The effects of intramammary cephalosporin antibiotic treatment at dry off on peripheral blood and milk mononuclear cell mitochondrial enzyme activity in Holstein cows

By

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CHAPTER ONE – REVIEW OF LITERATURE

INTRODUCTION

It is well understood that the organelle in eukaryotic cells responsible for metabolism and energy production is the mitochondrion; often referred to as the "powerhouse of the cell". Mitochondria possess their own circular genome known as mitochondrial DNA (**mtDNA**). Mitochondrial DNA is combined with nuclear DNA to code for protein complexes that are exclusive to mitochondria. The circular genome found within mitochondria (mtDNA) is a characteristic that this organelle shares with bacteria; meaning that both bacteria and mitochondria possess circular genomes. This similarity, among others has led scientists to believe that mitochondria arose from a bacterial ancestor due to an endosymbiotic relationship that was established when a mitochondrion was engulfed by a eukaryotic progenitor. Lane and Martin (2010) describe this as "endosymbiotic theory". Mitochondria have further been described as dynamic organelles that influence a variety of cellular processes, and their dysfunction has shown to influence ovarian function, neural tissue function, and various disease states across species (Nunnari and Suomalainen, 2012). Within the dairy industry mitochondrial oxygen consumption, quantification, biogenesis, gene expression, and enzyme activity have shown to play a crucial role in reproduction (Ferreira *et al*., 2016), milk production (Brown *et al*., 1988; Schultz *et al*., 1994; Alex *et al*., 2015), and calf growth potential (Niesen and Rossow, 2019). The common ancestry that was likely shared between mitochondria and bacteria implies that cephalosporin antibiotics may negatively affect mitochondrial enzyme activity. Cephalosporins act on enzyme complexes within bacteria, which may be similar to those found in mitochondria within mammalian cell types. However, it is unclear if cephalosporins used within the dairy industry inhibit mitochondrial function. The assessment of mitochondrial function combined with cow

health and performance records may provide key insights to better understand how mitochondrial function influences different aspects of bovine physiology. Finally, if cephalosporins inhibit mitochondrial function (i.e. enzyme activity), it will be necessary to determine the lasting impacts this interaction has on milk production and quality, which may ultimately lead to changes in disease management practices.

MITOCHONDRIA INFLUENCE ANIMAL HEALTH & PERFORMANCE

Mitochondria are the primary energy producers within eukaryotic cells that generate adenosine triphosphate molecules (**ATP**) derived from glucose, fatty acids, and amino acids (Rambold and Pearce, 2018). Molecules of ATP are generated by the function of enzymatic complexes located within the inner mitochondrial membrane (**IMM**) and the mitochondrial matrix. Enzymes associated with the tricarboxylic acid cycle (**TCA**) are housed within the mitochondrial matrix and function to supply electron carrier molecules (NADH and $FADH_2$) to the respiratory chain (**RC**, Lanza and Nair, 2009). Enzymatic complexes: complex I (**CI**), complex II (**CII**), complex III (**CIII**), complex IV (**CIV**) and complex V (**ATP synthase**) are housed in the IMM. Complexes I - IV are commonly referred to as the electron transport chain (**ETC**) or RC. Once electrons are supplied to the RC a series of coupled reactions produces a proton gradient that is utilized by ATP synthase to phosphorylate molecules of ADP, generating molecules of ATP; this process is known as oxidative phosphorylation.

When the proton gradient is formed there is an electric charge potential of approximately 175 mV across the IMM (Acetoze *et al*., 2016). If the electric charge potential is not utilized by ATP

synthase to produce molecules of ATP, protons and electrons will leak through the IMM generating heat and reactive oxygen species (**ROS**, Ramsey *et al*., 2004). The ROS are produced primarily within mitochondria and are formed when molecular oxygen receives a single electron from the RC (Valko *et al*., 2007). It was initially thought that ROS induced cellular damage and was associated with loss of cellular function; implying that all ROS production was unfavorable and was characteristic of mitochondrial dysfunction (Sordillo and Aitken, 2009). However, more recent evidence demonstrated that mitochondrial ROS act as signaling molecules, which are essential to normal cellular function (Sena and Chandel, 2012). This has been demonstrated in T lymphocytes where mitochondrial ROS generated from CI and CIII initiate T cell activation (Nunnari and Suomalainen, 2012; Sena *et al*., 2013; Mehta *et al*., 2017). Furthermore, ROS are involved in the release of cytochrome c from the IMM, which signals apoptosis (Guo *et al*., 2013). These examples show that mitochondria serve as sensory beacons within in a cell and that their function/dysfunction has influence on a range of cellular processes.

Mitochondrial research has involved various tissues such as systemic blood, skeletal muscle, cardiac muscle, liver, and neural tissue to identify the role mitochondria have in animal performance, cell function, disease states, and whole-body metabolism. The oxidative capacity of mitochondria varies across tissues due to differences in energetic demands. Fernàndez-Vizarra *et al*. (2011) validated this concept through the comparison of mitochondrial enzyme activities of heart, brain, liver, skeletal muscle, and kidney tissue samples from male (n = 6) and female (n = 6) rats. Citrate synthase (**CS**) and CIV enzyme activities were used to assess mitochondrial function as the activity of these enzymes would be correlated with an increase in cellular oxidative capacity. Differences in enzyme activity across tissues were observed with heart

mitochondrial enzyme activity of CS and CIV being greater when compared with all other tissues. Furthermore, the data show that the enzyme activity of CS and CIV did not differ between neural tissue and skeletal muscle. However, skeletal muscle showed increased CS and CIV activity when compared with liver and kidney tissue samples. These results demonstrate that mitochondria function differently across tissues to accommodate for energetic demands.

Bovine research evaluated hepatic mitochondrial oxygen consumption to determine the relationship between mitochondrial respiration and animal performance (Brown *et al*., 1988; Lancaster *et al*., 2014). Measurements of mitochondrial oxygen consumption performed on liver homogenates were thought to be representative of whole-body metabolism, due to the liver's central role in metabolism, particularly in ruminants. The relationship between mitochondrial oxygen consumption and cattle performance was first studied by Brown *et al*. (1988). This study included two different groups of cows and utilized hepatic mitochondrial oxygen consumption to assess mitochondrial function. Group one consisted of lactating (n = 10) Holstein cows. Group two consisted of three different breeds of cattle (Angus, Brangus, and Hereford) with a sample size of 13 cows for each breed. No differences in mitochondrial oxygen consumption were observed between cattle in group two. It was reported that the small variation in performance characteristics found within the beef breeds of cattle was likely the reason for this result. Interestingly, group one mitochondrial respiration rates were correlated with milking traits in Holstein cows, which implied that lactation performance could be predicted if the effects of maternal cytoplasmic inheritance were considered. In agreement with Brown *et al*. (1988), Schutz *et al*. (1994) reported that mtDNA sequences were associated with milk production, and reproduction when mtDNA of 36 different maternal lineages were sequenced from whole blood.

However, the association between mtDNA sequence and cow health remained unclear (Schutz *et al*., 1994). Together these reports indicate that mitochondrial enzyme complex structure and function (measured by oxygen consumption and the combination of nuclear DNA with mtDNA) may affect the organelles ability to efficiently produce energy, suggesting that a desirable nuclear and mitochondrial genome may be associated with metabolically efficient mitochondria, which would then be associated with lactation performance.

The relationship between mitochondrial quantity and lactation performance was elucidated by Alex *et al*. (2015), who found that milk yield differences between 1x and 4x milking were associated with changes in mitochondrial number within mammary tissue. Holstein cows (n = 6) were enrolled prior to parturition and two quarters of the mammary gland were milked 1x daily and the other two quarters were milked 4x daily. Ipsilateral quarters (2) were the experimental unit of interest. Mammary tissue biopsies were taken at 15, 60, 120, and 230 DIM and mitochondria were quantified from mammary tissue biopsies. Quarters that were milked 4x daily increased milk production by a factor of two throughout the entire lactation when compared with quarters that were milked 1x daily. Mammary mitochondrial numbers were highest (approximately 5 to 10 more mitochondria per cell) throughout the entire lactation in quarters that were milked 4x daily and mitochondrial numbers per cell decreased as milk production decreased in both groups. This study demonstrates that mitochondrial quantity is correlated with lactation performance and mammary tissue function.

