Title

Peripheral blood mononuclear cell mitochondrial enzyme activity is associated with parity and lactation performance in early lactation Holstein dairy cows

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Peripheral blood mononuclear cell mitochondrial enzyme activity in calves is associated with average daily gain, reproductive outcomes, lactation performance, and survival

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The ARRIVE guidelines 2.0: author checklist

The ARRIVE Essential 10

These items are the basic minimum to include in a manuscript. Without this information, readers and reviewers cannot assess the reliability of the findings.

Item		Recommendation	Section/line number, or reason for not reporting
Study design	1	For each experiment, provide brief details of study design including: a. The groups being compared, including control groups. If no control group has been used, the rationale should be stated. b. The experimental unit (e.g. a single animal, litter, or cage of animals).	91-93 210
Sample size	2	a. Specify the exact number of experimental units allocated to each group, and the total number in each experiment. Also indicate the total number of animals used. b. Explain how the sample size was decided. Provide details of any *a priori* sample size calculation, if done.	91-93 93-97
Inclusion and exclusion criteria	3	a. Describe any criteria used for including and excluding animals (or experimental units) during the experiment, and data points during the analysis. Specify if these criteria were established *a priori.* If no criteria were set, state this explicitly. b. For each experimental group, report any animals, experimental units or data points not included in the analysis and explain why. If there were no exclusions, state so. c. For each analysis, report the exact value of *n* in each experimental group.	107 93 579-611
Randomisation	4	a. State whether randomisation was used to allocate experimental units to control and treatment groups. If done, provide the method used to generate the randomisation sequence. b. Describe the strategy used to minimise potential confounders such as the order of treatments and measurements, or animal/cage location. If confounders were not controlled, state this explicitly.	Observational study Observational Study
Blinding	5	Describe who was aware of the group allocation at the different stages of the experiment (during the allocation, the conduct of the experiment, the outcome assessment, and the data analysis).	NA
Outcome measures	6	a. Clearly define all outcome measures assessed (e.g. cell death, molecular markers, or behavioural changes). b. For hypothesis-testing studies, specify the primary outcome measure, i.e. the outcome measure that was used to determine the sample size.	82-85 122-128 85-89
Statistical methods	7	a. Provide details of the statistical methods used for each analysis, including software used. b. Describe any methods used to assess whether the data met the assumptions of the statistical approach, and what was done if the assumptions were not met.	210-282 229-232
Experimental animals	8	a. Provide species-appropriate details of the animals used, including species, strain and substrain, sex, age or developmental stage, and, if relevant, weight. b. Provide further relevant information on the provenance of animals, health/immune status, genetic modification status, genotype, and any previous procedures.	91-93 120-123
Experimental procedures	9	For each experimental group, including controls, describe the procedures in enough detail to allow others to replicate them, including: a. What was done, how it was done and what was used. b. When and how often. c. Where (including detail of any acclimatisation periods). d. Why (provide rationale for procedures).	88-207 120-128 131-132 91 99-104
Results	10	For each experiment conducted, including independent replications, report: a. Summary/descriptive statistics for each experimental group, with a measure of variability where applicable (e.g. mean and SD, or median and range). b. If applicable, the effect size with a confidence interval.	579-611 NA

The Recommended Set

These items complement the Essential 10 and add important context to the study. Reporting the items in both sets represents best practice.

Item		Recommendation	Section/line number, or reason for not reporting
Abstract	11	Provide an accurate summary of the research objectives, animal species, strain and sex, key methods, principal findings, and study conclusions.	14-40
Background	12	a. Include sufficient scientific background to understand the rationale and context for the study, and explain the experimental approach. b. Explain how the animal species and model used address the scientific objectives and, where appropriate, the relevance to human biology.	44-85 50-62
Objectives	13	Clearly describe the research question, research objectives and, where appropriate, specific hypotheses being tested.	82-85
Ethical statement	14	Provide the name of the ethical review committee or equivalent that has approved the use of animals in this study, and any relevant licence or protocol numbers (if applicable). If ethical approval was not sought or granted, provide a justification.	88-89
Housing and husbandry	15	Provide details of housing and husbandry conditions, including any environmental enrichment.	107-117
Animal care and monitoring	16	a. Describe any interventions or steps taken in the experimental protocols to reduce pain, suffering and distress. b. Report any expected or unexpected adverse events. c. Describe the humane endpoints established for the study, the signs that were monitored and the frequency of monitoring. If the study did not have humane endpoints, state this.	134-135 NA NA
Interpretation/ scientific implications	17	a. Interpret the results, taking into account the study objectives and hypotheses, current theory and other relevant studies in the literature. b. Comment on the study limitations including potential sources of bias, limitations of the animal model, and imprecision associated with the results.	288-438 328-331 432-434
Generalisability/ translation	18	Comment on whether, and how, the findings of this study are likely to generalise to other species or experimental conditions, including any relevance to human biology (where appropriate).	NA
Protocol registration	19	Provide a statement indicating whether a protocol (including the research question, key design features, and analysis plan) was prepared before the study, and if and where this protocol was registered.	NA
Data access	20	Provide a statement describing if and where study data are available.	7
Declaration of interests	21	a. Declare any potential conflicts of interest, including financial and non-financial. If none exist, this should be stated. b. List all funding sources (including grant identifier) and the role of the funder(s) in the design, analysis and reporting of the study.	5 6, 10-12

Title:

Peripheral blood mononuclear cell mitochondrial enzyme activity in calves is associated with average daily gain, reproductive outcomes, lactation performance, and survival

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INTERPRETATIVE SUMMARY

This research highlights the dairy industry's need for exploring novel technologies such as mitochondrial function to assess cow performance and energy status. Use of mitochondrial enzyme activities could provide greater insight into predicting cow health, survival, reproductive performance, and milk production.

ABSTRACT

Mitochondria are central to metabolism and are the primary energy producers for all biosynthesis. The objective of this study was to determine if the mitochondrial enzyme activity of peripheral blood mononuclear cells in heifers were associated with ADG, reproductive outcomes, first lactation milk production and survival. Twenty-three Holstein and 23 Jersey heifer calves were enrolled, and blood and body weight data were collected at 1, 2, 8, 36, 52 and 110 wk of age. Respiratory and fecal scores were recorded daily for the first 30 d of life. Milk production data were collected from herd management software through first lactation and health events were tracked to the fourth lactation on surviving animals. Mitochondrial isolation and enzyme activities for citrate synthase, complex I, complex IV, and complex V were determined using kits from Abcam. Data were analyzed using GLM and the Logistic procedure of SAS (Version 9.4). Multivariate regression analyses were conducted to determine if calf mitochondrial enzymatic activity and covariate health indices (fecal and respiratory scores, number of treatments, hematology) were associated with ADG (8, 36, 52 and 110 wk), lactation performance (milk yield, fat yield, solids yield, ECM, 305ME and relative value) and reproduction (age at first service, age at first conception, age at first calving and number of services). For Holsteins and Jerseys, mitochondrial enzyme activities and health indices were correlated to all ADG and milk production outcomes ($R^2 \geq 0.63$, and $R^2 \geq 0.45$, respectively). Reproduction outcomes were correlated with bodyweight gain, mitochondrial function and red blood cell traits for Holsteins and Jerseys ($R^2 \geq 0.47$, and $R^2 \geq 0.55$, respectively). Logistic regression analyses were performed to determine if early life enzymatic activity impacted survival outcomes in the herd. Calves below the median for complex V enzyme activity at 1 wk were more likely to be removed from the herd compared to calves above the median by lactation

1, 2, 3 and 4 (odds ratio = 4.7, 7.7, 7.0 and 6.9, respectively). Calves below the median for the difference in hematocrit from 2 to 1 wk were more likely to be removed from the herd compared to calves above the median by lactation 1, 2, 3 and 4 (odds ratio = 13, 10, 5.2 and 4.7, respectively). These findings suggest that predictions of cow performance could be improved by considering the impact of early life mitochondrial enzymatic activity and health indices.

