Peripheral blood mononuclear cell mitochondrial enzyme activity associations with embryo transfer success in dairy cattle recipients

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ABSTRACT

Mitochondria produce energy vital for health, reproduction, and metabolism and contain their own DNA, which is inherited exclusively from the mother. The objective of this study was to assess how mitochondrial enzyme activity (MEA) of Complex I (CI), Complex (IV), and Complex V (CV) varies with pregnancy outcome and other physiological and management factors influencing reproductive efficiency. Whole blood was collected from 40 Holstein cows from June to Sept 2024. Recipients were assigned either Embryo A (Donor 1/Sire 1, n=20) or Embryo B (Donor 2/Sire 2, n=20) based on donor embryo production and recipient availability. Crude mitochondrial extracts were isolated from PBMC and analyzed for MEA of CI, CIV, and CV (Abcam, Cambridge, UK). Pregnancy outcome was checked at approximately 32 days carried calf (DCC) and confirmed at 45 and 90 DCC. Management, genomic, and ET outcome data were collected from the herd management software DairyComp 305 (VAS, Tulare, CA). MEA for each enzyme was regressed on the independent variables of pregnancy outcome confirmed through 90 DCC, embryo treatment, days in milk (DIM) at enrollment, age at enrollment, transfer date, lactation, embryo transfer technician, service number, other enzyme activities (CI, CIV, CV), hematological variables, and performance variables using the GLM procedure of SAS (SAS 9.4, SAS Institute Inc., Cary, NC, USA). Hematological, management and genomic variables were also included because they influence reproductive success. The MEA of CI was associated with DIM at transfer, CIV, age and weight at first calving, and genomic daughter pregnancy rate. CIV was associated with CI and genomic daughter pregnancy rate, and CV was associated with lymphocyte number and genomic Dairy Wellness Profit Dollars® (Zoetis). Associations between CI and CIV reflect their joint role in creating the proton gradient in the electron transport system, and the relationships with age and weight at first

calving, and DIM at transfer suggest that metabolic state influences MEA of CI. Associations with genomic predictors indicate that genetic value contributes to differences in mitochondrial activity through broader effects on metabolism and physiology. Complex I, IV and V activities were not associated with success or failure of pregnancy up to 90 DCC. While mitochondrial enzyme activity was shaped by genetic and physiological factors, pregnancy outcome did not significantly influence MEA in ET recipients.

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ABBREVATIONS

ADG	average daily gain
ADP	adenosine diphosphate
AGEFC	age at first calving
ATP	adenosine triphosphate
CI	Complex I
CIV	Complex IV
CS	citrate synthase
CV	Complex V
DCC	days carried calf
DIM	days in milk
DIMT	days in milk at transfer
ECM	energy corrected milk
ET	embryo transfer
ETS	Electron transport system
FCM	fat corrected milk
HB	hemoglobin
HCG	human chorionic gonadotropin
HCT	hematocrit
IVF	in-vitro fertilization
IVP	in-vitro production
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MEA	mitochondrial enzyme activity
MPV	mean platelet volume
mtDNA	mitochondrial DNA
OPU	ovum pick up
PBMC	peripheral blood mononuclear cells
PLT	platelets
RBC	red blood cell count
RCR	respiratory control ratios
RDW	red cell distribution width
RFI	residual feed intake
SCC	somatic cell count
TCA	Tri Carboxylic Acid
TS	total solids
WBC	white blood cell count
WGTFC	weight at first calving

CHAPTER ONE - REVIEW OF LITERATURE

Introduction

Commonly referred to as the "powerhouse of the cell", mitochondria are organelles in eukaryotic cells that are responsible for the creation of energy for cellular processes. Mitochondria contain their own circular genome without histones, leaving DNA vulnerable to potential damage and higher mutation rates. Combined with nuclear DNA, mitochondrial DNA (mtDNA) contains a varying number of genes that encode for proteins, transfer RNAs, and ribosomal RNA. Cooperation between nuclear DNA and mtDNA allows the mitochondria to create proteins that are not entirely encoded in the cell's DNA. The endosymbiotic theory implies that mitochondria were once bacteria because of their similarities to bacteria. According to the theory, they were engulfed by a primitive eukaryotic cell and after many generations eventually became symbiotic and evolved into mitochondria (Lane and Martin, 2010).

Unlike the genetic inheritance of nuclear DNA, where offspring inherit 50 percent of their genes from each parent, mtDNA is exclusively inherited from the mother in the oocyte. This allows mtDNA to function as a female surname that identifies maternal relatives and relationships. Within dairy cattle, mitochondrial research presents an interest given the historically predominant emphasis of males in dairy cattle genetic selection. This aligns with the need for heightened female selection intensity due to the widespread adoption of sexed semen, increased utilization of embryo transfer and in-vitro fertilization (IVF) technologies, and the expanding market for dairy-beef crossbred terminal calves.

Recent research studies have shown mitochondrial number and enzyme activity can significantly impact calf growth (Niesen and Rossow, 2023), production (Niesen et al., 2022), and reproductive performance (Ferreira et al., 2016). Given the association of higher mitochondrial

enzyme activity (MEA) with higher milk producing cows in early lactation (Niesen and Rossow, 2022) it is of interest to explore if a similar association exists with reproduction. Prior studies have compared mitochondrial quantity and reproductive success, but to our knowledge, no studies to date have explored the association between MEA and reproductive success. If a positive association is present, MEA could be used as a selection tool to identify ideal recipient candidates for reproductive technologies like embryo transfer.

Mitochondrial Structure and Processes

Mitochondria are typically rod-shaped with two membranes, an inner and outer membrane. The mitochondrial outer membrane separates the mitochondrion from the cytosol and protects it from the rest of the cell. The inner mitochondrial membrane is characterized by elaborate folds called cristae and surrounds the matrix (Munn, 2014). Mitochondria produce energy through a series of stages called cellular respiration which consists of glycolysis, the Tri-Carboxylic Acid (TCA) cycle, and oxidative phosphorylation through the Electron Transport System (ETS).

Glycolysis occurs in the cytosol and produces 2 net energy molecules of Adenosine Triphosphate (ATP), 2 NADH coenzymes, and 2 pyruvates. The pyruvates are oxidized to produce 2 additional NADH molecules, and 2 Acetyl CoA molecules. The Acetyl CoA molecules are later utilized in the Citric Acid (TCA) cycle, also referred to as the Krebs cycle , in the inner mitochondrial matrix. In the TCA cycle, Acetyl CoA molecules are further oxidized producing 6 NADH, 2FADH2, and 2 ATP. Lastly, oxidative phosphorylation occurs via the ETS in the inner mitochondrial membrane and will use the NADH and FADH2 produced during glycolysis and the TCA cycle to create a proton gradient of hydrogen ions. This gradient powers ATP synthase that adds a phosphate group to Adenosine Diphosphate (ADP), thereby producing ATP. Compared to glycolysis, oxidative phosphorylation during the ETS produces a significantly

greater number of ATP, roughly 28 ATP (Hill, 2014). However, oxidative phosphorylation requires adaptation in times of increased energy demand and is sensitive to stress. Difficulty adapting can excessively expose mitochondria to proton leak and damage by reactive oxygen species (ROS). Reactive oxygen species can impair mitochondrial structure, components, and DNA, compromising mitochondrial function and cellular health (Paradies et al., 2010). A clear example of this concept has been witnessed in poultry, in which broilers that were not exposed to a coccidiosis challenge had lower proton leak, and higher respiratory control ratios (RCR), indicating higher efficiency of converting ADP to ATP (Acetoze et al., 2016).

