2 in milk leukocytes; *SOD1* mRNA = superoxide dismutase 1 [Cu-Zn] in milk leukocytes; *CXCL8* mRNA = interleukin-8 in milk leukocytes; *IL6* mRNA = interleukin-6 in milk leukocytes; FRAP = plasma ferric reducing antioxidant power; ROM = plasma reactive oxygen metabolite; BAP = plasma biological antioxidant potential; plasma GSH-Px = plasma glutathione peroxidase activity; erythrocyte GSH-Px = erythrocyte glutathione peroxidase activity. It was necessary to manage missing data to draw this PCA, and this was achieved by imputation using a 2-dimensional PCA model and applying a good compromise.

10^9/L respectively; Jain, 1993), indicating a mild inflammation in multiparous cows. The blood neutrophil count observed in wk 2 in multiparous cows agreed with the findings of a previous study (Meglia et al., 2001).

Throughout the experiment, and as observed by others, blood eosinophil counts were higher in multiparous cows than in primiparous cows, probably because of the increased exposure to parasites related to age (Greatorex, 1957; Vallejo-Timarán et al., 2020).

The Functional Capacities of Neutrophils and Cytokines More Impaired in Multiparous Cows than in Primiparous Cows

The further characterization of the neutrophil population by flow cytometry analysis enabled a distinction between the recently described regulatory ($MHCII^+$) and classical ($MHCII^-$) neutrophils (Rambault et al., 2021). This analysis showed, for the first time, that multiparous cows had a higher percentage of classical neutrophils and a lower percentage of regulatory neutrophils than primiparous cows at the onset of lactation. Regulatory neutrophils are able to reduce lymphocyte T proliferation in vitro (Rambault et al., 2021), and their presence correlates with T-cells in the milk of cows suffering from subclinical mastitis, and not only in blood (Rambault et al., 2023). The lower percentage of regulatory neutrophils in multiparous cows than in primiparous cows could translate into a decreased capacity to modulate adaptive immunity in multiparous cows during early lactation (Rambault et al., 2021).

Another approach to determining the immune status of cows was to evaluate the functional capacities of immune cells using 2 types of ex vivo tests. The first of these consisted in measuring ROS production by $MHCII^-$ and $MHCII^+$ neutrophils, with or without TBHP stimula-

tion. Tert-butyl hydroperoxide is known to destabilize membrane lipids in all neutrophils; this creates lesions that induce the production of intracellular ROS (van der Zee et al., 1985; Moya et al., 2008). This test showed that after TBHP stimulation, regulatory neutrophils produced less ROS in multiparous cows than in primiparous cows. Moreover, without TBHP stimulation, both classical and regulatory neutrophils tended to produce less ROS in multiparous cows than in primiparous cows, indicating weaker functional capacities of neutrophils in multiparous cows. The second ex vivo test consisted in measuring cytokine production by blood cells with or without stimulation by HK *E. coli*. In both cases, a tendency toward lower total mean cytokine production and significantly lower levels of pro-inflammatory cytokines and chemokines were measured in blood cells from multiparous cows than from primiparous cows. However, these results need to be confirmed given that they contrasted with those of a previous study, which showed that activated immune cells from multiparous cows produced more TNF-α and nitric oxide than those from primiparous cows (Lessard et al., 2004). Altogether, these results showed that immune response of primiparous cows is stronger than the one of multiparous with or without stimulation. This may be related to the lower peripartum inflammation disturbing less the immune response during lactation for primiparous than for multiparous cows. This indicates that multiparous cows may be less able to respond effectively to a pathogen attack than primiparous cows.

No Major Local Inflammation Showed that Mammary Epithelium Integrity Was Preserved in Both Multiparous and Primiparous Cows.

We further investigated the immune capacity of cows by analyzing the expression of 2 genes encoding cytokines in milk leukocytes. Despite the lack of a parity effect on *IL6* mRNA levels, a tendency toward higher *CXCL8* mRNA levels suggested limited local inflammation in multiparous cows when compared with primiparous cows. To further characterize inflammatory status in the mammary gland, we evaluated epithelium integrity because it has been shown that inflammation can induce its loss (Horst et al., 2021). During our study, mammary epithelium integrity, monitored by measuring MEC exfoliation, the milk Na^+:K^+ ratio and blood lactose, was gradually restored after calving, as previously observed elsewhere (Gasselin et al., 2020). No effect of parity on these variables was observed. To conclude, at the local scale of the mammary gland, parity did not appear to induce major local inflammation, even in multiparous cows that produced more milk than primiparous cows.