Key words

Mitochondria, survival, production, reproduction, growth

INTRODUCTION

One opportunity for cutting expenses and maintaining profitability on dairy farms is to focus resources on heifers with high-performing mitochondria. Mitochondria are central to metabolism and health and offer a novel approach to assess cow performance. Mitochondrial traits have been shown to influence bovine bodyweight gain and milk production (Brown et al., 1988; Niesen and Rossow, 2019; Niesen and Rossow, 2022) and reproduction (Iwata et al., 2010; Ferreira et al., 2016; Kansaku et al., 2017). Additionally, early works by Bell et al. (1985) and Brown et al. (1988) suggested that cow cytoplasmic inheritance could indicate future milk production in progeny, since mitochondria are maternally inherited. The use of peripheral blood mononuclear cells (PBMC) offers a high throughput method of assessing mitochondrial function in cattle, as the mitochondria can be obtained from blood samples (Niesen and Rossow, 2019; Niesen and Rossow, 2022). Assays of PBMC mitochondrial enzymes of the respiratory chain complexes and citric acid cycle enzymes are minimally invasive and can identify mitochondrial impairment (Rustin et al., 1994; Hsiao et al., 2018). Dysfunction of the respiratory chain complexes can result from mutations in mitochondrial or nuclear DNA, aging, and may result in increased reactive oxygen species, cell death and disease (DiMauro and Schon, 2003; Balaban et al., 2005; Moran et al., 2012). The mitochondrial enzymes of the respiratory chain complexes and citric acid cycle enzymes are central to the production of ATP and impact an animal's ability to produce the energy necessary to meet the demands of growth, health, and production.

If mitochondria could be screened for performance, heifer merit could be determined early in life and improve farm economic outcomes by meeting production goals with fewer heifers raised. Care and management for a replacement heifer can be as high as 20% of the total cost associated with dairy production (Fetrow, 1987; Lehenbauer & Oltjen; 1998; Gabler et al.,

2000) and has been estimated to be between $1700 – $2400 per heifer (Overton & Dhuyvetter, 2020). Heifer culling and mortality are highest in the first 2 years of life. Producers often battle high pre-wean calf mortality, where 13 – 22 % of heifers fail to reach first calving and up to 26% are culled after their first lactation (Hadley et al., 2006; Brickell and Wathes, 2011; Cooke et al., 2013).

The selection of dairy cows based on genetic milk yield traits, has adversely affected their lifespan, due to the increased metabolic demand (Essl, 1998; Ingvartsen et al., 2003; Oltenacu and Broom, 2010). When cows undergo negative energy balance, they are more susceptible to metabolic problems, exhibit poor physical condition, have decreased reproductive ability, and are present in the herd for a shorter period (Bauman and Currie, 1980; Rauw et al., 1998; Walsh et al., 2011). Since mitochondrial respiratory chain enzymes are central to energy production pathways, heifer selection based on mitochondrial enzyme function may select for animals that are less prone to metabolic problems. Mitochondrial function assays could be used as a screening tool to help farms make strategic breeding and culling decisions before costs associated with feed, treatments and labor are incurred. Therefore, the objective of this study was to determine if PBMC mitochondrial enzyme activities of citrate synthase, complex I, complex IV and complex V in Holstein and Jersey dairy cows change with time and are associated with ADG, reproductive outcomes, first lactation milk production and survival.

MATERIALS AND METHODS

Study design

This prospective observational study was approved by the University of California, Davis Animal Care and Use Committee, Protocol # 21157.

Twenty-three Holstein and 23 Jersey heifer calves from a California commercial dairy were enrolled between December 2016 and February 2017 and data were collected from 1 to 110 wk on animals that survived to each timepoint. This study did not interfere with farm management practices or cow culling, reasons cows were removed from the herd are shown in Table 1. A minimum sample size of 8 cows per treatment as estimated based on a two tailed test with a difference of 30% between electron transport chain enzyme complex activities with a power of 0.90 and an alpha of 0.05 using data from past studies involving mitochondrial measurements (Lancaster et al., 2014; Acetoze et al., 2015).

Cows were sampled at 5 timepoints throughout the study. The first samples were collected at 1 wk, as this was the earliest window that PBMC could be obtained for mitochondrial enzyme analyses due to immature cell differentiation. The second timepoint, 2 wk, was selected as it was near the onset of immune challenge in the form of diarrhea. The third timepoint was at 8 wk, before weaning and the fourth timepoint was at 52 wk, before the first breeding. Lastly, the fifth timepoint was 110 wk of age in early lactation (55 to 75 DIM).

Animal management and housing

Detailed pre-wean calf management and housing methods were presented in Niesen and Rossow (2019). In short, calves were enrolled with inclusion criteria being a respiratory score of 1, general appearance score of 1 and fecal score of 3 or less following the CalfTrack scoring system (Heinrichs et al., 2003). Calves were housed in raised individual wooden hutches with cement flush lanes and ad libitum access to water. Weaning occurred at roughly 60 d at the discretion of the calf manager and depending on heifer size. Upon leaving the hutches, post-wean heifers were grouped in mixed breed pens according to frame size in dry lots with shade covers

and fed a TMR once daily at approximately 0700 h. Heifers nearing parturition were moved to a close-up pen and remained there from approximately -21 to 0 DIM where they were fed a TMR at approximately 0530 h. Upon leaving the close-up pen, heifers were moved into milking pens sorted by stage of lactation and fed a TMR at approximately 0600 h. Both the close-up and milking pens had freestalls with attached flush lanes and were mixed by breed.

Health events, treatments, milk production, and body weight measurements

Respiratory and fecal scoring were performed daily for the first 30 d of life in pre-wean calves following methods defined by Niesen and Rossow (2019) to be used as model covariates. Pre-wean treatments were collected from treatment records on the hutches. Post-wean events (treatments, breeding, conception, illness, sold, died) and first lactation milk production data were collected from DairyComp305 (Valley Ag Software). Milk production data were collected through the first lactation and events were tracked to fourth lactation on surviving animals. Production data were recorded once monthly by Tulare DHIA and analyzed for milk yield, total fat yield, total solids yield, ECM, 305ME and relative value. Pre-wean calves were weighed at 1, 2, and 8 wk according to Niesen and Rossow (2019). Post-wean body weight measures were measured at 36, 52, and 110 wk with a Coburn breed specific weigh tape (Coburn Company Inc).

Blood collection, hematology and PBMC isolation

Blood samples were collected at 1, 2, 8 and 52 wk via jugular venipuncture and 110 wk via coccygeal tail vein. Two sets of whole blood (30 mL and 4 mL) were collected into vacutainer tubes (BD Biosciences) containing K2 EDTA as an anticoagulant at each timepoint and processed within 2 h of sample collection. Samples were taken as quickly as possible to ensure minimal stress to the animals.

Well mixed blood (4 ml) from a K2 EDTA tube was used to determine hematocrit (%), mean corpuscular hemoglobin (pg), mean corpuscular volume (fL), and neutrophil yield (K/μL) using the Drew Scientific Hemavet® 950 Hematology Analyzer System (Erba Diagnostics). Prior to evaluating samples, quality control samples were run to ensure that equipment was functioning within specification (Multi-Trol, Drew Scientific).