Enzymes involved in the ETS were selected for analysis due to their crucial role in ATP production, as the ETS produces the majority of ATP during cellular respiration. Complex I, also referred to as NADH dehydrogenase, is embedded in the inner mitochondrial membrane. Complex I serves as the primary entry point for electrons and is responsible for pumping protons from the mitochondrial matrix into the intermembrane space to establish the proton gradient used for ATP synthesis (Grba et al., 2020). Complex II, also known as succinate dehydrogenase, serves as a direct link between the TCA and ETS by oxidizing succinate to fumarate and transferring the resulting electrons to the ubiquinone pool. The ubiquinone pool in the ETS facilitates the transfer of electrons between the various complexes ensuring a continuous flow and efficiency of energy production (Ackrell, 2000). Complex III, also referred to as the Cytochrome bc1 Complex, receives electrons from ubiquinol in the ubiquinone pool and transfers them to cytochrome c. It also pumps protons from the inner mitochondrial matrix into the intermembrane space, contributing to the proton gradient (Banerjee et al., 2022). Complex IV, also referred to as cytochrome c oxidase, is the terminal enzyme of the ETS. After receiving electrons from cytochrome c, it reduces molecular oxygen to water and pumps protons from the

mitochondrial matrix into the intermembrane space (Capaldi, 1990). Once electrons are received from cytochrome c, Complex IV reduces oxygen molecules to create water (Capaldi, 1990). Complex V, also known as ATP synthase, uses the proton gradient created by Complexes I, III, and IV to catalyze the formation of ATP from ADP and inorganic phosphate. The combined processes of electron transport and ATP synthesis are collectively referred to as oxidative phosphorylation (Neupane et al., 2019).

Methods of Mitochondrial Analysis

Methods of mitochondrial analysis include the use of isolated mitochondria, whole cells, and tissue. Most studies rely on indirect measurements of reactants and products as proxies for electron flow, such as spectrophotometric assays that detect changes in color associated with substrate consumption or product formation. Early mitochondrial research focused on measuring oxygen consumption with tools like the Clark-type oxygen electrode, which, like spectrophotometry, provides an indirect assessment of mitochondrial function by tracking changes in substrates or products of enzymatic reactions. Using isolated mitochondria within cells and tissues, oxygen was measured with a Clark oxygen electrode set to a polarizing voltage in a small incubation chamber. The chamber was maintained at a consistent temperature with reactants added and frequently stirred. While the Tang (2005) study used a chamber temperature of 25 C, similar Clark-type oxygen electrode experiments have widely been conducted at physiological chamber temperatures of approximately 37 C (Kann et al., 2022; Takahashi et al., 2017). Oxygen consumption was measured at different pH levels. Oxygen was measured when only substrate was present (state IV), and when substrate and ADP were present (state III). The ratio between these two states is referred to as the respiratory control ratio (RCR) and indicates the efficiency and integrity of the isolated mitochondria (Tang, 2005). Oxygen consumption can

also be measured in whole cells as seen with the Seahorse (Agilent, Santa Clara, CA). However, since this method utilizes whole cells, oxygen consumption reflects ATP production in the entire cell, including both mitochondrial respiration and cytoplasmic glycolysis, rather than mitochondria alone.

Mitochondrial function can be directly assessed by measuring mitochondrial enzyme activity utilizing cells, tissue, or isolated mitochondria. Enzyme activity is expressed relative to total mitochondrial protein content to ensure differences reflect functional differences rather than variation in mitochondrial quantity. Mitochondrial protein content can be determined using a bicinchoninic acid (BCA) assay, in which proteins reduce copper ions to the cuprous state, generating a color change that is proportional to protein concentration. Whole cells and tissue are homogenized to break open the membranes, releasing mitochondrial enzymes and proteins for measurement, providing a crude assessment of MEA and mitochondrial protein. In contrast, isolated mitochondria from cells or tissue can be obtained using cold, high-speed centrifugation, allowing for a more specific assessment of mitochondrial function and protein content. Samples from homogenates or isolated mitochondria are then added to assay plates with reaction solution and placed in a plate reader for a kinetic assay, where measurements are taken at a specific absorbance wavelength over time and are expressed relative to the mitochondrial protein concentration.

Mitochondrial Dynamics in Cells and Tissues

Mitochondria are present in higher amounts in cells and tissues with a high energy demand, such as the liver, brain, skeletal muscle, and mammary system. The oxidative phosphorylation capacity of cells within a tissue to perform metabolic tasks is dependent on the function and physiological demand of the tissue (Kunz, 2003). This concept was demonstrated with

mammalian tissue in Fernández-Vizarra et al (2011) comparing brain, heart, liver, kidney, and skeletal muscle mtDNA levels and OXPHOS capacity in rats. Mitochondria were isolated from the tissue samples and MEA was measured using spectrophotometric methods. Complex IV (CIV) and citrate synthase (CS) activities were measured in each tissue to measure the capacity of ATP production as well as the total amount or volume of mitochondria present. COX was highest in heart tissue, and lowest in liver and skeletal muscle. Similar results were seen for CS with the exception that liver had the lowest levels in comparison to other tissues. Higher amounts of mtDNA were found in cardiac and skeletal muscle.

Previous studies have associated liver mitochondrial function with performance as seen in poultry (Iqbal, 2005) and cattle (McKenna, 2020). Mckenna (2020) explored mitochondrial quantity and function in both liver and skeletal muscle in Simmental beef cattle and its association with residual feed intake (RFI). Liver and skeletal muscle samples were collected from purebred Simmental heifers (n=24) and bulls (n=28) in addition to growth, and individual dry matter intake over a 70-day period. Enzyme activity of electron transport complex enzymes and Citrate synthase were measured in biopsy samples using spectrophotometry. Animals were ranked into terciles within sex by RFI from high (least feed efficient) to low (most feed efficient). No effect of RFI on mitochondrial quantity in liver or muscle tissue was detected. As for Electron Transport Complex enzyme activity, an RFI x sex interaction was present for Complex I within muscle ($P < 0.05$), and high RFI animals had increased Complex IV activity in liver tissue compared to low RFI animals ($P < 0.05$) (McKenna, 2020).