Linking Energy Metabolism, Redox, and Immune Status to Explain Variations as a Function of Parity or Week of Lactation

This global data analysis showed that certain variables, such as metabolic biomarkers (NEFA, BHB, glucose, and calcium), neutrophil ROS production, and antioxidant and cytokine gene expression in milk leukocytes were highly correlated according to the first dimension, which described the main differences linked to parity. Week −3 and wk 4 were located at the opposing ends of this axis, indicating that dimension 1 was related to modifications between a lactating phase and gestational phase. This axis may indicate a change in the partitioning of nutrients between pregnancy and lactation (Bauman and Currie, 1980). This reinforces the idea previously mentioned that the availability of glucose and calcium, and elevated NEFA or BHB levels may affect neutrophil functions in the blood (LeBlanc, 2020). In addition, dimension 1 allowed us to observe a correlation between metabolic biomarkers and the expression of genes related to antioxidant and immune functions in milk leukocytes, these variables being diametrically opposed to those of ROS production by circulating neutrophils. This might indicate that the same forces do not drive the response of immune cells at the systemic and local levels. This is in line with the reduced leucocyte functions in a milk environment as previously reported (Dosogne et al., 2001; Denis et al., 2006). From the result of this PCA, multiparous cows may appear to have an energy profile that promoted milk synthesis and a local immune response, whereas primiparous cows may exhibit a systemic ability of immune cells to be activated. These 2 distinct profiles imply different energy needs, and distinct sensitivities to pathologies during early lactation.

According to dimension 2, variables other than those in correlation with dimension 1 also made it possible to distinguish primiparous and multiparous cows. This dimension mainly highlighted the variations between wk 2 and 8. The variables in correlation along this second axis were related to biomarkers for redox balance (plasma ROM, FRAP, BAP and GSH-Px) and inflammation (white blood cells, haptoglobin). Haptoglobin did not vary with parity or week, but analysis was related to the redox status and white blood cell count. The effects of peripartum on inducing oxidative stress (Castillo et al., 2005) and inflammation (Odegaard and Chawla, 2013) have long been known. Others have shown that OSI in plasma, established with ROM and either FRAP or BAP, was correlated with plasma GSH-Px activity to resolve the redox balance (Sharma et al., 2011; Chen et al., 2022). It should be noted that a few weeks after calving in our study, one element disrupted the redox status in wk 8. Indeed, redox status differed according to parity

in only wk 8 of lactation, where 100% of the primiparous cows were in the luteal phase. This explanation is reminiscent of the fact that the luteal phase may reduce the oxidant and antioxidant response (Aydilek et al., 2014). The potential link between the lower oxidative stress index and GSH-Px activity in plasma and the ovarian cycle is original and may result of our longitudinal design over 12 wk, whereas other studies closer to calving did not observe this (Bernabucci et al., 2002; Abuelo et al., 2013; Elischer et al., 2015). Consequently, in plasma, we cannot clearly state that the difference observed between primiparous and multiparous cows with respect to redox status and GSH-Px activity was entirely due to parity. Finally, vitamin E (α-tocopherol) and GSH-Px activity in erythrocytes were correlated on the PCA. Vitamin E can react with the antioxidant reaction of GSH-Px, and erythrocytes contain an antioxidant enzyme to protect oxygen transport (Liao et al., 2022).

Nevertheless, we were able to highlight that parity and week of lactation are elements that must be taken into account in studies of energy metabolism, redox, and immune status, while pointing out that other variables, such as reproduction, may influence the results.

CONCLUSIONS

In healthy cows, variations in metabolic, redox, and immune status may be dependent on parity, week of lactation, or both. Our hypothesis that multiparous cows would have higher metabolic solicitations to produce milk and more oxidant biomarkers was confirmed, and we showed that redox metabolism was stronger in multiparous cows. However, in our study, multiparous cows displayed lower functional capacities of their circulating immune cells than primiparous cows. At the same time, these characteristics may depend on the week of lactation. Whatever the parity, cows experienced more inflammation and oxidative stress without symptoms of illness in wk 2 and 4, which may be linked to calving or the ovarian cycle. During the early lactation period, the physiological profile of dairy cows is complex, and there is no single physiological profile of a healthy cow. It is necessary to understand the physiological requirements of primiparous and multiparous cows to ensure management and nutrition that are adapted according to parity.

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Nonstandard abbreviations used: BAP = biological antioxidant potential; FRAP = ferric reducing antioxidant power; GSH = glutathione; GSH-Px = glutathione peroxidase; HK = heat-killed; MEC = mammary epithelial cells; MFI = median fluorescence intensities; MHCII = class II major histocompatibility complex; NEFA = nonesterified free fatty acids; OSI = oxidative stress index; OSI_{BAP} = OSI with ROM:BAP ratio; OSI_{FRAP} = OSI with ROM:FRAP ratio; PAR = effect of parity; PCA = principal component analysis; ROM = reactive oxygen metabolite; ROS = reactive oxygen species; TBHP = tert-butyl hydroperoxide; WK = week effect.

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