Platelet-rich plasma (PRP) and buffy coat were separated from the remaining whole blood (30 mL) by centrifugation at 2,000 *g* for 20 min at 20°C. Plasma total protein was determined from the PRP using a handheld clinical ATC refractometer (Index Instruments) at 1 to 8 wk and the remaining PRP was discarded. The buffy coat was diluted (1:4) with autoMACS Rinsing Solution (phosphate-buffered saline, pH 7.2, and 2 m*M* EDTA, MiltenyiBiotec) and applied to a Histopaque density gradient (specific gravity 1.077, Sigma Chemical Cat #10771) and centrifuged without application of the brake at 2,000 *g* for 20 min at 20°C. The PBMC were collected and pelleted at 300 *g* for 10 min at 20°C and washed with autoMACS Rinsing Solution three times. Prior to the second wash, red cell contaminants were lysed via osmotic shock using distilled water, vortexed and immediately diluted with autoMACS Rinsing Solution. The washed PBMC were then pelleted at 300 *g* for 10 min at 4°C and the supernatant discarded. All subsequent steps utilized kits from Abcam and followed the manufacturer's instructions.

Mitochondrial isolation and protein quantification

Mitochondria were extracted from PBMC using the Mitochondria Isolation Kit for Cultured Cells (Abcam, ab110170). Protein concentration of PBMC lysate was measured by BCA assay (Abcam, ab102536) and pellets were frozen at -80°C for 10 min to weaken cellular membranes then supplemented with 0.2 μL of universal nuclease (Fisher Scientific Co.,

PI88700) to reduce viscosity. Samples were re-suspended to 5 mg/mL in Reagent A followed by homogenization. The homogenate was centrifuged at 1,000 *g* for 10 min saving the supernatant and re-suspending the pellet in Reagent B. Homogenization and spin steps were repeated and the supernatants were combined and further centrifuged at 12,000 *g* for 15 min. The resulting supernatant was discarded, and the crude mitochondrial pellet dissolved in Reagent C supplemented with protease inhibitor (Abcam, ab201111), aliquoted and stored at -80°C. The crude mitochondrial protein concentration of one aliquot per sample was measured by bicinchoninic acid assay and used to correct the final activities of each sample (Abcam, ab102536).

Measurement of mitochondrial complex I, complex IV, complex V and citrate synthase enzyme activities

All mitochondrial enzyme activities were measured in duplicate using crude mitochondrial extracts. Microplates were incubated for 3 h prior to the collection of absorbance data using a VersaMax tunable microplate reader (Molecular Devices) in kinetic mode. Prior to evaluating samples, a calibration test plate (Bio-Tek Instruments Inc.) was used to ensure the spectrophotometer was within specification. All enzymatic assays were performed the day after blood sample collection and mitochondria isolation. All assay kits were bovine species reactive and the intra-assay CV for controls and samples was < 5%, and the inter-assay CV for all kits was < 15%. Assay sensitivity data, where appropriate, can be found in the manufacturer's protocol. Spontaneous product conversion (background) was determined for each kit by measuring the slope of blank wells containing only the reaction solution. This activity was determined for each plate and subtracted from the activity of each sample run per plate. Each enzymatic activity was determined with the following assay kits.

Complex I (EC 1.6.5.3) Enzyme Activity Microplate Assay Kit (Abcam, ab109721) was used to determine the activity of complex I via immunocapture and spectrophotometric analysis. In short, activity was determined by an increase in absorbance at 450 nm following the oxidation of NADH to NAD+ and the simultaneous reduction of dye. Kinetic readings were measured at room temperature, 450 nm, and 20 s intervals for 30 min with shaking between readings.

Complex IV (EC 1.9.3.1) activity was measured using the Complex IV Human Enzyme Activity Microplate Assay Kit (Abcam, ab109909). Complex IV was immunocaptured and activity was determined by decreased absorbance at 550 nm resulting from the oxidation of reduced cytochrome c. Kinetic readings were measured at 30°C at 3 min intervals for 60 min without shaking between readings.

Complex V (EC 3.6.3.14) enzyme activity was determined using the ATP Synthase Enzyme Activity Microplate Assay Kit (Abcam, ab109714). The hydrolysis of ATP to ADP facilitated by immunocaptured ATP-Synthase was coupled to the oxidation of NADH to NAD+ resulting in reduced absorbance at 340 nm. Kinetic readings were measured at 30°C at 1 min intervals for 60 min without shaking between readings.

The activity of citrate synthase (EC 4.1.3.7) was measured spectrophotometrically by increased absorbance at 412 nm via the development of 1,3,5-Trinitrobenzene from 5,5'-dithiobis-2-nitrobenzoic acid using the Citrate Synthase Activity Assay Kit (Abcam, ab119692). Kinetic readings were measured at room temperature at 20 s intervals for 15 min with shaking between readings.

Statistical analysis

Cow was the experimental unit of interest and enzyme activity was defined as the linear rate of change of the absorbance per min per μg crude mitochondrial protein loaded into the well. Only pre-steady state kinetics were evaluated. The slope for each sample was determined using the GLM procedure of SAS (Version 9.4) to regress absorbance on time with outlier removal set at 2 standard deviations and final activities corrected by crude mitochondrial protein. The model was, $Y_{OD} = \beta_0 + \beta_1 Time + \varepsilon_{OD}$, in which Y_{OD} = optical density, β_0 = y intercept, β_1 = regression coefficient of time and ε_{OD} = the error.

Enzymatic activity and hematological variables were modeled two ways, the first as a single timepoint and the second as the difference between two timepoints. This allowed mitochondrial and hematological outcomes to be evaluated at a given stage of life and also explored how they changed in response to age. Variables that represent a difference between two timepoints (in weeks) are noted with the delta symbol (Δ) between the timepoints, e.g., variable_2Δ1, the difference in the variable from 2 wk to 1 wk, while single timepoint variables are expressed with a single timepoint following the variable e.g., variable_1, the variable at 1 wk. For data analysis, respiratory score covariates were defined as days with a score ≥ 3 and fecal score covariates were defined as days with a score > 3. The number of pre-wean treatments covariate was a count of all individual treatments administered to a calf (lactated ringers, electrolytes, and antibiotics). Calculations of ADG were determined using the body weight measurement from 1 wk as the starting weight for all subsequent ADG calculations. Only covariates with $P \leq 0.05$ were included in the models. All models were visually assessed for fit and residual uniformity, covariates were assessed for collinearity and removed from the models if they had a variance inflation factor greater than five.

Multivariate regression analyses were conducted to determine which mitochondrial and health covariates were associated with the dependent variables ADG (8, 36, 52, and 110 wk) and first lactation milk production (milk yield, fat yield, solids yield, ECM yield, 305ME and relative value) using backward elimination. Average daily gain and milk production outcomes were regressed on the independent covariates; mitochondrial enzyme activities citrate synthase_2Δ1, complex I_2Δ1, complex V_2Δ1, respiratory scores (days with a score ≥ 3), number of pre-wean treatments, fecal scores (days with a score > 3), hematocrit _2, mean corpuscular hemoglobin_8, mean corpuscular hemoglobin_2Δ1, and neutrophils_2Δ1 using the GLM procedure of SAS (Version 9.4). The model was, $Y_{ADG\text{-}Prod} = \beta_0 + \beta_1 Enz_1 + \beta_2 Enz_2 + \beta_3 Enz_3 + \beta_4 RESP + \beta_5 TRT + \beta_6 FEC + \beta_7 HCT + \beta_8 MCH + \beta_9 NE + \varepsilon$, in which $Y_{ADG\text{-}Prod}$ = Dependent variables ADG (8, 36, 52, 110 wk), milk yield, fat yield, solids yield, ECM yield, 305ME or relative value, where β_0 = y - intercept, β_1 = regression coefficient of enzyme activity for citrate synthase (Enz_1), β_2 = regression coefficient of enzyme activity for complex I (Enz_2), β_3 = regression coefficient of enzyme activity for complex V (Enz_3), β_4 = regression coefficient of respiratory score (RESP), β_5 = regression coefficient of number of pre-wean treatments (TRT), β_6 = regression coefficient of fecal score (FEC), β_7 = regression coefficient of hematocrit (HCT), β_8 = regression coefficient of mean corpuscular hemoglobin (MCH), β_9 = regression coefficient of neutrophils (NE) and ε = the error.