Mitochondrial analysis offers numerous potentially valuable data points for management decisions, yet it also presents certain limitations. A limitation to most mitochondrial analysis is that it only represents the energy status at the time the sample was collected, and these levels can

change with age or parity in liver tissue (Meng et al., 2007; Kwong and Sohal, 2000) mammary tissue (Hadsell et al., 2011; Alex et al., 2015), and peripheral blood mononuclear cells (Niesen and Rossow, 2019; Niesen and Rossow, 2022). Additionally, extracting tissue samples for mitochondrial analysis from liver or mammary tissue can be labor-intensive and is typically a highly invasive procedure resulting in small sample sizes. Utilizing Peripheral blood mononuclear cells (PBMC) has emerged as a minimally invasive alternative to traditional tissue samples. A correlation between PBMC mtDNA content and tissue mtDNA content in early lactation cows was established by Laubenthal and collaborators (2016). Additionally, several novel studies involving lactating cows (Niesen and Rossow, 2022) and calves (Niesen et al., 2019; Niesen and Rossow, 2023) have assessed mitochondrial function utilizing PBMC. In a 2022 study, Niesen and Rossow associated parity common milk production test-day results of average milk (kg/d), peak milk (kg/d), energy-corrected milk (ECM) (kg/d), fat-corrected milk (FCM) (kg/d), lactose (kg/d), milk fat (kg/d), milk protein (kg/d), and total solids (TS) (kg/d) with MEA of Complex I, Complex IV, Complex V, and Citrate Synthase in 56 Holstein primiparous (n=21) and multiparous (n=35) cows. Enrolled cattle were 70 ± 11 days in milk (DIM) and required to have four functional quarters with no prior cases of *Staphylococcus aureus* mastitis infection. Cows were also housed in the same pen and fed the same diet. Parity related differences were observed in all mitochondrial enzymes apart from Complex V. Both Complex I and Complex V exhibited higher activity in high-production cows across multiple production parameters, including, ECM, FCM, milk fat yield, and TS yield.

Similar results were observed in calves when associating MEA in PBMC with average daily gain, reproductive outcomes, future lactation performance, and survival in Niesen and Rossow's 2019 study. Forty-six calves, comprising 23 Holsteins and 23 Jerseys, were enrolled between 3

and 6 days of age. Calves were required to meet respiratory, general appearance, and fecal scoring criteria, and were evaluated at multiple time points: 1, 2, 8, 36, 52, and 110 weeks of age. The PBMC enzyme levels for Citrate synthase, Complex I, Complex IV, and Complex V, changed relative to age, immune challenge and relative gain (ADG corrected for difference in birth weight). Complex I was higher in higher gain Jersey calves and tended to be higher in Holstein calves, indicating a potential marker for calf growth.

Both studies demonstrate PBMC mitochondrial activity associations with lactation and calf growth performance, however, no study has examined an association with reproductive performance. In addition, MEA levels have been observed to change with age, parity, stage of lactation, and immune status, creating inconsistency depending on the time point collected. The optimal time point and environment in which animals should be sampled to best utilize and interpret mitochondrial activity data has yet to be defined.

Genetic Inheritance of Mitochondria

Numerous prior studies have examined mtDNA sequences and their correlation with production outcomes such as milk and component yields (Bell et al., 1985; Huizinga et al. 1986; Schutz et al., 1992; and Schutz et al., 1994), reproduction (Bell et al., 1985; Huizinga et al. 1986), and health (Schutz et al., 1994). In each of these studies, unique maternal lineages were identified for enrolled animals using Holstein-Friesian herd books. In an analysis of 4,461 cows from 102 maternal lineages, the Bell study concluded that cytoplasmic inheritance, the inheritance of genes outside of the nucleus such as mitochondria DNA, explained 2.0, 1.8, and 3.5% of the phenotypic variation of milk yield, milk fat yield, and percentage of fat in milk (Bell et al., 1985).

Following this study, higher phenotypical values for production variables were reported. Huizinga witnessed 0-13% variation attributed to cytoplasmic inheritance for combined milk fat and protein yield, while Schutz's 1992 paper recorded 4.1, 5.2, and 10.5% variation due to maternal lineage and mtDNA for milk yield, fat yield, and fat percentage. Regarding reproduction, Bell found cytoplasmic inheritance in one model with 83 maternal lines accounting for a 2% variation. Huizinga explored the association between number of inseminations and cytoplasmic inheritance for 79 maternal lines but did not find an effect.

In Schutz et al. (1994), 36 maternal lineages were identified by isolating mitochondrial DNA from whole blood samples. Blood was collected from heifers across various herds in Iowa to identify potential mitochondrial DNA polymorphisms. The findings were consistent with prior studies; associations with production and reproduction were observed, but associations with health and health costs remained unclear. These studies investigating genetic inheritance and the effect of maternal lineage have provided valuable insights into a potential influence on performance, although conclusive findings remain varied and warrant further investigation. It is important to note, however, that mitochondrial DNA alone does not entirely determine mitochondrial activity, as most mitochondrial proteins are encoded by nuclear DNA.

Mitochondria and Reproduction

Mitochondria are of interest in evaluating reproductive success because of their role in cellular energy metabolism. Like other biological processes, reproduction requires energy. For associations with reproduction performance, most mitochondrial research has focused on mitochondrial count and ATP in oocytes as impacted by the age of the donor or season. Research in humans (Bentov, 2011) and cattle (Kansaku et al., 2017; Tamassia et al., 2004; Iwata et al., 2011) have shown that mitochondrial count and ATP content positively impacts the competency

of oocytes and the outcomes of in vitro derived embryos. In humans and cows, younger animals have oocytes with higher mitochondrial counts and ATP compared to older animals, which is associated with greater reproductive success. A similar relationship is seen when comparing bovine oocyte competency in different seasons as demonstrated by Ferreira (2016). Oocytes collected during the summer had lower mitochondrial DNA counts by eight-fold and lower blastocyst rates compared to those collected in the winter (Ferreria et al., 2016).

While most studies have focused on mitochondrial counts and ATP in oocytes, research in mice and humans have explored mitochondrial function directly through ETS enzyme activity. These studies which were conducted in oocytes and preimplantation embryos demonstrated that reduced enzyme activity can compromise oocyte quality, early embryo development, and fertility outcomes (Cecchino et al., 2018; Zou et al., 2021). To date, no studies have examined ETS enzyme activity in relation to reproductive outcomes in dairy cattle, despite its key role in ATP production.

Mitochondrial research has also been conducted in uterine cells and tissues, including immune cells such as neutrophils and macrophages, which are accessible through techniques like uterine cytobrush and biopsy. In dairy cows, factors such as postpartum inflammation, uterine infections, or high metabolic demand, can reduce ATP production and increase signals that promote cell death in endometrial epithelial cells. For instance, an in vitro study by Yang et al. (2022) exposed bovine endometrial epithelial cells to hydrogen peroxide and observed mitochondrial dysfunction, including decreased ATP levels and increased pro-apoptotic proteins, indicating compromised mitochondrial integrity under inflammatory conditions. Such impairment of mitochondrial function may compromise the uterus's capacity to support early embryo development and implantation, ultimately affecting fertility in dairy cattle.