Mitochondrial function has been used to evaluate cell function and animal performance. Due to the invasiveness of tissue biopsies and the laborious methods required by oxygen consumption measurements using a Clark electrode; mitochondrial research has been limited to small, controlled trials that have merit, but lack inference (Rossow *et al*., 2018). These limitations led Niesen and Rossow, (2019) to introduce a novel method to assess mitochondrial function across large populations of cattle. The method needed to be minimally invasive to facilitate high sample throughput, so large-scale studies evaluating mitochondrial function could be performed. Previous research by Rustin *et al*. (1994) compared intact cellular respiration and mitochondrial enzyme activity in human lymphocytes, muscle, liver, and heart homogenates to identify suitable methods for the initial identification of RC deficiencies. They found that measurements of mitochondrial enzyme activity obtained from lymphocytes could be used to characterize RC disorders. Furthermore, their results indicate that impaired RC activity can be caused by a defect in one or more enzyme complex. These results alluded to the idea that peripheral blood mononuclear cell (**PBMC**) mitochondrial enzyme activity may be reflective of whole-body metabolism. This method could be used to elucidate the relationship between mitochondrial function and production potential on large populations of cattle due to the low level of invasiveness. Niesen and Rossow, (2019) applied this method to evaluate the effects of age and body weight gain on PBMC mitochondrial enzyme activity in pre-weaned calves. Holstein and Jersey calves (n = 20 for each breed) were sampled at 1, 2, and 8 wk of age. Results from the study indicated that CI mitochondrial enzyme activity may serve as a marker for calf growth potential and that ATP synthase activity may be associated with immune challenge in the form of calf diarrhea. Currently, there is no literature available to help explain why CI activity is related to calf growth. However, Acetoze *et al*. (2015) reported that mitochondrial oxygen consumption

changes in response to systemic inflammation, which supports the conclusion that ATP synthase activity may be influenced by immune challenge. The literature discussed above demonstrates that mitochondrial function is associated with lactation performance, reproduction, cow health and calf growth potential. However, these are general associations, and optimum mitochondrial function at various stages of life and exogenous factors that may affect mitochondrial function have not been defined. These discoveries have the potential to help explain or identify an animal's growth, performance, their ability conceive, and their susceptibility to disease.

CEPHALOSPORINS & MITOCHONDRIAL FUNCTION

Cephalosporins are a class of β-lactam antibiotics commonly used to treat bacterial infections. Nickerson *et al*. (1986) hypothesized that antibiotics and anti-inflammatory agents will be used prophylactically within the dairy industry to reduce somatic cell counts (**SCC**) in milk and to prevent intramammary infections (**IMI**). This hypothesis held true as research in dry cow therapy elucidated the effects of prophylactic intramammary (**IM**) antibiotic treatment with cephalosporins such as ceftiofur hydrochloride and cephapirin benzathine. The specific aim of research in prophylactic IM antibiotic treatment at dry off was to determine if IM antibiotic treatment would treat and prevent IMI that may occur during the dry period or within the animal's subsequent lactation (Hallberg *et al*., 2006; Pinedo *et al*., 2012; Arruda *et al*., 2013). However, research in this area neglects previous reports that cephalosporin antibiotics can negatively affect polymorphonuclear neutrophilic leukocyte (**PMNL**) function (Nickerson et al., 1986). More recently, Cameron *et al*. (2015) and McParland *et al*. (2019) produced data that indicate low SCC cows (< 200,000 cells/ml) that were treated with an IM cephalosporin antibiotic at the time of dry off may be at risk for developing an IMI in the subsequent lactation,

which was supported by the increase in SCC in the subsequent lactation observed in both studies. These data produce evidence that cephalosporin antibiotics may inhibit mitochondrial function and this interaction may interfere with the cow's innate ability to fight infection, further linking mitochondrial function/dysfunction to animal health and performance.

To date, research has yet to determine the effects IM cephalosporins infused at dry off have on mitochondrial function in dairy cattle. While these antibiotics prove effective for the treatment and prevention of IMI, cephalosporin antibiotics have shown to inhibit mammalian cell mitochondrial function (Tune *et al*., 1988; Kiyomiya *et al*., 2000; Kalghatgi *et al*., 2013). Tune *et al*. (1988) compared the effects cephaloglycin, and cephalexin (both are cephalosporin antibiotics) had on renal cortical mitochondrial function *in vitro* and *in vivo*, by measuring the rate of oxygen consumption. Their study was performed because cephalosporin antibiotics were thought to cause nephrotoxicity in humans. Three groups of female rabbits were administered clinically relevant doses of cephaloglycin, cephalexin, and an antibiotic vehicle (no antibiotic; control treatment) intravenously. Mitochondria from these rabbits were also used to evaluate the effects of *in vitro* exposure to cephalosporins. Renal cortical mitochondrial tissue samples were obtained and processed from decapitated rabbits 1 hr after receiving cephalosporin or vehicle treatment. Both, *in vitro* and *in vivo* rates of oxygen consumption behaved similarly within treatment, suggesting that renal cortical mitochondrial functions similarly in both environments. Rabbits treated with cephaloglycin showed a decrease in mitochondrial oxygen consumption when compared with control rabbits, indicating that cephaloglycin inhibited mitochondrial function and may cause nephrotoxicity in humans. Interestingly, cephalexin had no effect on

mitochondrial respiration when compared with the control, which implies that not all cephalosporins inhibit mitochondrial function.

Kiyomiya *et al*. (2000) assessed *in vitro* effects of the cephalosporin; cephaloidine (**CLD**), using cultured kidney cells from swine (cell line: LLC-PK) and measured mitochondrial function using an enzymatic approach. Cultured cells were incubated at 37°C until confluent; then dosed with CLD (0.5 and 1.0 mM). Control cells were not treated. Both doses of CLD decreased the enzymatic activity of cytochrome c oxidase (complex IV), which suggests that cellular metabolism and mitochondrial function was impaired by CLD treatment (Kiyomiya *et al*., 2000). Results from Kiyomiya *et al*. (2000) and Tune *et al*. (1988) indicated that mammalian cell mitochondrial function appeared to behave similarly across species, both *in vivo* and *in vitro,* in response to CLD treatment. Research in this area is extremely limited across species and has yet to be performed in dairy cattle. Combining measurements of mitochondrial enzyme activities with cow health and performance records could ultimately provide key insights to better understand how mitochondria influence different aspects of bovine physiology. Finally, if cephalosporins negatively affect mitochondrial function, this research may be able to identify the lasting impact this interaction has on milk production/quality and lead to a shift in disease management practices.

CONCLUSION

Mitochondrial research as it relates to lactation performance is scarce. The literature across various species and cell types shows that mitochondria are central to metabolism and health, and

their function impacts many aspects of physiology. Novel methods to assess mitochondrial function from the PBMC fraction of whole blood introduced by Niesen and Rossow, (2019) make it possible to evaluate mitochondrial function in large populations of cattle due to a low level of invasiveness. Utilizing this method to determine the extent to which cephalosporins influence mitochondrial function in dairy cattle could lead to key insights that affect dairy management practices and could ultimately improve cow health and lactation performance.

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CHAPTER TWO

INTERPRETIVE SUMMARY

Batchelder, The effects of intramammary cephalosporin antibiotic treatment at dry off on peripheral blood and milk mononuclear cell mitochondrial enzyme activity were measured just before dry off, a week after dry off, and between 55 - 75 DIM in the next lactation in Holstein cows. Our results show that intramammary cephalosporin treatment did not influence mitochondrial enzyme activity. The changes in enzyme activity were time related, indicating that physiological differences accompanied by stage of lactation influences mitochondrial enzyme activity in peripheral blood and milk mononuclear cells.

Title: **The effects of intramammary cephalosporin antibiotic treatment at dry off on peripheral blood and milk mononuclear cell mitochondrial enzyme activity in Holstein cows**

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Running header: Cephalosporins and mitochondrial function

ABSTRACT

Cephalosporin antibiotics are commonly used at dry off to treat and prevent intramammary infections (**IMI**). Research in murine and other mammalian cell types showed that cephalosporin antibiotics negatively affect mitochondrial respiratory chain activity, which impacts cellular function. The objective of this study was to evaluate the effects of intramammary (**IM**) cephalosporin (Cefitiofur Hydrochloride and Cephapirin Benzathine) antibiotic treatment administered at dry off on peripheral blood mononuclear cell (**PBMC**) and milk mononuclear cell (**MMC**) mitochondrial enzyme activity 4 h prior to dry off (**TPT1**), 7 d post dry (**TPT2**), and between 55 – 75 DIM in the subsequent lactation (**TPT3**). Thirty-seven Holstein cows from a commercial dairy were enrolled at TPT1 and assigned to 1 of 4 treatments: 1) Low SCC control (**LCON**), 2) High SCC Control (**HCON**), 3) High SCC Ceftiofur Hydrochloride (**CH**), (Spectramast DC; Zoetis Inc., Kalamazoo, MI), and 4) High SCC Cephapirin Benzathine (**CB**), (ToMorrow; Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO). Control treatments received no antibiotics. Low and high SCC cows were defined as < 100,000 cells/mL (low), and > 200,000 cells/mL (high) at TPT1. Whole blood and milk samples were collected at TPT1, TPT2, and TPT3. Enzyme activities of citrate synthase (**CS**), complex I (**CI**), complex IV (**CIV**), and ATP synthase were performed on crude mitochondrial extracts using kits from Abcam (Cambridge, MA). An index representing the ratio of electron transport activity to ATP synthase activity (**OXPHOS**) was calculated from the sum of CI and CIV enzyme activities divided by ATP synthase enzyme activity. Data were analyzed by REML ANOVA with repeated measures in R (Version 4.0.4). The PBMC CIV enzyme activity was highest at TPT1 in the LCON treatment compared with the HCON, CH, and CB treatments, but was not different at TPT2 and TPT3, indicating that PBMC CIV enzyme activity was influenced by IMI. The PBMC and MMC

CS, CI, ATP synthase and OXPHOS changed with time. These data indicate that MMC and PBMC mitochondrial enzyme activities and OXPHOS were not influenced by cephalosporin treatment. The time related changes in OXPHOS and enzyme activities were likely influenced by diet composition, milk production, oxidative stress, and fetal growth.