Multivariate regression analyses were conducted to determine which growth, mitochondrial and hematological covariates were associated with the dependent reproductive variables (age at first service, age at first conception, age at first calving, and number of services) using backward elimination. Reproductive outcomes were regressed on the independent covariates; ADG (8, 36 wk), mitochondrial enzyme activities citrate synthase_8Δ1, complex

IV_52Δ8, complex V_2Δ1, complex V_8Δ2, complex V_52Δ1, mean corpuscular volume_8 and mean corpuscular hemoglobin_52Δ8, using the GLM procedure of SAS (Version 9.4). The model was, $Y_{Repro} = \beta_0 + \beta_1 ADG + \beta_2 Enz_1 + \beta_3 Enz_2 + \beta_4 Enz_3 + \beta_5 MCV + \beta_6 MCH + \varepsilon$, in which $Y_{ADG\text{-}Prod}$ = Dependent variables age at first service, age at first conception, age at first calving, and number of services, where β_0 = y - intercept, β_1 = regression coefficient of ADG (ADG), β_2 = regression coefficient of enzyme activity for citrate synthase (Enz_1), β_3 = regression coefficient of enzyme activity for complex IV (Enz_2), β_4 = regression coefficient of enzyme activity for complex V (Enz_3), β_5 = regression coefficient of mean corpuscular volume (MCV), β_6 = regression coefficient of mean corpuscular hemoglobin (MCH) and ε = the error.

Logistic regression analyses were conducted to evaluate if mitochondrial function and pre-wean health indices impacted survivability of calves using the LOGISTIC procedure of SAS (Version 9.4). Survivability was defined as 0 = removed from the herd, or 1 = survived to lactation (lactation 1, 2, 3, and 4). Removal from the herd was determined by farm records and only cows that died or were culled for disease or reproductive failure were included in the analysis. Single timepoint mitochondrial enzyme activity, difference in mitochondrial enzyme activities, respiratory scores, number of pre-wean treatments, fecal scores, single timepoint hematological values, and differences in hematological values were assessed as risk factors by splitting each variable into halves (above and below the median) and assessing if calves below the median had increased odds of being removed from the herd when compared to calves above the median. The model was, $Logit(p) = \beta0 + \beta1ENZ + \beta2RESP + \beta3TRT + \beta4FEC + \beta5HEM$, where p is the probability of being removed from the herd, $\beta0$ = y - intercept, $\beta1$ = regression coefficient of enzyme activity (ENZ), $\beta2$ = regression coefficient of respiratory score (RESP), $\beta3$

= regression coefficient of number of pre-wean treatment (TRT), β4 = regression coefficient of fecal score (FEC), and β5 = regression coefficient of hematological value (HEM).

RESULTS AND DISCUSSION

This study explored how the mitochondrial enzymatic activities of citrate synthase, complex I, complex IV and complex V in Holstein and Jersey dairy cows change with time and are associated with ADG, reproductive outcomes, first lactation milk production and survival.

Mitochondrial enzyme activity and changes with age

To evaluate how PBMC mitochondrial enzyme activities changed from birth to first lactation, the least squares mean of citrate synthase, complex I, complex IV, and complex V from each timepoint were plotted for Holstein (Figure 1) and Jersey cows (Figure 2). For both breeds, there was a trend of increased enzymatic activity from weaning (8 wk) to first lactation (110 wk), where each enzyme has maximal activity at 110 wk. The activity of citrate synthase has been associated with mitochondrial number (Holloszy et al., 1970, Williams et al., 1986) and complexes I and IV are two of the three enzymes in the electron transport chain that form the electrochemical gradient that produces ATP through complex V. The maximal activity observed at 110 wk for all enzymes likely resulted from the increased metabolic pressure the cows faced, as this timepoint was between 55 – 75 DIM in their first lactation. These results agree with Niesen and Rossow (2022), where differences in mitochondrial enzymatic activity were observed between high and low producing lactating cows (55 – 75 DIM), indicating that metabolic pressure can impact mitochondrial response. Similarly, Brown et al. (1988) observed a positive association between lactation performance and mitochondrial respiration activities. In addition to lactational pressure, these heifers were still growing, and increased activity of enzymes

interrelated to ATP output may help them meet their energy requirements during this metabolically demanding time.

At 52 wk there was a decrease in activity of citrate synthase, complex IV and complex V compared to 8wk for both Holsteins (Figure 1A, 1C, 1D) and Jerseys (Figure 2A, 2C, 2D). Complex I increased from 8 wk to 110 wk for both Holsteins (Figure 1B). and Jerseys (Figure 2B). Since citrate synthase, complex IV, and complex V had a decrease in activity at 52 wk, selected hematological values were plotted to determine if the cows experienced shifts in blood cell traits near this time (Figure 3). For both Holstein and Jerseys, lymphocyte number increased, and neutrophil number decreased at 52 wk. Increased lymphocytes can result from viral, bacterial, or parasitic pressure and decreased neutrophils limit the ability to fight off infection. There were no health events in farm records that explained the shifts in white blood cell populations. However, nutritional deficiencies can impact neutrophil differentiation (Robertson et al., 1992; Tsai and Collins, 1993) and negatively impact mitochondrial homeostasis (Acin-Perez et al., 2010). The increase in lymphocyte number and decrease in neutrophil number were within the equipment's normal ranges for adult cows (2.5 – 7.5 K/μL and 0.6 – 0.41 K/μL respectively) and agree with adult reference ranges observed in Roland et. al (2014), but these heifers were not fully grown. The shift in cell populations seen at this time could indicate that the cows were experiencing immunological or nutritional stress and explain the decreased mitochondrial activity of citrate synthase, complex IV and complex V at 52 wk. Conversely, it is possible that heifers were minimally challenged at this time, as they were past the immune challenge events faced in the hutches and are not yet experiencing the pressures of pregnancy and lactation. Further research is needed to explain whether a decrease in mitochondrial activity at this timepoint is normal and explore shifts in blood cell parameters near breeding. Complex I

activity was not impacted by this perturbance that was reflected in lymphocyte and neutrophil populations.

ADG and milk production

Since ADG and milk production can be influenced by a variety of factors, multivariate regression models were developed to identify variables that correlate to ADG and first lactation milk production in Holstein and Jersey cows (Table 2, Table 3). For Holsteins, mitochondrial enzymes, pre-weaning health indices and red blood cell hematological traits were present in the models for 8, 36, 52 and 110 wk ADG ($R^2 = 0.63$, $R^2 = 0.72$, $R^2 = 0.70$, and $R^2 = 0.99$, respectively, Table 2). Jersey models were similar, and mitochondrial enzymes, pre-wean health indices, red blood cell hematological traits, and neutrophils were correlated to 8, 36, 52 and 110 wk ADG ($R^2 = 0.64$, $R^2 = 0.77$, $R^2 = 0.70$, and $R^2 = 0.76$, respectively). Complex I, fecal scores, and mean corpuscular hemoglobin appeared more frequently in the models of Holstein ADG compared to Jerseys. While neutrophils and respiratory scores were more frequently included in Jersey growth models compared to Holsteins.

