# ADAPTACIONES METABÓLICAS EN VACAS LECHERAS DE DISTINTOS GENOTIPOS HOLSTEIN BAJO DOS ESTRATEGIAS DE ALIMENTACIÓN

María Mercedes GARCÍA-ROCHE SARACCO

TESIS presentada como uno de los requisitos para obtener el título de Doctora en Ciencias Agrarias – opción Ciencias Animales

MONTEVIDEO, URUGUAY
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TABLA DE CONTENIDO

RESUMEN

El objetivo del trabajo fue profundizar en los mecanismos moleculares relacionados con el metabolismo energético hepático en vacas lecheras Holstein-Friesian de distinto origen genético, sometidas a dos estrategias nutricionales a lo largo de la lactancia. Modelo 1: vacas Holstein-Friesian multíparas sometidas a dos estrategias de alimentación. De los 0 a los 180 días en leche (DEL) las vacas fueron alimentadas con una dieta totalmente mezclada (DTM) *ad libitum* (G0) o pastorearon *Festuca arundinacea* o *Medicago sativa* y fueron suplementadas con concentrado (G1); de los 180 a 250 días todas las vacas pastorearon *Festuca arundinacea* y fueron suplementadas con una DTM. Modelo 2: vacas Holstein-Friesian de origen genético neozelandés (NZH) o norteamericano (NAH) sometidas a dos estrategias de alimentación, la de máximo pastoreo (MaxP), donde el forraje representó el 67 % del consumo ofrecido (70:30 relación de *Medicago sativa* y *Dactylis glomerata* o *Festuca arundinacea* a forraje conservado) y el 33 % restante se ofreció como concentrado, y la estrategia FixP donde el pastoreo representó el 32 % del consumo ofrecido y el restante se ofreció como DTM. Para el modelo 1 se tomó plasma y biopsias de hígado a los -14, 35, 60, 110, 180 y 250 DEL y para el modelo 2 a los -14, 21, 100 y 180 DEL con el objetivo de caracterizar los perfiles metabólico-endócrinos, la expresión génica de enzimas de las vías metabólicas, la función mitocondrial, los niveles de proteína y las modificaciones postraduccionales. Nuestro trabajo evidenció una disfunción mitocondrial hepática durante la lactancia temprana asociada a los niveles de acetilación de lisinas en proteínas mitocondriales en vacas sometidas a estrategias pastoriles, con una recuperación hacia la lactancia media tardía. Sin embargo, las vacas NAH en la estrategia MaxP no lograron recuperar su función mitocondrial durante la lactancia media tardía y mostraron una más alta expresión de genes asociados a la gluconeogénesis sugiriendo una mayor persistencia que las vacas NZH.

Palabras clave: función mitochondrial, sistemas mixtos, acetilación, metabolismo energético.

SUMMARY

The aim of this work was to focus on the molecular mechanisms related to hepatic energy metabolism in Holstein-Friesian dairy cows of different genetic origin subjected to two nutritional strategies throughout lactation. Model 1: Multiparous Holstein-Friesian cows subjected to two feeding strategies. From 0 to 180 days in milk (DIM) the cows were fed a totally mixed ration (TMR) *ad libitum* (G0, without grazing) or grazed *Festuca arundinacea* or *Medicago sativa* and were supplemented with concentrate (G1, with grazing). From 180 to 250 days, all cows grazed *Festuca arundinacea* and were supplemented with a DTM. Model 2: Holstein cows of New Zealand (NZH) or North American (NAH) genetic origin subjected to two feeding strategies, maximum grazing (MaxP) where forage represented 67 % of the offered dry matter intake (70:30 direct grazing of *Medicago sativa* and *Dactylis glomerata* or *Festuca arundinacea* to conserved forage ratio) and the remaining 33 % of the dry matter intake was offered as concentrate, and the fixed pasture strategy (FixP) where grazing represented 32 % of the DMI offered and the remaining was offered as a TMR. For model 1 plasma and liver biopsies were collected at -14, 35, 60, 110, 180 and 250 DIM and for model 2 liver biopsies were taken at -14, 21, 100 and 180 dry matter intake with the objective to characterize the metabolic-endocrine profiles, gene expression of metabolic pathways, mitochondrial function, protein levels and post-translational modifications by western blot. Our results showed that there was a hepatic mitochondrial dysfunction during early lactation associated with lysine acetylation levels in mitochondrial proteins in cows subjected to grazing strategies which is later reinstated towards late mid-lactation. However, NAH cows in the MaxP strategy failed to recover their mitochondrial function during late mid-lactation and showed higher expression of genes associated with gluconeogenesis suggesting greater persistence than NZH cows.

Key words: mitochondrial function, energy metabolism, mixed systems, acetylation.

# 1. INTRODUCCIÓN

## 1.1 PLANTEO DEL PROBLEMA

La producción de leche en el Uruguay ha tenido un incremento sostenido durante los últimos 15 años, a una tasa del 5% anual (DIEA, 2015). Este resultado se debió tanto a una mayor producción individual como a un aumento en la carga animal, jugando los concentrados y las reservas forrajeras un papel fundamental, ya que la cosecha directa de forraje por parte de los animales ha permanecido sin cambios (Chilibroste, 2015). La lechería en Uruguay representa un importante sector agropecuario, donde se exporta el 70% de la leche producida, y el volumen de las ventas al exterior posiciona a Uruguay como el séptimo país exportador mundial de leche. Actualmente, debido a la crisis sanitaria, la producción mundial de leche se ha desacelerado y la demanda mundial se ve fortalecida por el ingreso de nuevos mercados como China (Instituto Nacional de la Leche, Uruguay). A pesar de que la actual situación mundial presente una importante oportunidad de crecimiento para Uruguay, es necesario levantar restricciones relacionadas con la infraestructura, la dinámica de los rodeos y el bienestar animal (Fariña y Chilibroste, 2019) para asegurar una mayor competitividad.

Uruguay tiene un perfil exportador y tomador de precios frente a un mercado internacional fuertemente intervenido, competitivo y fluctuante. Esto determina que el mantenimiento (o reducción) de los costos de producción sea clave para la competitividad de los sistemas de producción y del sector en su conjunto. En este sentido, los sistemas de producción que incluyen una mayor proporción de pastura directamente cosechada en la dieta, en contraste con sistemas estabulados que se basan en el uso de reservas forrajeras y concentrados, presentarían un menor costo de producción y necesidades de inversión, a la vez que tienen el potencial de presentar beneficios para la

calidad de producto y el impacto en el medio ambiente (Dillon, 2006; Fariña y Chilibroste, 2019; Stirling et al., 2021).

Sin embargo, los sistemas con mayor inclusión de pastura directamente cosechada tienen más dificultades para asegurar un flujo estable de nutrientes a los animales a lo largo del tiempo, debido a las importantes variaciones temporales tanto en cantidad como calidad del forraje, respecto a sistemas estabulados. El consumo de materia seca (MS) en sistemas pastoriles es usualmente más bajo que en sistemas de confinamiento y podría ser insuficiente para sostener la alta producción de leche que podría lograrse con el potencial genético (Chilibroste et al., 2012; Kolver y Muller, 1998). En investigaciones nacionales, el análisis de las curvas de lactancia (Chilibroste et al., 2011; Chilibroste et al., 2002) sugiere que los animales no logran expresar su potencial productivo, seguramente en respuesta a la diferencia entre requerimientos-oferta de nutrientes y ambiente productivo. Esto es importante cuando se manejan animales de elevado potencial genético con altos requerimientos de nutrientes.

Se ha demostrado que cuando la concentración de nutrientes es insuficiente, las vacas pueden mostrar un pobre estado metabólico, ya que los marcadores del balance energético negativo, como los ácidos grasos no esterificados, el beta-hidroxibutirato plasmático y los niveles de triglicéridos hepáticos, se exacerban (García-Roche et al., 2021; Meikle et al., 2013). Por otro lado, la menor expresión de genes relacionados con el crecimiento embrionario y la función uterina en vacas bajo un sistema pastoril (Astessiano et al., 2017), así como la ciclicidad ovárica atrasada (Meikle et al., 2013), son indicativos de un pobre rendimiento reproductivo.

En los últimos años se han reportado un número importante de trabajos de investigación que apuntan a generar pautas de manejo del pastoreo y estrategias de alimentación pre y posparto que permitan mejorar la respuesta

animal desde un punto de vista productivo y reproductivo (Meikle et al., 2013). El uso de la combinación de forraje fresco cortado con dieta totalmente mezclada (DTM) ha mostrado efectos beneficiosos sobre el consumo de MS y la producción de leche con valores de inclusión de forraje fresco de hasta un 30% de la materia seca total (Mendoza et al., 2016). El uso estratégico de DTM en lactancia temprana (Fajardo et al., 2015; Meikle et al., 2013) tiene un impacto positivo sobre la producción de leche, grasa, proteína y sobre las variaciones de peso vivo (PV) y condición corporal (BCS) al inicio de la lactancia, así como en la respuesta reproductiva de las vacas lecheras (Astessiano et al., 2017; Meikle et al., 2013). Un estudio que comparó vacas primíparas alimentadas con DTM con y sin inclusión de pasturas demostró que la energía retenida en leche y tejido fue menor para las vacas con inclusión de pasturas, probablemente debido a los gastos de mantenimiento asociados a las actividades de pastoreo y caminata (Jasinsky et al., 2019).

La estrategia de alimentación, pero también el genotipo del animal a utilizar, constituyen componentes centrales en la definición de cualquier sistema lechero (Garcia, 2002). En particular, dentro del ganado Holstein-Friesian, estudios demostraron que la línea genética con mayor porcentaje de genes provenientes de ancestros norteamericanos (Holstein Norteamericano, NAH por su sigla en inglés) donde predominan los sistemas estabulados basados en el uso de DTM, se caracteriza por tener un alto PV y por ser capaz de producir más litros de leche por la mayor partición de nutrientes consumidos a la glándula mamaria (Kolver et al., 2000; Fulkerson et al., 2008). En contraste, la literatura sugiere (Kolver et al., 2002; Fulkerson et al., 2008; Lucy et al., 2009) que las vacas Holstein de origen neozelandés (NZH por su sigla en inglés) se adaptan mejor en base pastoril que las vacas NAH, ya que si bien no tuvieron diferencias en rendimiento de sólidos, contenido proteico de la leche y persistencia de la lactancia, las de genotipo NAH no lograron mantener un índice de BCS y PV aceptables, mientras que las NZH sí (Kolver et al., 2002; Fulkerson et al., 2008; Lucy et al., 2009). Estudios nacionales

recientes demostraron que cuando se manejan vacas de genotipo NZH y NAH en un sistema de base pastoril con suplementación de concentrado, durante lactancia tardía, las vacas NZH tienen menores costos de mantenimiento que las vacas NAH (Talmón et al., 2020), además de mantener valores más altos de BCS, PV (Stirling et al., 2021) y de eficiencia alimenticia en un sistema donde se alimentaron únicamente con pasturas de cosecha directa (Talmón et al., 2022). A su vez, Lucy et al. (2009) explican que estas diferencias se deben al desacople del eje somatotrópico que conduce la partición de nutrientes, demostrando que las vacas NAH tienen un alto desacople del eje mientras que las NZH mantuvieron un eje somatotrópico acoplado. Es así que adecuar el tipo de vaca al sistema de producción (interacción genotipo-ambiente) es clave a la hora de maximizar la eficiencia de producción de los sistemas lecheros.

## 1.2 METABOLISMO ENERGÉTICO HEPÁTICO

Las vacas lecheras de alta producción sufren adaptaciones metabólicas extremas durante la lactancia. El período de transición de la gestación tardía a la lactancia temprana representa un momento de aumento de casi cuatro veces en los requerimientos de energía, proteínas y minerales, cuando múltiples órganos orquestan adaptaciones metabólicas que serán críticas para definir el éxito de la lactancia (Bauman, 2000; Roche et al., 2013). Particularmente, durante esta etapa se desencadena la mayor parte de las enfermedades y trastornos metabólicos, debido a la movilización exacerbada de reservas necesarias para acompasar los crecientes requerimientos (Drackley, 1999). En este contexto, el hígado juega un rol clave en la coordinación de las adaptaciones metabólicas, ya que participa del metabolismo de glúcidos, proteínas y lípidos (Wei et al., 2008), en la regulación del metabolismo del colesterol y triglicéridos y en el mantenimiento de la homeostasis y los niveles de glucosa en sangre (Knebel et al., 2015).

En un contexto donde la entrada de ácidos grasos no esterificados (AGNE) al hígado es muy alta, aumenta la acumulación de triglicéridos y la vaca lechera puede desarrollar esteatosis hepática, que, a su vez, puede evolucionar hacia una esteatohepatitis (Gao et al., 2018). En otros modelos animales, como ratas y ratones, se plantea que la disfunción mitocondrial (Cimen et al., 2010; Kendrick et al., 2011), el aumento en los niveles de citoquinas y adipoquinas y el estrés del retículo endoplásmico (Nassir e Ibdah, 2014) cumplen un rol importante en el desarrollo de esta enfermedad. Tanto la esteatosis como la esteatohepatitis son condiciones patológicas que afectan la función del hígado, lo cual amenaza tanto a la salud del animal como a su bienestar y, en consecuencia, el rendimiento productivo (Ingvartsen y Moyes, 2013).

Las mitocondrias son organelos que tienen un rol preponderante en el metabolismo energético, ya que participan de la conversión de los nutrientes a una fuente energética utilizable por los procesos fisiológicos; este proceso se denomina respiración mitocondrial (Mavangira y Sordillo, 2017). Además, sus funciones incluyen: síntesis y catabolismo de metabolitos, formación de especies reactivas de oxígeno (ROS, del inglés, *reactive oxygen species*; pueden ser responsables del 90% de ROS producidos (Brand y Nicholls, 2011; Nassir e Ibdah, 2014)) y regulación del calcio citoplasmático y mitocondrial. La mitocondria también está involucrada en la vía de apoptosis intrínseca, donde la liberación de citocromo *c* conduce a la formación del apoptosoma y la activación de caspasas (Radi, 2004). La alteración en cualquiera de estos procesos puede considerarse una disfunción mitocondrial (Brand y Nicholls, 2011).

Como mencionamos, en el hígado, los ácidos grasos no esterificados (AGNE) pueden ser esterificados a triglicéridos u oxidados en la mitocondria. La β-oxidación mitocondrial involucra cuatro reacciones donde se generan NADH, $FADH_2$ y acetil-coenzima A (acetil-CoA) como producto (Lehninger et al., 2005). Esta vía está regulada en gran parte por la enzima carnitina palmitoil

transferasa I (CPT-I) que regula la entrada de ácidos grasos a la mitocondria. Esta enzima cataliza la formación de acil-carnitina a partir del ácido graso activado (acil-CoA) y la carnitina. La CPT-I es inhibida por el malonil-CoA, un precursor de la síntesis de los ácidos grasos (Drackley et al., 2001). Una alta concentración de este metabolito inhibe la actividad de la enzima CPT-I y disminuye el catabolismo de los ácidos grasos. En contraste, cuando los niveles de malonil-CoA son bajos, en estados de ayuno o de alta exigencia energética, la CPT-I se activa y promueve la oxidación de ácidos grasos (Wei et al., 2008).

La β-oxidación mitocondrial es eficiente pero poco flexible en situaciones de adaptación metabólica. Se ha demostrado que en los casos de alta lipidemia, cuando existe acumulación de ácidos grasos en el hígado, el peroxisoma tiene mayor actividad β-oxidativa. Esto está dado en gran parte por la permeabilidad de la membrana peroxisomal a los ácidos grasos y la ausencia de un regulador como CPT-I. Sin embargo, algunos estudios indican que a pesar de que el peroxisoma juega un rol compensatorio en la oxidación de ácidos grasos, también puede verse desbordado por el flujo de estos. En estas condiciones, los ácidos grasos que no son degradados se reesterifican y forman triglicéridos, dando lugar a la condición de hígado graso (Poirier et al., 2006; Knebel et al., 2015).

El acetil-CoA resultante tiene dos posibles destinos: 1) la oxidación completa a dióxido de carbono ($CO_2$) en el ciclo de Krebs o 2) la producción de cuerpos cetónicos como β-hidroxibutirato y acetoacetato.
La síntesis de cuerpos cetónicos ocurre el hígado en situaciones de ayuno, alta exigencia física o balance energético negativo (Youssef y El-Ashker, 2017). Cuando los niveles de glucosa en sangre son bajos, aumenta la síntesis de cuerpos cetónicos como 3-hidroxibutirato y acetoacetato en el hígado. Estos se utilizan como fuente de energía alternativa en tejidos extrahepáticos como el corazón y cerebro (Wei et al., 2008).

Durante la lactancia temprana se observa un gran aumento en la producción de cuerpos cetónicos. Una posible razón para esto es que en este período escasean los intermediarios del ciclo de Krebs, ya que están volcados a la síntesis de glucosa en la gluconeogénesis para la lactogénesis (White, 2015). En estas condiciones el acetil-CoA es direccionado a la síntesis de cuerpos cetónicos y puede ocasionar una cetosis (Ghanta et al., 2013).

El acetil-CoA generado a partir de la oxidación de ácidos grasos (Pougovkina et al., 2014) también es utilizado para la acetilación de lisinas, una modificación postraduccional (MPT) reversible de proteínas. La acetilación participa en la regulación de la adaptación metabólica en estados de obesidad, malnutrición o sobrenutrición (Ghanta et al., 2013), ya sea modificando actividades de enzimas metabólicas claves, involucradas en la β-oxidación, el ciclo de la urea, la fosforilación oxidativa y el ciclo de Krebs, tanto inhibiéndolas como activándolas, o actuando también a nivel transcripcional, modificando la acetilación de histonas que regula la expresión de genes. (Choudhary et al., 2009, Anderson y Hirschey, 2012, Wagner y Payne, 2011, Drazic et al., 2016). Por tanto, se cree que la acetilación de lisinas es un amplio modulador de señales en las más diversas funciones celulares (Sun et al., 2017).

En la última década, se ha descubierto que la acetilación de lisinas juega un papel importante regulando reacciones y procesos que ocurren dentro de la mitocondria, en particular en vías metabólicas, afectando la actividad de enzimas. Esto se ha visto claramente en modelos de restricción calórica (Sun et al., 2017).

En la mitocondria, las reacciones de acetilación pueden darse de forma no enzimática o catalizadas por acetilasas como la acetil-transferasa (GCN5L1) (Thapa et al., 2017). Por otro lado, las reacciones de desacetilación son catalizadas por enzimas, entre las que encontramos a las sirtuinas (Ansari et

al., 2017), desacetilasas dependientes de NAD+ que regulan numerosos procesos metabólicos (Ghanta et al., 2013, Anderson y Hirschey, 2012, Wagner y Payne, 2011). En el ámbito mitocondrial, la sirtuina 3 desacetila y activa la superóxido dismutasa de manganeso (MnSOD), una importante enzima antioxidante mitocondrial (Hirschey et al., 2010; Wagner y Payne, 2011), la enzima 3-hidroxi-metilglutaril CoA sintasa 2 de la síntesis de cuerpos cetónicos (Shimazu et al., 2010), las enzimas de la β-oxidación acil-CoA deshidrogenasa de cadena larga y la beta-hidroxiacil-CoA deshidrogenasa (Hirschey et al., 2010; Alrob et al., 2014), la enzima ornitina transcarbamoilasa del ciclo de la urea (Hallows et al., 2006) y la enzima succinato deshidrogenasa que participa tanto del ciclo de Krebs como de la fosforilación oxidativa (Cimen et al., 2010; Finley et al., 2011).