Keywords: mitochondria, electron transport chain enzymes, cephalosporin

INTRODUCTION

Mitochondria possess their own circular genome known as mitochondrial DNA (**mtDNA**). Mitochondrial DNA is combined with nuclear DNA to code for protein complexes that are exclusive to the mitochondrion. This characteristic led scientists to believe that mitochondria arose from a bacterial ancestor due to an endosymbiotic relationship that was established when a mitochondrion was engulfed by a eukaryotic progenitor. Lane and Martin (2010) describe this as "endosymbiotic theory". The common ancestry that was shared between mitochondria and bacteria implies that cephalosporin antibiotics may negatively affect mitochondrial enzyme activity. Ceftiofur hydrochloride and cephapirin benzathine are two common intramammary (**IM**) cephalosporins used at dry off to treat existing intramammary infections (**IMI**), prevent IMI that may occur throughout the dry period, or in the subsequent lactation. Cephalosporins such as ceftiofur hydrochloride and cephapirin benzathine act on enzyme complexes within bacteria, which are similar to those found in mitochondria within mammalian cell types. However, it is unknown if IM infusion of ceftiofur hydrochloride or cephapirin benzathine inhibits mitochondrial function.

The organelle in eukaryotic cells responsible for metabolism and energy production is the mitochondria; often referred to as the "powerhouse of the cell". Mitochondria are dynamic organelles that influence a variety of cellular processes, and their dysfunction has shown to influence ovarian function, neural tissue function, and various disease states across species (Nunnari and Suomalainen, 2012). Within the dairy industry, mitochondrial oxygen consumption, quantity, biogenesis, gene expression, and enzyme activity were shown to play a crucial role in reproduction (Ferreira *et al*., 2016), milk production (Brown *et al*., 1988; Schultz

et al., 1994; Alex *et al*., 2015), and calf growth potential (Niesen and Rossow, 2019). Measurements of mitochondrial enzyme activities with and without cephalosporin treatment at dry off combined with cow health and performance records may provide key insights to better understand the impact mitochondrial function has on cow health and production. If cephalosporins negatively affect mitochondrial function (i.e. enzyme activity), research in this area may be able to identify the impacts this interaction has on cow health and performance, which may ultimately lead to advancements in disease management practices. Therefore, the objectives of this study were to 1) determine if IM infusion of ceftiofur hydrochloride, or cephapirin benzathine at dry off affects peripheral blood mononuclear cell (**PBMC**) and milk mononuclear cell (**MMC**) mitochondrial enzyme activity 7 d into the dry period, and 2) characterize how mitochondrial enzyme activity changes 4 h prior to dry off (**TPT1**), 7 d post dry (**TPT2**), and between 55 – 75 DIM in the subsequent lactation (**TPT3**).

MATERIALS & METHODS

Experimental design

All study protocols and procedures involving cows were approved by the University of California, Davis Institutional Animal Care and Use Committee.

Holstein cows (n = 37) from a 1,000 cow commercial dairy located in Strathmore, CA were enrolled in a cohort study between August 2019 and May of 2020. The enrollment criteria included primiparous and multiparous Holstein cows that were not clinically ill or lame at TPT1. Whole blood and milk samples were collected at three time points: TPT1, TPT2, and TPT3. At

TPT1 treatments had yet to be administered to all cows allowing each cow to serve as its own control. At TPT2 cephalosporins should have progressed into systemic blood and have acted on bacteria and mitochondria. At TPT3 the effects of IM cephalosporin treatment would reveal lasting impacts treatment may have had in the subsequent lactation.

The dairy selected cows to end their lactation if they were greater than 210 d pregnant or if they produced < 20 kg of milk per d. At TPT1, milk samples from each quarter were obtained to group cows by quarter SCC using the California Mastitis Test (Immucell Corp., ME), and these results were confirmed by the Tulare DHI(A) (Bentley Instruments, Chaska, MN). Cows were placed in the low SCC group if SCC were < 100,000 cells/mL in all 4 quarters. If SCC were > 200,000 cells/mL in at least 1 quarter, cows were placed in the high SCC group. Cows that had more than 1 quarter SCC between 100,000 – 200,000 cells/mL were not eligible for enrollment since these cows may have been developing or clearing an IMI. Once cows were grouped by SCC, they were assigned to one of four treatments: 1) Low SCC Control (**LCON**), 2) High SCC Control (**HCON**), 3) High SCC Ceftiofur Hydrochloride (**CH**), and 4) High SCC Cephapirin Benzathine (**CB**). All treatments were administered by dairy employees. Prior to the infusion of IM cephalosporins, all teats were dipped with an antiseptic (pre-dip), dried with a clean towel, and cows were milked. Then, cephalosporins were administered to cows enrolled in the CH and CB treatments by gently inserting the nozzle of the treatment into the teat canal, the cephalosporin was infused, and the teat was briefly massaged. The CH treatment received 500 mg of Ceftiofur Hydrochloride (Spectramast DC; Zoetis Inc., Kalamazoo, MI) in each individual quarter and the CB treatment received 300 mg of Cephapirin Benzathine (ToMorrow;

Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO) in each individual quarter. Control treatments (LCON and HCON) received no cephalosporin antibiotics or placebo.

Cows and facilities

Throughout the dry period, cows were housed in a dry lot pen, where they remained until 30 d prior to their projected calving date. Cows were then moved to a dry lot close-up pen bedded with almond shells where they remained until parturition. During lactation, cows were housed in free stall barns that were bedded with dried manure solids, and equipped with flush lanes, and fans and soakers above the headlocks. Lactating cattle at this facility were milked twice daily in a flat barn. All pens at this facility were equipped with shade structures and cows had ad libitum access to water.

Milk sample collection

Milk was extracted by hand at all time points by first cleaning the external teat surface with gauze soaked in ethanol, wiping the teat end dry with a clean paper towel, and collecting milk into one 500 mL conical for mitochondrial analyses and one 50 mL conical that was analyzed by the Tulare DHI(A) for: milk fat (%), milk protein (%), lactose (%), solids not fat (%), milk urea nitrogen (%), and SCC (K/mL). Only 50 mL of milk were collected from cows assigned to the low SCC group because these samples were unable to yield measurable isolates of mitochondria from MMC due to the low number of somatic cells. Milk samples were labeled and immediately stored on ice until arriving at the laboratory for further processing. The remaining 500 mL of milk were centrifuged at 300 × *g* at 20°C to pellet somatic cells. The supernatant was discarded,

and the pellet was resuspended in 1 mL of autoMACS Rinsing Solution (phosphate-buffered saline, pH 7.2, and 2 m*M* EDTA, Miltenyi Biotec, Sunnyvale, CA). The resuspended pellet was then transferred to a 50 mL conical and diluted to a final volume of 50 mL with autoMACS Rinsing Solution. The samples were centrifuged once more at 300 × *g* for 10 min at 20°C; the supernatant liquid was discarded, and the pelleted somatic cells were transferred to a sodium citrate cell preparation tube (BD Biosciences, San Jose, CA) for subsequent isolation of MMC.

Blood collection and hematology

Whole blood (23 mL) was collected at each time point into 3 separate vacutainers (BD Biosciences. San Jose, CA) containing K2 EDTA as an anticoagulant via jugular or coccygeal venipuncture. Immediately after blood collection, tubes were inverted a minimum of 8 times to ensure that the anticoagulant was mixed thoroughly, labeled, and stored on ice. Samples were processed within 2 h of collection. Well-mixed whole blood (3 mL) was used to determine red blood cell count [**RBC**, M/µL], white blood cell count [**WBC**, K/µL], hemoglobin (g/dL), hematocrit (%), mean-corpuscular volume (**MCV**, fL), red cell distribution width (**RDW**, %), neutrophil K/µL, %, lymphocyte K/µL, %, monocyte K/µL, %, eosinophil K/µL, %, and basophil K/µL, %, yield using the Drew Scientific Hemavet 950 Hematology Analyzer (Erba Diagnostics, Oxford, CT). Prior to sample analysis, quality controls were tested to ensure the instrument was operating within specification (Multi-Trol, Drew Scientific, Miami Lakes, FL). The whole blood that remained (20 mL) was centrifuged at 2000 × *g* for 20 min at 20°C. After centrifugation, the plasma was discarded without disrupting the buffy coat or RBC and approximately 4 mL of

buffy coat and RBC were then transferred to a sodium citrate cell preparation tube (BD Biosciences, San Jose, CA) for subsequent isolation of PBMC.

Peripheral blood and milk mononuclear cell isolation

The fraction of whole blood and somatic cells that were transferred to sodium citrate cell preparation tubes described previously were diluted to a final volume of 8 mL with autoMACS Rinsing Solution, inverted 4 times, and centrifuged at 1800 × *g* for 30 min at 20°C. The supernatant was discarded without disrupting the mononuclear cell layer on the density gradient. The PBMC and MMC pellets were resuspended in 1 mL of autoMACS Rinsing Solution, transferred to a 50 mL conical, diluted to a final volume of 50 mL with autoMACS Rinsing Solution, and pelleted at 300 × *g* for 10 min at 4°C. The supernatant was discarded, and this step was repeated once more on all samples to wash PBMC and MMC. Prior to the second wash red cell contaminants from the PBMC were lysed with distilled water, vortexed and diluted to a final volume of 50 mL with autoMACS Rinsing Solution. After the final wash, the supernatant was discarded by pouring. The PBMC and MMC that were pelleted were resuspended in 1 mL of autoMACS Rinsing Solution and transferred to a microcentrifuge tube. Lysate protein concentration from the PBMC and MMC fractions were quantified following the Abcam® BCA Protein Assay Kit (Abcam, Cambridge, MA, ab102536). Prior to performing the BCA protein quantification assay, 30 µL of mononuclear cells were heat shocked in liquid nitrogen to weaken nuclear membranes and the assay was performed following the manufacture's protocol. The remaining aliquots that contained the mononuclear cells were pelleted at 300 × *g* for 10 min at

4°C, the supernatant was discarded, and mononuclear cell pellet was stored in liquid nitrogen for no more than 3 d before mitochondria were isolated.