In the models of Holstein milk production, complex I_2Δ1, complex V_2Δ1, respiratory scores, number of pre-wean treatments, and fecal scores correlated to milk yield, fat yield, solids yield, ECM, 305ME and relative value ($R^2 = 0.86$, $R^2 = 0.89$, $R^2 = 0.90$, $R^2 = 0.98$, $R^2 = 0.94$ and $R^2 = 0.88$, respectively, Table 3). For Jerseys, citrate synthase_2Δ1, complex I_2Δ1, complex V_2Δ1, respiratory scores, and number of pre-wean treatments were correlated to milk yield ($R^2 = 0.51$). For the remaining Jersey production models citrate synthase_2Δ1, complex I_2Δ1, and respiratory scores were correlated to fat yield, solids yield, ECM, 305ME and relative value ($R^2 = 0.48$, $R^2 = 0.45$, $R^2 = 0.50$, $R^2 = 0.47$ and $R^2 = 0.48$, respectively, Table 3). For both breeds,

complex I_2Δ1 was the covariate that had the greatest effect on milk production models, as indicated by the greatest model variable SSE. The model for Holstein milk yield was the only exception, where complex V_2Δ1 had the largest model variable SSE.

The breeds differed by the early life variables that were correlated to their first lactation milk production. Number of pre-wean treatments, fecal score, and complex V activity were included in Holstein production models more frequently than Jerseys, and citrate synthase activity was present in Jersey production models and not Holstein (Table 3). Jerseys had increased citrate synthase activity from 1 to 2 wk (Figure 2A) and Holsteins did not (Figure 1A). This could indicate differences in mitochondrial number (Kirby et al., 2007) and may explain why different mitochondrial enzymes are associated with future milk production across breeds. The repeated inclusion of complex I and complex V in the ADG and milk production models is likely the result of their role in the production of ATP. These results agree with previous works that found complex I is correlated to body weight gain in heifers and complex I and V are associated with high milk production (Niesen and Rossow, 2019; Niesen and Rossow, 2022). The number of pre-wean treatments, neutrophil number, hematocrit, mean corpuscular hemoglobin, fecal and respiratory scores in the models implicates the importance of calf health and nutrition metrics. Combined, these model variables could indicate the health, nutrition, and energy status of the heifers, which impacts production outcomes like treatments, mortality risk, ADG, increased age at first calving, and reduced first-lactation milk yield (Bach, 2011; Heinrichs and Heinrichs, 2011; Buczinski et al., 2021).

Mitochondrial enzyme activity reproduction and survival

To determine the impacts of growth, mitochondrial enzyme activity and hematological parameters on reproductive outcomes, multivariate regression models were developed (Table 4). For Holsteins, ADG, mitochondrial enzymes, and red blood cell hematological traits were present in the reproduction models for age at first service, age at first conception, age at first calving and number of services ($R^2 = 0.91$, $R^2 = 0.93$, $R^2 = 0.89$, and $R^2 = 0.47$, respectively). For the majority of the Holstein models, ADG_8 was the growth covariate correlated to reproductive outcomes, with the exception of age at first service which was correlated to ADG_36. These findings signal that pre-wean growth rather than post-wean growth was better at predicting reproductive success in Holstein heifers and agree with previous work showing growth rates are associated with reproductive outcomes (Gardner et al., 1977; Cooke et al., 2013). For mitochondrial enzymes, citrate synthase_8Δ1 was correlated to all reproductive outcomes, and complex IV_52Δ8, and complex V_8Δ2 were included in three out of the four. Ge et al. (2012) reported that mitochondrial metabolism impacted oocyte development and subsequent embryo development in mice, which could explain why mitochondrial enzymes linked to energy production correlate to reproductive outcomes in cattle. Lastly, all Holstein reproduction models included one or both red blood cell hematological covariates, mean corpuscular volume_8, and mean corpuscular hemoglobin_52Δ8. For Jerseys, ADG, mitochondrial enzymes, and red blood cell hematological parameters were also correlated to age at first service, age at first conception, age at first calving and number of services ($R^2 = 0.55$, $R^2 = 0.73$, $R^2 = 0.55$, and $R^2 = 0.70$, respectively). Jerseys differed from Holsteins in that ADG_36 was the growth covariate included in the models, indicating that post-wean growth better predicts Jersey reproductive outcomes. Similar to Holsteins, Jersey models included citrate synthase_8Δ1, complex IV_52Δ8, complex V_2Δ1, and complex V_8Δ2. These results differ

368 from the models of ADG and milk production, in that mitochondrial changes later in life (8Δ1,
369 8Δ2, 52Δ8) were correlated to reproductive outcomes and early life mitochondrial changes
370 (2Δ1) were correlated to ADG and milk production (Table 2, Table 3). For red cell variables,
371 Jerseys differed from Holsteins in that mean corpuscular volume_8 was included in all
372 reproductive models rather than mean corpuscular hemoglobin (Table 4). Mean corpuscular
373 volume estimates the average size of red blood cells, and mean corpuscular hemoglobin is an
374 estimate of the average hemoglobin held per red cell. The covariates in these models signify the
375 importance of body weight gain, mitochondrial function and oxygen carrying capacity to
376 reproductive outcomes in Holstein and Jersey heifers. They are similar to the models of ADG,
377 and milk production, in that they include mitochondrial covariates integral to energy production,
378 and hematological variables that are linked to oxygen carrying capacity.

379 Logistic regression analyses were performed to determine if being below the median for a
380 particular variable increased risk of dying or being culled across lactations. Being below the
381 median for complex V_1, complex V_8Δ2, mean corpuscular hemoglobin_2Δ1, and
382 hematocrit_2Δ1 were correlated to removal from the herd (Figure 4). For complex V_1, calves
383 below the median were more likely to be removed from the herd compared to calves above the
384 median by lactation 1, 2, 3 and 4 (Figure 4A, odds ratio = 4.7, 7.7, 7.0 and 6.9, respectively). For
385 complex V_8Δ2, the majority of calves below the median showed no change or a decrease in
386 activity during this time range (Figure 4B). These calves were more likely to be removed from
387 the herd compared to calves above the median by lactation 2 and 3 (odds ratio = 6.9, 5.2,
388 respectively). These findings indicate that increased complex V activity near birth, and the
389 calves' ability to increase their complex V activity across the pre-wean period is protective
390 against early culling or death. The majority of calves below the median for mean corpuscular

hemoglobin_2Δ1 showed no change or a decrease in mean corpuscular hemoglobin during this time range (Figure 4C). These calves were more likely to be removed from the herd compared to calves above the median by lactation 1, 2, 3 and 4 (Figure 4C, odds ratio = 4.7, 5.0, 4.2 and 4.1), respectively). Panousis et al. (2017) have reported decreases in mean corpuscular hemoglobin from 1 d to 9 d which align with what was observed in calves below the median (Figure 4C). However, in this study, increasing mean corpuscular hemoglobin per red cell was protective when compared to calves that show no change. Lastly, for hematocrit_2Δ1, calves below the median were more likely to be removed from the herd compared to calves above the median by lactation 1, 2, 3 and 4 (Figure 4D, odds ratio = 13, 10, 5.2 and 4.7, respectively). Similar to mean corpuscular hemoglobin, these findings indicate that calves that are able to increase their hematocrit percentage early in life are protected against early removal from the herd. To our knowledge, no previous research has explored the relationship between calf complex V activity, mean corpuscular hemoglobin, and hematocrit to survival outcomes. Hematocrit and mean corpuscular hemoglobin are linked to cellular oxygen, the final electron acceptor in the electron transport chain, and complex V is the site of ATP production. Therefore, it is logical to conclude that reduced performance of these variables would result in energetic stress to the cow and impact her health and survivability.