Conclusion

Mitochondria are central to cellular energy metabolism and reproductive physiology. In dairy cattle, mitochondrial number, ATP content, and maternal lineage have all been associated with reproductive outcomes. Research in mice and humans further demonstrates that reduced activity of electron transport system (ETS) enzymes can impair oocyte quality and early embryo development. To date, however, no studies have directly examined the relationship between mitochondrial enzyme activity and reproductive performance in dairy cattle. Investigating mitochondrial function through ETS enzyme activity may provide biomarkers for fertility in dairy cattle and help explain variation in reproductive outcomes. Less invasive approaches, such as measuring mitochondrial function in peripheral blood mononuclear cells (PBMCs), oocytes, or uterine cells obtained via cytobrush or biopsy, present viable options for assessing mitochondrial health without compromising animal welfare.

CHAPTER TWO - Peripheral blood mononuclear cell mitochondrial enzyme activity association with embryo transfer success in dairy cattle recipients

Introduction

Mitochondria generate the energy essential for metabolism, production, health, and reproduction, and possess their own DNA, mtDNA, which is inherited solely from the mother. Despite this, genetic progress and selection in dairy cattle breeding have traditionally focused on paternal traits, often overlooking the potential contributions of maternal influences. With the growing use of beef semen and a shrinking domestic heifer inventory, selecting the best females for propagating the next generation has become more critical than ever. The dairy industry has advanced in identifying top-ranking females for sexed semen allocation, but less attention is given to those in lower percentiles, who are commonly designated for beef semen or serve as embryo transfer (ET) recipients. Reproductive technologies like embryo transfer enable the production of high-value, genetically superior animals from lower-value recipient animals. Recipient selection typically relies heavily on genetic merit, segmenting these females in a lower-ranking group. Within this group, limited attention is given to further identifying the most suitable candidates, which can affect the success of these costly pregnancies. Exploring the contribution of mitochondrial efficiency to reproductive outcomes could provide an opportunity to maximize economic inputs for breeding and selection.

Mitochondria are of interest in evaluating reproductive success because of their role in cellular energy metabolism. For associations with reproduction performance, most mitochondrial research has focused on mitochondrial count and ATP in oocytes as impacted by the age of the donor or season. Research in humans (Bentov, 2011) and cattle (Kansaku et al., 2017; Tamassia et al., 2004; Iwata et al., 2011) has shown that mitochondrial count and ATP content positively

impact the competency of oocytes and the outcomes of in vitro derived embryos. It has been observed in humans and cows that younger animals have oocytes with higher mitochondrial counts and ATP compared to older animals, resulting in greater reproductive success. A similar relationship is seen when comparing bovine oocyte competency in different seasons as demonstrated by Ferreira (2016). Oocytes collected during the summer had lower mitochondrial DNA counts by eight-fold and lower blastocyst rates compared to those collected in the winter (Ferreria et al., 2016). To date, no studies have correlated dairy cattle reproductive outcomes with MEA of electron transport system (ETS) enzymes, which is instrumental in ATP production.

Prior studies have also examined mtDNA sequences and their correlation with production outcomes such as milk and component yields (Bell et al., 1985; Huizinga et al. 1986; and Schutz et al., 1992;), reproduction (Bell et al., 1985; Huizinga et al. 1986), and health (Schutz et al., 1994). In each of these studies, unique maternal lineages were identified for enrolled animals using Holstein-Friesian herd books. In Schutz et al. (1994), 36 maternal lineages were identified by isolating mitochondrial DNA from whole blood samples. Blood was collected from heifers across various herds in Iowa to identify potential mitochondrial DNA polymorphisms. The findings were consistent with prior studies; associations with production and reproduction were observed, but associations with health and health costs remained unclear. The effect of maternal lineage has provided insights on correlations with performance; however, because mitochondrial processes rely on proteins encoded by both nuclear and mitochondrial DNA, further investigation of performance with mitochondrial processes and function is needed.

This study examines mitochondrial enzyme activities (MEA) within peripheral blood mononuclear cells (PBMC). Traditionally, enzyme activity has been measured in high-energy-

demand tissues like the liver or mammary gland, which is invasive and labor-intensive. In contrast, PBMC have emerged as a minimally invasive alternative as they can be isolated from whole blood. A correlation between PBMC mtDNA content and tissue mtDNA content in early lactation cows was established in (Laubenthal et al., 2016). Several novel studies involving lactating cows (Niesen and Rossow, 2022) and calves (Niesen et al., 2019; Niesen and Rossow, 2023) found higher activities of ETS enzymes Complex I and Complex V were associated with increased ECM, FCM, milk fat, and TS in lactating cows, and increased calf survivability. The hypothesis of this study was that MEA of Complex I (CI), Complex IV (CIV), and Complex V (CV) in PBMC at the time of ET, differ according to pregnancy outcome. The objective of this study was to assess how mitochondrial enzyme activity (MEA) of Complex I (CI), Complex (IV), and Complex V (CV) varies with pregnancy outcome and other physiological and management factors influencing reproductive efficiency.

Materials and Methods

The cows were housed on a commercial dairy in Wisconsin, and blood samples were shipped to the University of California, Davis laboratory in California. No direct contact with the cows occurred. Blood collection followed routine herd protocols, as samples are regularly taken from recipients and embryo transfer candidates for health testing.

Animal management

The study was conducted from June to September of 2024 on a commercial dairy herd of approximately 2,800 cows located in southern Wisconsin. Virgin yearling Holstein heifers (n=2) were used as oocyte donors for in-vitro production (IVP) of embryos. Donor animals were housed at one location (Wisconsin, USA) that specializes in donor management. Recipients were housed at a commercial dairy grouped with animals of the same age. Records were entered by farm personnel into a dairy management software program (DairyComp305; VAS, Tulare, CA). Genomic data was collected on all donor and recipient animals enrolled.

Power analysis

A minimum sample size of 12 cows per treatment was estimated based on a 2-tailed test with a difference of 50 % between electron transport chain enzyme complex activities with a power of 0.80 and an α of 0.05 based on data from past studies involving mitochondrial measurements (Niesen and Rossow, 2022).

Enrollment criteria

Embryo recipients (n = 40) were lactating Holstein cows between 70 and 120 DIM, mostly primiparous, and were selected by the farm based on their genetic merit percentile within the

herd, the number of eligible females available for breeding for the month, and the number of heifer calf pregnancies required for the month. Enrollment was based on the number of embryos produced each week and the availability of recipients selected by the farm, with a preference given to primiparous recipients whenever feasible.

Study design

This prospective observational study compares MEA in recipients with embryo transfer pregnancy outcomes (Figure 1). Recipients received one of two embryo matings (A or B) at 70-120 DIM. Of the 40 total recipients that received embryos, 20 were assigned embryo A, and 20 were assigned embryo B. Embryos were assigned based on which donor had embryos available that week and recipient availability.