Recientemente, estudios de proteómica, basada en espectrometría de masa, han identificado noveles proteínas acetiladas que participan de la oxidación de ácidos grasos y pueden ser críticas en la modulación del hígado graso en vacas lecheras (Le-Tian et al., 2020).

Por otra parte, un factor relevante en la regulación del metabolismo energético es la proteína quinasa dependiente de AMP (AMPK), un regulador maestro del metabolismo de la glucosa y los lípidos en el hígado y otros tejidos. Esta quinasa fosforila y regula la actividad de enzimas y factores reguladores de la transcripción, del metabolismo energético celular, en respuesta a señales celulares y hormonales (Herzig y Shaw, 2018). Entre las vías que regula la AMPK se encuentra la biogénesis mitocondrial (Scarpulla, 2011), una vía relevante para la adaptación de la célula ante un aumento de la demanda energética (Pesta et al., 2011). La AMPK actúa en concierto con otros sensores metabólicos, como la sirtuina 1 y el coactivador 1α del receptor activado por el proliferador de peroxisomas $\gamma$ (PGC-1α), estableciendo una respuesta coordinada frente al déficit de energía, que ayuda mantener la

homeostasis metabólica. En particular, PGC-1α aumenta tanto la masa como la respiración mitocondrial (Cantó y Auwerx, 2009).

El aumento de la masa mitocondrial hepática en el ganado se ha asociado con una mayor eficiencia alimenticia en varios sistemas de producción. Por ejemplo, los novillos de bajo consumo de alimento residual presentaron mayor masa mitocondrial y actividad de citrato sintasa que los novillos de alto consumo de alimento residual; esto sugiere que los novillos de alta eficiencia probablemente canalizaron los nutrientes de manera más eficiente hacia el metabolismo energético (Casal et al., 2018). Además, vacas lecheras con alta eficiencia alimenticia mostraron tener mayor número de copias de ADN mitocondrial comparado con vacas lecheras con baja eficiencia alimenticia en un sistema de confinamiento (Kennedy et al., 2021).

La AMPK también regula los procesos de dinámica mitocondrial; entre estos encontramos la fusión y la fisión mitocondrial, muy relevantes para el control de calidad y el funcionamiento de la mitocondria (Toyama et al., 2016; Herzig y Shaw, 2018).

El conocimiento cada vez mayor de los cambios metabólicos coordinados, que ocurren durante el período de transición y de la lactancia, ha llevado a mejoras en la producción, reproducción y el estado de salud de las vacas lecheras. Específicamente, las adaptaciones del metabolismo de la glucosa (Bell y Bauman, 1997) y los eventos moleculares subyacentes a la resistencia a la insulina (De Koster y Opsomer, 2013), uno de los mecanismos más importantes en las vacas lecheras de alto rendimiento, han sido ampliamente estudiados y han conducido a plantear diferentes estrategias nutricionales (Grum et al., 1996). Paralelamente, el estudio del balance energético negativo de la lactancia temprana ha aclarado la base molecular de patologías asociadas al período de transición, como la cetosis (Adewuyi et al., 2005; McArt et al., 2013) y el hígado graso (Han van der Kolk et al., 2017). Estos

estudios han llevado, a su vez, a la investigación en nutrición (Ingvartsen, 2006; Ingvartsen y Moyes, 2013) y al suministro de alimentos específicos para preservar la salud del animal (Pinotti et al., 2003; Chung et al., 2009; Niehoff et al., 2009; Corbin y Zeisel, 2013; Jayaprakash et al., 2016; Panda et al., 2017).

# 2. HIPÓTESIS Y OBJETIVOS

## 2.1 HIPÓTESIS

Durante el balance energético negativo de la lactancia temprana, las vacas lecheras Holstein-Friesian movilizarán reservas corporales, lo que aumentará la acumulación de triglicéridos en el hígado y afectará negativamente la función mitocondrial. Las vacas sometidas a una estrategia de alimentación con mayor inclusión de pasturas vs. DTM o suplementación con DTM mostrarán una movilización más exacerbada. Además, en el sistema con mayor inclusión de pasturas, las vacas Holstein de origen genético neozelandés mostrarán un mejor estado metabólico (menores niveles de triglicéridos, mayor función mitocondrial, menores niveles de acetilación mitocondrial) en relación con las de origen norteamericano.

## 2.2 OBJETIVO GENERAL

Profundizar en los aspectos moleculares que explican las adaptaciones en el metabolismo energético hepático en vacas lecheras durante el BEN y realizar un estudio comparativo entre estrategias de alimentación y genotipos Holstein.

## 2.3 OBJETIVOS ESPECÍFICOS:

1. Caracterizar los cambios de composición del tejido hepático durante la lactancia.
2. Estudiar la regulación transcripcional de las adaptaciones en el metabolismo energético hepático durante la lactancia.
3. Estudiar la función mitocondrial, la β-oxidación y la regulación postraduccional de la vía por acetilación de lisinas en tejido hepático durante la lactancia.

## 3. ESTRUCTURA GENERAL DE LA TESIS

Consiste en cuatro artículos científicos que constituyen la estructura central de la tesis y un capítulo final de discusión general y conclusiones globales.

El artículo “Glucose and Fatty Acid Metabolism of Dairy Cows in a Total Mixed Ration or Pasture-Based System During Lactation” fue publicado en la revista *Frontiers in Animal Science* y constituye el cuarto capítulo de esta tesis.

El artículo titulado “Impaired Hepatic Mitocondrial Funtion During Early Lactation in Dairy Cows: Association with Protein Lysine Acetylation” fue publicado en la revista *PlosOne* y constituye el quinto capítulo de esta tesis.

El sexto capítulo de esta tesis está constituido por el artículo titulado “Differential Hepatic Mitochondrial Function and Gluconeogenic Gene Expression in Two Holstein Strains in a Pasture-Based System”, recientemente aceptado por la revista *Journal of Dairy Science*.

El artículo titulado “Hepatic Metabolism of Grazing Holstein Cows of Diverging Genetic Origin Supplemented with Total Mixed Ration or Concentrate During Lactation” se encuentra en formato de borrador.

## 4. GLUCOSE AND FATTY ACID METABOLISM OF DAIRY COWS IN A TOTAL MIXED RATION OR PASTURE-BASED SYSTEM DURING LACTATION

García-Roche M, Cañibe G, Casal A, Mattiauda DA, Ceriani M, Jasinsky A, Cassina A, Quijano C and Carriquiry M (2021)
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Este trabajo tuvo como objetivo caracterizar las adaptaciones del metabolismo de la glucosa y los ácidos grasos en las vacas del modelo 1 (DTM vs. un sistema de base pastoril). También se evaluaron los mecanismos moleculares subyacentes, tanto a nivel transcripcional como postraduccional, responsables de la regulación metabólica durante distintas etapas de la lactancia. Para ello, se tomaron biopsias de hígado y muestras de plasma a los -14, 35, 60, 110, 180 y 250 días en leche (DEL) y se realizaron distintos análisis. Se observó que la abundancia de ARNm de enzimas gluconeogénicas estaba aumentada durante lactancia temprana ($P < 0,05$), en particular la expresión génica de metilmalonil-CoA mutasa para las vacas G0 ($P < 0,05$) y piruvato carboxilasa (*PC*) para el grupo G1 ($P < 0,05$), apuntando al uso diferencial de precursores gluconeogénicos. La fosforilación de AMPK se vio aumentada durante lactancia tempana vs. lactancia tardía ($P < 0,01$), como también los niveles de ARNm del coactivador 1α del receptor activado por el proliferador de peroxisomas $\gamma$ (*PPARGC1A,* que codifica a PGC-1$\alpha$), el receptor alfa activado por el proliferador peroxisomal *(PPARA,* que codifica a PPAR-$\alpha$) y la desacetilasa nuclear sirtuina 1 (*SIRT1*). Además se observaron asociaciones positivas entre estos sensores metabólicos y factores de transcripción de genes clave de la gluconeogénesis y oxidación de los ácidos grasos. Estos resultados mostraron que las adaptaciones metabólicas asociadas al BEN de lactancia temprana pueden estar afectados por la estrategia de alimentación y además pueden estar regulados por el eje AMPK, *SIRT1* y PGC-1$\alpha$.

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# Glucose and Fatty Acid Metabolism of Dairy Cows in a Total Mixed Ration or Pasture-Based System During Lactation

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In this study, we explored mechanisms related to glucose and fatty acid metabolism in Holstein–Friesian multiparous dairy cows during lactation under two feeding strategies. From 0 to 180 days postpartum, cows were fed total mixed ration (TMR) *ad libitum* (non-grazing group, G0) or grazed *Festuca arundinacea* or *Medicago sativa* and were supplemented with 5.4 kg DM/d of an energy-protein concentrate (grazing group, G1). From 180 to 250 days postpartum, all cows grazed *F. arundinacea* and were supplemented with TMR. Plasma samples and liver biopsies were collected at −14, 35, 60, 110, 180, and 250 days in milk (DIM) for metabolite, hormone, gene expression, and western blot analysis. Our results showed increased levels of negative energy balance markers: plasma non-esterified fatty acids (NEFA), liver triglyceride and plasma $\beta$-hydroxybutyrate (BHB) ($P < 0.01$), triglyceride and $\beta$-hydroxybutyrate concentration were especially elevated for G1 cows. Also, hepatic mRNA expression of gluconeogenic enzymes was upregulated during early lactation ($P < 0.05$). In particular, methymalonyl-CoA mutase expression was increased for G0 cows ($P < 0.05$) while pyruvate carboxylase (*PC*) expression was increased for G1 cows ($P < 0.05$), suggesting differential gluconeogenic precursors for different feeding strategies. Phosphorylation of AMP-activated protein kinase was increased in early lactation vs. late lactation ($P < 0.01$) and negatively correlated with *PC* mRNA levels. The positive association of gluconeogenic genes with proliferator-activated receptor gamma coactivator 1-alpha *(PPARGC1A*) hepatic expression supported the importance of this transcription factor in glucose metabolism. The peroxisome proliferator-activated receptor alpha *(PPARA)* mRNA was increased during early lactation ($P < 0.05$), and was positively associated to *PPARGC1A,* carnitine palmitoyl-transferase 1, and hydroxymethylglutaryl-CoA synthase 2 (*HMGCS2)* mRNA expression. Alongside, hepatic mRNA expression of *FABP* was decreased for G1 vs. G0 cows ($P < 0.05$), possibly linked to impaired fatty acid transport

and related to accumulation of liver triglycerides, evidencing G1 cows fail to adapt to the demands of early lactation. In sum, our results showed that metabolic adaptations related to early lactation negative energy balance can be affected by feeding strategy and might be regulated by the metabolic sensors AMPK, SIRT1, and coordinated by transcription factors PPARGC1A and PPARA.

Keywords: AMPK, sirtuin 1, PGC1A, PPARA, dairy cows, grazing

## INTRODUCTION

High producing dairy cows undergo extreme metabolic adaptations during lactation. In particular, the transition period from late gestation to early lactation is the time where most diseases and metabolic disorders occur (Drackley, 1999). It represents a moment of almost 4-fold increases in energy, protein, and mineral requirements and when multiple organs orchestrate metabolic adaptations which will be critical in defining the success of the oncoming lactation (Bauman, 2000; Roche et al., 2013).

Recently, the inclusion of grazing in dairy cow systems achieved interest to farmers due to its reduced requirement for capital infrastructure and operating expenses per kilogram of milk, decreased environmental impact, and access to higher value markets where animal welfare is perceived as an added value (Gregorini et al., 2017; Roche et al., 2017). In dairy grazing systems, herbage is accounted for the primary diet component and unlike in total mixed rations (TMR) systems, in which feed quantity and quality can be controlled, manipulation of intake, and concentration of dietary nutrients are more complex to manage (Chilibroste et al., 2012). In addition, the challenges faced in grazing systems include assuring sufficient energy intake through quantity and quality of the forage and taking strategic decisions to account for a stable stocking rate during the different seasons of the year (Chilibroste et al., 2005; Mattiauda et al., 2013; Gregorini et al., 2017). Furthermore, previous research has shown that in order to achieve high milk yields or to maintain body reserves (i.e., body condition score) in Holstein cows on intensive grazing systems, supplemental energy is needed (Kolver and Muller, 1998).

Moreover, milk yield in grazing systems with supplementation of concentrate is reduced when compared to either TMR or partial TMR systems (60–70% of offered TMR; Bargo et al., 2002;Jasinsky et al., 2019). Pasture grazing, especially when herbage allowance, quality and access time may be limiting for herbage intake, may lead to excessive mobilization of energy reserves (Astessiano et al., 2015). Indeed, decreased body reserves has been pointed out to lead to poorer productive and reproductive performance (Astessiano et al., 2017).

Full lactation studies have reported that mobilization of body reserves, seen as changes in body weight and body condition score, reaches a plateau after peak of lactation at 60–85 days in milk (DIM) and replenishment of body stores is observed during mid-lactation (Buckley et al., 2000). However, this is highly dependent on the level of concentrate, as previous studies observed that Holstein–Friesian cows supplemented with high levels of concentrate started gaining body condition score 5–7 weeks prior to cows supplemented with lowlevels of concentrate (Veerkamp et al., 1994). Indeed, body condition score replenishment during mid-late lactation has been increasingly challenging for high yielding dairy cows in pasture based systems (Kolver and Muller, 1998; Wales et al., 2013). However, although there is information about body condition score and body weight changes, metabolism adaptation related to replenishment of body reserves in pasture based systems has been understudied.

Increasing knowledge of the coordinated metabolic shifts has led to improvements in production or the health status of dairy cows. Specifically, adaptations of glucose metabolism (Bell and Bauman, 1997) and the molecular events underlying insulin resistance (De Koster and Opsomer, 2013), one of the most significant mechanisms in high yielding dairy cows, have been extensively studied and led to different nutritional strategies (Grum et al., 1996). Alongside, the study of early lactation negative energy balance has clarified the molecular basis of pathologies associated to the transition period such as ketosis (Adewuyi et al., 2005; McArt et al., 2013) and fatty liver (Han van der Kolk et al., 2017) which have led to research in nutrition (Ingvartsen, 2006; Ingvartsen and Moyes, 2013) as well as supply of particular feedstuffs (Pinotti et al., 2003; Chung et al., 2009; Niehoff et al., 2009; Corbin and Zeisel, 2013; Jayaprakash et al., 2016; Panda et al., 2017).

Hence, the aim of this work was to characterize the adaptations in glucose and fatty acid metabolism in cows under two contrasting feeding strategies (TMR vs. pasture-based) during lactation as well as the underlying regulating mechanisms at both a transcriptional and post-transcriptional levels, elucidating plausible mechanisms involved in the regulation of glucose and lipid metabolism during different stages of lactation. We hypothesized that mechanisms of adaptation to lactation would differ between TMR and pasture-based strategies especially due to differential gluconeogenic precursors and differential energy balance during early lactation.

## MATERIALS AND METHODS

The experiment was conducted at the Experimental Station “Dr. Mario A. Cassinoni” of the Facultad de Agronomía, Universidad de la República, Paysandú, Uruguay. Animal use and procedures were approved by the Animal Experimentation Committee (CHEA) of the Universidad de la República, Uruguay (file number: 021130-001914-15).

## Experimental Design

The experiment was conducted in a randomized block design in which 24 Holstein cows (664 ± 65 kg BW and 3.0 ± 0.4 units of BCS; 18/08/2015 ± 11 of calving date) were blocked by calving date, parity, BW, and BCS and randomly assigned to two feeding strategies from calving to 180 ± 11 DIM. Feeding strategies were: cows fed 100% of a total-mixed ration (TMR) *ad libitum* (a non-grazing control group; G0, $n$ = 12) and cows with grazing and supplementation (G1; $n$ = 12).

The TMR (forage to concentrate ratio of 70:30, as fed basis and 40:60 forage to concentrate ratio as DM basis) was offered once a day after the morning milking to control cows (G0). The TMR was formulated for a milk production target of 35 kg per day and 15–20% refusals according to NRC DairyModel 2001 software (National Research Council, 2001). It was composed by corn silage and moha (*Setaria italica*) hay or alfalfa haylage and a concentrate that included sorghum grain (22.6%), corn grain (6.8%), barley grain (4.4%) sunflower expeller (7.5%), soybean expeller (13.6%), and minerals and vitamins (1.7%). Chemical composition was 43.1% DM, 12.9% crude protein (CP), 33.8% neutral detergent fiber (NDF), 21.3% acid detergent fiber (ADF), and 1.63 Mcal per kg of net energy of lactation (NEL) (DM basis, **Table 1**). Cows were housedin three pens (8 × 22.6 m each; 4 cows per pen) of a free stall facility (wood-frame barn) with wood shavings (>10 cm) for bedding, and access to shade, water, and a feeder (2.4 m long, 1.12 m wide in the top, 0.58 wide in the bottom and 0.50 m deep).

From 0 to 113 DIM, G1 cows grazed a tall fescue *(Festuca arundinacea)* pasture (2,500 ± 490 kg DM per ha, two grazing sessions from 08:00 to 16:00 h and from 18:00 to 04:00 h; mean herbage allowance, 4 cm above ground level, of 30 kg dry matter (DM)/d per cow in a 7-d rotational system). The chemical composition (DM basis) was 26.4% DM, 14.2% CP, 54.7% NDF, 30.1% ADF, and 1.58 Mcal per kg DM of NEL. Cows were also supplemented after the morning milking, in individual feeders, with 5.4 kg DM/d per cow of a concentrate containing corn grain (32%), barley grain (31%), and soybean expeller (32%) with an 87% DM, 16.8% CP, 28.5% NDF, 9.3% ADF, and 1.83 Mcal per kg of NEL (DM basis). From 114 to 180 DIM, cows grazed an alfalfa *(Medicago sativa)* pasture (1,380 ± 328 kg DM per ha, one grazing session from 18:00 to 04:00 h; mean herbage allowance−4 cm above ground level—of 20 kg DM/d per cow) in a 7-d rotational system. The chemical composition (DM basis) was 26.4% DM, 23.3% CP, 30.1% NDF, 24.7% ADF, and 1.68 Mcal per kg DM of NEL. During this period, cows received after the morning milking, in the free stall facility, TMR (50% of offered TMR to G0 cows) composed by corn silage (23.3%), alfalfa haylage (19%), sorghum grain (20.8%), corn grain (11.8%), barley grain (11.5%), soybean expeller (11.8%), and minerals and vitamins (1.8%) with a chemical composition (DM basis) of 41.5% DM, 11.1% CP, 32.1% NDF, 22.0% ADF, and 1.64 Mcal/kgDM of NEL. Management changed at 113 DIM as temperature-humidity index exceeded the value of 72 for more than 5 consecutive hours and for 3 consecutive days denoting heat stress (Johnson et al., 1961).

After 180 DIM until the end of the experiment (250 DIM), both, G0 and G1 cows grazed a *F. arundinacea* pasture (2,340 ± 291 kg DM per ha, one grazing session from 16:30 to 04:00 h, mean herbage allowance—above 4 cm of ground level—of 20 kg DM per cow per day in a 7-d rotational system. Herbage chemical composition was 28.3% DM, 10.1% CP, 56.6% NDF, 32.2% ADF, and 1.48 Mcal per kg of NEL, DM basis). After the morning milking, cows were supplemented in the free stall facility, with TMR (50% of offered TMR to G0 cows at 180 DIM; 23.4% corn silage, 12.4% alfalfa hay, 28.3% sorghum grain, 11.5% corn grain, 11.1% barley grain, 11.5% soybean expeller, and 1.8% minerals and vitamins) with 50% DM, 12.5% CP, 29.7% NDF, 18.8% ADF, and 1.76 Mcal per kg of NEL (DM basis).