Mitochondrial isolation

Mitochondria were extracted from the mononuclear cell fraction of whole blood and milk as described in Niesen and Rossow (2019) using the Mitochondrial Isolation Kit for Cultured Cells with modifications (Abcam, Cambridge, MA, ab110170). The mononuclear cell pellets were removed from liquid nitrogen and supplemented with 0.4 µL of universal nuclease (Fisher Scientific co., Fair Lawn, NJ, PI88700). Sample pellets were resuspended in 5 mg/mL of Reagent A and homogenized. The homogenate was transferred back into its corresponding microcentrifuge tube and centrifuged at 1,000 × *g* for 10 min at 4°C. The supernatant was saved, and the remaining pellet was resuspended in Reagent B (using the same volume of reagent A), homogenized, and centrifuged once more. Supernatants were combined and centrifuged at 12,000 × *g* for 15 min at 4°C. The mitochondrial pellet was collected, resuspended in 500 µL of Regent C supplemented with protease inhibitors (Abcam, Cambridge, MA, ab 201111), aliquoted, and stored at -80°C. Crude mitochondrial protein concentration was measured on one aliquot per sample following the BCA protein quantification assay (Abcam, Cambridge, MA, ab102536).

Measurement of mitochondrial enzyme activities, CS, CI, CIV, & ATP synthase

Mitochondrial enzyme activities of Citrate Synthase (**CS**, EC 4.1.3.7), Complex I (**CI**, EC 1.6.5.3), Complex IV (**CIV**, EC 1.9.3.1), and Complex V (**ATP synthase**, EC 3.6.3.14) were performed on separate aliquots of crude mitochondrial extracts as described in Niesen and Rossow (2019). Briefly, enzyme activity was measured using Enzyme Activity Microplate Assay Kits (Abcam, Cambridge, MA). Individual microplates for each enzyme were placed on an orbital shaker and incubated for 3 h at room temperature prior to collection of absorbance data. A VersaMax (Molecular Devices, Sunnyvale, CA) tunable microplate spectrophotometer in kinetic mode was used to capture absorbance data. To ensure the instrument was operating within specification, a calibration test plate (Bio-Tek Instruments Inc., Winooski, VT) was read by the device following manufacturer's protocol. All assay kits were bovine species reactive and were from the same manufacturer lot. Intraassay coefficients of variation for CS, CI, CIV, and ATP synthase were: < 7 %, < 15 %, < 10%, and < 13 %, respectively.

Statistical Methods

In all analyses, cow was the experimental unit of interest.

To ensure that cows were grouped properly according to quarter SCC at TPT1, an ANOVA confirmed that the LCON quarters had LSM SCC < 100,000 cells/mL and HCON, CH, and CB quarters had LSM SCC > 200,000 cells/mL. The LCON LSM SCC was lower compared with HCON, CH, and CB. Furthermore, there were no differences in LSM quarter SCC (**QSCC**) for HCON, CH, and CB indicating that enrollment of high and low SCC groups was successful.

Mitochondrial enzyme activity was defined as the linear rate of change of the absorbance (mili-optical density units) per minute per microgram of crude mitochondrial protein (mOD units / min / μg) loaded into the well and was calculated as described in Niesen and Rossow (2019). An index representing the ratio of electron transport activity to ATP synthase activity (**OXPHOS**) was created using CI, CIV, and ATP synthase enzyme activities in the following equation:

$$OXPHOS = \frac{(x + y)}{(z)}$$

where x = the enzyme activity of CI, y = the enzyme activity of complex IV, and z = the enzyme activity of ATP synthase.

To determine if CH and CB influenced mitochondrial enzyme activity, multivariate regression models with enzyme activity or OXPHOS as the outcome variables were built in R (version 4.0.4, R Core Team, Vienna, Austria). Hematological variables, QSCC, incidence of mastitis, milk yield (kg), DIM, and parity were predictor variables. Initially, Pearson correlation coefficients were obtained from univariate analyses that regressed the outcome variables on potential predictors. Pearson correlation coefficients > 0.20 (absolute value) from univariate analyses identified potential predictor variables. The backward model selection procedure using the LEAPS package in R (version 4.0.4, R Core Team, Vienna, Austria) identified the best subset of predictor variables that were included in the final regression models based on the lowest Bayesian Information Criteria. Each outcome variable was analyzed separately by REML ANOVA using the LME4 package in R (version 4.0.4, R Core Team, Vienna, Austria). The general model used to determine if CH or CB treatment influenced the outcome variables:

PBMC or MMC mitochondrial enzyme activity (CS, CI, CIV, and ATP synthase) or OXPHOS was as follows:

$$Y_i = \beta_0 + \beta_a T + \beta_b P + \beta_c Z + \beta_d I + e$$

Where the Y was outcome variable of interest, β_0 was the intercept coefficient, $\beta_a T$, $\beta_b P$, and $\beta_c Z$ were fixed effects of treatment, time point, and the treatment by time point interaction, respectively; $\beta_d I$ = random effects of cow for PBMC mitochondria, or random effects of quarter nested within cow for MMC mitochondria, and e = the residual error. The fixed effects and their respective partition of the sums of squares that were included in each model are summarized in **Table 1**.

The model used to determine if the LCON, HCON, CH, and CB treatments had differences in hematological variables was as follows:

$$Y_i = \beta_0 + \beta_a T + \beta_b P + \beta_c Z + \beta_d I + e$$

The Y was the hematological variable of interest, β_0 was the intercept coefficient, $\beta_a T$, $\beta_b P$, and $\beta_c Z$ were fixed effects of treatment, time point, and the treatment by time point interaction, respectively, $\beta_d I$ = random effects of cow, and e = the residual error.

To elucidate the relationship between MMC and PBMC OXPHOS and mitochondrial enzyme activity, univariate regression analyses were performed as follows:

$$Y_{MMC} = \beta_0 + \beta_{PBMC} X_{PBMC} + e$$

Where Y_{MMC} = MMC CI, CIV, ATP synthase or OXPHOS, β_0 = the intercept, β_{PBMC} = the slope coefficient, X_{PBMC}= PBMC CI, CIV, ATP synthase or OXPHOS and $e =$ the residual error.

Normality of outcome variables and in all models normality of residuals were evaluated using the Shapiro-Wilk test statistic and normal probability plots. Outcome variables that produced non-normal distributions were log transformed to comply with assumptions of normality. After transformation of the data, two outliers; one from the CB treatment and one from the HCON treatment, were excluded since they possessed enzyme activities that were > 3 standard deviations from the overall mean. A $P < 0.05$ was considered different, and $0.10 \geq P > 0.05$ was considered a tendency. Due to a defective CS assay kit, data for this enzyme were limited to TPT1 and TPT2.

RESULTS & DISCUSSION

Mitochondria share similar characteristics with bacteria and are thought to be related to a single bacterial ancestor (Lane and Martin, 2010). Cephalosporins have been shown to inhibit bacterial growth and mammalian cell mitochondrial function and enzyme activity (Tune *et al*., 1988; Kiyomia *et al*., 2000). The primary objective of this study was to determine if MMC mitochondrial enzyme activities for treatments CH or CB compared with HCON were different at TPT2, and if this difference persisted through to TPT3 compared with TPT1. **Table 2** shows that there was a tendency ($P < 0.06$) for lower MMC CIV activity in CH and CB compared with HCON implying that cephalosporins tended to decrease MMC CIV activity. However, there were no differences by time point. The LCON treatment did not yield enough MMC to isolate

mitochondria and measure mitochondrial enzyme activities. Since we could not measure MMC mitochondrial enzyme activity in LCON, we cannot conclude that cephalosporin treatment alone was responsible for the tendency towards lower MMC CIV activity in CB and CH. Also, cephalosporins were administered after samples were collected at TPT1. **Figure 1B** shows that HCON had numerically higher MMC CIV activity at TPT1 when compared with CH and CB. So, the tendency for increased MMC CIV activity in HCON at TPT1 may be explained by the variation in QSCC and RBC count. **Table 1** shows that the partition of the sums of squares for RBC count and QSCC were 1.80 and 12.91, respectively. The largest sums of squares in the model used to analyze MMC CIV activity was in QSCC, which means that QSCC explains more variation in MMC CIV enzyme activity than RBC count, treatment, time, and the treatment x time point interaction. Interestingly, there was a treatment by time point interaction for RBC count (**Table 3**). Tukey-Kramer adjusted pairwise comparisons for this interaction showed that treatment differences occurred at TPT1 (data not shown). The LCON and HCON treatments had higher RBC counts when compared with CB. Therefore, the tendency for treatment to decrease MMC CIV enzyme activity may be associated with level of IMI that corresponds with lower RBC counts and higher QSCC in CH and CB. The RBC count may have influenced MMC CIV activity as RBC supply tissues with oxygen and remove carbon dioxide. Molecular oxygen serves as the final electron acceptor at CIV in the electron transport chain (**ETC**), implying that CIV activity depends on oxygen delivery via RBC. The inclusion of QSCC as a fixed effect in the model used to analyze MMC CIV may be explained by metabolic adaptations mononuclear cell mitochondria acquire during infection (Buck *et al*., 2016). As QSCC increased there may be greater numbers of activated lymphocytes and monocytes that make up MMC. Upon activation, lymphocytes rapidly proliferate, fight infection, and shortly die via apoptosis. To support rapid

cell proliferation, lymphocytes favor aerobic glycolysis as their preferred metabolic pathway, which utilizes oxygen in the reoxidation of NADH to NAD^+. Rather than converting pyruvate to acetyl-CoA that enters the tricarboxylic acid (**TCA**) cycle for energy production via oxidative phosphorylation, pyruvate is converted to lactate in aerobic glycolysis. This characteristic of activated lymphocytes may lead to lower MMC CIV enzyme activity in quarters with lower QSCC, as molecular oxygen is utilized for lactate production rather than serving as the final electron acceptor at CIV in oxidative phosphorylation. Further research is needed to confirm that cephalosporins tend to decrease MMC CIV activity with or without infection.