Follicle Stimulation, Ovum Pick Up, and In Vitro Embryo Production

Two nulliparous donors were collected on a bi-weekly basis in alternating weeks. Prior to Ovum Pick Up (OPU) donor heifers underwent follicle stimulation. Donors were administered a 2 mL dose of Gonadotropin Releasing Hormone (GnRH; Fertagyl, Merck Animal Health, Rahway, NJ) 6 d prior to OPU, followed 48 h later by 6 follicle stimulating hormone (FSH; FOLLTROPIN®, Vetoquinol USA, Fort Worth, TX) injections. The FSH doses were administered in decreasing dosages, concluding 24 h before OPU. For the OPU procedure, donor animals were restrained in a squeeze chute and provided epidural anesthesia (5 mL of 2 % lidocaine hydrochloride; Clipper Distributing Company, St. Joseph, MO). Follicular aspiration was conducted using a transvaginal ultrasound-guided technique with the Evo III ultrasound (IBEX, E.I Medical Imaging, Loveland, CO) and an OPU ultrasound probe (E.I Medical Imaging, Loveland, CO, eC9OPU) for cattle. Follicles greater than 3 mm in diameter were aspirated using a 17-gauge, 600 mm needle. The

needle was connected via plastic tubing to a 50 mL conical tube with 5 mL of heparinized complete flush solution (ABT Complete Flush, ABT 360, Pullman, WA). The conical tube was held in a tube warmer maintained at 36.5 °C and was also separately linked to a vacuum pump consistently operated at 25 mL/min. The resulting follicular content was filtered through a 100 µm cell strainer and rinsed into a warmed grid-searching dish with complete flush solution. Cumulus oocyte complexes were located using a stereomicroscope. In vitro production methods were followed as described in Brown et al. (2024). Embryos in Stage 6-9 and Grade 1 per International Embryo Transfer Society guidelines (4^{th} Edition, 2010) were used for embryo transfer. Each embryo was loaded with holding medium into a 0.25 mL French straw (IMV Technologies, L'aigle, Basse-Normandie, France), placed into a portable straw incubator set at 36.5 °C, and transported to the farm for fresh embryo transfer with a holding time no greater than 3 h.

Recipient estrus synchronization, estrus detection, and embryo transfer

The farm identified animals as potential recipients for embryo transfer based on pedigree and genomic results of common-industry genetic indexes. Recipients were synchronized using a timed-ET double-ovsynch protocol 34 d prior to transfer. Estrus was not observed; cows with a palpable corpus luteum (CL) at time of transfer, had an embryo transferred into the ipsilateral uterine horn and received 2.5 mL of human chorionic gonadotropin (HCG; Chorulon, Merck Animal Health, Rahway, NJ) intramuscularly. Pregnancy was diagnosed via transrectal ultrasound at approximately 32, 45, and 88 d carried calf (DCC), and by palpation at 200 DCC. Since embryos were approximately 7 days old at transfer, the initial pregnancy check occurred around 25 days post-ET. In this context, DCC represents the embryonic age the embryo would be in a successfully maintained pregnancy. Calving ease was recorded and scored on a scale of 1-5

(1 = no problems encountered or unobserved birth; 5 = extreme difficulty) consistent with the National Association of Animal Breeders and United States Department of Agriculture program for calving ease evaluations (1978). Live and stillborn calves were recorded in the herd management software along with birth weight and sex.

Blood Collection and Hematology

Given PBMC are immune cells, hematology analysis was performed on whole blood samples to evaluate the immune status of enrolled animals. Three whole blood 10 mL samples and one whole blood 4 mL sample were obtained via coccygeal venipuncture at the time of OPU collection for donors, and embryo transfer for recipients. Blood samples were collected into tubes containing K2 EDTA anticoagulant (BD Biosciences), inverted to ensure proper mixing, labeled, and then stored on ice packs. Samples were taken as quickly as possible to minimize stress on the animals. These specific timepoints were chosen to evaluate the energy status of the animals precisely at time of oocyte collection and conception. Blood samples were refrigerated, shipped overnight, and processed within 24 h of collection.

Blood (4 mL) from a K2 EDTA tube 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, %, using the Drew Scientific Hemavet 950 Hematology Analyzer (Erba Diagnostics, Oxford, CT). Before evaluating samples, quality control samples (Multi-trol, Drew Scientific) were tested weekly to ensure equipment functioned within specification.

PBMC Isolation and Protein Quantification

Upon arrival to the lab, blood samples were gently mixed on a laboratory rocker and centrifuged at 2000 x *g* for 20 min at 20 °C. Following centrifugation, buffy coat was extracted and transferred to a sodium citrate cell preparation tube (BD Biosciences). Cell preparation tubes were centrifuged at 1800 x g for 30 min at 20 °C. After centrifugation, platelet rich plasma was aspirated as close as possible to the PBMC layer without disrupting the density gradient separating the PBMC. The PMBC were collected, transferred into a sterile 50 mL conical tube with autoMACS Rinsing Solution (PBS, pH 7.2, and 2 mM EDTA; Miltenyi Biotec) for the first wash, and centrifuged at 300 x g for 10 minutes at 20 °C. In the second wash, remaining red cell contaminants were lysed by osmotic shock by adding 1 mL of distilled water, vortexed for 5 s, and immediately diluted with autoMACS Rinsing Solution. PBMC was centrifuged to the final time at 300 x *g* for 10 min at 20 °C and the supernatant was discarded. The mononuclear cell pellet was resuspended in 1 mL of PBS, and 1 mL was transferred to a cryosafe microcentrifuge tube to be stored in liquid nitrogen for mitochondrial isolation. The remaining volume of cell suspension was aliquoted for cell viability and counting, and protein quantification. Cells were counted, and viability was assessed using a 1:200 dilution with a trypan blue and a hemocytometer. Protein concentration within the PBMC was quantified using a BCA protein quantification assay (Abcam, Cambridge, MA; ab102536) according to the manufacturer's protocol.

Mitochondrial Isolation

Samples were stored in liquid nitrogen for no more than 3 d prior to mitochondrial isolation following PBMC isolation. Mitochondria were isolated using the Mitochondrial Isolation Kit for Cultured Cells (Abcam, Cambridge, MA; ab110170) according to the manufacturer's protocol and as detailed by Niesen and Rossow (2022). Isolated PBMC were lysed under gentle

conditions to release cellular organelles and simultaneously preserve mitochondrial integrity. This was followed by centrifugation to pellet mitochondria and separate them from other cellular components. Once the mitochondrial pellet was isolated, resuspended in 500 µL of Reagent C, and supplemented with 2.5 µL of protease inhibitor (Abcam, Cambridge, MA, ab201111), samples were aliquoted and stored at -80 °C. The crude mitochondrial protein concentration of one aliquot per animal was measured by BCA and used to correct the final activities of each sample.