To determine diet composition, DM intake of TMR (based on difference between feed offered and refused) and pasture (based on NRC requirements) were determined and the proportion of diet components (DM basis) was calculated for each treatment. Diet was composed of 100% TMR from 0 to 180 DIM for G0 cows, and for G1 cows of 73.4% pasture and 26.6% concentratefrom 0 to 113 DIM and 32.7% pasture and 67.3% TMR from 114 to 180 DIM; from 180 to 250 DIM diet was composed of 28% pasture and 72% TMR for all cows (G0 and G1). Chemical composition of estimated diets is presented on **Table 1**.

Cows were milked twice a day and milk yield was recorded daily. Milk samples were collected weekly until 90 DIM, every 14 d from 90 to 180 DIM and every 28 d from 180 to 250 DIM. Cow BCS (score 1–5; Edmonson et al., 1989) and BW were determined every 14 d.

## Plasma and Liver Biopsies

Blood samples were collected at −14, 35, 60, 110, 180, and 250 DIM by venipuncture of the coccygeal vein using BD Vacutainer® tubes with heparin (Becton Dickinson, Franklin Lakes, NJ, USA). Samples were centrifuged at 2,000 g for 15 min at 4 °C within 1 h after collection and plasma was stored at −20 °C until analyses were performed.

Liver biopsies were collected at −14, 35, 60, 110, 180, and 250 DIM using a 14-gauge biopsy needle (Tru-Core-II Automatic Biopsy Instrument; Angiotech, Lausanne, Switzerland) after the local intramuscular administration of 3 mL of 2% lidocaine HCl (Carriquiry et al., 2009) and snap frozen in liquid nitrogen and stored at −80 °C until analysis.

## Metabolite and Hormone Assays in Plasma Samples

zGlucose concentrations were determined with a kit from Biosystems S.A. (Costa Brava, Barcelona, Spain), following manufacturer instructions. In this assay glucose oxidase oxidizes glucose yielding hydrogen peroxide, which forms a colored quinonoid in the reaction with 4-aminoantipyrine and phenol catalyzed by a peroxidase. Absorbance was measuredat 505 nm. Concentrations of $\beta$-hydroxybutyrate (BHB) were determined with a kit from Randox Laboratories Ltd. (Ardmore, UK) following manufacturer instructions. The assay measures NADH formation spectrophotometricallyat 340 nm as $\beta$-hydroxybutyrate dehydrogenase catalyzes

TABLE 1 | Estimated nutrient composition of diets according to feeding strategy during lactation.

| | G0[a] | G1 | | All cows |
|---|---|---|---|---|
| Days postpartum | 0–180 | 0–113 | 114–180 | >180 |
| Dry matter, % | 43.1 | 42.4 | 36.5 | 43.9 |
| Crude protein, %DM | 12.9 | 14.9 | 15.1 | 11.8 |
| Neutral detergent fiber, %DM | 33.8 | 47.6 | 31.4 | 37.2 |
| Acid detergent fiber, %DM | 21.3 | 24.5 | 22.9 | 22.6 |
| Net energy of lactation, Mcal/kg DM[c] | 1.68 | 1.64 | 1.65 | 1.68 |
| Metabolizable protein, g/d[b] | 1,854 | 1,762 | 1,914 | 1,749 |

[a] *Feeding strategies were a non-grazing group (control group; G0) fed 100% of a total-mixed ration (TMR) ad libitum and a grazing group (G1), which grazed on pasture and received supplementation.*

[b] *Net energy of lactation and metabolizable protein (MP) were estimated according to National Research Council (2001). Estimated MP balances indicates diets provided, at least 85% of MP requirement for both, G0 and G1, from 0 to 180 DIM and 95% of MP requirement after 180 DIM.*

[c] *Diets were formulated to supply micronutrients according with requirements at all times of the lactation curve, thus they included a minerals and vitamin premix composed (% of DM or ppm, IU, g per animal) of 0.15% S, 19.31% Ca, 2.33% P, 2.98% Cl, 7.87% Na, 0.11% K, 3.59% Mg, 0.21 ppm Co, 5.7 ppm Cu, 12.9 ppm Fe, 8.8 ppm Mn, 0.08 ppm Se, 0.02 ppm Y, 18.7 Zn, 14.4 ppm chelated Zn, 5.04 ppm chelated Cu, 0.04 ppm chelated Se, 2,000.00 IU Vitamin A, 202.00 IU Vitamin D3, 2.10 IU Vitamin E, 12.16 ppm monensin, 0.56 g yeast, 0.28 g betaglucan, 0.28 g mannan-oligosaccharides.*

the oxidation of BHB to acetoacetate (Porter et al., 1997). Concentrations of non-esterified fatty acids (NEFA) were determined spectrophotometrically with a kit from FUJIFILM Wako Diagnostics USA Corp. (Mountain View, CA, USA), following manufacturer instructions. In this method, NEFA is incubated with acyl-CoA synthetase and ATP and yields acyl-CoA, finally acyl-CoA is oxidized in a reaction catalyzed by acyl-CoA oxidase producing hydrogen peroxide, which reacts with 4-aminoantipyrine and 3-methyl-N-ethyl-N-(β-hydroxyethil)-aniline in the presence of peroxidase forming a purple colored end-product with an absorption maximum at 550 nm (Chilliard et al., 1984). For all metabolite assays intra and inter-assay coefficient of variation (CV) were <10%.

Insulin concentrations were measured using immunoradiometric assays (IRMA) with a commercial kit(INS-IRMA; DIA Source Immune Assays S.A., Belgium) asdescribed by previously Astessiano et al. (2015); the assay detection limit was 1.18 $\mu$IU/ml. Leptin concentrations were determined by a liquid-phase radioimmunoassay (RIA) using a commercial Multi-Species Leptin kit (RIA kit, Merck Millipore Billerica, MA, USA) reported previously in bovines (Adrienet al., 2012; Astessiano et al., 2015); the assay detection limit was 1.8 ng/ml. Concentrations of adiponectin were measured with a human RIA kit (HADP-61 HK, Merck Millipore) using undiluted plasma samples (Astessiano et al., 2015); the sensitivity of the assay was 1.01 ng/ml. All samples were determined ina single assay. The intra-assay CVs for all the assays were <8%. The Revised Quantitative Insulin Sensitivity Check Index (RQUICKI) was calculated as 1/(log(fasting insulin) + log(fasting glucose) + log(free fatty acids) (Holtenius and Holtenius, 2007).

## Triglyceride Quantification in Liver Samples

Triglyceride quantification was performed in liver homogenates as described in García-Roche et al. (2019). Briefly, lipids were extracted, and an internal standard was added to the samples; then, lipid extracts were spotted manually on thin layer chromatography plates using a microsyringe (Hamilton) along with the internal standard and a triglyceride standard. Finally, densitometry quantification analysis of the bands was performed using ImageJ software.

## Free Glucose and Glycogen Quantification in Liver Samples

For liver glycogen and free glucose, homogenization was performed using 500 $\mu$L 2N HCl and glass Dounce homogenizers. Glycogen was digested to glucose by acid- heat hydrolysis (Bancroft and Fry, 1933), for this, samples were subjected to 100°C during an hour. Finally, free liver glucose and digested liver glycogen were determined using the aforementioned glucose detection kit after neutralizing acid samples with an equal amount of 2M NaOH.

## Western Blots

Liver homogenates were performed as previously described by García-Roche et al. (2019), the lysis buffer was complemented with protease and phosphatase inhibitors: 1 mM PMSF, supplemented with SigmaFAST protease inhibitor cocktail and Calbiochem phosphatase inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA). Protein content was determined with the Bradford assay using bovine serum albumin as standard (Bradford, 1976) and samples were kept at −80°C until analyzed. Liver homogenates were resolved (25 $\mu$g) in 12%Tris-Glycine-SDS polyacrylamide gels (SDS/PAGE), along withprotein ladders (SDS7B2; Sigma-Aldrich) and proteins were transferred overnight to nitrocellulose membranes. Membranes were blocked with blocking buffer (Tris buffered saline with 0.1%Tween 20 and 0.5% skimmed milk) and incubated overnightat 4°C with primary antibodies against: $\beta$-actin (1:1,000, sc-81178; Santa Cruz Biotechnology, Santa Cruz, CA, USA), AMPK$\alpha$ (1:1,000; #2532, Cell Signaling Technology, Danvers, MA, USA), and phosphorylated AMPK$\alpha$ (1:1,000, #2535; Cell Signaling Technology).

Membranes were washed and probed with secondary antibodies from LI-COR Biosciences, Lincoln, NE, USA: anti-mouse (1:10,000, IRDye 680, 926–68070) or anti-rabbit (1:20,000, IRDye 800, 926–32211). Immunoreactive proteins were detected with an infrared fluorescence detection system (Odyssey, LI-COR Biosciences) and bands were quantified by densitometry with ImageStudio software (LI-COR Biosciences).

## RNA Extraction and qPCR Analysis

Isolation of total RNA from liver tissue and cDNA synthesisby reverse transcription was performed by Carriquiry et al. (2009). Briefly, total RNA was isolated using the Trizol reagent and concentration was quantified using a spectrophotometer (NanoDrop ND-1000 Spectrophotometer; Nanodrop Technologies, Wilmington, DE, USA). Quantified

samples were subject to lithium chloride precipitation and DNase treatment using Ambion™ DNA-free™ DNA Removal Kit following manufacturer instructions (ThermoFisher Scientific, Waltham, MA, USA). Finally, concentrationof RNA was determined by measuring the absorbance at 260 nm (NanoDrop ND-1000 Spectrophotometer; Nanodrop Technologies, Wilmington, DE, USA), and purity and integrity of RNA isolates were assessed from 260/280 and 260/230 absorbance ratios (>1.9 and 1.8, respectively). Samples of RNA were stored at −80°C. Retrotranscription of RNA was performed using the SuperScript™ III Reverse Transcriptase kit (Invitrogen™ from ThermoFischer Scientific) using random hexamers and 1 $\mu$g of total RNA as a template. The cDNA was stored at −20°C until its use in the real-time PCR. Primers (**Supplementary Information 1**) to specifically amplify cDNA of target genes: acetyl-CoA carboxylase (*ACACA*), $\beta$-actin (*ACTB*), adiponectin receptor 1 (*ADIPOR1*), carnitine palmitoyl-transferase (*CPT1A*), liver fatty acid binding protein (*FABP1*), glucose-6-phosphatase (*G6PC*), hydroxymethylglutaryl-CoA synthase 2 (*HMGCS2*), hypoxanthine phosphoribosyl transferase (*HPRT1*), methylmalonyl-CoA mutase (*MMUT*), leptin receptor (*LEPR*), pyruvate carboxylase (*PC*), phosphoenolpyruvate carboxykinase (*PCK1*), peroxisome proliferator-activated receptor gamma coactivator 1-alpha *(PPARGC1A*), peroxisome proliferator-activated receptor alpha (*PPARA*), and sirtuin 1 (*SIRT1*) were obtained from the literature or specifically designed using the Primer3 website (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and bovine nucleotide sequences available from NCBI (http://www.ncbi.nlm.nih.gov/).

Real time PCR reactions were performed in a total volume of 15 $\mu$l using Maxima SYBR Green/ROX qPCR Master Mix 2X (ThermoFisher Scientific), using the following standard amplification conditions: 10 min at 95°C and 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C in a 48-well StepOne™ Real-Time PCR System (Applied Biosystems™ from ThermoFischer Scientific). Melting curves were run on all samples to detect primer dimers, contamination, or presence of other amplicons. Each plate included a pool of total RNA from bovine liver samples analyzed in triplicate to be used as the basis for the comparative expression results (exogenous control) and duplicate wells of non-template control (water). Gene expression was measured by relative quantification with respect to the exogenous control (Pfaffl, 2004) and normalized to the geometric mean expression of the endogenous control genes (*ACTB* and *HPRT*). Expression stability of two selected housekeeping genes was evaluated using MS-Excel add-in Normfinder (MDL, Aarhus, Denmark). The stability values obtained with Normfinder were 0.004 for *ACTB* and 0.003 for *HPRT*. Amplification efficiencies or target and endogenous control genes were estimated by linear regression of a cDNA dilution curve (**Supplementary Information 1**). Intra and inter-assay CV-values were 1.9 and 5.9% ($n$ = 5 dilutions, from 100 to 6.25 ng/well), respectively.

## Statistical Analysis

Data were analyzed in a randomized block design using the SAS System program (SAS Academic Edition; SAS Institute Inc., Cary, NC, USA), where the cow was the experimental unit and they were blocked according to calving date, previous lactation yield, initial BW and initial BCS. Univariate and linear regression analyses were performed with all variables to identify outliers and inconsistencies and to verify normality of residuals. When data did not have normal distribution (Shapiro-Wilk $P$-value $< 0.10$) logarithmic transformations were performed in order to more closely approximate to normality requirements or outliers were removed when the studentized residual $r$ was between −3 and 3, no more than three values per variable were excluded. Least square means and pooled standard error values of all variables shown were done with the non-transformed data, so as to aid in the comparison with variables that were not transformed.

Data were analyzed as repeated measures using the MIXED procedure, the model included treatment, stage of lactation (DIM), and their interaction as fixed effects, block as random effect and calving date, initial BW and BCS as covariates when $P < 0.20$. The interaction between pen and block was included in the model as a random effect, as covariance parameter estimates were zero or close to zero it was removed from the model. Spatial power [SP (POW)] was used as the covariance structure and the Kenward-Rogers procedure was used to adjust the denominator degree of freedom. Tukey-Kramer tests were conducted to analyze differences between groups. Means were considered to differ when $P < 0.5$ and a trend was declared when $0.05 < P < 0.10$. The CORR procedure was performed for correlation analyses, Pearson correlations were used for variables with normal distribution while Spearman correlations were used for variables with non-normal distribution.

# RESULTS

Energy corrected milk yield (**Figure 1A**) decreased from 35 to 250 DIM ($P < 0.001$), without differences between treatments. On the other hand, an interaction between DIM and treatment ($P < 0.05$) was observed for body condition score (**Figure 1B**) as it increased with the progression of lactation ($P < 0.001$) and was higher for G0 than G1 from 110 to 180 DIM. A drop in body condition score was observed at 180 DIM, probably due to heat stress conditions recorded during the summer season.

## Glucose Metabolism and the AMPK, PGC-1$\alpha$, and SIRT1 Axis

Plasma glucose concentrations decreased sharply during early lactation (60 DIM) and gradually increased during the progress of lactation reaching levels similar to pre-partum at late lactation (250 DIM; **Figure 1C**). However, neither effect of treatment nor its interaction with stage of lactation were observed for plasma glucose. In contrast, for insulin concentration there was an interaction between DIM and treatment ($P < 0.01$, **Figure 1D**) where plasma insulin increased from early to mid-lactation (60–180 DIM) only in G0 cows. Since the increase in insulin coincided with the decrease in glucose concentrations, the revised quantitative insulin sensitivity index (RQUICKI), indicative of insulin sensibility, was calculated (**Figure 1E**). Although RQUICKI was not affected by the interaction between

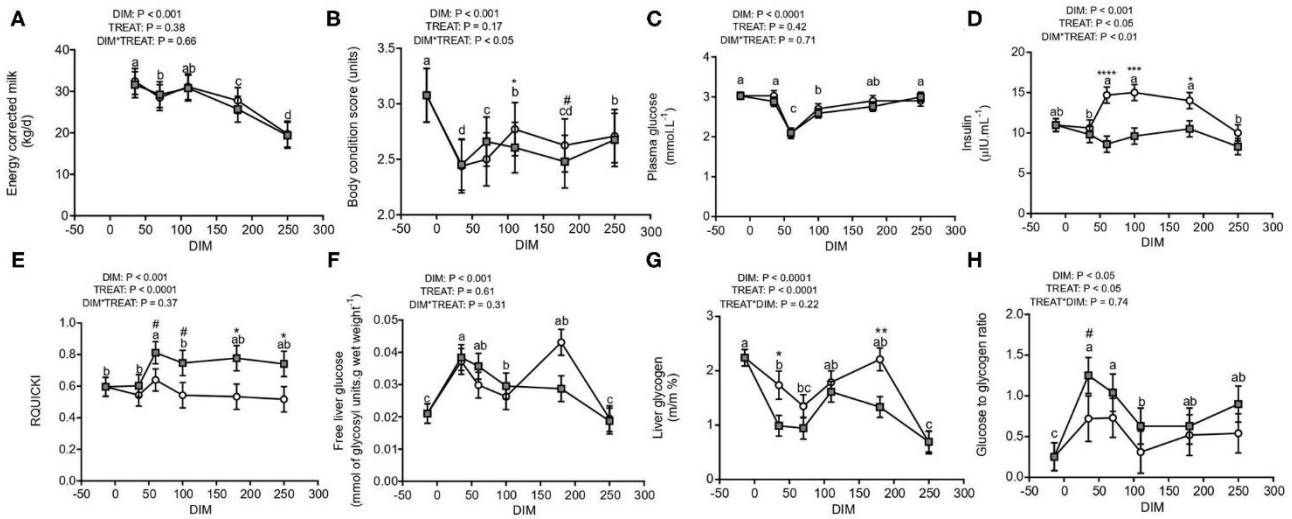


FIGURE 1 | Energy corrected milk yield and glucose metabolism from pre-partum to late lactation in cows from G0 (white) and G1 (gray) ($N$ = 8–12). Different letters (a and b for $P < 0.05$) and asterisks or numerals (* for $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$; # for trends, $0.05 < P < 0.10$) depict differences between means; letters for DIM effect and asterisks or numerals for treatment effect. Graphs show energy corrected milk yield (A), body condition score (B), concentrations of plasma glucose (C), plasma insulin (D), RQUICKI (E), free liver glucose (F), liver glycogen (G), and glucose to glycogen ratio (H). G0: Cows were fed TMR *ad libitum* from calving to 180 DIM. G1: Cows grazed *Festuca arundinacea* plus a commercial concentrate or *Medicago sativa* supplemented with TMR (50% of G0 offer), depending on heat stress conditions, from calving to 180 DIM. From 180 to 250 DIM both groups grazed *Medicago sativa* and were supplemented with TMR (50% of G0 offer at 180 DIM).

stage of lactation and treatment, it was affected by stage of lactation ($P < 0.001$) and treatment ($P < 0.0001$) as it increased only for G1 cows from 35 DIM onwards.

Regarding liver composition, both, free liver glucose (**Figure 1F**) and liver glycogen (**Figure 1G**) were affected by DIM ($P < 0.001$). Free hepatic glucose peaked in early lactation(35 DIM) and returned to pre-partum (−14 DIM) levels at 250 DIM where minimum concentrations were observed. Hepatic glycogen reserves dropped dramatically from pre-partum to early lactation, increased from 60 to 180 DIM and decreased again in late lactation (250 DIM). Additionally, liver glycogen was higher for G0 than G1 cows. Free glucose and liver glycogenwere negatively correlated at 35 DIM ($r = -0.42$ and $P < 0.05$) while liver glycogen and insulin concentrations were positively correlated ($r = 0.30$, $P < 0.05$). Free liver glucose to liver glycogen ratio (**Figure 1H**) peaked during early lactation ($P < 0.05$) and was higher for G1 cows ($P < 0.05$), especially at 35 DIM ($P = 0.07$).

Phosphorylated AMPK was 1.8-fold greater at 35 than 250 DIM ($P < 0.01$), while no changes in total AMPK protein expression were observed ($P > 0.13$). This yielded a pAMPK to AMPK ratio more in favor of phosphorylated AMPK, at 35 than 250 DIM (**Figures 2E,F**, $P < 0.01$), evidencing AMPK activation during early lactation. Plasma adiponectin was not affected by DIM, treatment, or their interaction (**Figure 2A**), while plasma leptin tended to be higher for G0 than G1 cows ($P = 0.09$, **Figure 2C**). But, both hepatic *ADIPOR1* ($P = 0.08$, **Figure 2B**) and *LEPR* ($P < 0.05$; **Figure 2D**) mRNA tended to decrease at 110DIM. Gene expression of hepatic *ADIPOR1* and *LEPR* correlated positively ($r = 0.43$ and $P < 0.001$).