For PBMC CIV activity, we were able to include a negative control, LCON, in the analysis **(Table 2 and Figure 1A)**. The PBMC CIV activity was highest in LCON compared with HCON, CH and CB at TPT1 implying CIV was not influenced by cephalosporins since treatments were not administered until after samples were collected at TPT1. But, PBMC CIV activity may have been influenced by level of SCC similar to MMC CIV activity. This is supported by the inclusion of WBC count as a fixed effect for the model used to analyze PBMC CIV. While different from the fixed effects included in MMC CIV model, WBC count is a marker of systemic infection, suggesting that CIV activity was influenced by level of IMI. There were no differences in CIV activity at other time points. These results conflict with those previously reported by Kiyomiya *et al*. (2000) who showed that cephalosporidine treatment inhibits *in vitro* CIV enzyme activity in cultured kidney cells from swine. The conflicting results could be due to the differences in cell type, the *in vitro* exposure to cephalosporins, time at which samples were collected, or the type of cephalosporin used. In this study, MMC and PBMC CIV

enzyme activities were probably influenced by IMI since the activities of these enzymes were lower in the HCON, CH, and CB treatments, all of which were high SCC cows at TPT1.

Hematology

Cows belonging to the HCON, CH, and CB treatments had quarter SCC > 200,000 cells/mL suggesting that cows within these treatments may have experienced local or systemic infection at the time of sample collection (Carvalho-Sombra *et al*., 2021). Mitochondrial metabolism in mononuclear cells changes in response to infection. Buck *et al*. (2016) showed that the onset of infection metabolically reprograms mitochondria in activated lymphocytes to favor aerobic glycolysis as the preferred metabolic pathway to support rapid cell proliferation needed to overcome infection. The metabolic switch from oxidative phosphorylation to aerobic glycolysis may be accompanied by a decrease in PBMC and MMC mitochondrial enzyme activity. Hematological profiles may change with level of IMI infection and these differences may influence PBMC and MMC mitochondrial enzyme activities. To determine if hematological profiles were different among treatments, hematology data were analyzed across all time points. There were no differences in most hematological variables among treatments (**Table 3**). The variables that were influenced by the treatment by time point interaction were RBC count, hemoglobin concentration, and hematocrit percent. Tukey-Kramer adjusted pairwise comparisons of these effects revealed that each of these variables were within normal reference ranges and the differences occurred at TPT1.

Peripheral blood mononuclear cells vs. milk mononuclear cells

MMC and PBMC mitochondrial enzyme activities should behave similarly because they are the same cell type. Rustin *et al*. (1994) reported that mononuclear cell mitochondrial function is reflective of whole-body metabolism. Similar trends in PBMC and MMC mitochondrial enzyme activities over time and treatments suggested that the activities of these enzymes were correlated with one another. MMC and PBMC OXPHOS, ATP synthase, and CS activities had relatively high coefficients of determination (**Table 4**) indicating that MMC mitochondria functions similarly to PBMC mitochondria. Interestingly, MMC and PBMC CI, and CIV activities were not related to each other. The key role CI and CIV have in cell signaling pathways, particularly in ROS generation and apoptosis, may explain these differences. Complex IV facilitates the release of cytochrome c from the inner mitochondrial membrane, which signals apoptosis, and may be marked by a decrease in CIV enzyme activity (Guo *et al*., 2013). The shift in mitochondrial metabolism from oxidative phosphorylation to aerobic glycolysis may cause a decrease in the enzyme activity of CIV, since molecular oxygen is no longer acting as the final electron acceptor for CIV, but rather being utilized in aerobic glycolysis. Therefore, the lower PBMC CIV activity at TPT1 in the HCON, CH, and CB treatments compared with the LCON treatment is likely attributed to the existing IMI, suggesting that infection influence PBMC CIV enzyme activity.

MMC and PBMC CI activity

Both MMC and PBMC CI activity were influenced by WBC count (**Table 1**), suggesting that CI activity changes due to level of IMI. The additional fixed effects: hemoglobin concentration,

mean platelet volume (**MPV**) and MMC ATP synthase activity were included in the model to analyze MMC CI. Together, WBC count, hemoglobin concentration and MPV can all be used as markers of systemic inflammation and further support that CI enzyme activity changed with level of infection. The inclusion of MMC ATP synthase in this model provided evidence that electron transport activity and ATP synthase activities are related as these reactions are coupled. However, it remains unclear why ATP synthase activity was specific to MMC CI activity and not PBMC CI activity.

Complex I activity was different across time points (**Table 2**). In MMC and PBMC, CI activity decreased from TPT1 to TPT2 representing a change from late lactation milk production (TPT1) and third trimester gestation to dry cow treatment (none, CH or CB) and udder involution 1 wk into the dry period with a cessation of lactation (TPT2; **Figure 2A and Figure 3A**). Putman *et al*. (2018) reported that cows transitioning from late lactation to the dry period experience high oxidative or metabolic stress that appears to be elevated days before milk cessation. Complex I is the first enzyme in the ETC that reduces NADH to NAD^+. This enzyme also contributes to the majority of ROS produced by mitochondria, which is utilized in several cell signaling pathways allowing mitochondria to serve as the metabolic signaling centers (Hamanaka and Chandel, 2010). The ROS produced by CI facilitates the release of cytochrome c from CIV of the ETC to signal apoptosis. Increased mitochondrial ROS production is characteristic of oxidative stress and may be the result of increased CI activity and a decrease in CIV activity. The higher levels of MMC CI activity at TPT1 in comparison to TPT2 may be explained by the role of CI in ROS production associated with cellular oxidative stress signals in late lactation.

The main difference between MMC and PBMC CI activities was at TPT3, which occurred during peak milk production in the next lactation. There was no difference in TPT3 MMC CI activity compared with TPT1 or TPT2 but, PBMC CI activity was lower at TPT3 and higher at TPT1. The MMC that are recruited to the mammary gland from peripheral blood play a role in mammary gland health and maintenance throughout lactation. But, the lack of difference in MMC CI activity at TPT3 compared with TPT1 and TPT2 may reflect that level of milk production does not influence MMC CI activity. In contrast, PBMC CI activity is decreased at TPT3 compared with TPT1. Previous research found an association between increased CI activity in PBMC and increased milk fat yield, peak milk yield, and SCC (Niesen *et al*., 2021 submitted) but did not measure PBMC CI activity in dry cows. In this study, PBMC CI activity may be more reflective of fetal growth or pregnancy.

MMC and PBMC ATP synthase activity

Hemoglobin concentration and RBC count were included as a fixed effects in the regression model used to analyze PBMC ATP synthase activity (**Table 1**). The partitioning of the sums of squares showed that time, RBC count, and hemoglobin concentration had a greater effect on this enzyme than treatment or the treatment by time point interaction. Together RBC and hemoglobin function to supply cells with oxygen and remove carbon dioxide. Since molecular oxygen serves as the final electron acceptor at CIV in the ETC, and the activity of ATP synthase depends on respiratory chain activity, it is reasonable to assume the variables RBC count and hemoglobin concentration would influence the activity of PBMC ATP synthase. Interestingly, the model used to analyze MMC ATP synthase did not indicate these variables were associated with the activity of this enzyme. Yet, in this study ATP synthase activity in MMC and PBMC were highest at 55 -

75 DIM in the next lactation (**Figures 2B and 3B**). This agrees with Niesen *et al*. (2021 submitted) who showed that PBMC ATP synthase activity increased with increased peak milk yield and milk fat yield. The ATP synthase enzyme complex is the last enzyme involved in oxidative phosphorylation and utilizes the proton gradient formed by the ETC to phosphorylate molecules of ADP. Lactogenesis involves nearly every tissue and is accompanied by large energetic demands during early lactation compared with cows in late lactation or non-lactating cows (Anderson *et al*., 1985). These circumstances may explain the elevated MMC and PBMC ATP synthase enzyme activity at TPT3, as this is the last enzyme involved in the coupled reaction that produces ATP. Consistent with this interpretation, Long *et al*. (2015) reported that ATP synthase activity will increase as cellular energy demands increase. In contrast, DMI and starch intake are typically highest in early lactation. As starch intake increases, the molar proportion of propionate produced by the rumen will be greater in cows during early lactation (TPT3) compared with cows in late lactation or non-lactating cows. Given that propionate is transported to the liver and utilized as a precursor in gluconeogenesis, the increase in PBMC ATP synthase activity may be highest at TPT3 when compared with TPT1 and TPT2 due to differing dietary intakes, and nutrient composition that accompanies stage of lactation (Anderson *et al*., 1985).