Measurement of Mitochondrial Complex I, Complex IV, and Complex V Activities

Enzymatic activity assays were conducted following the collection and processing of all samples into crude mitochondrial extracts, which were subsequently used for measuring enzyme activities. Mitochondrial protein content in the crude extracts was quantified using a BCA protein assay (Abcam, Cambridge, MA; ab102536) according to the manufacturer's protocol, allowing enzyme activities to be expressed relative to protein content. Enzyme activity was measured using microplate assay kits (Abcam, Cambridge, MA) following the manufacturer's protocol and as detailed by Niesen and Rossow (2022). All kits were bovine species reactive and from the same lot. Negative background controls were prepared using the buffer solution provided in the enzyme kits, while positive controls consisted of bovine heart mitochondria (Abcam, Cambridge, MA; ab110338) standardized to 5.5 mg/mL of total protein. The microplates were incubated for 3 hours at room temperature before absorbance measurements were taken using a VersaMax tunable microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) in kinetic mode. Readings were taken every 30-60 seconds for one hour, dependent on the enzyme kit.

Statistical Analysis

Cow was the experimental unit of interest. Difference was determined by $P < 0.05$ with tendencies considered as $0.05 < P \leq 0.10$. All residuals were examined for normality. All data were expressed as least square means with standard error of the mean. Backwards selection was used to remove independent variables that did not contribute to the regression analysis.

Cows were grouped by pregnancy outcome for each embryo. Enzyme activity was defined as the linear rate of change of absorbance per minute, adjusted to the micrograms of crude mitochondrial protein in the well (mOD units/min/μg mitochondrial protein). For each sample, absorbance data timepoints were used to calculate a single slope, which represented the enzyme activity value for that animal. The slope (enzyme activity) was estimated using the General Linear Models procedure to regress optical density on time (SAS 9.4, SAS Institute Inc. 2024, Cary, NC, USA). Outliers beyond 2 standard deviations were excluded and final activities were corrected by the amount of crude mitochondrial protein in the sample. The model used in analysis was $Y_{OD} = \beta_0 + \beta_1 Time + \varepsilon$, in which Y_{OD} = optical density, β_0 = y-intercept, β_1 = regression coefficient for time, and ε = residual error.

Animal performance, hematology, and MEA analysis were also conducted using the General Linear Models procedure of SAS. To assess initial differences between recipient groups, a model was run for each of the following animal performance metrics as the dependent variable: pre-wean average daily gain (ADG), heifer days to conception, weight at first calving, age at first calving, week 4 daily milk average, week 8 daily milk average, week 12 daily milk average, genomic Dairy Wellness Profit Dollars® (Zoetis), genomic Daughter Pregnancy Rate, and genomic Cow Conception Rate. In each model, embryo, age at transfer, DIM at transfer, and the other performance metrics were included as independent variables in the preliminary model. Each trait was analyzed once as the dependent variable, and also included as an independent

variable in the models for the other traits. Backward selection was then used to create the final models.

For hematology analysis, each hematological variable was regressed on the independent variables of pregnancy outcome confirmed through 90 DCC, embryo treatment, DIM at enrollment, age at enrollment, transfer date, lactation, embryo transfer technician, service number, calving ease of prior calving, sex of calf of prior calving, and hematological variables. Hematological variables included: white blood cell count (WBC), neutrophil count, lymphocyte count, monocyte count, eosinophil count, basophil count, red blood cell count (RBC), hemoglobin (HB), hematocrit % (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelets (PLT), and mean platelet volume (MPV). Each hematological variable was initially included as an independent variable in the preliminary model prior to backward elimination. A separate model was run for each hematological variable as a dependent variable. Hematology variables were included because MEA were measured in PBMC allowing for a better understanding of the immune state and its potential impact on MEA. Statistical differences within the clinically normal range were defined as not different.

For MEA analysis, MEA for each enzyme was regressed on the independent variables of pregnancy outcome confirmed through 90 DCC, embryo treatment, DIM at enrollment, age at enrollment, transfer date, lactation, embryo transfer technician, service number, calving ease of prior calving, sex of calf of prior calving, other enzyme activities (CI, CIV, CV), hematological variables, and performance variables. These variables were selected due to their connection with reproductive outcomes. Variables were then subjected to backward selection to obtain the final models. The model for CI was $CI = \beta0 + \beta1DIMT + \beta2CIV + \beta3AGEF1 + \beta4WGHT1 +$

β5gDPR + ε, in which CI = enzyme activity for Complex I, β0 = intercept, β1 = regression coefficient for DIMT, β2 = regression coefficient for CIV, β3 = regression coefficient for AGEF1, β4 = regression coefficient for WGHT1, β5 = regression coefficient for gDPR, and ε = residual error. The model for CIV was CIV = β0 + β1CI + β2gDPR + ε, in which CIV = enzyme activity for Complex IV, β0 = intercept, β1 = regression coefficient for CI, β2 = regression coefficient for gDPR, and ε = residual error. The model for CV was CV = β0 + β1gDWP$® + β2lynum + ε, in which CV = enzyme activity for Complex V, β0 = intercept, β1 = regression coefficient for gDWP$®, β2 = regression coefficient for lymphocyte number (lynum), and ε = residual error. Residuals for these regressions were normally distributed.

Results and Discussion

Of the total 40 recipients, 12 conceived: 6 that received embryo A and 6 that received embryo B. All 12 recipients remained pregnant from the initial pregnancy diagnosis at 32 DCC through 90 DCC.

Initial Differences between Recipient Groups

To assess potential initial differences in recipient uniformity on PBMC MEA, prior performance variables were compared between recipient groups (Table 1). These variables included pre-wean growth performance (ADG), parturition metrics (heifer days to conception, age at first calving, weight at first calving), early lactation milk production (weekly average for week 4,8, and 12 of current lactation), and genomic values (DWP$®, DPR, CCR). Age and DIM at time of enrollment were also evaluated. No differences were observed among any of the variables between recipient groups, indicating that the quality of recipients was consistent between recipient groups.

This herd utilized a precise genetic strategy that calculated the number of heifers required each month and strategically ranked breeding eligible animals monthly on the DWP$® selection index (Table 1). High-ranking females received sexed semen to create the next generation of replacements, and lower-ranking females were assigned to beef semen, embryo transfer, or in a few cases conventional Holstein semen. Compared to their herd mates of the same age and lactation stage who were not in the trial, enrolled females averaged over 60 points lower on DWP$®, were the same for DPR and CCR, and had less than 0.5kg variation in milk yield at 4 wk, 8 wk , and 12 wk of lactation. However, enrolled females calved 35 days earlier and weighed 9.55 kg less at first calving. Despite their lower genetic merit which categorized them as

recipients, enrolled heifers required 0.5 fewer services per conception compared to their herd mates, explaining their earlier age and lighter weight at calving.