CONCLUSIONS

Models including mitochondrial enzyme activities of citrate synthase, complex I, complex IV and complex V as well as early life health indices and hematological values were associated with ADG, reproductive outcomes, future milk production and survival across breeds. When considering the models of ADG, milk production, reproduction, and survival together, all include variables indicative of health, nutrition, and energy status of the heifers. By monitoring

mitochondrial function, early life health traits and hematological parameters, farms could identify high risk animals and make informed and strategic breeding and culling decisions about their youngstock. Focusing financial resources on long living high producing heifers would maintain profitability and reduce environmental expenses such as manure and methane.

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552 **Table 1. The number of Holstein and Jersey cows removed at each timepoint and reasons for exiting the herd**

Item	8 wk	52 wk	110 wk
Holstein (n = 23)			
Death	1	2	1
Sold Illness	-	-	3
Sold Repro	-	-	4
Sold Unknown[1]	-	-	2
Sold Farm[2]	-	-	-
Jersey (n = 23)			
Death	4	-	-
Sold Illness	-	-	1
Sold Repro	-	-	-
Sold Unknown	-	-	-
Sold Farm	-	-	1

553 [1]Farm records do not indicate a reason for sale

554 [2]Farm records indicate sold to another dairy

Table 2. Multivariate regression of mitochondrial enzyme activities and health indices that contribute to ADG in Holsteins and Jerseys

			Model Variable SSE[1]									
Item	n	LSM[2]	CI_2△1[3]	CV_2△1[4]	RESP[5]	TRT[6]	FEC[7]	HCT_2[8]	MCH_8[9]	MCH_2△1[10]	NE_8△2[11]	R^2
Holstein												
ADG 8 wk, kg/d	22	0.44	0.125	0.180	-	0.212	0.080	-	-	0.570	-	0.63
ADG 36 wk, kg/d	22	0.87	0.131	-	-	0.266	0.056	0.076	-	0.658	-	0.72
ADG 52 wk, kg/d	20	0.91	0.074	-	-	0.123	0.021	0.160	-	0.030	-	0.70
ADG 110 wk, kg/d	10	0.77	0.001	0.006	0.013	0.033	0.002	-	-	-	-	0.99
Jersey												
ADG 8 wk, kg/d	19	0.41	-	0.188	0.135	-	-	-	0.178	-	0.149	0.64
ADG 36 wk, kg/d	19	0.65	0.011	0.067	0.004	0.056	-	0.056	-	-	0.024	0.77
ADG 52 wk, kg/d	19	0.69	0.007	0.029	0.011	0.013	0.006	-	-	-	0.052	0.70
ADG 110 wk, kg/d	17	0.51	-	-	0.006	0.028	0.030	0.007	-	0.006	0.006	0.76

[1]The model was YADG-Prod = β0 + β1Enz1 + β2Enz2 + β3Enz3 + β4RESP + β5TRT+ β6FEC + β7HCT + β8MCH + β9NE + ε, in which YADG-Prod = Dependent variables ADG (8, 36, 52, 110 wk), milk yield, fat yield, solids yield, ECM yield, 305ME or relative value, where β0 = y - intercept, β1 = regression coefficient of enzyme activity for citrate synthase (Enz1), β2 = regression coefficient of enzyme activity for complex I (Enz2), β3 = regression coefficient of enzyme activity for complex V (Enz3), β4 = regression coefficient of respiratory score (RESP), β5 = regression coefficient of number of pre-wean treatments (TRT), β6 = regression coefficient of fecal score (FEC), β7 = regression coefficient of hematocrit (HCT), β8 = regression coefficient of mean corpuscular hemoglobin (MCH), β9 = regression coefficient of neutrophils (NE) and ε = the error, with the criteria for inclusion being $P \leq 0.05$

[2]The least squares mean of the item

[3]The difference in complex I enzyme activity from 2 to 1 wk, units are mOD/min/µg mitochondrial protein

[4]The difference in complex V enzyme activity from 2 to 1 wk, units are mOD/min/µg mitochondrial protein

[5]The number of days with a respiratory score ≥ 3 during the first month of life

[6]The number of treatments administered by farm staff during the pre-wean period

[7]The number of days with a fecal score > 3 during the first month of life

[8]Hematocrit at 2 wk, units are %

[9]Mean corpuscular hemoglobin at 8 wk, units are pg

[10]The difference in mean corpuscular hemoglobin from 2 to 1 wk, units are pg

[11]The difference in neutrophils from 8 to 2 wk, units are K/µL

Table 3. Multivariate regression of mitochondrial enzyme activities and health indices that contribute to first lactation milk production in Holstein and Jersey cows

		Model Variable SSE[1]						
Item	LSM[2]	CS_2△1[3]	CI_2△1[4]	CV_2△1[5]	RESP[6]	TRT[7]	FEC[8]	R^2
Holstein (n = 9)[9]								
Milk yield, kg	12,710	-	2.3E+07	3.2E+07	1.6E+07	2.8E+06	3.5E+06	0.86
Milk fat yield, kg	497	-	3.0E+04	8.3E+03	2.4E+04	1.6E+04	1.3E+04	0.89
Milk solids yield, kg	417	-	2.3E+04	1.8E+04	1.4E+04	4.7E+03	9.4E+02	0.90
ECM yield, kg	13,789	-	2.5E+07	1.6E+07	1.8E+07	7.4E+06	5.2E+06	0.98
305ME, kg	15,837	-	2.7E+07	5.7E+06	1.9E+07	1.1E+07	6.7E+06	0.94
Relative Value[10], %	112	-	1.3E+03	2.5E+02	9.6E+02	5.7E+02	3.1E+02	0.88
Jersey (n = 17)								
Milk yield, kg/d	8,776	1.6E+07	3.8E+07	3.2E+06	2.2E+07	4.2E+06	-	0.51
Milk fat yield, kg/d	431	1.6E+04	7.8E+04	-	3.8E+04	-	-	0.48
Milk solids yield, kg/d	330	2.2E+04	4.4E+04	-	3.0E+04	-	-	0.45
ECM yield, kg/d	10,982	1.9E+07	5.2E+07	-	2.8E+07	-	-	0.50
305ME, kg/d	12,429	2.0E+07	3.8E+07	-	1.8E+07	-	-	0.47
Relative Value, %	91	1.1E+03	2.1E+03	-	1.0E+03	-	-	0.48

[1]The model was YADG-Prod = β0 + β1Enz1 + β2Enz2 + β3Enz3 + β4RESP + β5TRT+ β6FEC + β7HCT + β8MCH + β9NE + ε, in which YADG-Prod = Dependent variables ADG (8, 36, 52, 110 wk), milk yield, fat yield, solids yield, ECM yield, 305ME or relative value, where β0 = y - intercept, β1 = regression coefficient of enzyme activity for citrate synthase (Enz1), β2 = regression coefficient of enzyme activity for complex I (Enz2), β3 = regression coefficient of enzyme activity for complex V (Enz3), β4 = regression coefficient of respiratory score (RESP), β5 = regression coefficient of number of pre-wean treatments (TRT), β6 = regression coefficient of fecal score (FEC), β7 = regression coefficient of hematocrit (HCT), β8 = regression coefficient of mean corpuscular hemoglobin (MCH), β9 = regression coefficient of neutrophils (NE) and ε = the error, with the criteria for inclusion being $P \leq 0.05$