Hepatic mRNA expression levels of the *PPARGC1A* tended to be affected by DIM ($P = 0.06$) as it was 1.4-fold greater ($P < 0.05$) at 35 than 250 DIM (**Figure 2G**). Alongside, for hepatic *SIRT1* mRNA an interaction between DIM and treatment was observed ($P < 0.05$) with higher expression, roughly above 40%, for G0 than G1 cows at 35 DIM and the increase from 35 to 110 DIM for G1 cows, reinforcing the lower levels that these cows suffered at 35 DIM (**Figure 2H**). Although the pAMPK to AMPK ratio did not correlate with hepatic *PPARGC1A* or *SIRT1* mRNA levels, *PPARGC1A* and *SIRT1* mRNA expression levels did correlate positively ($r = 0.43$, $P < 0.01$).

Pyruvate carboxylase and methylmalonyl-CoA mutase catalyze reactions involved in recruiting different metabolites to the gluconeogenic pathway. Both, *PC* and *MMUT* mRNA were affected by the interaction between DIM and treatment ($P < 0.05$; **Figures 2I,J**). Hepatic *PC* decreased from 35 to 110 DIM to increase again at 250 DIM, its expression was 1.6-fold greater for G1 than G0 at 35 DIM (**Figure 2I**). Instead, expression of *MMUT* mRNA was 1.7-fold greater in G0 than G1 cows at 35 DIM ($P < 0.05$; **Figure 2J**).

In addition, expression of genes coding for phosphoenolpyruvate carboxykinase and glucose-6-phosphatase, key enzymes of the gluconeogenic pathway, was assessed. Hepatic expression of *PCK1* mRNA was affected by DIM ($P < 0.01$) and tended to be affected by treatment ($P = 0.06$; **Figure 2K**). Hepatic *PCK1* mRNA decreased from 35 to 110 DIM to increase again at 250 DIM, however, its expression was 2.7-fold higher for G1 than G0 at 110 DIM (**Figure 2K**). While *G6PC* mRNA was only affected by DIM ($P = 0.05$), its levels were 1.6-fold greater at 35 than 110 and 250 DIM (**Figure 2L**).

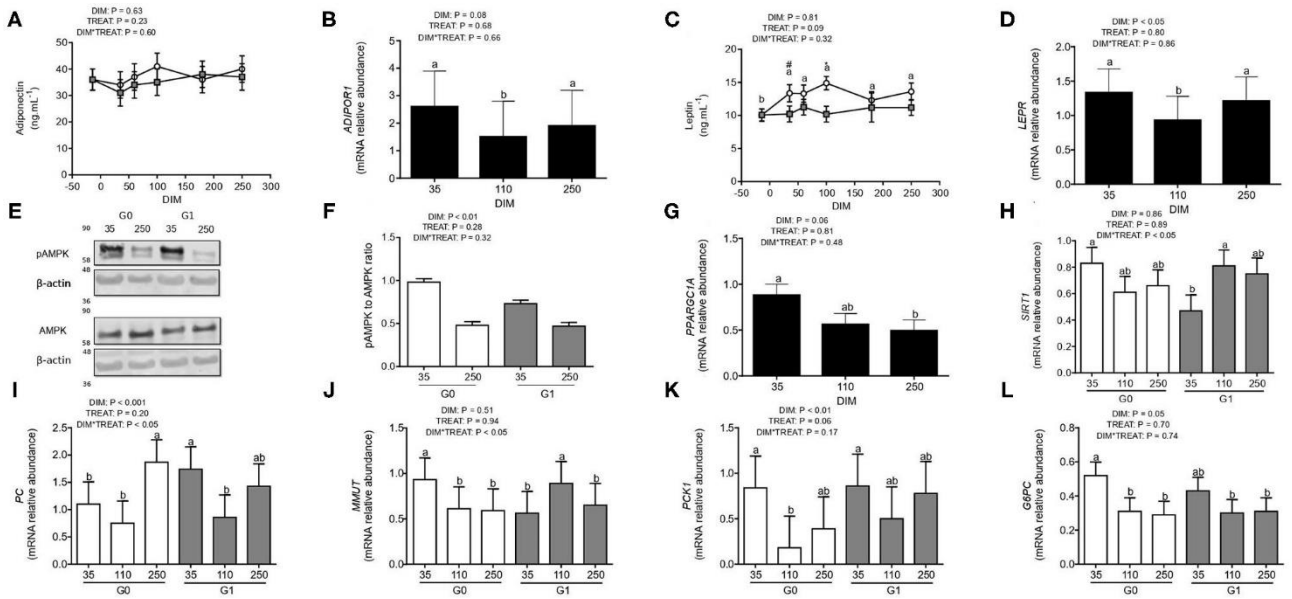


FIGURE 2 | AMPK, PGC-1$\alpha$, and SIRT1 axis and expression of gluconeogenic genes during 35, 110, and 250 DIM in cows from G0 (white) and G1 (gray) ($N$ = 8–12). Different letters (a and b for $P < 0.05$) and asterisks or numerals (* for $P < 0.05$ and # for trends, $0.05 < P < 0.10$) depict differences between means; letters for DIM effect and asterisks or numerals for treatment effect. Graphs show plasma concentrations of adiponectin (A) and leptin (C) and gene expression of *ADIPOR1* (B), *LEPR* (D), *PPARGC1A* (G), *SIRT1* (H), *PC* (I), *MMUT* (J), *PCK1* (K), and *G6PC* (L). (E) A representative western blot of pAMPK and AMPK in liver homogenates is shown. (F) pAMPK and AMPK levels were quantified by densitometry from blots like shown in (E), normalized by protein levels of the loading control ($\beta$-actin) and the pAMPK/AMPK ratio was calculated and expressed in relation to the average value of the G0 group at 35 DIM. G0: Cows were fed TMR *ad libitum* from calving to 180 DIM. G1: Cows grazed *Festuca arundinacea* plus a commercial concentrate or *Medicago sativa* supplemented with TMR (50% of G0 offer), depending on heat stress conditions, from calving to 180 DIM. From 180 to 250 DIM both groups grazed *Medicago sativa* and were supplemented with TMR (50% of G0 offer at 180 DIM).

Hepatic *PPARGC1A* mRNA correlated positively with mRNA expression of all gluconeogenic genes (*PPARGC1A* and *PC:* $r = 0.30$ and $P < 0.05$, *PPARGC1A* and *MMUT:* $r = 0.53$ and $P < 0.0001$, *PPARGC1A* and *PCK1:* $r = 0.33$ and $P < 0.05$, *PPARGC1A* and *G6PC:* $r = 0.26$ and $P = 0.06$) and *SIRT1* mRNA correlated positively only with expression of *MMUT* and *PCK1* mRNA ($r = 0.30$ and $P < 0.05$). Instead, the pAMPK/AMPK ratio correlated negatively with hepatic *PC* mRNA ($r = -0.42$ and $P < 0.05$). Both *PC* and *MMUT* mRNA correlated positively with *PCK1* ($r = 0.30$ and $P < 0.05$ and $r = 0.51$ and $P < 0.0001$,respectively) and *G6PC* mRNA ($r = 0.34$ and $P < 0.05$ and $r = 0.30$ and $P < 0.05$, respectively) and *PC* and *PCK1* mRNA correlated negatively with liver glycogen ($r = -0.30$ and $P < 0.05$ and $r = -0.35$ and $P < 0.01$, respectively) while *PCK1* mRNA correlated positively with energy corrected milk ($r = 0.37$ and $P < 0.05$). Finally, *PPARGC1A* mRNA correlated positively with free liver glucose ($r = 0.28$ and $P < 0.05$).

## Fatty Acid Metabolism and PPARA Mediated Pathway

Plasma concentrations of BHB (**Figure 3A**) and NEFA (**Figure 3B**), and liver triglyceride (**Figure 3C**) were quantified. Concentrations of BHB were affected by stage of lactation, treatment, and their interaction ($P < 0.05$) as plasma BHB reached its highest concentration during early lactation (between 35 and 60 DIM) mainly due to the 2-fold increase observed for G1 with respect to G0 cows. On the other hand, although no interaction was observed, plasma NEFA was affected ($P < 0.0001$) by DIM as it peaked in early lactation, decreasing at +60 DIM and thereafter. However, concentrations of NEFA tended ($P = 0.08$) to be affected by treatment, as they were greater for G1 than G0 cows especially during 35 and 180 DIM. Additionally, liver triglyceride was affected by DIM ($P < 0.0001$) and treatment ($P < 0.05$) and tended ($P = 0.06$) to be affected by the interaction between DIM and treatment (**Figure 3C**). While liver triglyceride concentrations remained unchanged for G0 cows during the progress of lactation, they peaked at 35 DIM for G1 cows.

Indeed, hepatic gene expression of PPARA was affected by DIM ($P < 0.05$) as it was higher at 35 and 250 DIM than during 110 DIM (**Figure 3D**). Expression of genes downstream PPARA mediated pathway were studied such as *FABP1*, *CPT1A*, *HMGCS2* (**Figures 3E–G**). Both, expression of *FABP1* and *CPT1A* mRNA tended to be affected ($P = 0.07$) or were affected($P < 0.01$) by DIM as *FABP1* mRNA decreased from 35 to 250 DIM and *CPT1A* mRNA decreased at 110 DIM when compared to 35 and 250 DIM. Expression of *FABP1* mRNA was affected by treatment ($P < 0.01$) as it was 2-fold higher for G0 than G1 cows while *HMGCS2* mRNA tended to be 1.6-fold higher forG1 than G0 cows ($P = 0.08$). Hepatic *PPARA* mRNA correlated positively with both *CPT1A* and *HMGCS2* mRNA ($r = 0.42$, $P < 0.01$ and $r = 0.64$, $P < 0.001$). Additionally, the expression of *CPT1A* and *HMGCS2* mRNA were positively correlated ($r = 0.50$, $P < 0.001$).

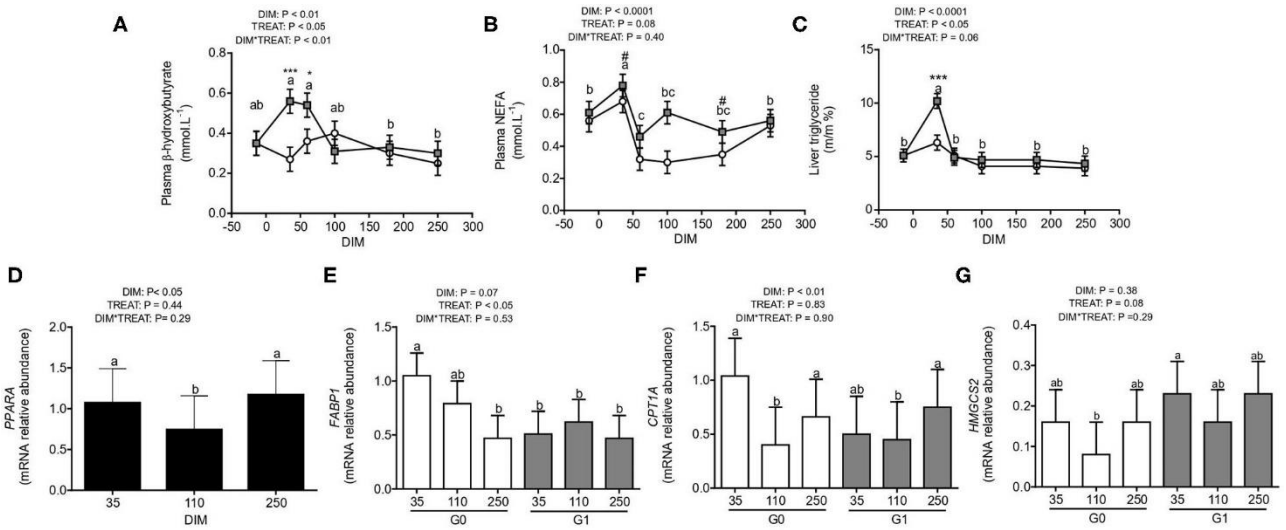


FIGURE 3 | Fatty acid metabolism and PPARA in pre-partum to late lactation in cows from G0 (white) and G1 (gray) ($N$ = 8–12). Different letters (a and b for $P < 0.05$) and asterisks or numerals (* for $P < 0.05$, *** for $P < 0.001$ and # for trends, $0.05 < P < 0.10$) depict differences between means; letters for DIM effect and asterisks or numerals for treatment effect. Graphs show concentrations of plasma BHB (A), plasma NEFA (B), and liver triglyceride (C), and gene expression of *PPARA* (D), *FABP1* (E), *CPT1A* (F), and *HMGCS2* (G). G0: Cows were fed TMR *ad libitum* from calving to 180 DIM. G1: Cows grazed *Festuca arundinacea* plus a commercial concentrate or *Medicago sativa* supplemented with TMR (50% of G0 offer), depending on heat stress conditions, from calving to 180 DIM. From 180 to 250 DIM both groups grazed *Medicago sativa* and were supplemented with TMR (50% of G0 offer at 180 DIM).

Besides, positive correlations between the pAMPK to AMPK ratio and *HMGCS2* expression ($r$ = 0.46 and $P < 0.05$), and between *PPARGC1A* and *HMGCS2* mRNA levels ($r$ = 0.34 and $P < 0.05$); as well as between *PPARGC1A* and *PPARA* mRNA levels ($r$ = 0.31 and $P < 0.05$) were observed.

## DISCUSSION

During early lactation glucose metabolism changes drastically as a response to maximal lactogenesis, from pre-partum to 60 DIM insulin peaked, especially for G0 cows as glucose dropped and free liver glucose increased while glycogen decreased. In addition to this, although blood and free liver glucose concentrations did not differ between treatments glycogen stores increased for G0 vs. G1 cows at 60 DIM probably due to increased gluconeogenesis to replenish glycogen stores or a decrease in glycogen hydrolysis. Alongside, glucose to glycogen ratio shows that at 35 DIM G1 is primarily focused in glycogen catabolism. Although *PCK1* mRNA was similar for both groups, indicative of similar enzyme activity (Greenfield et al., 2000), we found G0 and G1 cows were likely using different precursors for gluconeogenesis which was evidenced by *MMUT* and *PC* differential mRNA expression (**Figure 4A**). Indeed, *MMUT* expression, the point of entry of propionate to gluconeogenesis (Aschenbach et al., 2010), was greater for G0 than G1 cows during early lactation. Propionate is the primary precursor for gluconeogenesis in fed cows, however, when propionate from dietary origin is insufficient, other gluconeogenic precursors are used such as: lactate, glycerol, and amino acids (Kristensen, 2005; Larsen and Kristensen, 2013). These alternative precursors are converted to pyruvate, hence their point of entrance to the gluconeogenic pathway is commonly assumed to be via the conversion of pyruvate to oxaloacetate catalyzed by PC (Greenfield et al., 2000). Previous studies have demonstrated that mRNA abundance and enzyme activity of PC were strongly correlated, therefore gene expression of this enzyme is suggestive of enzyme activity (Greenfield et al., 2000). In line with this, we found grazing cows (G1 cows) had greater *PC* mRNA expression than cows fed TMR (G0 cows) during early lactation. Hence, our results suggest that G0 cows sustained glucose synthesis for lactose production during early lactation more on propionate as the precursor while G1 cows used more non-propionate precursors. In addition, since propionate is the most abundant precursor (Larsen and Kristensen, 2013), G0 cows had increased availability of substrates for gluconeogenesis and in turn produced more glucose which would probably account for their increased glycogen stores at 60 DIM.

During the latter part of early lactation to mid-lactation, insulin concentrations peaked only for TMR-fed cows (G0) consistent with the greater nutrient availability in the TMR diet (Meikle et al., 2013) and the reduced energy requirements for walking and grazing (Jasinsky et al., 2019). On the other hand, grazing and walking activities for pasture based cows could have an insulin sensitizing effect (Astessiano et al., 2015) as seen from the RQUICKI index which peaked at 60 DIM and remained higher during all the course of lactation only for these cows (G1).

Hepatic glycogen reserves were mobilized during late lactation (from 180 to 250 DIM) at the same time that expression of *PC* and *PCK1 mRNA* were high. Nevertheless, these changes would not probably be associated with a need for glucose for lactogenesis as energy corrected milk yield reached its lowest point at 250 DIM. Also, the decrease in plasma insulin concentration suggests

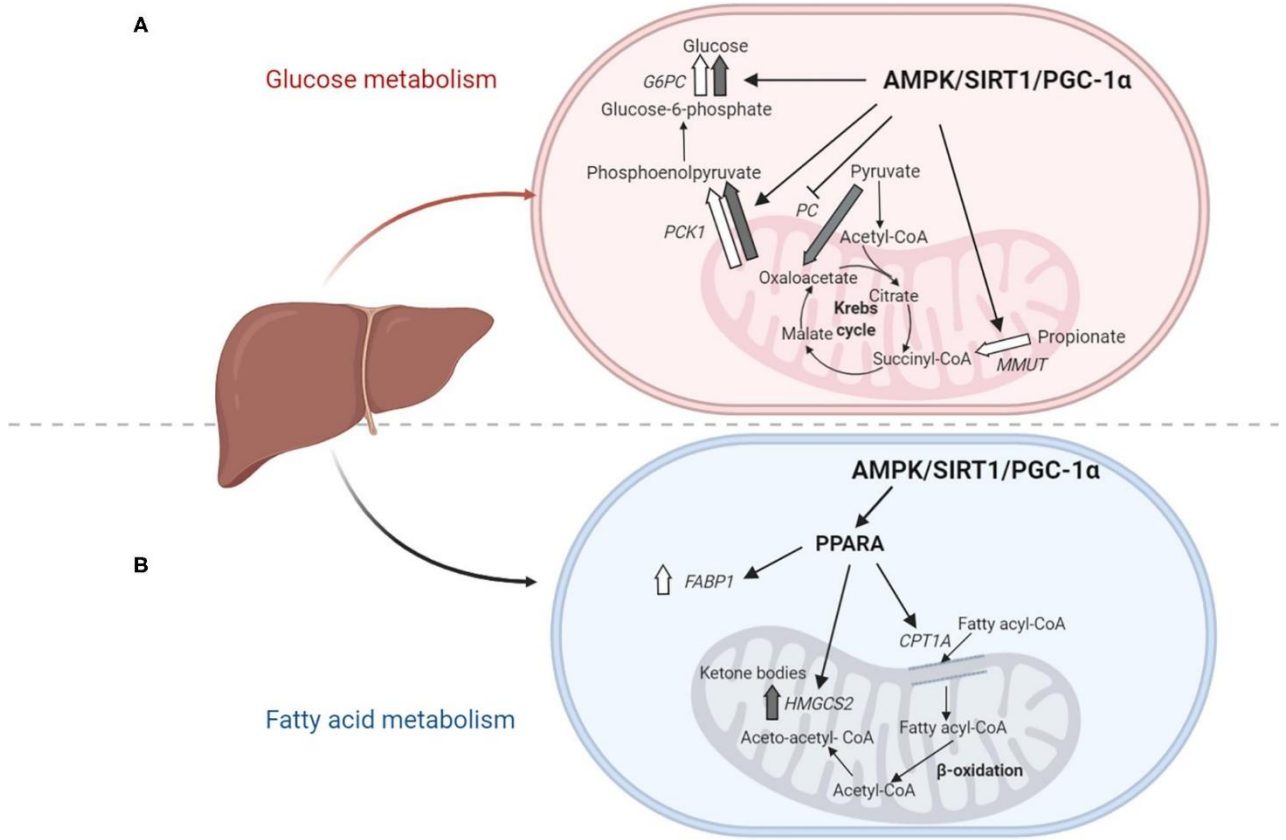


FIGURE 4 | (A) Plausible mechanism behind differential use of gluconeogenic precursors. During early lactation when milk yield is maximum, the expression of gluconeogenic gene *G6P* and *PCK1* is high in all the animals. However, G0 cows (white) increase *MMUT* expression and use propionate over pyruvate as gluconeogenic precursor; while G1 cows (gray) increase *PC* expression and use pyruvate over propionate as gluconeogenic precursor. Based on our results we hypothesize that AMPK, SIRT1, and PGC1-$\alpha$ induce the expression of gluconeogenic genes *G6P* and *PCK1* and *MMUT*, while suppressing *PC* expression, (B) Plausible mechanism behind metabolic adaptations of fatty acid metabolism in TMR vs. grazing cows. During early lactation, lipid reserves are mobilized and fatty acids reach the liver. In G0 cows (white) the expression of genes *CPT1A* and *FABP1* increases and probably accounts for an increase in fatty acid oxidation. As opposed to G1 cows (gray) where this metabolic adaptation does not occur and fatty acids accumulate as liver triglycerides. We hypothesize that AMPK, SIRT1, PGC1-$\alpha$ activate PPARA which in turn induces the expression of fatty acid oxidation genes *FABP1*, *HMGCS2,* and *CPT1A*, but differential regulation occurs in G0 and G1 cows that present different levels of hepatic BHB synthesis and triglycerides. In panels (A) and (B) metabolites are written in black, metabolic pathways in bold, genes in italics, master regulators in bold letters, and thick arrows represent higher transcription of genes.

a decrease in intake which could be due to less availability of dietary nutrients since both groups were on a pasture-based system from 180 DIM onwards and were subject to heat stress. Previous authors have suggested that metabolic adaptations related to heat stress are initiated due to decreased dry matter intake representing an inadequate plane of nutrition (Baumgard et al., 2011). Additionally, when heat stressed cows are under similar planes of nutrition as their thermal-neutral cohorts, heat stressed cows do not mobilize adipose tissue as much and increase their reliance on glucose as a fuel source in orderto remain euthermic (Wheelock et al., 2010). Lipid oxidation increases body temperature and thus heat stressed cows mobilize skeletal muscle to a greater extent than their thermal–neutral cohorts, hence yielding alanine and lactate as gluconeogenic precursors (Rhoads et al., 2011; Baumgard and Rhoads, 2012). In addition to this and consistent with our findings, previous reports have observed increased glycogenolysis and gluconeogenesis, in particular higher expression of PC (Rhoads et al., 2011).