Least square means were also compared between pregnancy diagnosis confirmed through 90 DCC (Table 1). Age and DIM at enrollment were different between pregnant and non-pregnant animals. Of the 40 cows enrolled, 37 were first lactation, 2 were second lactation, and 1 was third lactation. The third lactation cow conceived to embryo transfer, making her an outlier for age and DIM. However, since this involved only one cow and no differences were found in age and DIM among first lactation cows, the significance was not considered. All other management variables were not different by pregnancy diagnosis.

Hematological differences between pregnancy outcome and recipient group

Hematology variables were measured for each recipient using samples taken at the time of embryo transfer (Table 2). Given that MEA was measured in PBMCs, hematology parameters were evaluated to account for the animal's immune status, which could influence MEA outcomes. All cows enrolled as potential recipients were deemed healthy based on the absence of clinical illnesses such as mastitis, lameness, or reproductive diseases. Hematology results were defined by pregnancy outcome confirmed through 90 DCC. Hematological values were not included for one pregnant animal due to poor blood sample quality and values that were beyond three standard deviations. Hematology parameters were compared by pregnancy outcome, embryo assigned, and relative to clinically normal reference ranges. Reference ranges were obtained from the manufacturer of the HemaVet analyzer (Erba Diagnostics, Oxford, CT) used to perform hematological assessments.

Recipients were in the normal reference ranges for all hematological parameters except for neutrophil count, which was slightly higher than the reference range. The neutrophil count was not different between embryo assigned ($P = 0.71$). A slightly elevated neutrophil count could suggest a mild inflammatory response, sub-clinical infection, or stress. Given the experiment was conducted during the summer, this mild-inflammatory state could potentially be in response to higher temperatures. Somatic cell count (SCC) data was collected monthly through routine milk testing on the herd. The second and third monthly tests, conducted around the time of embryo transfer, did not indicate evidence of subclinical infection, with the highest SCC recorded among recipients being 102,000 cells/mL. However, higher mean neutrophil concentrations have been found to be significantly different between reproductive states ($P < 0.001$; Subandrio et al., 2000). Progesterone can have an immunosuppressive effect to protect the developing embryo, which is partially foreign or semi-allogenic to the maternal immune system (Herath et al., 2006; Cui et al., 2020).

The platelet count was higher in pregnant animals at 304 compared to 210 in non-pregnant cows ($P = 0.03$). However, although the platelet values are statistically different, they are within the reference range and were not considered different in terms of clinical significance. No significant differences were observed between embryo assignments, except for MCV. Mean corpuscular volume, which reflects the average size of red blood cells, was 0.04 fL greater in pregnant cows compared to those that were non-pregnant, although both values were in the normal reference range. Overall, the immune status was comparable between pregnancy outcome and embryo assignment.

Mitochondrial enzyme activity differences between pregnancy outcome and recipient groups

Mitochondrial enzymes from the electron transport system were analyzed due to their key role in ATP production during cellular respiration. Complexes I and IV help create the proton gradient, while CV (ATP Synthase) uses that gradient to generate ATP through oxidative phosphorylation. Mitochondrial enzyme activities between pregnant and non-pregnant recipients are summarized in Table 3. Ratios between C1:CV, CIV:CV, and CI:CIV were also evaluated but were not different.

Complex I and IV

Complex I was associated with DIM at transfer (DIMT), CIV activity, age at first calving (AGEF1), weight at first calving (WGHT1), and genomic daughter pregnancy rate (gDPR). Complex IV was associated with CI activity and gDPR. The observed positive association between CI and CIV is supported by their interdependent roles within the ETS. As the primary entry point for electrons, CI initiates electron flow, while CIV serves as the terminal complex, reducing oxygen to water. Together, they establish and maintain the proton gradient required for ATP synthesis.

Both CI and CIV were associated with gDPR, implying that nuclear-encoded genes regulating mitochondrial function are represented to some extent within the genomic markers used for fertility evaluations. Interestingly, the associations were in opposite directions for CI (positive) and CIV (negative), suggesting that selection for fertility may capture some aspects of mitochondrial function, though the relationship is complex. These findings highlight the potential practical value of selecting for fertility. Genomic trait values such as gDPR may reflect both reproductive performance and underlying energy metabolism important for cow health and longevity, even though MEA was not directly linked to pregnancy outcome in this study.

Complex I (CI) activity was solely associated with DIM at transfer (DIMT), age at first calving (AGEF1), and weight at first calving (WGHT1), suggesting that metabolic state influences mitochondrial enzyme activity. Associations with AGEF1 and WGHT1 are critical for farm profitability, as they are key indicators of heifer development and future performance. CI activity was positively associated with higher age at first calving and negatively associated with weight at first calving. These results suggest that heifers reaching an adequate age at first calving while maintaining moderate body weight may have more optimal metabolic efficiency. However, it is not possible to determine whether differences in weight reflect overall size or body condition, as body condition score or stature data were not collected. The association with DIMT likely reflects differences in metabolic state depending on stage of lactation. Niesen and Rossow (2022), showed that CI activity varies with proximity to peak DIM. The positive association with DIMT likely reflects differences in metabolic state depending on the stage of lactation. CI activity may vary as cows approach or move past peak lactation, reflecting shifts in energy demand and substrate availability. This idea is reflected in Niesen and Rossow (2022), which demonstrated that CI activity changes with proximity to peak DIM, where higher activity was observed near peak milk production.

Despite these associations and prior observations, no differences in MEA were observed at the time of transfer between cows that subsequently maintained pregnancy through 90 DCC and those that did not for CI or CIV in this study. Embryo assignment also did not result in differences in MEA between recipient groups. While previous work by Niesen and Rossow (2022) found that CI activity was higher in high-producing cows and associated with various milk production metrics, and their earlier study (2019) found similar trends for growth in calves, no relationship was observed between MEA and milk production or average daily milk yield in

this population. Average daily milk yield for 4, 8, and 12 wk of lactation were included in the preliminary MEA model but were removed from the final model during backwards elimination.

Complex V

Complex V activity was associated with lymphocyte number (lynum) and genomic Dairy Wellness Profit $® (gDWP$®; Zoetis). The association of lynum was negative (estimate = -0.16924), indicating that higher lymphocyte counts corresponded to slightly lower CV activity. A slightly elevated lymphocyte count could suggest a mild inflammatory response, sub-clinical infection, or stress. Since enzyme activity was measured in PBMCs, this may reflect shifts in the metabolic state or composition of immune cells rather than systemic energy availability, as MEA results from PBMC are sensitive to changes in immune status.

Complex V activity was also negatively associated with gDWP$® (estimate = -0.000045286). However, the magnitude of this estimate is extremely small relative to the large variability in gDWP$® values, which spanned approximately 800 points, indicating that this directional trend is unlikely to have meaningful consequences for herd profitability or longevity.

No differences were detected between pregnancy outcome or recipient groups. In Niesen and Rossow (2022), higher-producing cows had increased CV activity and CV activity was different between production level groups, but not between parities as seen with CI, making CV unique as a potential marker for lactation performance. This trend was also observed in the earlier study (Niesen and Rossow, 2019), however CV was not different when comparing high- and low-gain groups for both Holstein and Jersey calves.