[2]The least squares mean of the item

[3]The difference in citrate synthase enzyme activity from 2 to 1 wk, units are mOD/min/µg mitochondrial protein

[4]The difference in complex I enzyme activity from 2 to 1 wk, units are mOD/min/µg mitochondrial protein

[5]The difference in complex V enzyme activity from 2 to 1 wk, units are mOD/min/µg mitochondrial protein

[6]The number of days with a respiratory score ≥ 3 during the first month of life

[7]The number of treatments administered by farm staff during the pre-wean period

[8]The number of days with a fecal score > 3 during the first month of life

[9] One Holstein was culled mid lactation

[10] Relative value is the mature equivalent 305 expressed as a percentage of the herd average mature equivalent

Table 4. Multivariate regression of ADG, mitochondrial enzyme activities, and health indices that contribute to reproductive performance in Holsteins and Jerseys

			Model Variable SSE[1]								
Item	n	LSM[2]	ADG_8[3]	ADG_36[4]	CS_8△1[5]	CIV_52△8[6]	CV_2△1[7]	CV_8△2[8]	MCV_8[9]	MCH_52△8[10]	R^2
Holstein											
Age at first service, d	10	417	-	127	111	845	1396	-	1836	262	0.91
Age at first conception, d	10	434	4794	-	1516	1263	-	6261	-	12716	0.93
Age at first calving, d	10	708	3071	-	1321	1194	-	6036	-	12225	0.89
Services, #	10	2	45	-	46	-	-	141	-	25	0.47
Jersey											
Age at first service, d	19	409	-	-	7963	-	-	1407	2104	5358	0.55
Age at first conception, d	18[11]	446	-	4896	1947	7780	2792	-	16095	2312	0.73
Age at first calving, d	19	726	-	4613	-	4730	3079	-	23793	-	0.55
Services, #	18[12]	2	-	35	2	20	-	-	10	-	0.70

[1]The model was YRepro = β0 + β1ADG + β2Enz1 + β3Enz2 + β4Enz3 + β5MCV + β6MCH + ε, in which YADG-Prod = Dependent variables age at first service, age at first conception, age at first calving, and number of services, where β0 = y - intercept, β1 = regression coefficient of ADG (ADG), β2 = regression coefficient of enzyme activity for citrate synthase (Enz1), β3 = regression coefficient of enzyme activity for complex IV (Enz2), β4 = regression coefficient of enzyme activity for complex V (Enz3), β5 = regression coefficient of mean corpuscular volume (MCV), β6 = regression coefficient of mean corpuscular hemoglobin (MCH) and ε = the error, with the criteria for inclusion being $P \leq 0.05$

[2]The least squares mean of the item

[3]ADG at 8 wk, units are kg/d

[4]ADG at 36 wk, units are kg/d

[5]The difference in citrate synthase enzyme activity from 8 to 1 wk, units are mOD/min/µg mitochondrial protein

[6]The difference in complex IV enzyme activity from 52 to 8 wk, units are mOD/min/µg mitochondrial protein

[7]The difference in complex V enzyme activity from 2 to 1 wk, units are mOD/min/µg mitochondrial protein

[8]The difference in complex V enzyme activity from 8 to 2 wk, units are mOD/min/µg mitochondrial protein

[9]Mean corpuscular volume at 8 wk, units are fL

[10]The difference in mean corpuscular hemoglobin from 52 to 8 wk, units are pg

[11]One Jersey heifer was serviced by a bull so age at first conception was not in farm records

[12]One Jersey heifer was serviced by a bull so number of services was not in farm records

Figure 1. Enzymatic activity of peripheral blood mononuclear cells in Holstein cows from birth to first lactation Citrate synthase activity vs time (Figure A), complex I activity vs time (Figure B), Complex IV activity vs time (Figure C), Complex V activity vs time (Figure D) where n = 23, 23, 22, 20, and 10 at 1, 2, 8, 52, and 110 wk respectively

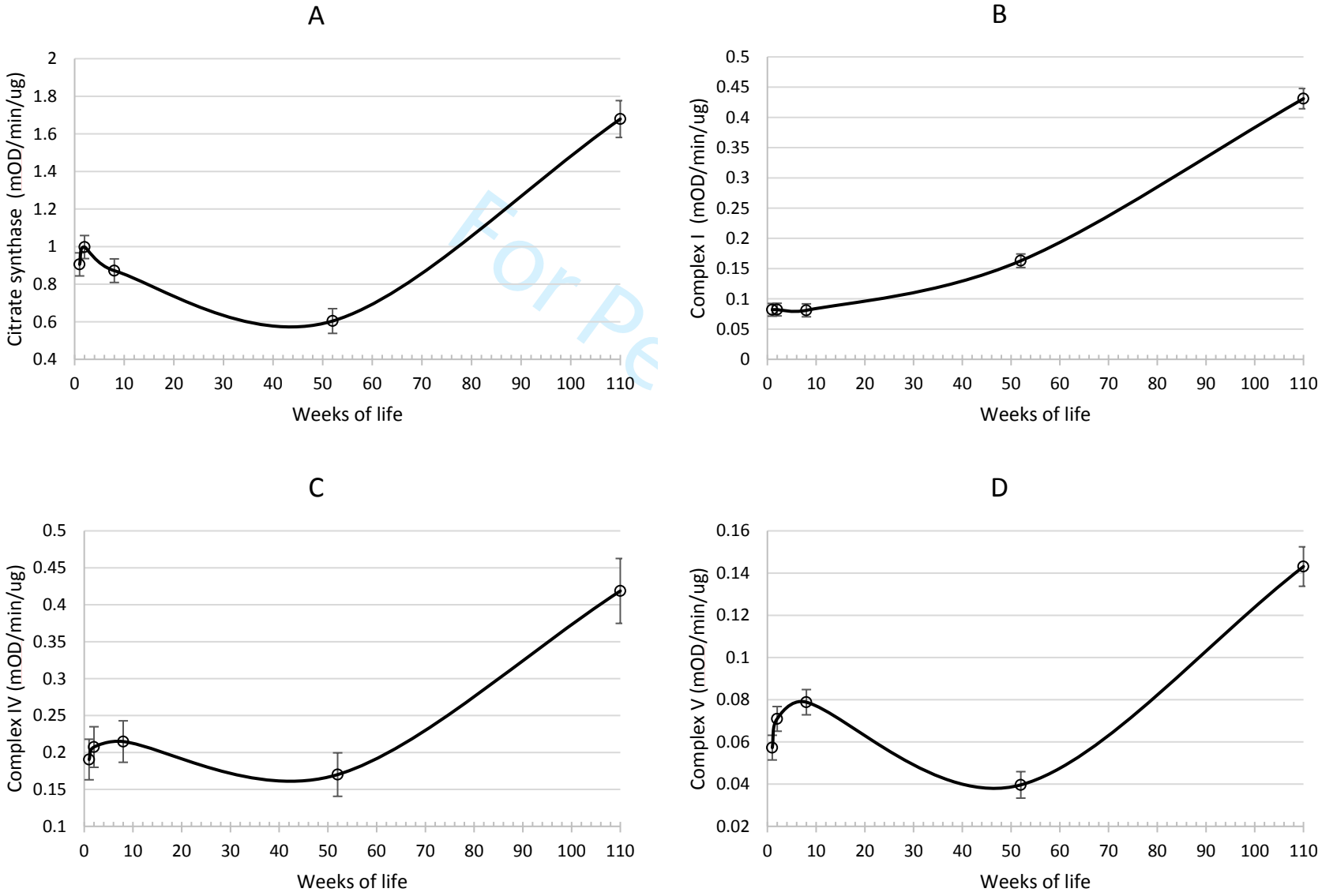

Figure 2. Enzymatic activity of peripheral blood mononuclear cells in Jersey cows from birth to first lactation
Citrate synthase activity vs time (Figure A), complex I activity vs time (Figure B), Complex IV activity vs time (Figure C), Complex V activity vs time (Figure D) where n = 23, 23, 19, 19, and 17 at 1, 2, 8, 52, and 110 wk respectively