Apart from insulin concentrations, we studied adiponectin and leptin, plasma concentrations of these hormones did not change throughout lactation but the increased mRNA expression of *LEPR* at 35 and 250 DIM could point out to higher tissue sensitivity during early and late lactation. Indeed, previous authors have observed that hypoinsulinemia could trigger hepatic leptin receptor expression (Thorn et al., 2008), this could be the case for both 35 and 250 DIM where insulin is decreased. Interestingly, leptin plasma concentrations tended to remain higher in G0 cows, probably due to increased energy balance and improved adipocyte size (Ehrhardt et al., 2000; Laubenthal et al., 2016).

Increased mobilization of lipid reserves reflected in increased plasma BHB and NEFA as well as liver triglyceride concentrations were observed during early lactation, especially in grazing cows (G1 cows). The trend to higher mRNA expression of *HMGCS2* in G1 cows together with higher plasma BHB and liver triglyceride concentrations indicated these cows may have mobilized more

lipid reserves than G0 cows in agreement with previous research (Astessiano et al., 2015). In addition, in early lactation the expression of *FABP1* and *CPT1A* was increased with respect to mid-lactation only for G0 cows, suggesting that G1 cows fail to adapt their metabolism to higher levels of fatty acid oxidation, and therefore accumulate triglycerides in the liver (**Figure 4B**). The FABP participates in intracellular fatty acid transport and facilitates fatty acid oxidation (Nuño-lámbarri et al., 2016). Previous authors, have found that *FABP1* is downregulated in cows with severe negative energy balance as well as early postpartum cows and propose that this may limit long chain fatty acid $\beta$-oxidation and result in the accumulation of hepatic triacylglycerols (Loor, 2010; McCarthy et al., 2010; Guzmán et al., 2013; Albrecht et al., 2019). Indeed, previous studies have shown that *FABP1* mRNA expression was downregulated in cows during early lactation or under physiological imbalance (Loor, 2010; Moyes et al., 2013). Additionally, in mice models, targeted deletion of FABP1 has shown to induce hepatic triglyceride accumulation (Nassir and Ibdah, 2014).

Finally, toward mid to late lactation, both groups had similar and low levels of lipid mobilization as plasma BHB, NEFA, and liver triglyceride reach pre-partum levels. However, *PPARA* and *CPT1A* expression increases from 110 to 250 DIM, probably unrelated to mobilization of body reserves.

Associated to early lactation negative energy balance, we previously found an impairment in mitochondrial function in early lactation in G1 vs. G0 cows (García-Roche et al., 2019), this could lead to an increase in AMP to ATP ratios promoting the phosphorylation and activation of the energy sensing AMPK (Jenkins et al., 2013; Hardie, 2018; Herzig and Shaw, 2018). Regarding adiponectin and leptin, that have also been reported as activators of the AMPK pathway (Kelly et al., 2006; Ohtani et al., 2012; Yamauchi et al., 2014; Gamberi et al., 2018), we observed that the plasma levels of these hormones did not differ during lactation; while the expression of the receptors *LEPR* and *ADIPOR1* differed or tended to differ, respectively, during lactation probably increased sensibility to these hormones. However, we did not observe a correlation between *ADIPOR1* or *LEPR* mRNA and pAMPK to AMPK.

The AMPK also phosphorylates and activates PGC1-$\alpha$. Alongside AMPK is SIRT1, that is activated by an AMPK-dependent increase in $NAD^+$ levels. The SIRT1 deacetylates PGC1-$\alpha$ and further stimulates the activity of this transcription factor coactivator (Cantó and Auwerx, 2009), the positive correlation between hepatic *SIRT1* and *PPARGC1A* mRNA in our study is suggestive of the interplay between these two molecules. Besides AMPK promotes the activation of transcription factor EB that directly binds the promoter and activates the transcription of the *PPARGC1A* gene (Herzig and Shaw, 2018). In agreement, both AMPK phosphorylation and hepatic expression of the *PPARGC1A* were higher ($P < 0.05$) at 35 than 250 DIM.

The transcriptional coactivator PGC-1$\alpha$ interacts with transcription factors, such as forkhead box protein O1, glucocorticoid receptor, and hepatocyte nuclear factor 4 alpha, promoting the transcription of gluconeogenic genes in the liver in response to several hormones (Herzig et al., 2001; Yoonet al., 2001; Puigserver et al., 2003; Rowe and Arany, 2014). In particular, the transcriptional coactivator PGC-1$\alpha$ regulates glucose metabolism inducing *G6PC* and *PCK1* transcription and increasing gluconeogenesis and glucose production from pyruvate (Herzig et al., 2001; Yoon et al., 2001; Puigserver et al., 2003; Rodgers et al., 2005; Rowe and Arany, 2014). Moreover, SIRT1 is required for the PGC-1$\alpha$ regulation of these gluconeogenic genes (Rodgers et al., 2005). In this sense, our results showed an increase in both *G6PC and PCK1* mRNA levels in early lactation with respect to mid and late lactation which positively correlate with *PPARGC1A* expression, suggesting that the increase in gluconeogenesis and glucose output in early lactation might be regulated by the AMPK/SIRT1/PGC-1$\alpha$ axis. Increases in the expression of *G6PC* and *PCK1* during early lactation have been reported by others (Aschenbach et al.,2010), our results contribute to elucidate the factors regulating gluconeogenesis in the dairy cow.

However, when assessing the first steps of gluconeogenesis, related to the point of entry of gluconeogenic precursors, we found that the pAMPK to AMPK ratio correlated negativelywith hepatic *PC* mRNA expression levels and especially during early lactation, pyruvate is formed from alternative gluconeogenic precursors, other than propionate (Aschenbach et al., 2010). Gluconeogenesis from alternative precursors is a costly adaptation for the metabolism of the dairy cow sinceit requires the mobilization of reserves instead of utilizing dietary propionate, hence we hypothesize that the AMPK pathway may be inhibiting *PC* mRNA expression in order to inhibit gluconeogenesis driven by non-propionate precursors (Winder et al., 2000; Zhou et al., 2001; Hardie et al., 2017). Three promoters drive the transcription of *PC* in bovine liver, in response to nutritional and environmental stimuli (Aschenbach et al., 2010). Putative binding sites for transcription factors nuclear factor-Y, and TATA binding protein (TBP) and Sp1/Sp3, have been identified (Aschenbach et al., 2010) in these promoters. Interestingly Sp1 is reported to undergo severalpost-translational modifications, including phosphorylation and acetylation (Chang and Hung, 2012). Besides, both AMPK activation and SIRT1 overexpression are reported to reduce Sp1 levels in mouse and human liver cells, respectively (Yie et al., 2014; Rada et al., 2018). Thus, Sp1 might link AMPK activation with *PC* expression, though this remains to be investigated. On the other hand, we found both *SIRT1* and *PPARGC1A* mRNA were positively associated with the hepatic expression of the gluconeogenic gene, *MMUT* suggesting that the SIRT1/PGC- 1$\alpha$ axis could be favoring gluconeogenesis from propionate in early lactation.

The AMPK also has an effect on lipid metabolism (Hardie, 2018; Herzig and Shaw, 2018). In this sense, we observed AMPK activation was increased during early lactation when levels of NEFA, BHB, and liver triglycerides were elevated compared to late lactation, this is in accordance with other authors that have found an increase in AMPK phosphorylation in bovine hepatocytes treated with increasing doses of BHB and acetic acid (Li et al., 2013; Deng et al., 2015). Also, AMPK has been reported to increase the expression of *PPARA* which in turn upregulates the expression of lipid oxidation genes *CPT1A, HMGCS2, FABP1* (Guzmán et al., 2013; Khan et al., 2014; Grabacka et al., 2016;

Li et al., 2020). In fact, we found a positive correlation between the pAMPK to AMPK ratio and mRNA *HMGCS2* expression. In addition to this, *PPARGC1A*, mRNA expression levels were also positively associated with the expression of *HMGCS2* mRNA. Hence AMPK could be using PGC-1$\alpha$ as its effector in order to alter the expression of fatty acid oxidation genes (Finck and Kelly, 2006; Cantó and Auwerx, 2009).

The coactivator PGC-1$\alpha$ interacts with PPARA and contributes to the transcriptional control of genes encoding fatty acid oxidation enzymes (Vega et al., 2000; Finck and Kelly, 2006; Gastaldi et al., 2007; Gerhart-Hines et al., 2007; Song et al., 2010) and indeed we found a positive association between these two factors. In the liver, PPARA is proposed to be activated by fatty acids to induce enzymes related to peroxisomal and mitochondrial fatty acid oxidation and ketogenesis (Schoonjans et al., 1996; Drackley, 1999). Our results are consistent with these findings given the positive association between hepatic mRNA expression levels of *PPARA* and *CPT1A* and *HMGCS2*. Previous studies in dairy cows have shown inconsistent results regarding *PPARA* mRNA expression levels, on one hand,*PPARA* mRNA remained unchanged when cows consumed more than 100% their energy requirements (Carriquiry et al., 2009; Khan et al., 2014). On the other hand, its expression decreased continuously after parturition (Han van der Kolk et al., 2017), which is consistent with our findings that *PPARA* was upregulated during 35 DIM and downregulated during 110 DIM, however its increased expression during 250 DIMis a novel result suggesting a metabolic response related toheat stress which may shift metabolism toward lipolysis and inhibition of insulin-mediated lipogenesis and glucose utilization (Baumgard and Rhoads, 2012).

Additionally, the negative correlation ($r = -0.50$ and $P < 0.01$) between hepatic expression of *SIRT1* studied in this work and protein acetylation in liver homogenates observed in García-Roche et al. (2019), suggests a possible regulatory effect of *SIRT1* on protein deacetylation (García-Roche et al., 2019). Deacetylation of PGC-1$\alpha$ mediated by SIRT1 has been shown to activate PPARA with the consequent transcription mitochondrial fatty acid oxidation genes (Purushotham et al., 2009). Indeed, in this work hepatic *SIRT1* and *CPT1A* expression positively correlated ($r = 0.41$ and $P < 0.01$), and althoughno correlation was confirmed between *PPARGC1A* and *CPT1A* mRNA expression levels, PGC-1$\alpha$ could have been activated by deacetylation and not increased expression, as aforementioned.

## CONCLUSION

In sum, this work elucidates plausible regulatory mechanisms for hepatic glucose and fatty acid metabolism during lactation in TMR vs. grazing dairy cows. Our results confirm the extreme energy demands and synchronized metabolic adaptations imposed by lactogenesis during early lactation, especially in grazing dairy cows.

In addition, we found an implication of AMPK, PGC-1$\alpha$, and SIRT1 in the regulation of the gluconeogenic pathway; as well as in a PPARA dependent regulation of fatty acid oxidation. Mechanistic information on whole lactation phenomena, presented in this study, could be useful to assertively manage dairy cattle nutrition.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

## ETHICS STATEMENT

The animal study was reviewed and approved by Animal use and procedures were approved by the Animal Experimentation Committee (CHEA) of the Universidad de la República, Uruguay (file number: 021130-001914-15).

## AUTHOR CONTRIBUTIONS

MG-R conceived and designed lab experiments, performed field and lab experiments, analyzed the results, and wrote the original draft and revised versions of the manuscript. MCa conceptualized and designed field experiment, conceived and designed lab experiments, supervised field and lab experiments and results analyses, obtained funding and administrated the project, and reviewed and edited the manuscript. CQ conceived, designed and supervised lab experiments, and reviewed and edited the manuscript. ACass conceived, designed, and supervised lab experiments. DM conceptualized and designed field experiment and obtained funding. ACasa and GC performed field and lab experiments. MCe and AJ participated in field experiments and sample collection. All authors reviewed the manuscript and approved the final version of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fanim.2021.622500/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5. IMPAIRED HEPATIC MITOCHONDRIAL FUNCTION DURING EARLY LACTATION IN DAIRY COWS: ASSOCIATION WITH PROTEIN LYSINE ACETYLATION

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Este trabajo tuvo como objetivo determinar la función mitocondrial a lo largo de lactancia y explorar los mecanismos moleculares asociados a las alteraciones observadas en vacas del modelo 1. Nuestros resultados mostraron que durante lactancia temprana (35 DEL) los niveles de marcadores de balance energético negativo (BEN), como triglicéridos en hígado y ácidos grasos no esterificados y beta-hidroxiburtirato en plasma, se vieron aumentados ($P < 0,01$). En las vacas G1 estos marcadores, se vieron especialmente aumentados, en particular los niveles de beta-hidroxibutirato ($P < 0,01$). A su vez, la función mitocondrial se vio disminuida durante el BEN de lactancia temprana, especialmente para el grupo G1 ($P < 0,05$); esta variable tuvo una correlación negativa con beta-hidroxibutirato en plasma y los triglicéridos en hígado ($r < -0,4$, $P < 0,05$). Asimismo, se observaron niveles de acetilación más elevados en las proteínas mitocondriales hepáticas durante el BEN de lactancia temprana en vacas G1 ($P < 0,05$), esta variable correlacionó negativamente con la función mitocondrial ($r = -0,6$, $P < 0,05$) y positivamente con beta-hidroxibutirato en plasma y los triglicéridos en hígado ($r = 0,5$, $P < 0,01$).

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RESEARCH ARTICLE

# Impaired hepatic mitochondrial function during early lactation in dairy cows: Association with protein lysine acetylation

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## Abstract

Early lactation is an energy-demanding period for dairy cows which may lead to negative energy balance, threatening animal health and consequently productivity. Herein we studied hepatic mitochondrial function in Holstein-Friesian multiparous dairy cows during lactation, under two different feeding strategies. During the first 180 days postpartum the cows were fed a total mixed ration (70% forage: 30% concentrate) *ad libitum* (non-grazing group, G0) or grazed *Festuca arundinacea* or *Mendicago sativa* plus supplementation (grazing group, G1). From 180 to 250 days postpartum, all cows grazed *Festuca arundinacea* and were supplemented with total mixed ration. Mitochondrial function was assessed measuring oxygen consumption rate in liver biopsies and revealed that maximum respiratory rate decreased significantly in grazing cows during early lactation, yet was unchanged in non-grazing cows during the lactation curve. While no differences could be found in mitochondrial content or oxidative stress markers, a significant increase in protein lysine acetylation was found in grazing cows during early lactation but not in cows from the non-grazing group. Mitochondrial acetylation positively correlated with liver triglycerides and β-hydroxybutyrate plasma levels, well-known markers of negative energy balance, while a negative correlation was found with the maximum respiratory rate and sirtuin 3 levels. To our knowledge this is the first report of mitochondrial function in liver biopsies of dairy cows during lactation. On the whole our results indicate that mitochondrial function is impaired during early lactation in grazing cows and that acetylation may account for changes in mitochondrial function in this period. Additionally, our results suggest that feeding total mixed ration during early lactation may be an efficient protective strategy.

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## Introduction

High yielding dairy cows are greatly challenged by the onset of lactation. Lactogenesis results in a dramatic increase in total energy requirements, and insufficient dry matter intake may lead to negative energy balance [1]. In addition to the physiological changes attributed to early lactation, the environment, particularly nutrition, is determinant in negative energy balance. Pasture-based systems are an economically advantageous alternative widely used in temperate regions [2]. However, pasture dry matter intake is highly dependent on cow physiology and behavior as well as sward characteristics and, in addition, may result in increased energy expenditure due to activity (grazing and walking)[2–4]. Previous studies have shown that limited pasture allowance may lead to higher mobilization of energy reserves, poor reproductive performance and limit the productive responses of dairy cows [5–8].

During early lactation, gut, liver, mammary gland and adipose tissue undergo adaptations to support lactation [9], in particular, a sharp increase in gluconeogenesis can be observed [10]. In order to meet energy demands and increase the availability of lactogenic precursors dairy cows mobilize body reserves [11,12], resulting in a decrease in body weight (BW) and body condition score (BCS). Excessive mobilization of adipose tissue triglycerides results in high levels of circulating non-esterified fatty acids (NEFA). The liver takes up NEFA [13]; that are either completely oxidized to carbon dioxide, partially oxidized to ketone bodies (an alternative fuel for non-hepatic tissues) or re-esterified into triglycerides and packaged into very low density lipoproteins for transport [14,15].

Imbalances in oxidation/re-esterification routes along with low synthesis and export rate of very low density lipoproteins can give place to hepatic steatosis also known as fatty liver [1,14,16], that can in turn progress towards steatohepatitis [17–19]. In humans and mice models with fatty liver decreased activity of the respiratory chain and β-oxidation enzymes, ultrastructure abnormalities, and increased mitochondrial reactive oxygen species (ROS) have been reported [18,20,21]. Although the molecular events behind mitochondrial impairment and ROS formation are not fully established, protein lysine acetylation appears as a relevant post-translational modification, capable of regulating both mitochondrial energy metabolism and redox status [22], and has been shown to increase in fatty liver of mice receiving a high-fat diet [20].