Conclusion

Mitochondrial enzyme activities in PBMCs were assessed in Holstein dairy cattle recipients during early lactation. Complex I, IV, and V activities were associated with genomic and physiological indicators of metabolic status, growth, and immune function, including DIM at transfer, age and weight at first calving, lymphocyte counts, and genomic measures of fertility and profitability. However, no differences in enzyme activities were observed between cows that maintained pregnancy and those that did not following embryo transfer, up to 90 DCC. These results suggest that while MEA in PBMC reflects aspects of metabolic and immune function, it may not be a reliable marker for predicting reproductive success in recipient cows. Additional biomarkers or combinations of physiological and metabolic measures may be needed to better identify the most suitable candidates for embryo transfer. Further research with more animals should be conducted to further investigate these concepts.

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Table 1. Least-squared means comparison of recipient age and DIM at enrollment and prior performance metrics between recipient groups and pregnancy diagnosis through 90 DCC.

Metric	Embryo Group		Pregnancy Diagnosis		SEM	*P*-Value[3]	
	Recipients A[1] (n=20)	Recipients B[2] (n=20)	Pregnant (n=12)	Non-Pregnant (n=28)		Embryo[4]	Preg[5]
Age at Enrollment, d	860	839	889	810	30	0.47	0.03
DIM at Enrollment, d	96	94	98	92	2.5	0.30	0.03
ADG to Weaning (kg/d)	0.84	0.82	0.84	0.83	0.014	0.16	0.66
Age at First Calving (d)	700	700	700	700	1.23	0.87	0.78
Weight at First Calving (kg)	644	648	652	639	7.2	0.58	0.18
4 wk Milk Avg (kg)[6]	40.1	39.8	40.0	39.9	0.54	0.54	0.93
8 wk Milk Avg (kg)[7]	43.2	43.5	43.2	43.5	0.43	0.42	0.71
12 wk Milk Avg (kg)[8]	43.1	42.5	42.9	42.8	0.62	0.32	0.93
gDWP$®$®[9]	771	806	762	815	46	0.45	0.31
gDPR[10]	-0.8	-0.8	-0.8	-0.7	0.14	0.93	0.53
gCCR[11]	0.4	0.4	0.4	0.4	0.17	0.59	0.97

[1]Cows that received Embryo A.
[2]Cows that received Embryo B.
[3]Significance determined at a *P*-value of < 0.05.
[4]*P*-Value comparison of recipients that received Embryo A vs Embryo B.
[5]*P*-Value comparison of pregnant vs non-pregnant recipients.
[6]DC305 average milk per day in wk 4 of lactation.
[7]DC305 average milk per day in wk 8 of lactation.
[8]DC305 average milk per day in wk 12 of lactation.
[9]August 2024 genomic value for Zoetis Dairy Wellness Profit Dollars Index®.
[10]August 2024 genomic value for Daughter Pregnancy Rate genetic trait.
[11]August 2024 genomic value for Cow Conception Rate genetic trait.

Table 2. Least square means of hematological variables by pregnancy diagnosis confirmed through 90 DCC.

Variable	Reference Range[1]	Pregnancy Diagnosis		SEM	*P*-Value[2]	
		Pregnant (n=11)[3]	Non-pregnant (n=28)		Preg[4]	Embryo[5]
Leukocytes						
WBC, K/µL	4.0 - 12.0	7.25	7.25	0.0042	0.53	0.51
Neutrophils, K/µL	0.6 - 4.1	4.32	4.32	0.0021	0.12	0.71
Lymphocytes, K/µL	2.5 - 7.5	2.63	2.63	0.0021	0.13	0.70
Monocytes, K/µL	0.0 - 1.2	0.183	0.186	0.0020	0.11	0.74
Eosinophils, K/µL	0.0 - 2.4	0.086	0.089	0.0020	0.18	0.43
Basophils, K/µL	0.0 - 0.4	0.021	0.024	0.0022	0.16	0.94
Erythrocytes						
Red Blood Cells, M/µL	5.0 - 10.0	6.30	6.31	0.0090	0.34	0.27
Hemoglobin, g/dL	8.0 - 15.0	9.26	9.26	0.019	0.76	0.81
Hematocrit, %	24.0 - 46.0	27.1	27.1	0.036	0.67	0.97
Mean Corpuscular Volume, (fL)	40.0 - 60.0	43.1	43.1	0.037	0.34	0.01
Mean Corpuscular Hemoglobin, pg	11.1 - 17.0	14.7	14.7	0.026	0.45	0.73
Mean Corpuscular Hemoglobin Concentration, g/dL	28.2 - 36.0	34.2	34.2	0.047	0.83	0.14
Red Cell Distribution Width, %	12.0 - 27.0	21.5	22.0	0.38	0.25	0.16
Thrombocytes						
Platelets, K/µL	200 - 800	304	210	41	0.03	0.79
Mean Platelet Volume, fL	5.0 - 20.0	7.36	7.12	0.35	0.50	0.74

[1]Normal cow hematology reference ranges preset on the Hemavet 950 FS analyzer.
[2]Significance determined at a *P*-value of < 0.05.
[3]One pregnant animal was not included in the analysis due to poor blood sample quality.
[4]*P*-Value comparison of pregnant vs non-pregnant recipients.
[5]*P*-Value comparison of recipients that received Embryo A vs Embryo B.

Table 3. Least square means of recipient mitochondrial enzyme activity by pregnancy diagnosis confirmed through 90 DCC.

Variable	Pregnancy Diagnosis		SEM	*P*-Value[2]								
	Pregnant (n=12)	Non-pregnant (n=28)		Preg[3]	DIMT[4]	CI[5]	CIV[6]	AGEF1[7]	WGHT1[8]	gDPR[9]	gDWP$®[10]	lynum[11]
Enzyme												
Complex I	0.0142	0.0123	0.0022	0.5678	0.0113	-	<.0001	0.0031	0.0315	0.0016	-	-
Complex IV	0.186	0.249	0.044	0.1695	-	<.0001	-	-	-	0.0067	-	-
Complex V	0.0714	0.0708	0.0078	0.9428	-	-	-	-	-	-	0.05	0.0085

[1]Units of enzyme activity are milli optical density/min per µg of mitochondrial protein.
[2]Significance determined at a *P*-value of < 0.05.
[3]*P*-Value comparison of pregnant vs non-pregnant recipients.
[4]*P*-Value comparison of DIM at time of embryo transfer.
[5]*P*-Value comparison of Complex I activity.
[6]*P*-Value comparison of Complex IV activity.
[7]*P*-Value comparison of age at first calving.
[8]*P*-Value comparison of weight at first calving.
[9]*P*-Value comparison of genomic daughter pregnancy rate value.
[10]*P*-Value comparison of genomic Dairy Wellness Profit $® value.
[11]*P*-Value comparison of lymphocyte number.

Figure 1: Breakdown of experimental design for recipient sample and data collection.