MMC and PBMC OXPHOS

OXPHOS was highest in late lactation just before dry off and decreased across time points to be lowest around peak milk yield in the next lactation (**Figures 2C and 3C**). Since OXPHOS represents the balance between electron transport and the transfer of electrons into energy to generate ATP, it would be expected that the balance between electron transport and ATP

production was optimal during the most energetically demanding period in the lactation cycle, peak lactation. Therefore, values of MMC and PBMC OXPHOS between 1 – 5 may be representative of optimum mitochondrial function as this would indicate that CI, CIV and ATP synthase were functioning proportionally with one another. Values of OXPHOS larger than 5 may be associated with decreased mitochondrial energy efficiency, mitochondrial dysfunction, or oxidative stress. Cows in late lactation have impaired energy, immune and hormonal states which contribute to oxidative stress in late lactation (Zobel *et al*., 2015; Putman *et al*., 2018). Mitochondria from cows at TPT1 and TPT2 may have had more uncoupling between the ETC and ATP synthase with corresponding proton leak. Proton leak may be due to higher energy intakes relative to lower energy requirements i.e., anabolic metabolism during third trimester pregnancy in TPT1 or, oxidative stress due to the cessation of lactation followed by the involution of the mammary gland (Putman *et al*., 2018) as in the state of the mammary gland at TPT2. The majority of MMC residing in the mammary gland could have been subject to cell death via apoptosis, leading to smaller values of MMC OXPHOS at TPT2 in comparison to TPT1. The values of MMC and PBMC OXPHOS were lowest at TPT3 and fell between 2 – 5, which would suggest that these mitochondria were more efficient at this time point. The values of MMC and PBMC OXPHOS appear to be inversely related when compared to MMC and PBMC ATP synthase activity, suggesting that either of these measurements may serve as indicators of mitochondrial function and milk production. However, more research is needed to better understand if MMC and PBMC OXPHOS and ATP synthase activity can serve as markers of mitochondrial function or dysfunction.

MMC and PBMC CS enzyme activity

Citrate synthase is the first enzyme in the TCA cycle that converts acetyl-CoA and oxaloacetate into citrate and has been used as a marker for TCA cycle activity and mitochondrial content (Kiyomiya *et al*., 2000; Fernàndez-Vizarra *et al*., 2011; Larsen *et al*., 2012). We could not utilize CS as an indicator of mitochondrial content due to a defective CS assay kit that was used on the TPT3 samples therefore TPT3 is missing from the analyses. Both hemoglobin concentration and monocyte count were included in the models used to analyze PBMC and MMC CS activity (**Table 1**), which suggests that CS activity may be influenced by metabolic adaptations of mitochondria as TCA cycle activity is regulated by the respiratory chains ability to reduce NADH and produce ATP. There were no differences among treatments for CS, but CS activity decreased from TPT1 to TPT2 in MMC and PBMC (**Figure 4**). Time related changes in PBMC and MMC CS activity may be explained by cellular energy demands. It has been reported that CS may determine the rate of the entire TCA cycle (Ciccarone *et al*., 2019). An increase in CS activity would then correspond with an increase in TCA cycle activity, ultimately increasing the production of NADH and $FADH_2$ molecules that will feed respiratory chain complexes. This is reflected in TPT1 vs. TPT2 CS activities in MMC and PBMC because cows are still lactating at TPT1. The rate of the TCA cycle would be greater (TPT1) compared with cows that are dry (TPT2), as cellular energy demands would be higher during lactation.

SUMMARY

Differences in MMC and PBMC CIV activities may be due to low vs high SCC. Activities of ATP synthase, OXPHOS, and CS activity were correlated between PBMC and MMC across time

points and treatments. However, MMC and PBMC CI and CIV activities were not correlated, which may be due to their role in ROS generation and apoptosis. Dry cow treatment using ceftiofur hydrochloride and cephapirin benzathine did not affect MMC and PBMC mitochondrial enzyme activities at TPT2 and TPT3. These results are from cows at a single dairy so mitochondrial electron transport and ATP synthase enzyme activities may respond differently on different dairies with different management practices. Therefore, more research is needed to understand the effects of SCC and cephalosporin treatment on mitochondrial enzyme activities during the lactation cycle.

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Table 1. Partition of the sums of squares for fixed effects included in multivariate regression models used to analyze mitochondrial enzyme activity or OXPHOS

	Fixed Effects[1]										
Outcome Variable	WBC	MOK	RBC	HB	MPV	PBMC CI	MMC CV	QSCC	Trt	Time[2]	Trt x Time
PBMC											
Complex I	3.58	…	…	…	…	…	…	…	0.72	3.12	1.59
Complex IV	2.73	…	…	…	…	2.99	…	…	2.70	0.43	3.32
ATP Synthase	…	…	3.11	8.67	…	…	…	…	0.25	27.12	1.37
OXPHOS[3]	0.10	…	…	…	…	…	…	…	0.84	74.43	5.57
Citrate Synthase	…	12.22	…	24.80	…	…	…	…	0.50	9.32	0.76
MMC											
Complex I	2.54	…	…	3.92	6.14	…	2.47	…	1.46	3.24	2.07
Complex IV	…	…	1.80	…	…	…	…	12.91	3.60	1.57	1.01
ATP Synthase	…	…	…	…	…	…	…	…	0.08	25.55	0.48
OXPHOS	…	…	…	…	…	…	…	…	1.09	28.34	0.23
Citrate Synthase	…	8.32	…	16.32	…	…	…	…	2.13	24.70	0.16

[1]Fixed effects from REML ANOVA are shown and their respective partition of the sums of squares are reported. Abbreviations and units: White blood cell count (**WBC**, K/μL), Monocyte count (**MOK**, K/μL), Red blood cell count (**RBC**, M/μL), Hemoglobin concentration (**HB**, g/dL), Mean platelet volume (**MPV**, fL), peripheral blood mononuclear cell (**PBMC**), milk mononuclear cell (**MMC**), quarter somatic cell count (**QSCC**, K/mL), treatment (**Trt**). Treatment included high SCC cows administered intramammary (**IM**) Ceftiofur Hydrochloride (**CH**) or Cephapirin Benzathine (**CB**) at dry off. Control treatments received no IM cephalosporins and included low SCC Control (**LCON**) and high SCC Control (**HCON**). Low SCC were defined as < 100,000 cells/mL in all four quarters and high SCC as > 200,000 cells/mL in a minimum of one quarter 4 h prior to dry off (**TPT1**), before IM cephalosporins were administered. Low SCC were defined as < 100,000 cells/mL in all four quarters and high as > 200,000 cells/mL in a minimum of one quarter 4 h prior to dry off (**TPT1**), before treatment was

[2]Time is defined as time point and includes TPT1, 7 d into the dry period (**TPT2**), and between 55 – 75 d in milk in the subsequent lactation (**TPT3**).

[3]OXPHOS was calculated by taking the sum of complex I and complex IV enzyme activities; divided by ATP Synthase enzyme activity.

Table 2. Least square mean peripheral blood mononuclear cell (PBMC) and milk mononuclear cell (MMC) mitochondrial enzyme activity by treatment across all time points

	Trt[1]				SEM	*P*-value[2]		
Enzyme	LCON	HCON	CH	CB		Trt	Time[3]	Trt x Time
PBMC								
Complex I	0.193	0.213	0.190	0.180	0.024	0.47	0.01	0.47
Complex IV	0.192[a]	0.135[ab]	0.099[b]	0.098[b]	0.024	0.01	0.38	0.03
ATP Synthase	0.050	0.057	0.064	0.046	0.007	0.68	< 0.01	0.25
OXPHOS[4]	10.14	11.99	9.56	9.39	1.59	0.75	< 0.01	0.25
MMC								
Complex I	…	0.16	0.11	0.17	0.03	0.16	0.02	0.27
Complex IV	…	0.26	0.13	0.12	0.04	0.06	0.29	0.81
ATP Synthase	…	0.05	0.06	0.05	0.01	0.93	< 0.01	0.94
OXPHOS	…	12.41	12.19	15.19	3.79	0.44	< 0.01	0.99

[a,b] Tukey-Kramer pairwise comparisons for treatment least square mean differ *P*-value < 0.05.

[1]Treatment (**Trt**) included high SCC cows administered intramammary (**IM**) Ceftiofur Hydrochloride (**CH**) or Cephapirin Benzathine (**CB**) at dry off. Control treatments received no IM cephalosporins and included low SCC Control (**LCON**) and high SCC Control (**HCON**). Low SCC were defined as < 100,000 cells/mL in all four quarters and high SCC as > 200,000 cells/mL in a minimum of one quarter 4 h prior to dry off (**TPT1**), before IM cephalosporins were administered. The LCON treatment is not listed for MMC because there were not enough somatic cells to isolate mononuclear cells and yield measurable mitochondrial isolates. Enzyme activity is expressed in mOD units per min per µg of crude mitochondrial protein.