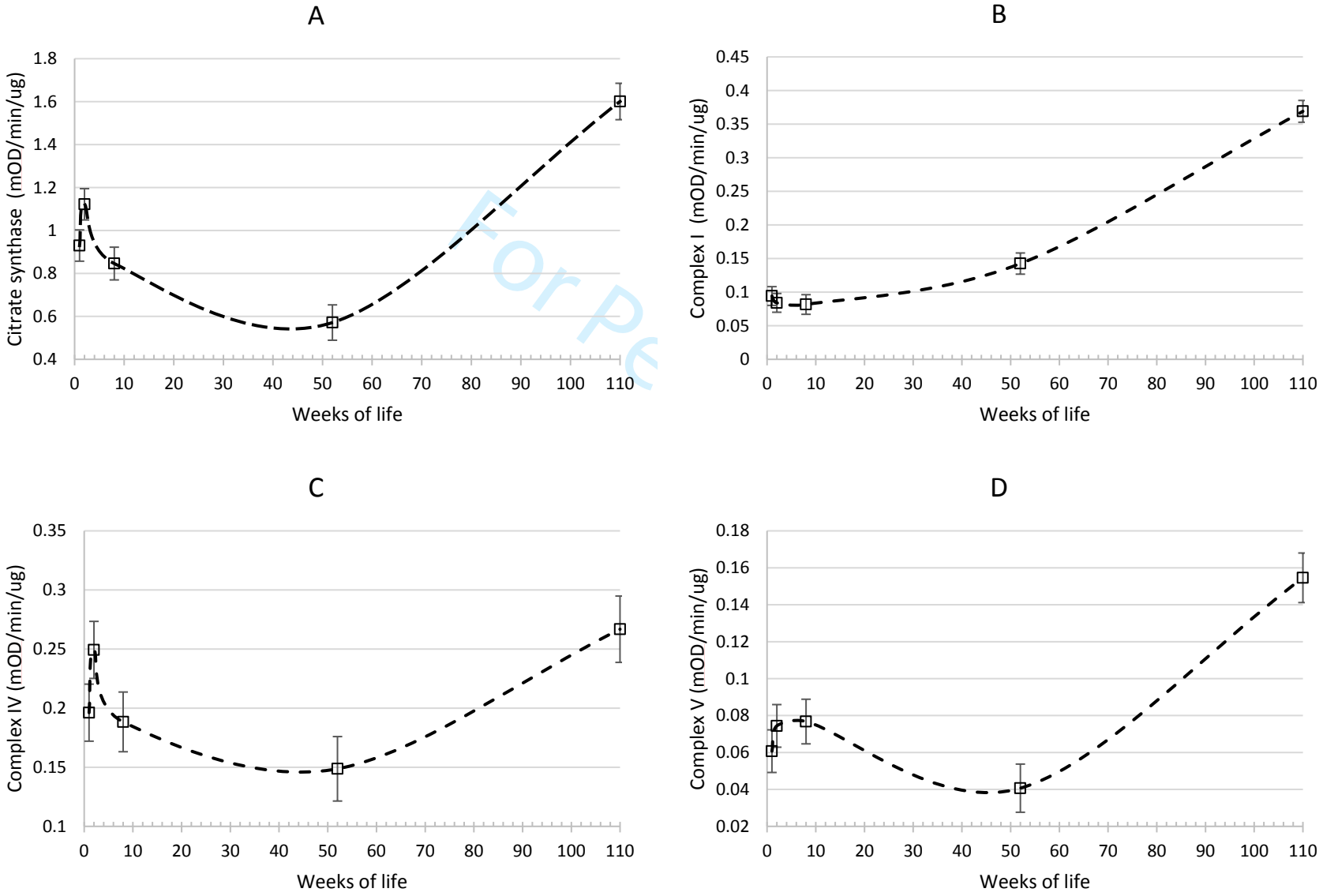

Figure 3. Lymphocyte and neutrophil yields of Holstein and Jersey cows from birth to first lactation
Lymphocyte number vs time (Figure A), Neutrophil number vs time (Figure B) for Holstein (○, solid line) and Jersey (□, dashed line) cows

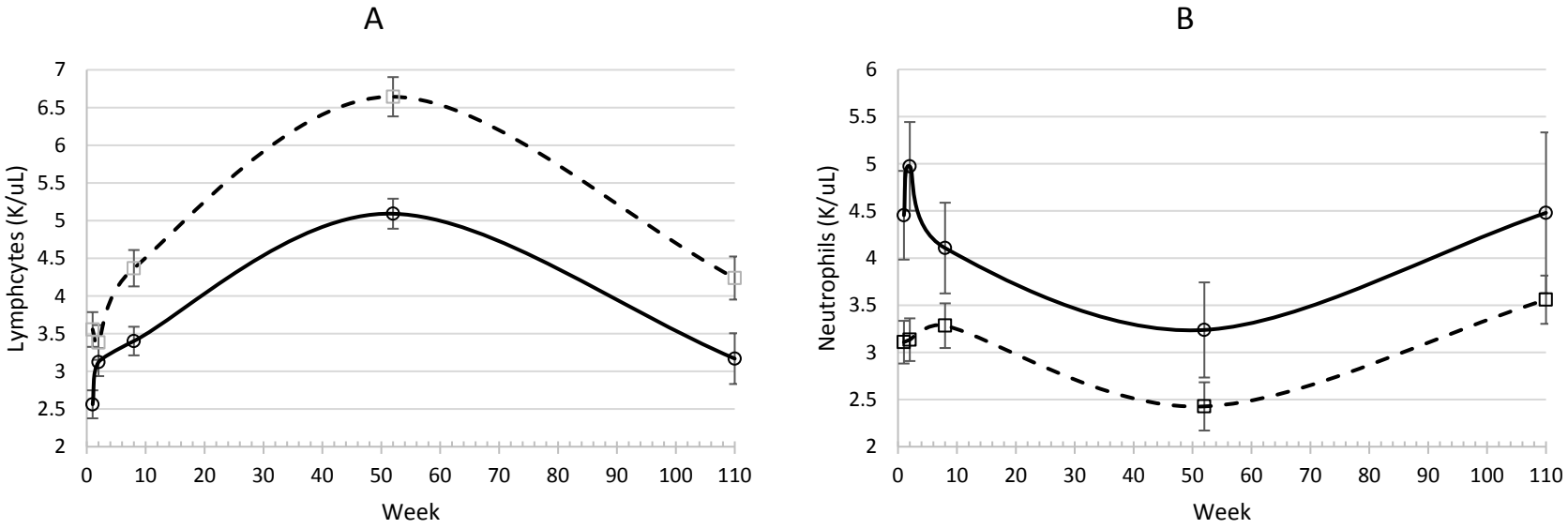

Figure 4. Box and whisker plots of variables correlated to calf survival with odds ratios of calves below the median being removed from the herd by lactation number

Only significant odds ratios are presented. Complex V activity at 1 wk, (Figure A), the difference in complex V activity from 8 to 2 wk (Figure B), the difference in mean corpuscular hemoglobin from 2 to 1 wk (Figure C), the difference in hematocrit from 2 to 1 wk (Figure D)