Although fatty liver syndrome is one of the most important metabolic diseases in high yielding dairy cows in early lactation [16,23], the pathogenesis of this disease is not thoroughly explored in ruminants; in particular the role of mitochondria has not been established. Pioneering studies assessing fatty acid oxidation in dairy cows showed that carnitine palmitoyltransferase I activity and β-oxidation were impaired in cows with hepatic steatosis [24,25]. Recently, Gao *et al*. [26] reported a decrease in expression and activity in several subunits of respiratory complexes and of relevant regulators of mitochondrial biogenesis and fusion, in cows with signs of steatohepatitis. However much remains to be explored, in particular a systematic functional analysis of mitochondrial electron transport and oxidative phosphorylation during the different stages of the lactation curve is lacking.

Furthermore, given that most of hepatic ATP is synthesized by oxidative phosphorylation [27] the study of mitochondrial function is essential to understand the adaptations of energy metabolism during lactation. The high-energy demands of gluconeogenesis faced by the liver during this crucial period underscore the relevance of assessing mitochondrial function, yet few reports can be found on this matter.

In this work we aimed to quantify hepatic mitochondrial oxygen consumption rate in liver biopsies by high-resolution respirometry, of dairy cows during early and late lactation (35 and 250 days post partum, respectively); and to explore molecular mechanisms affecting

respiration, focusing on mitochondrial content, lipoperoxidation and protein acetylation. Two different feeding strategies (TMR and pasture-based diet) were used the first 180 days of lactation to assess if hepatic energy metabolism, in this crucial period, is affected by diet.

## Materials and methods

### Ethics statement

The use of animals and all animal procedures were approved by the Animal Experimentation Committee (CHEA) of the Universidad de la República, Montevideo, Uruguay (file number: 021130-001914-15).

### Animals, feeding strategy and experimental design

Twenty-four multiparous Holstein-Friesian dairy cows calved in spring (664 ± 65 kg BW and 3.0 ± 0.4 units of BCS; 18/08/2015 ± 11 of calving date) grouped according to their due calving date, parity, BW and BCS were used in a randomized block design with two feeding strategies from calving to 180 ± 11 days postpartum (DPP): a non-grazing group (control group; G0) fed 100% of a total-mixed ration (TMR) *ad libitum* and a grazing group (G1) which grazed on pasture and received supplementation.

Cows in the non-grazing group (G0) (N = 12) were offered TMR once a day after the morning milking. The TMR had a forage to concentrate ratio of 70:30 (as fed basis) and was formulated according to NRC Dairy Model 2001 software [28] for a milk production target of 40 kg per day and 15–20% refusals. The TMR was composed by corn silage and moha (*Setaria italica*) hay or alfalfa haylage and a concentrate that included sorghum grain (22.6%), corn grain (6.8%), barley grain (4.4%) sunflower expeller (7.5%), soybean expeller (13.6%) and minerals and vitamins (1.7%). Cows were housed in a free stall facility (wood-frame barn) and wood shavings (> 10 cm) for bedding. Cows were allocated in three pens (8 x 22.6 m each; 4 cows per pen) and each pen had access to shade, water and a feeder (2.4 m high, 1.12 m wide in the top, 0.58 wide in the bottom and 0.50 m deep).

Cows in the grazing group (G1) (N = 12) grazed from 0 to 113 DPP a *Festuca arundinacea* pasture (2500 ± 490 kg DM per ha, 18 h of pasture access from 08:00 to 16:00 h and from 18:00 to 04:00 h) in a 7-d rotational system with a mean herbage allowance of 30 kg dry matter (DM) per cow per day (4 cm above ground level) and a chemical composition (DM basis) of 26.4% DM, 14.2% crude protein (CP), 54.7% neutral detergent fiber (NDF), 30.1% acid detergent fiber (ADF) and 1.58 Mcal per kg DM of net energy of lactation (NEL). In addition, after the morning milking, cows received, in individual feeders, 5.4 kg DM per cow per day of a concentrate containing corn grain (32%), barley grain (31%) and soybean expeller (32%) with a 87% DM, 16.8% CP, 28.5% NDF, 9.3% ADF and 1.83 Mcal per kg of NEL (DM basis). From 113 to 180 DPP they grazed a *Medicago sativa* pasture (1380 ± 328 kg DM per ha, 10 h of pasture access from 18:00 to 04:00 h) in a 7-d rotational system with a mean herbage allowance of 20 kg DM per cow per day (4 cm above ground level) and a chemical composition (DM basis) 26.4% DM, 23.3% CP, 30.1% NDF, 24.7% ADF and 1.68 Mcal per kg DM of NEL. During this period, after the morning milking, cows received, in the free stall facility, TMR (50% of offered TMR to G0 cows) composed by corn silage (23.3%), alfalfa haylage (19%), sorghum grain (20.8%), corn grain (11.8%), barley grain (11.5%), soybean expeller (11.8%) and minerals and vitamins (1.8%) with a chemical composition (DM basis) of 41.5% DM, 11.1% CP, 32.1% NDF, 22.0% ADF and 1.64 Mcal/kgDM of NEL. Diet change at 113 DPP was due to heat stress, since the temperature-humidity index exceeded the value of 72 for more than 5 consecutive hours and for 3 consecutive days [29].

After 180 DPP until the end of lactation, all cows (G0 and G1) grazed a *Festuca arundinacea* pasture (7-d rotational system; 11.5 h of pasture access from 16:30 to 04:00 h; with a herbage mass, above 4 cm of ground level, of 2340 ± 291 kg DM per ha and a herbage allowance of 20 kg DM per cow per day) with 28.3% DM, 10.1% CP, 56.6% NDF, 32.2% ADF and 1.48 Mcal per kg of NEL, DM basis) and were supplemented, after the morning milking, in the free stall facility, with TMR (50% of offered TMR to G0 cows at 180 DPP; 23.4% corn silage, 12.4% alfalfa hay, 28.3% sorghum grain, 11.5% corn grain, 11.1% barley grain, 11.5% soybean expeller and 1.8% minerals and vitamins) with 50% DM, 12.5% CP, 29.7% NDF, 18.8% ADF and 1.76 Mcal per kg of NEL (DM basis).

The proportion of pasture and TMR in the diet (DM basis) calculated for each treatment after the DM intake of TMR (based on difference between feed offered and refused) and pasture (based on NRC requirements) was determined. Diet was composed of 100% TMR from 0 to 180 DPP for G0 cows, and for G1 cows of 73.4% pasture and 26.6% concentrate from 0 to 113 DPP and 32.7% pasture and 67.3% TMR from 114 to 180 DPP; from 180 to 250 DPP diet was composed of 28% pasture and 72% TMR for all cows (G0 and G1). Nutrient composition of estimated diets is presented on Table 1.

Throughout the experiment, cows were milked twice a day and milk production was determined daily. Cow BCS (score 1 to 5)[30] and BW were recorded every two weeks.

## Liver tissue collection and blood samples

Liver biopsies were collected using a 14-gauge biopsy needle (Tru-Core-II Automatic Biopsy Instrument; Angiotech) after the local intramuscular administration of 3 mL of 2% lidocaine HCl, as described previously [31] at -14, 35, 60, 100, 180 and 250 DPP for oxygen consumption rate measurements. Two dates representative of early and late lactation (35 and 250 DPP, respectively) were taken into consideration for further molecular studies. Biopsies for oxygen consumption rate measurements were cryopreserved as described previously [32]. Biopsies for

**Table 1. Estimated nutrient composition of diets according to feeding strategy during lactation.**

| | G0[1] | G1 | | All cows |
|---|---|---|---|---|
| **Days postpartum** | **0 to 180** | **0 to 113** | **114 to 180** | **>180** |
| *Chemical composition*[2] | | | | |
| Dry matter, % | 43.1 | 42.4 | 36.5 | 43.9 |
| Crude protein, %DM | 12.9 | 14.9 | 15.1 | 11.8 |
| Neutral detergent fiber, %DM | 33.8 | 47.6 | 31.4 | 37.2 |
| Acid detergent fiber, %DM | 21.3 | 24.5 | 22.9 | 22.6 |
| Net energy of lactation, Mcal/kg DM[3] | 1.68 | 1.64 | 1.65 | 1.68 |
| Metabolizable protein, g/d[3] | 1854 | 1762 | 1914 | 1749 |

[1]Feeding strategies were a non-grazing group (control group; G0) fed 100% of a total-mixed ration (TMR) *ad libitum* and a grazing group (G1), which grazed on pasture and received supplementation.

[2]Diets were formulated to supply micronutrients according with requirements at all times of the lactation curve, thus they included a minerals and vitamin premix composed (% of DM or ppm, IU, g per animal) of 0.15% S, 19.31% Ca, 2.33% P, 2.98% Cl, 7.87% Na, 0.11% K, 3.59% Mg, 0.21 ppm Co, 5.7 ppm Cu, 12.9 ppm Fe, 8.8 ppm Mn, 0.08 ppm Se, 0.02 ppm Y, 18.7 Zn, 14.4 ppm chelated Zn, 5.04 ppm chelated Cu, 0.04 ppm chelated Se, 2,000.00 IU Vitamin A, 202.00 IU Vitamin D3, 2.10 IU Vitamin E, 12.16 ppm monensin, 0.56 g yeast, 0.28 g betaglucan, 0.28 g mannan-oligosaccharides.

[3]Net energy of lactation and metabolizable protein (MP) were estimated according to NRC (2001). Estimated MP balances indicates diets provided, at least 85% of MP requirement for both, G0 and G1, from 0 to 180 DPP and 95% of MP requirement after 180 DPP.

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western blot analysis and enzyme activity assays were immediately frozen in liquid nitrogen. All samples were stored at -80 ˚C until analysis. Although biopsies were taken from all cows, oxygen consumption measurements, mitochondrial isolation, Western Blots, triglycerides and activity measurements were performed for 8–10 cows of each treatment, due to tissue quantity.

Blood samples were collected at 35 and 250 DPP by venipuncture of the coccygeal vein using BD Vacutainer tubes with heparin (Becton Dickinson). Samples were centrifuged at 2000 g for 15 min at 4 ˚C within 1 hour after collection and plasma was stored at -20 ˚C until metabolite analyses were performed.

## Mitochondrial isolation

Mitochondria were isolated as described previously [33]. Liver tissue was homogenized in homogenization buffer (250 mM sucrose, 50 mM Tris-HCl, 5 mM $MgCl_2$) with protease inhibitors (SigmaFast Protease Inhibitor Cocktail and 1 mM phenylmethylsulfonyl fluoride) and deacetylase inhibitors, (1 μM trichostatin A and 5 mM nicotinamide, pH 7.4) using a Potter-Elvehjem homogeneizer set to 600–1000 rpm. Homogenates were centrifuged at 800 g for 15 minutes twice to remove large pieces of tissue and nuclei. Then mitochondria were isolated by centrifugation at 11,000 g for 10 min. The pellet containing mitochondria was washed thoroughly three times centrifuging at 11,000 g and finally resuspended in 50–100 μL of 50 mM Tris HCl, 1 mM EDTA, 0.5% Triton-X-100 with protease and deacetylase inhibitors, pH 6.8. Subcellular fractions enriched in mitochondria were stored at -80 ˚C until analyzed. All procedures were carried out in the cold (4 ˚C). The enrichment and purity of the mitochondrial fraction was verified by Western blot (S1 Fig).

## Mitochondrial oxygen consumption rate

Mitochondrial function was studied measuring oxygen consumption rate in a high-resolution respirometer OROBOROS Oxygraph—2k at 37 ˚C as described previously [32,34]. Electrodes were calibrated in modified MIR05 respiration medium (0.5 mM EGTA, 3mM $MgCl_2 \bullet 6H_2O$, 60 mM MOPS, 20 mM taurine, 10 mM $KH_2PO_4$, 20 mM HEPES, 110 mM sucrose, 1 $g.L^{-1}$ BSA, pH 7.1) with a calculated saturated oxygen concentration of 191 μM at 100 kPa barometric pressure at 37 ˚C [34]. Respiratory rates (pmol $O_2.min^{-1}.mL^{-1}$) were calculated using the DatLab 4 analysis software. Liver biopsies (2–10 mg) were weighed, added to the chamber and oxygen consumption measurements were obtained before and after the sequential addition of specific substrates of the respiratory chain, 10 mM glutamate plus 5 mM malate (complex I) or 20 mM succinate (complex II), followed by 4 mM adenosine diphosphate (ADP), 2 μM oligomycin (ATP synthase inhibitor), 2–4 μM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, an uncoupler of oxidative phosphorylation). Maximum uncoupling was obtained titrating FCCP concentrations used in the assay. Finally, respiration was inhibited with 0.5 μM rotenone (complex I inhibitor) or 2.5 μM antimycin A (complex III inhibitor).

All respiratory parameters and indices were obtained as described in [27,32]. Briefly, the non-mitochondrial oxygen consumption rate was determined after adding antimycin A or rotenone and subtracted from all other values before calculating the respiratory parameters. State 4 respiration was the baseline measurement obtained with substrates before the addition of ADP and state 3 respiration was determined after addition of ADP. Oligomycin-resistant respiration (ATP-independent) was measured after oligomycin injection and oligomycin-sensitive respiration (ATP-dependent) was calculated as the difference between state 3 and oligomycin-resistant respiration. Maximum respiratory capacity was determined after the addition of FCCP.

## Citrate synthase activity

Citrate synthase is a constitutive mitochondrial enzyme frequently used as a marker for mitochondrial content [35]. To determine its activity, liver tissue (100 mg) was homogenized using a Potter-Elvehjem homogenizer in 10 volumes of homogenization buffer (5 mM $KH_2PO_4$, 1 mM EGTA, 5 mM MOPS, 300 mM sucrose at pH 7.1). Enzyme activity was measured in homogenates following the formation of 5-thio-2-nitrobenzoic acid at λ = 412 nm ($\varepsilon_{412}$ = 13,700 $M^{-1}.cm^{-1}$) in the presence of 20 mM Tris-HCl pH 8, 300 μM acetyl-CoA, 500 μM oxaloacetate, 100 μM 5,5'-dithio-bis (2-nitrobenzoic acid), and 60 $\mu g.mL^{-1}$ of liver protein [35]. Specific activity was calculated after determining the protein concentration of the samples with the Bradford assay using bovine serum albumin as standard [36].

## Western blots

Liver tissue (10–20 mg) was disrupted using a Potter-Elvehjem homogenizer in 10 volumes of cold lysis buffer (150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 0.1% SDS with protease and deacetylase inhibitors).

After homogenization, samples were placed on a rotator at 4 ˚C for 1 hour. Samples for western blots with antibodies against mitochondrial respiratory chain subunits were spun at 12,000 g for 10 min at 4 ˚C and the supernatants containing soluble proteins were stored. The last step was avoided when preparing samples for western blots with antibodies against acetyl lysine (AcK) and 4-hydroxynonenal (4-HNE). Protein content was determined with the Bradford assay using bovine serum albumin as standard [36] and samples were kept at -80 ˚C until analyzed.

Liver homogenates (30–40 μg) and subcellular fractions enriched in mitochondria (20 μg) were resolved in 10 to 12% Tris-Glycine-SDS polyacrylamide gels (SDS/PAGE), along with protein ladders (LI-COR Biosciences 928–60000 or Thermo Fisher Scientific 26616), and proteins were transferred overnight to nitrocellulose membranes. Membranes were blocked with blocking buffer (Tris buffered saline with 0.1% Tween 20 and 0.5% skimmed milk) and incubated overnight at 4 ˚C with primary antibodies against: GAPDH (1:1000, Abcam, ab9484), β-actin (1:1000, Santa Cruz, sc-81178), α-tubulin (1:1000, Santa Cruz, sc-8035), succinate dehydrogenase subunit A (SDHA, 1:2000, Abcam, ab14715), α subunit of ATP synthase (ATP5A, 1:1000, Abcam, ab14748), acetylated lysine (1:1000, Cell Signaling Technology, 9441), protein-4-HNE adducts (1:1000, Abcam, ab46544), sirtuin 3 (1:1000, Cell Signaling Technology, 5490), sirtuin 5 (1:1000, Cell Signaling Technology, 8782), 3-nitrotyrosine (1:1000, a kind gift from Dr. Rafael Radi, CEINBIO, Departamento de Bioqu´ımica, Facultad de Medicina, Universidad de la República, Uruguay) and histone H3 (1:1000, Cell Signaling Technology, 4620). Membranes were washed and probed with secondary antibodies from LI-COR Biosciences: anti-mouse (1:10,000, IRDye 680, 926–68070), anti-rabbit (1:20,000, IRDye 800, 926–32211) or anti-goat (1:20,000, IRDye 800, 925–32214). Immunoreactive proteins were detected with an infrared fluorescence detection system (Odyssey, LI-COR Biosciences) and bands were quantified by densitometry with ImageStudio software (LI-COR Biosciences).

## Hepatic triglycerides

Lipids were extracted from liver homogenates (6 $mg.mL^{-1}$), in hexane/isopropanol/1 M acetic acid (30:20:2, v/v/v), in a 1:2.5 sample to solvent ratio. After vortexing for 30 seconds, 2.5 volumes of hexane were added, vortexed and the mixture was centrifuged at 1800 g for 5 min at 4 ˚C and the organic phase, containing the lipids, was separated from the aqueous phase. Hexane was added to the aqueous phase and centrifuged at 1800 g for 5 min at 4 ˚C to increase lipid recovery. Before extraction 1-dodecanol (Sigma, 75544) was added to the samples and used as

an internal standard for normalization in semi-quantification analyses. Finally hexane phases were pooled and subjected to solvent evaporation under vacuum in a RapidVap Vacuum Evaporation System (Labconco) [37].

Lipid extracts were dissolved in chloroform and spotted manually on thin layer chromatography plates using a microsyringe (Hamilton) along with the internal standard and a triglyceride standard. The triglyceride standard was an olive oil sample containing more than 98% triglycerides, characterized at the Instituto Nacional de Investigación Agropecuaria (INIA), Uruguay, by gas chromatography under the reference of the Intenational Oil Council (Norma COI/T.20/Doc. n˚ 24 2001). Lipids were separated using hexane/diethyl-ether/acetic acid (80:20:1, v/v/v) as mobile phase [38] and lipid bands visualized after spraying with 5% sulphuric acid (v/v) in ethanol and heating. Densitometry quantification analysis of the bands was performed using ImageJ software.

## Plasma biochemical assays

Plasma β-hydroxybutyrate concentrations were determined with a kit from Randox Laboratories Ltd. following manufacturer instructions. The assay measures NADH formation spectrophotometrically at 340 nm during β-hydroxybutyrate dehydrogenase catalyzed oxidation of β-hydroxybutyrate to acetoacetate [39].

Plasma NEFA concentrations were determined spectrophotometrically with a kit from FUJIFILM Wako Diagnostics, following manufacturer instructions. In this method, NEFA incubated with acyl-CoA synthetase and ATP yield acyl-CoA. Acyl-CoA is oxidized in a reaction catalyzed by acyl-CoA oxidase producing hydrogen peroxide, which in the presence of peroxidase forms a purple colored end-product with an absorption maximum at 550 nm [40].

Plasma aspartate aminotransferase (AST) catalytic activity was determined with a kit from Biosystems, following manufacturer instructions. Aspartate aminotransferase catalyzes the transference of an amino group from aspartate to 2-oxoglutarate, forming oxaloacetate and glutamate. The assay measures the decrease of NADH spectrophotometrically at 340 nm in the malate dehydrogenase coupled reaction [41].

All plasma biochemical assays were performed using a Vitalab Selectra 2 autoanalyzer (Vital Scientific).