[2]The effects of Trt, Time, and the Trt by Time interaction were determined at P-value < 0.05 by REML ANOVA.

[3]Time is defined as the three time points where sample collection took place: TPT1, 7 d post dry (**TPT2**), and 55 - 75 DIM in the subsequent lactation (**TPT3**).

[4]OXPHOS was calculated by taking the sum of complex I and complex IV enzyme activities; divided by ATP synthase enzyme activity.

Table 3. Least square means of hematological variables by treatment across all time points

Variable	Reference Range[2]	Trt[1]				SEM	*P*- value[3]		
		LCON	HCON	CH	CB		Trt	Time[4]	Trt x Time
Leukocytes									
WBC, K/µL	4.0 - 12.0	10.76	10.74	9.62	10.87	1.27	0.884	<0.01	0.263
Neutrophils, K/µL	0.6 - 4.1	4.28	4.20	3.97	3.91	0.21	0.496	<0.01	0.602
Lymphocytes, K/µL	2.5 - 7.5	4.75	4.52	4.19	5.63	1.02	0.776	0.101	0.301
Monocytes, K/µL	0.0 - 1.2	0.92	0.80	0.87	0.87	0.17	0.961	0.018	0.765
Eosinophils, K/µL	0.0 - 2.4	0.77	1.11	0.56	0.43	0.23	0.150	0.149	0.502
Basophils, K/µL	0.0 - 0.4	0.03	1.11	0.03	0.02	0.04	0.246	0.169	0.275
Erythrocytes									
Red Blood Cells, M/µL	5.00 - 10.00	6.34	6.35	6.08	5.84	0.17	0.101	<0.01	0.006
Hemoglobin, g/dL	8.0 - 15.0	8.51	8.05	7.83	7.84	0.30	0.303	<0.01	0.066
Hematocrit, %	24.0 - 46.0	28.8	29.0	27.0	27.0	0.84	0.156	<0.01	0.004
Mean Corpuscular Volume, (fL)	40.0 - 60.0	45.4	45.7	44.4	46.1	0.94	0.620	0.139	0.151
Mean Corpuscular Hemoglobin, pg	11.1 - 17.0	13.4	12.7	12.9	13.9	0.41	0.136	0.051	0.268
Mean Corpuscular Hemoglobin Concentration, g/dL	28.2 - 36.0	29.7	27.8	29.2	30.5	1.08	0.332	0.028	0.152
Red Cell Distribution Width, %	12.0 - 27.0	21.3	21.7	22.5	21.9	0.37	0.139	0.002	0.147
Thrombocytes									
Platelets, K/µL	200 - 800	353	346	369	364	34.5	0.958	0.014	0.602
Mean Platelet Volume, fL	5.0 - 20.0	6.47	6.18	6.32	6.84	0.13	0.130	<0.01	0.142

[1]Treatments (Trt) included high SCC cows administered intramammary (IM) Ceftiofur Hydrochloride (CH) or Cephapirin Benzathine (CB) at dry off. Control treatments received no IM cephalosporins and included low SCC control (LCON) and high SCC Control (HCON). Low SCC were defined as < 100,000 cells/mL in all four quarters and high SCC as > 200,000 cells/mL in a minimum of one quarter 4 h prior to dry off (TPT1) before treatments were administered.

[2]Normal hematology reference ranges present on the Hemavet 950 FS Hematology Analyzer.

[3]Significance comparing the effects of Trt, Time, and the Trt x Time interaction was determined at a *P*-value < 0.05 by REML ANOVA.

[4]Time is defined as the three time points where sample collection took place: TPT1, 7 d post dry (**TPT2**), and 55 - 75 DIM in the subsequent lactation (**TPT3**).

Table 4. Univariate regression analysis comparing PBMC and MMC enzyme activities or OXPHOS

Model[1]	R^2	Confidence Interval	Slope
Complex I	0.03	0.011, 0.533	0.272
Complex IV	-0.01	-0.406, 0.331	-0.040
ATP synthase	0.43	0.631, 1.001	0.820
OXPHOS[2]	0.41	0.555, 0.912	0.734
Citrate Synthase	0.73	0.731, 0.951	0.841

[1]Models regressed milk mononuclear cell (**MMC**) mitochondrial enzyme activities or OXPHOS on peripheral blood mononuclear cell (**PBMC**) mitochondrial enzyme activities or OXPHOS for each respective enzyme complex.
[2]OXPHOS was calculated by the sum of complex I and complex IV divided by ATP synthase activity.

Figure 1. LSM mitochondrial Complex IV (**CIV**) enzyme activity (y-axis) by treatment at 4 h prior to dry off (**TPT1**), 7 d post dry (**TPT2**) and between 55 – 75 DIM in the subsequent lactation (**TPT3**, x-axis). (**A**) Peripheral blood mononuclear cell CIV activity. (**B**) Milk mononuclear cell CIV activity. Treatments included low SCC Control (**LCON**), high SCC Control (**HCON**), high SCC Ceftiofur Hydrochloride (**CH**), and high SCC Cephaphirin Benzathine (**CB**). Treatments received intramammary (**IM**) Ceftiofur Hydrochloride, or Cephapirin Benzathine at dry off after TPT1 samples were collected. Control (LCON and HCON) treatments received no IM cephalosporins. Low SCC were defined as < 100,000 cells/mL in all four quarters and high SCC as > 200,000 cells/mL in a minimum of one quarter at TPT1. The LCON treatment is not shown in (**B**) since low SCC cows did not yield measurable quantities of mitochondria from milk mononuclear cells. Tukey-Kramer adjusted pairwise comparisons for the treatment differences by time point are shown by differing letters (A, B) determined at $P < 0.05$.

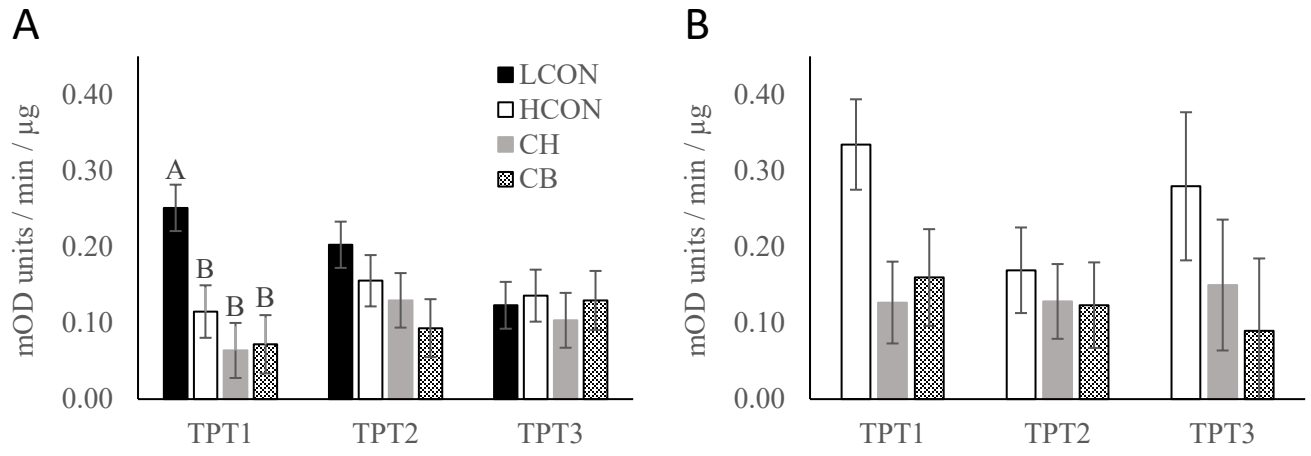

Figure 2. LSM peripheral blood mononuclear cell mitochondrial enzyme activities of complex I (**CI**), ATP synthase, and OXPHOS (y-axis) by treatment 4 h prior to dry off (**TPT1**), 7 d post dry (**TPT2**), and between 55 – 75 DIM in the subsequent lactation (**TPT3**, x-axis). (**A**) CI activity. (**B**) ATP synthase activity. (**C**) OXPHOS. Treatments included low SCC Control (**LCON**), high SCC Control (**HCON**), high SCC Ceftiofur Hydrochloride (**CH**), and high SCC Cephapirin Benzathine (**CB**). Treatments received intramammary (**IM**) Ceftiofur Hydrochloride, or Cephapirin Benzathine at dry off, after TPT1 samples were collected. Control (LCON and HCON) treatments received no IM cephalosporins. Low SCC were defined as < 100,000 cells/mL in all four quarters and high SCC as > 200,000 cells/mL in a minimum of one quarter at TPT1. Tukey-Kramer adjusted pairwise comparisons for time point differences are shown by differing letters (A, B) determined at $P < 0.05$.