## Statistical analyses

Data were analyzed in a randomized block design using the SAS System program (SAS Academic Edition; SAS Institute Inc., Cary, NC, USA). Univariate and linear regression analyses were performed with all variables to identify outliers and inconsistencies and to verify normality of residuals. Outliers were removed when the residual had a Studentized residual $< -4$ or $> 4$. In the case of β-hydroxybutyrate and AST natural logarithmic transformations were performed and back transformed values were used to calculate means, standard errors and graph data. Data were analyzed as repeated measures using the MIXED procedure, the model included treatment, DPP and their interaction as fixed effects, block and cow as random effects and calving date, initial BW and BCS as covariates when $P < 0.20$. Tukey-Kramer tests were conducted to analyze differences between groups. BCS was analyzed using the GENMOD procedure, the model included treatment, DPP and their interaction as fixed effects. Means were considered to differ when $P < 0.05$ and indicated with different letters in tables or asterisks in graphs ($^{\square}P < 0.05$, $^{\square\square}P < 0.01$, $^{\square\square\square}P < 0.001$ and $^{\square\square\square\square}P < 0.0001$) and trends were identified when $0.05 < P < 0.10$. Correlation analyses between variables were performed using the CORR procedure.

# Results

## Productive and metabolic parameters

Since our aim was to compare hepatic mitochondrial function during early and late lactation we verified the productive parameters of the animals at this two dates of the lactation curve. No interaction between DPP and feeding strategy was found for milk yield or BCS (Table 2). As expected milk production was higher ($P < 0.001$) while BCS was lower ($P < 0.001$) at 35 than at 250 DPP, but no differences were observed between feeding strategies and (Table 2).

To assess the general metabolic status of the cows, under different diets, during lactation we measured liver triglycerides, concentrations of β-hydroxybutyrate and NEFA in plasma (Fig 1) and AST activity in plasma.

The interaction of DPP and treatment was significant for plasma β-hydroxybutyrate and for liver triglyceride (DPP X Treat: $P< 0.05$), as average concentrations were two-fold higher during early lactation for G1 versus G0 cows (Fig 1A and 1B), while remaining unchanged by diet in late lactation. These observations suggest that feeding strategy impacts fatty acid metabolism in lactation. No significant interactions were found between DPP and treatment for plasma NEFA (Fig 1C), but values were higher during early than late lactation ($P < 0.05$). Additionally, the correlation coefficient between β-hydroxybutyrate and NEFA was positive and significant and tended to be significant between triglyceride and β-hydroxybutyrate (Fig 1D and 1E), while the correlation between liver triglyceride and NEFA was not significant.

We then measured AST activity in plasma to assess liver damage. No interaction between treatment and dates was found, neither significant differences between groups or DPP (50 ± 6 $U.L^{-1}$ in the G0 group versus 63 ± 6 $U.L^{-1}$ in the G1 group at 35 DPP; and 68 ± 6 $U.L^{-1}$ in the G0 group versus 60 ± 6 in the G1 group at 250 DPP; N = 12).

Overall our results indicate that average values of β-hydroxybutyrate and NEFA of the cows in our study were below pathological threshold (<1.2 $mmol.L^{-1}$ and <1 $mmol.L^{-1}$ respectively) [42]. Nevertheless, these markers of negative energy balance were higher during early than late lactation [42], in particular in the G1 group. Liver triglycerides indicated that in average cows had moderate fatty liver (triglyceride 5–10% of wet weight [16,42,43]) during early lactation, and mild fatty liver in late lactation (triglyceride 1–5% of wet weight [16,42,43]). Average values of AST activity were below the cut off value (<110 $U.L^{-1}$) for both groups during both lactation moments [43].

**Table 2. Productive parameters.**

| | Treat | DPP | | P-value | | |
|---|---|---|---|---|---|---|
| | | 35 | 250 | DPP | Treat | DPP x Treat |
| Milk yield (kg/d) | G0 | 35.8 ± 0.8[a] | 19.2 ± 0.8[b] | < 0.001 | 0.34 | 0.59 |
| | G1 | 36.2 ± 0.8[a] | 20.3 ± 0.8[b] | | | |
| BCS (units) | G0 | 2.44 ± 0.05[b] | 2.71 ± 0.05[a] | < 0.001 | 1.00 | 0.77 |
| | G1 | 2.46 ± 0.05[b] | 2.69 ± 0.05[a] | | | |

Average weekly milk yield and body condition score (BCS) were determined at 35 and 250 days postpartum (DPP). Milk yield and body condition score (BCS) were determined at 35 and 250 days postpartum (DPP) in cows under two different feeding strategies or treatments (Treat), G1 and G0. All data is shown as least square means ± standard error (N = 12).

[ab] Different letters denote differences between rows and columns ($P < 0.05$) according to Tukey-Kramer test. G0: Cows were fed TMR *ad libitum* from calving to 180 DPP G1: Cows grazed *Festuca arundinacea* plus a commercial concentrate or *Medicago* supplemented with TMR (50% of G0 offer), depending on heat stress conditions, from calving to 180 DPP. From 180 to 250 DPP both groups grazed *Medicago sativa* and were supplemented with TMR (50% of G0 offer at 180 DPP).

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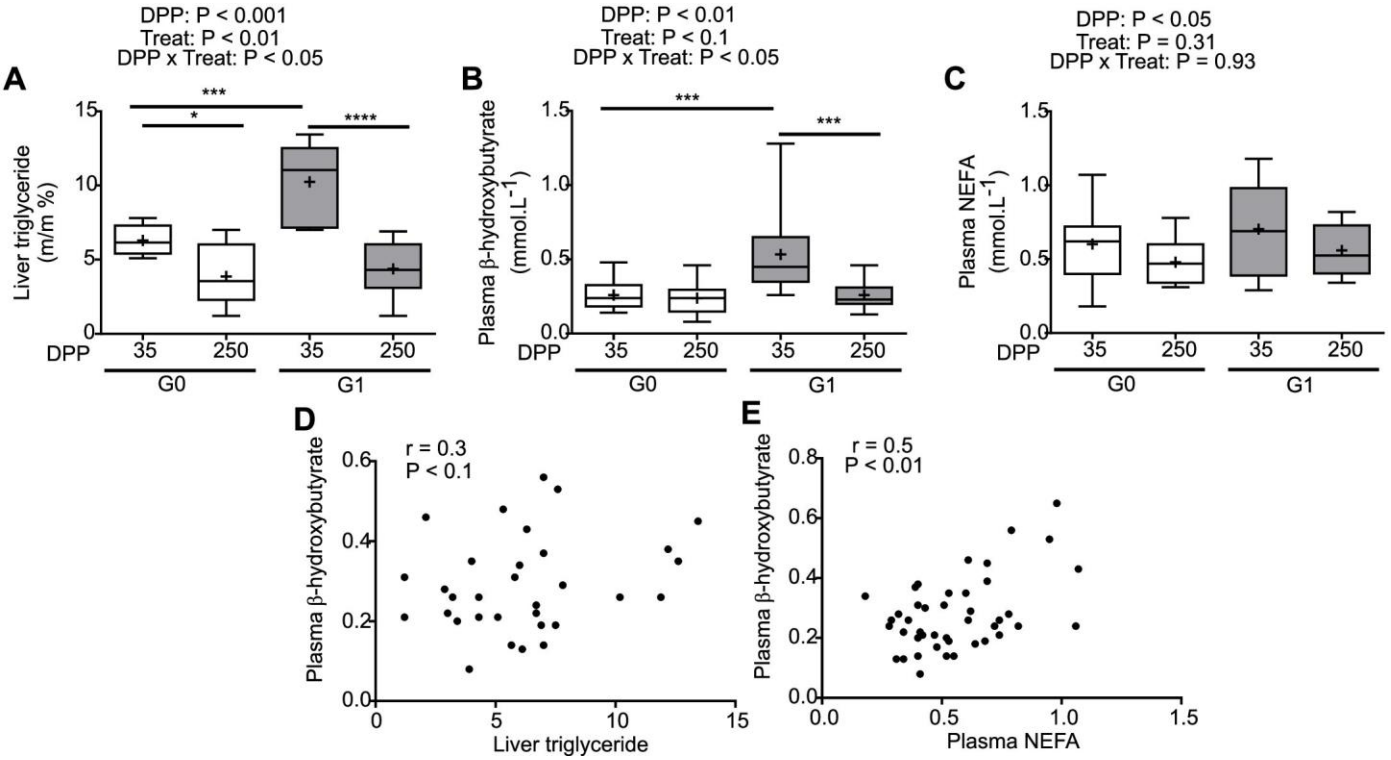


**Fig 1. Metabolic parameters during lactation.** Liver biopsies and blood samples were obtained at 35 and 250 DPP from cows in the G0 (white) and G1 (grey) groups. Graphs show the concentrations of **(A)** liver triglyceride, **(B)** plasma β-hydroxybutyrate and **(C)** plasma NEFA. Results are shown with box plots, the box extends from the 25th to 75th percentile, the line in the middle of the box is the median, the cross is the mean and the whiskers represent the minimum and maximum values (N = 8–12), * P < 0.05, *** P <0.001, **** P <0.0001. In graphs (**D**) and (**E**) the correlations between β-hydroxybutyrate, triglycerides and NEFA are shown (N = 8–12). G0: Cows were fed TMR *ad libitum* from calving to 180 DPP. G1: Cows grazed *Festuca arundinacea* plus a commercial concentrate or *Medicago* supplemented with TMR (50% of G0 offer), depending on heat stress conditions, from calving to 180 DPP. From 180 to 250 DPP both groups grazed *Medicago sativa* and were supplemented with TMR (50% of G0 offer at 180 DPP).

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## Mitochondrial function

Since fatty acid catabolism occurs in mitochondria in strict coordination with energy demands and relies heavily on mitochondrial function [44], respiratory analyses were carried out in liver biopsies, to assess electron transport chain activity and oxidative phosphorylation. Oxygen consumption rates were measured after addition of substrates of the respiratory chain, ADP, inhibitors and an uncoupler of oxidative phosphorylation (Fig 2 and Tables 3 and 4).

While complex I dependent respiration remained unchanged in G0 cows between the different DPP (Fig 2A), it was considerably lower in early lactation than in late lactation for the G1 group (Fig 2B). Significant interactions ($P < 0.05$) between DPP and treatment were found for complex I respiratory parameters related to respiratory chain activity and ATP synthesis. State 3, maximum and oligomycin sensitive respiration decreased in hepatic biopsies from G1 cows during early lactation when compared with late lactation, while remaining unchanged for G0 at the different dates (Table 3 and Fig 2C). Assessment of the maximum respiratory rate at different moments during the lactation curve (Fig 2D) revealed that this parameter had similar values from -14 to 180 DPP, becoming significantly higher at 250 DPP; and that significant differences between treatments could be detected only at 35 DPP. Maximum respiratory rate correlated negatively with liver triglyceride (Fig 2G) and β-hydroxybutyrate (Fig 2H), suggesting that decreased mitochondrial function is linked to liver steatosis and ketone body synthesis in dairy cows.

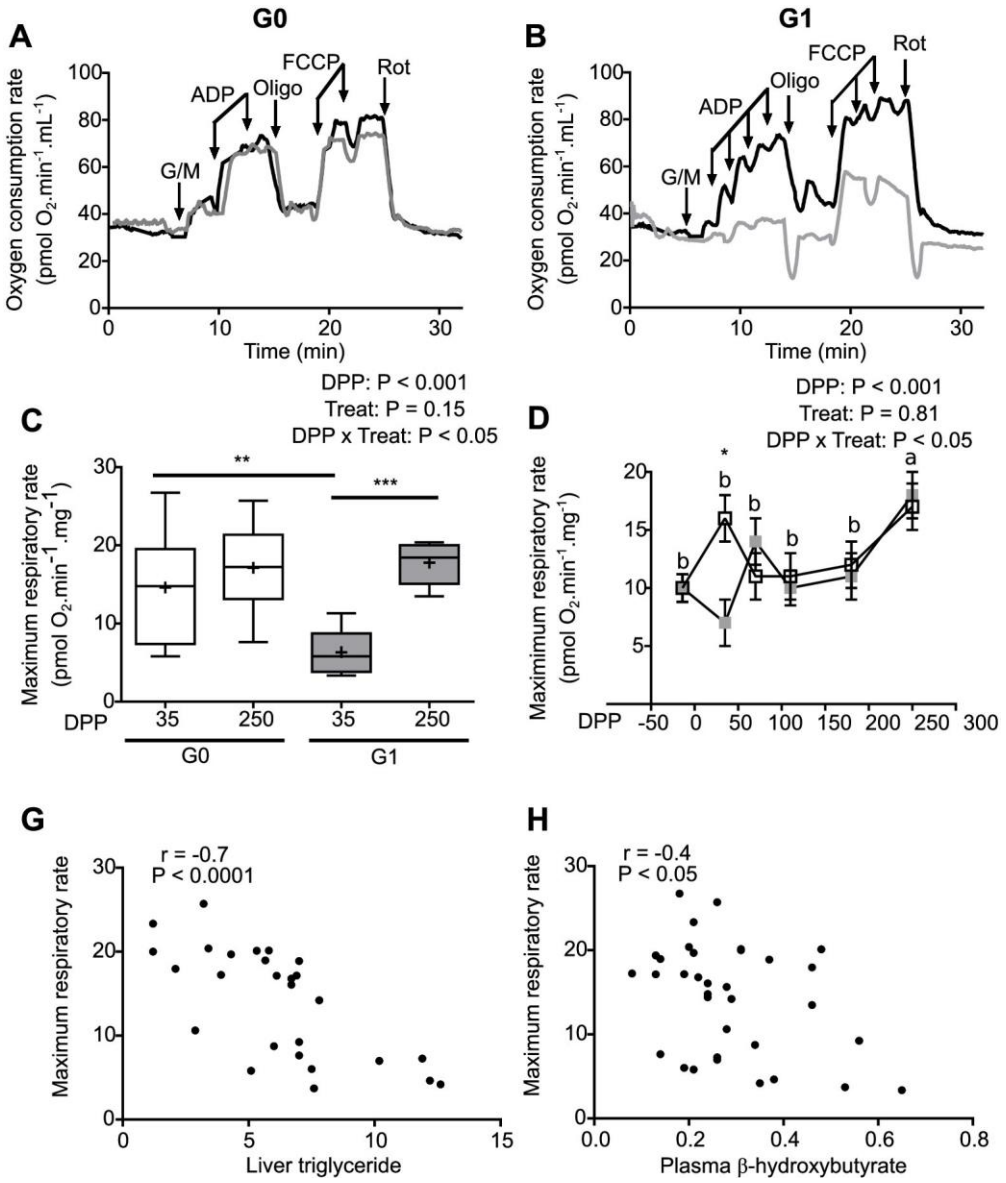


**Fig 2. Mitochondrial function decreases in pasture-fed dairy cows during early lactation.** Oxygen consumption rates were measured in liver biopsies before and after the sequential addition of 10 mM glutamate and 5 mM malate (Glu/Mal), 4 μM ADP, 2 μM oligomycin (Oligo), up to 4 μM FCCP and 0.5 μM rotenone (Rot). **(A and B)** Show representative traces of oxygen consumption rates obtained for liver biopsies of cows in the G0 group **(A)** and G1 group **(B)** at 35 DPP (grey) and 250 DPP (black). **(C)** Maximum respiratory rate, obtained from oxygen consumption rate measurements performed as described in A and B, of liver biopsies from cows in the G0 (white) and G1 (grey) groups. The box extends from the 25th to 75th percentile, the line in the middle of the box is the median, the cross is the mean and the whiskers represent the minimum and maximum values (N = 9–10), ** $P < 0.01$, and *** $P < 0.001$. **(D)** Maximum respiratory rate of liver biopsies obtained at different points during the lactation curve for both G0 (empty squares) and G1 (grey squares) cows. Data represent least square means ± SEM (N = 9–10). Different letters denote differences between dates ($P < 0.05$) and * denotes a difference between treatments ($P < 0.05$) according to Tukey-Kramer test. **(G)** and **(H)** show the correlation between maximum respiratory rate and liver triglyceride and plasma β-hydroxybutyrate, respectively (N = 8–12). G0: Cows were fed TMR *ad libitum* from calving to 180 DPP. G1: Cows grazed *Festuca arundinacea* plus a commercial concentrate or *Medicago* supplemented with TMR (50% of G0 offer), depending on heat stress conditions, from calving to 180 DPP. From 180 to 250 DPP both groups grazed *Medicago sativa* and were supplemented with TMR (50% of G0 offer at 180 DPP).

**Table 3. Complex I-dependent respiratory parameters.**

| Respiratory parameters | Treat | DPP | | P-value | | |
|---|---|---|---|---|---|---|
| | | 35 | 250 | DPP | Treat | DPP x Treat |
| State 3 | G0 | 12 ± 2$^{ab}$ | 15 ± 2$^{a}$ | < 0.001 | 0.34 | < 0.05 |
| | G1 | 7 ± 2$^{b}$ | 16 ± 2$^{a}$ | | | |
| State 4 | G0 | 4 ± 1$^{c}$ | 6 ± 1$^{b}$ | < 0.001 | 0.21 | < 0.05 |
| | G1 | 3 ± 1$^{c}$ | 9 ± 1$^{a}$ | | | |
| Maximum | G0 | 15 ± 2$^{a}$ | 17 ± 2$^{a}$ | < 0.001 | 0.15 | <0.05 |
| | G1 | 8 ± 2$^{b}$ | 18 ± 2$^{a}$ | | | |
| Oligomycin-resistant | G0 | 3 ± 1$^{c}$ | 8 ± 1$^{b}$ | < 0.001 | 0.22 | 0.06 |
| | G1 | 3 ± 1$^{c}$ | 11 ± 1$^{a}$ | | | |
| Oligomycin-sensitive | G0 | 7 ± 1$^{a}$ | 7 ± 1$^{a}$ | 0.09 | 0.17 | <0.05 |
| | G1 | 3 ± 1$^{b}$ | 7 ± 1$^{a}$ | | | |
| Non-mitochondrial | G0 | 8 ± 0.8$^{a}$ | 6 ± 0.8$^{b}$ | < 0.01 | 0.66 | 0.95 |
| | G1 | 8 ± 0.8$^{a}$ | 6 ± 0.8$^{b}$ | | | |

Respiratory parameters were determined at 35 and 250 DPP in biopsies from cows under two different feeding strategies or treatments (Treat), G1 and G0. Oxygen consumption rates were measured after the sequential addition of 10 mM glutamate and 5 mM malate, 4 μM ADP, 2 μM oligomycin, up to 4 μM FCCP and 0.5 μM rotenone (as shown in Fig 2). Respiratory parameters were calculated as described in Materials and Methods. All data is shown as least square means ± standard error (N = 8–10). Oxygen consumption rates are expressed as pmol $O_2$.min$^{-1}$.mg wet weight$^{-1}$.

$^{abc}$ Different letters denote differences between rows and columns ($P < 0.05$) according to Tukey-Kramer test. G0: Cows were fed TMR *ad libitum* from calving to 180 DPP. G1: Cows grazed *Festuca arundinacea* plus a commercial concentrate or *Medicago* supplemented with TMR (50% of G0 offer), depending on heat stress conditions, from calving to 180 DPP. From 180 to 250 DPP both groups grazed *Medicago sativa* and were supplemented with TMR (50% of G0 offer at 180 DPP).

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Interestingly, the interaction between dates and treatments for state 4 was significant ($P < 0.05$) and presented a trend for oligomycin-resistant respiration ($P = 0.06$) (Table 3). These parameters are associated with events that dissipate the mitochondrial membrane potential (e.g. ion transport across the inner mitochondrial membrane) but not with ATP synthesis. Non-mitochondrial oxygen consumption was increased in early lactation with respect to late lactation ($P < 0.01$) (Table 3); while no differences were detected between treatments (Table 3).