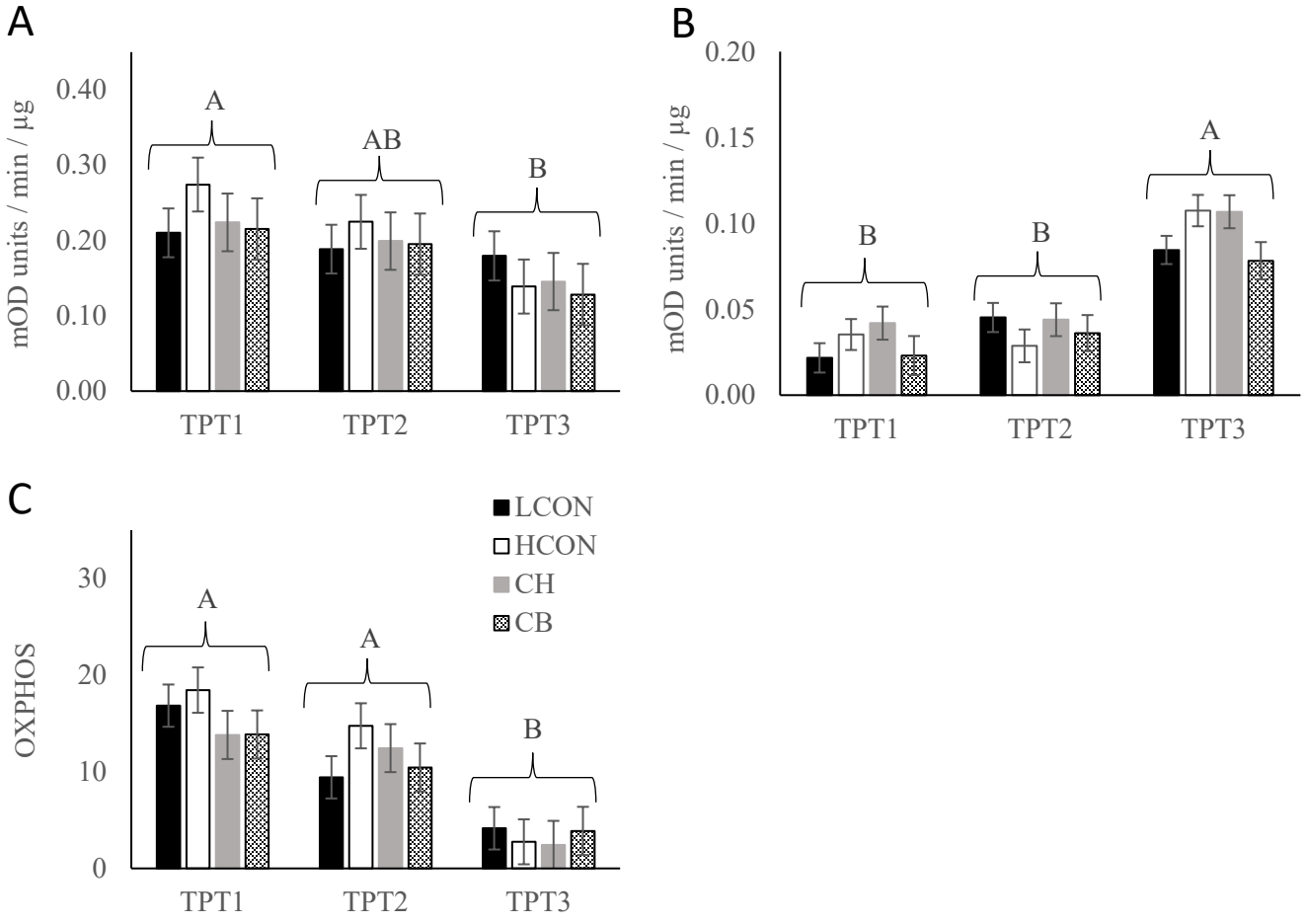

Figure 3. Treatment LSM mononuclear cell (**MMC**) mitochondrial enzyme activity of complex I (**CI**), ATP synthase, and OXPHOS (y-axis) in high SCC cows sampled 4 h prior to dry off (**TPT1**), 7 d post dry (**TPT2**), and between 55 – 75 DIM in the subsequent lactation (**TPT3**, x-axis). (**A**) MMC CI activity. (**B**) MMC ATP synthase activity. (**C**) MMC OXPHOS. Treatments included low SCC Control (**LCON**), high SCC Control (**HCON**), high SCC Ceftiofur Hydrochloride (**CH**), and high SCC Cephapirin Benzathine (**CB**). Treatments received intramammary (IM) Ceftiofur Hydrochloride, or Cephapirin Benzathine at dry off, after TPT1 samples were collected. Low SCC were defined as < 100,000 cells/mL in all four quarters and high SCC as > 200,000 cells/mL in a minimum of one quarter at TPT1. The LCON treatment is not listed since low SCC cows did not yield measurable quantities of mitochondria from MMC. Tukey-Kramer adjusted pairwise comparisons for time point differences are shown by differing letters (A, B) determined at $P < 0.05$.

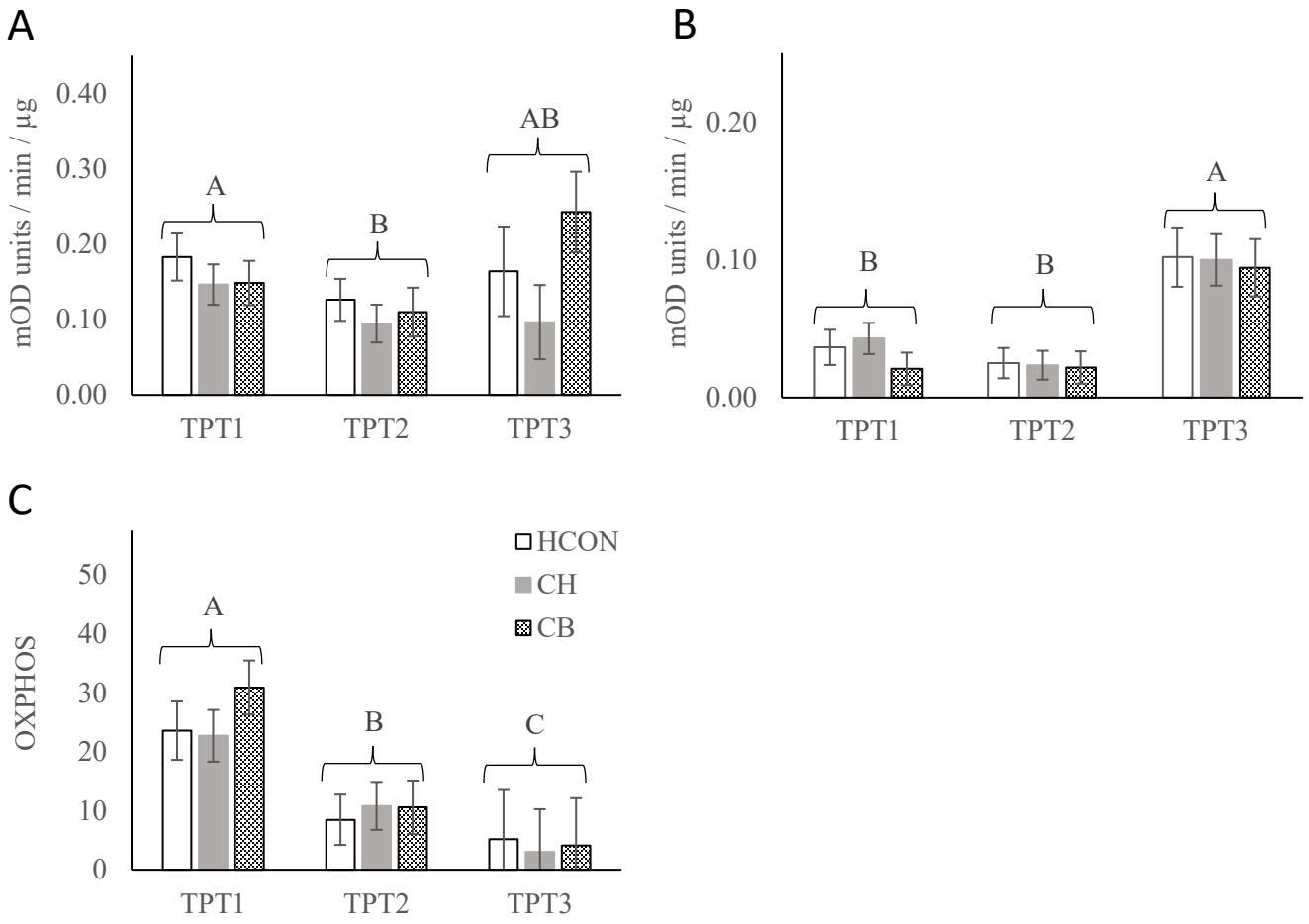

Figure 4. LSM peripheral blood and milk mononuclear cell citrate synthase (**CS**) enzyme activity (y-axis) by treatment 4 h prior to dry off (**TPT1**), and 7 d post dry (**TPT2**) (x-axis). (**A**) Peripheral blood mononuclear cell (**PBMC**) CS activity. (**B**) Milk mononuclear cell (**MMC**) CS activity. Treatments included low SCC Control (**LCON**), high SCC Control (**HCON**), high SCC Ceftiofur Hydrochloride (**CH**), and high SCC Cephapirin Benzathine (**CB**). Treatments received intramammary (**IM**) Ceftiofur Hydrochloride, or Cephapirin Benzathine at dry off, after TPT1 samples were collected. Control (LCON and HCON) treatments received no IM cephalosporins. Low SCC were defined as < 100,000 cells/mL in all four quarters and high SCC as > 200,000 cells/mL in a minimum of one quarter at TPT1. The LCON treatment is not shown in (**B**) since low SCC cows did not yield measurable quantities of mitochondria from milk mononuclear cells. Tukey-Kramer adjusted pairwise comparisons for time point differences are shown by differing letters (A, B) determined at $P < 0.05$.