No interaction between DPP and treatment was found for respiratory parameters obtained with complex II substrates were used (Table 4). However, as observed for complex I, maximum respiratory rate was lower ($P < 0.05$) while non-mitochondrial oxygen consumption was higher ($P < 0.001$) at 35 DPP than 250 DPP (Table 4).

## Oxidative stress markers

Since non-mitochondrial oxygen consumption has been associated with reactive oxygen species (ROS) formation [45,46], we looked for oxidative modifications in the tissue. We analyzed the levels of 4-HNE-protein adducts, a product of lipid peroxidation [47], both in homogenates and subcellular fractions enriched in mitochondria and no significant interaction between DPP and treatment were found; nor were differences between dates or treatments detected (Fig 3). Levels of 4-HNE-protein adduct did not correlate with maximum respiratory capacity, however, the correlation between 4HNE-protein adducts and non-mitochondrial oxygen consumption rate was positive and significant ($r = 0.4$, $P < 0.05$).

We also tried to measure 3-nitrotyrosine levels, a marker of oxidative events involving nitric oxide derived radicals and oxidant species [48]; but protein tyrosine nitration could not be

**Table 4. Complex II-dependent respiratory parameters.**

| Respiratory parameters | Treat | DPP | | P-value | | |
|---|---|---|---|---|---|---|
| | | 35 | 250 | DPP | Treat | DPP x Treat |
| State 3 | G0 | 43 ± 6 | 56 ± 6 | 0.08 | 0.76 | 0.79 |
| | G1 | 43 ± 6 | 52 ± 6 | | | |
| State 4 | G0 | 28 ± 4 | 37 ± 4 | 0.08 | 0.97 | 0.83 |
| | G1 | 30 ± 4 | 36 ± 4 | | | |
| Maximum | G0 | 57 ± 9$^{b}$ | 77 ± 8$^{a}$ | < 0.05 | 0.95 | 0.96 |
| | G1 | 58 ± 9$^{b}$ | 77 ± 8$^{a}$ | | | |
| Oligomycin-resistant | G0 | 34 ± 5 | 42 ± 4 | 0.09 | 0.85 | 0.74 |
| | G1 | 33 ± 5 | 42 ± 4 | | | |
| Oligomycin-sensitive | G0 | 10 ± 2 | 14 ± 2 | 0.09 | 0.23 | 0.87 |
| | G1 | 7 ± 2 | 10 ± 2 | | | |
| Non-mitochondrial | G0 | 8 ± 1$^{a}$ | 4 ± 1$^{b}$ | < 0.001 | 0.43 | 0.78 |
| | G1 | 8 ± 1$^{a}$ | 4 ± 1$^{b}$ | | | |

Respiratory parameters were determined at 35 and 250 DPP in biopsies from cows under two different feeding strategies or treatments (Treat), G1 and G0. Oxygen consumption rate measurements of liver biopsies were obtained after addition of 20 mM succinate, 4 μM ADP, 2 μM oligomycin, up to 4 μM FCCP and 2.5 μM antimycin. Respiratory parameters were calculated as described in Materials and Methods. All data is shown as least square means ± standard error (N = 8–10). Oxygen consumption rates are expressed as pmol $O_2$.min$^{-1}$.mg wet weight$^{-1}$.

$^{ab}$Different letters denote differences between rows and columns ($P < 0.05$) according to Tukey-Kramer test. G0: Cows were fed TMR *ad libitum* from calving to 180 DPP. G1: Cows grazed *Festuca arundinacea* plus a commercial concentrate or *Medicago* supplemented with TMR (50% of G0 offer), depending on heat stress conditions, from calving to 180 DPP. From 180 to 250 DPP both groups grazed *Medicago sativa* and were supplemented with TMR (50% of G0 offer at 180 DPP).

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identified in the tissue (S2 Fig). Controls were performed exposing liver homogenates to the strong oxidizing and nitrating agent peroxynitrite (S2 Fig).

## Mitochondrial content

Since changes in mitochondrial content could be accountable for changes in mitochondrial respiration rates, levels of mitochondrial proteins were assessed (i.e. ATP5A, SDHA) and citrate synthase activity was measured in whole tissue homogenates. No interaction was found between DPP and treatments and there were no significant differences in the levels of these mitochondrial proteins or in citrate synthase activity, between dates or between treatments either (S3 Fig). Correlations between these three markers and maximum respiratory rate were not significant.

## Protein lysine acetylation

Since neither an increase in oxidative stress nor changes in mitochondrial content could explain the decay in mitochondrial respiration we looked into protein lysine acetylation, since it has been recently described as a key regulator of energy metabolism [49–51]. Evaluation of AcK levels in isolated mitochondria (Fig 4) showed the existence of a significant interaction of DPP and treatment ($P < 0.01$). Protein acetylation was higher during early than late lactation in G1 (70% increase approximately), while remaining unchanged in G0 cows during lactation (Fig 4B). Mitochondrial AcK levels displayed a significant and positive correlation with liver triglycerides (Fig 4C) and with β-hydroxybutyrate (Fig 4D), in agreement with previous reports on regulation of liver lipid metabolism by acetylation [49]. In addition mitochondrial AcK levels and maximum respiratory rate presented a negative correlation (Fig 4E) suggesting

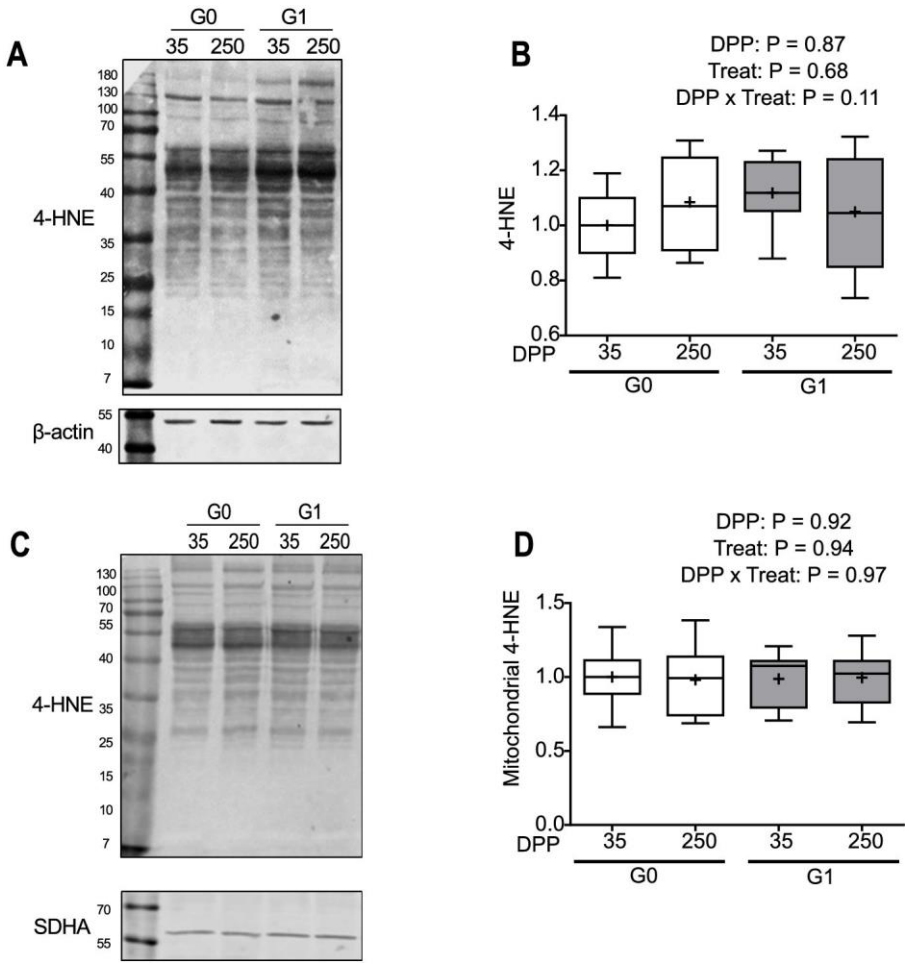


**Fig 3. Evaluation of 4-HNE-protein adducts formation in liver homogenates and mitochondria. (A and C)** Representative western blots of 4-HNE-protein adducts in liver homogenates **(A)** and isolated mitochondria **(C)** from cows in the G0 and G1 groups at 35 and 250 DPP; β-actin and SDHA were used as loading controls, respectively. **(B and D)** Quantification by densitometry of 4-HNE-protein adduct levels normalized by protein levels of loading control and expressed in relation to the average value of the G0 group at 35 DPP. In box plots the box extends from the 25th to 75th percentile, the line in the middle of the box is the median, the cross is the mean and the whiskers represent the minimum and maximum values (N = 8–10). G0: Cows were fed TMR *ad libitum* from calving to 180 DPP. G1: Cows grazed *Festuca arundinacea* plus a commercial concentrate or *Medicago* supplemented with TMR (50% of G0 offer), depending on heat stress conditions, from calving to 180 DPP. From 180 to 250 DPP both groups grazed *Medicago sativa* and were supplemented with TMR (50% of G0 offer at 180 DPP).

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that AcK could be responsible for the decrease in mitochondrial function observed during early lactation in pasture fed cows.

To further assess the extent of protein acetylation in the liver we studied AcK levels in tissue homogenates (Fig 5). The interaction between DPP and treatment tended to be significant ($P < 0.1$). AcK levels in liver homogenates of G0 cows were 30% lower during late lactation compared to early lactation and to G1 cows in the same period. No significant correlation was found between acetylated lysine levels in liver homogenates and maximum respiratory rate. These results indicate that the differences in mitochondrial acetylation are organelle specific and not a consequence of general changes in acetylation in the tissue.

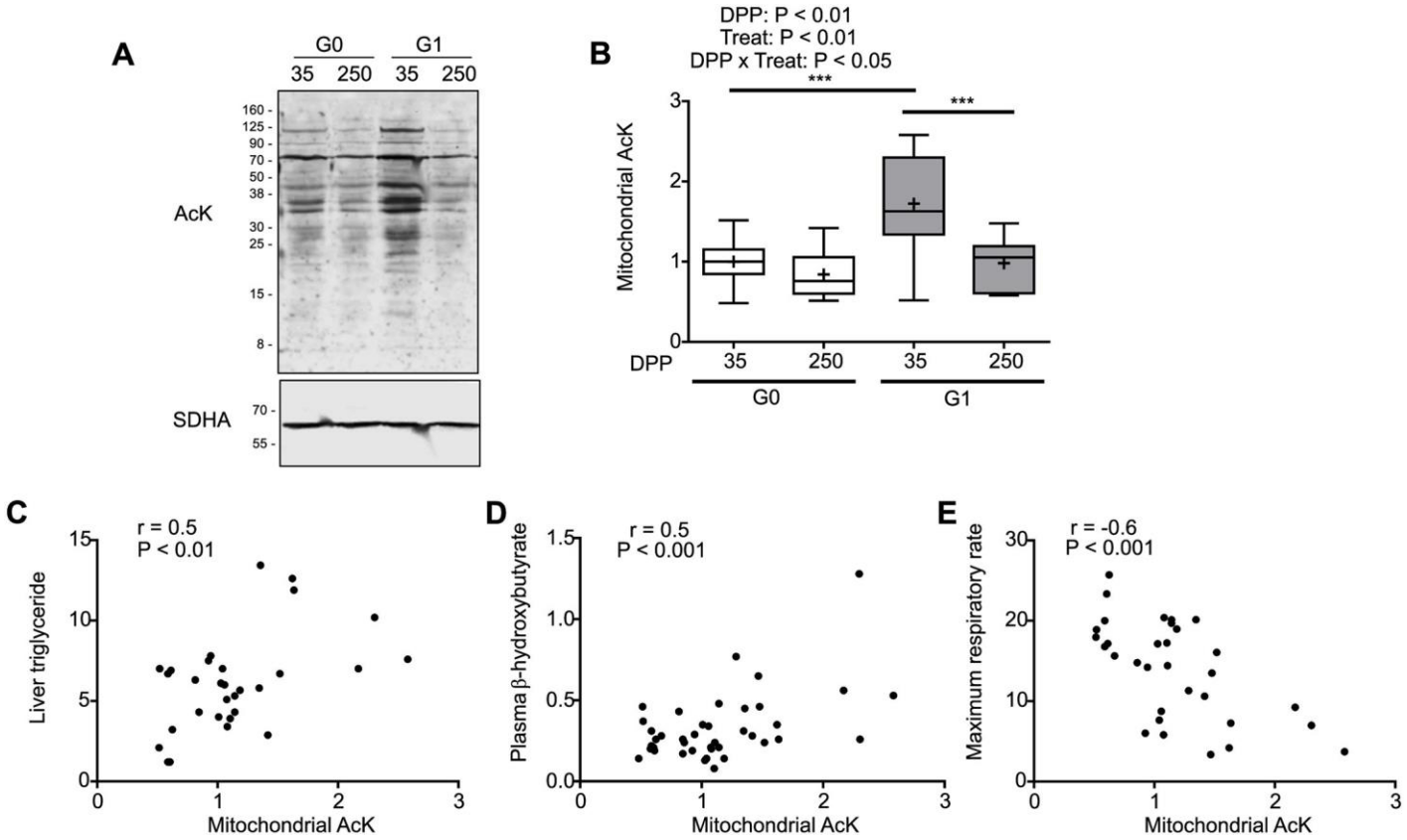


**Fig 4. Protein acetylation increases in liver mitochondria from pasture-fed dairy cows during early lactation. (A)** Representative western blots for AcK and SDHA (loading control) in liver subcellular fractions enriched in mitochondria from cows of both G0 and G1 groups at 35 and 250 DPP. **(B)** Independent western blots were quantified by densitometry. AcK levels were normalized with the loading control and expressed in relation to the average value of the G0 group at 35 DPP. The box extends from the 25th to 75th percentile, the line in the middle of the box is the median, the cross is the mean and the whiskers represent the minimum and maximum values (N = 10), □□□ P <0.001. **(C)**, **(D)** and **(E)** show the correlations between AcK levels and liver triglyceride, plasma β-hydroxybutyrate and maximum respiratory rate, respectively (N = 8–10). G0: Cows were fed TMR *ad libitum* from calving to 180 DPP. G1: Cows grazed *Festuca arundinacea* plus a commercial concentrate or *Medicago* supplemented with TMR (50% of G0 offer), depending on heat stress conditions, from calving to 180 DPP. From 180 to 250 DPP both groups grazed *Medicago sativa* and were supplemented with TMR (50% of G0 offer at 180 DPP).

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## Mitochondrial sirtuins

We then studied the levels of the mitochondrial sirtuins 3 and 5 (Fig 6). These enzymes catalyze the NAD dependent deacetylation of mitochondrial proteins and have been reported to regulate the activity of proteins involved in oxidative phosphorylation [52,53].

No significant interactions of treatment and dates were observed for either of the sirtuins (Fig 6B and 6D). Besides, in the case of sirtuin 5 no differences were observed between DPP or treatments (Fig 6A and 6B). However, sirtuin 3 levels were lower for the G1 cows than the G0 cows in both lactation moments ($P < 0.05$) (Fig 6C and 6D). The correlation between sirtuin 3 levels and mitochondrial AcK was negative and significant (Fig 6E) while a positive and significant correlation was found between sirtuin 3 levels and mitochondrial maximum respiratory rate (Fig 6F). These correlations suggest that a decrease in sirtuin 3 levels could be behind the observed increase in protein acetylation and the decay in mitochondrial function.

## Discussion

This study presents evidence of impairment in hepatic mitochondrial respiration during early lactation in pasture-fed cows, with increased markers of negative energy balance. Several

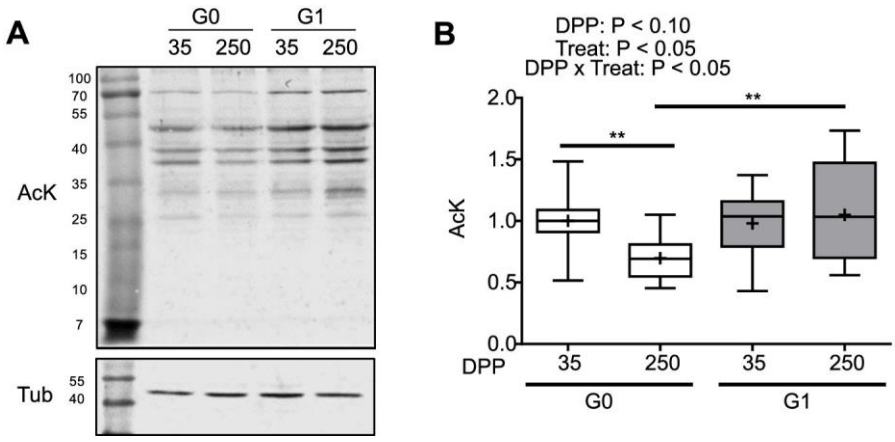


**Fig 5. Protein lysine acetylation in liver homogenates. (A)** Representative Western blot of AcK levels in liver homogenates from cows of both G0 and G1 groups at 35 and 250 DPP; tubulin was used as loading control. **(B)** Quantification by densitometry of total AcK levels normalized with the loading control and expressed in relation to the average value of the G0 group at 35 DPP. The box extends from the 25th to 75th percentile, the line in the middle of the box is the median, the cross is the mean and the whiskers represent the minimum and maximum values (N = 10). $^{**}$ $P < 0.01$. G0: Cows were fed TMR *ad libitum* from calving to 180 DPP. G1: Cows grazed *Festuca arundinacea* plus a commercial concentrate or *Medicago* supplemented with TMR (50% of G0 offer), depending on heat stress conditions, from calving to 180 DPP. From 180 to 250 DPP both groups grazed *Medicago sativa* and were supplemented with TMR (50% of G0 offer at 180 DPP).

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respiratory parameters indicative of mitochondrial function (state 3, maximum, oligomycine sensitive respiration) were decreased in early lactation in pasture fed cows, but not in cows in the TMR diet. In particular, maximum respiratory rates were affected, indicating a decrease in the capacity to adapt to energy demands or to withstand damaging insults [27,32]. Additionally we observed that maximum respiratory rate correlated negatively with ketone bodies and liver triglycerides and with mitochondrial protein acetylation; pointing towards a relation between acetylation, mitochondrial function and fatty acid catabolism in bovine liver during early lactation and negative energy balance (Fig 7).

Our results are in agreement with previous reports of impaired mitochondrial fatty acid oxidation in cows, mice and human patients with fatty liver and ketosis [14,21,54]; but differ with those by Koliaki *et al*., where an increase in maximum electron transport activity was observed in hepatic biopsies from subjects with non-alcoholic fatty liver (NAFL) when compared to healthy subjects [55]. However, since most of the reported data about fatty liver disease come from humans and mouse models, it is not clear if their conclusions can be extrapolated to ruminants.

In dairy cows during lactation oxaloacetate, an intermediary of the Krebs cycle, is channeled towards glucose synthesis, limiting complete oxidation of acetyl-CoA [10] [15]. The increase in acetyl-CoA levels can lead to ketone body synthesis [15], as well as protein acetylation [56,57]. Thus, nutritional management is extremely important during early lactation, when the cow may experience a shortage of glucogenic precursors because demands cannot be fully met by feed intake [8,10]. Pasture-based and TMR systems present different advantages and caveats [58,59]. From a metabolic/nutritional point of view it is generally accepted that TMR systems contribute to an increase in dry matter intake and contain higher levels of non-fiber carbohydrates than pasture based feeding strategies, therefore might result in higher production of propionate in the rumen [8]. Using the NDS Professional software (from RUM&N and Cornell University Department of Animal Science, Reggio Emilia, Italy) based on the

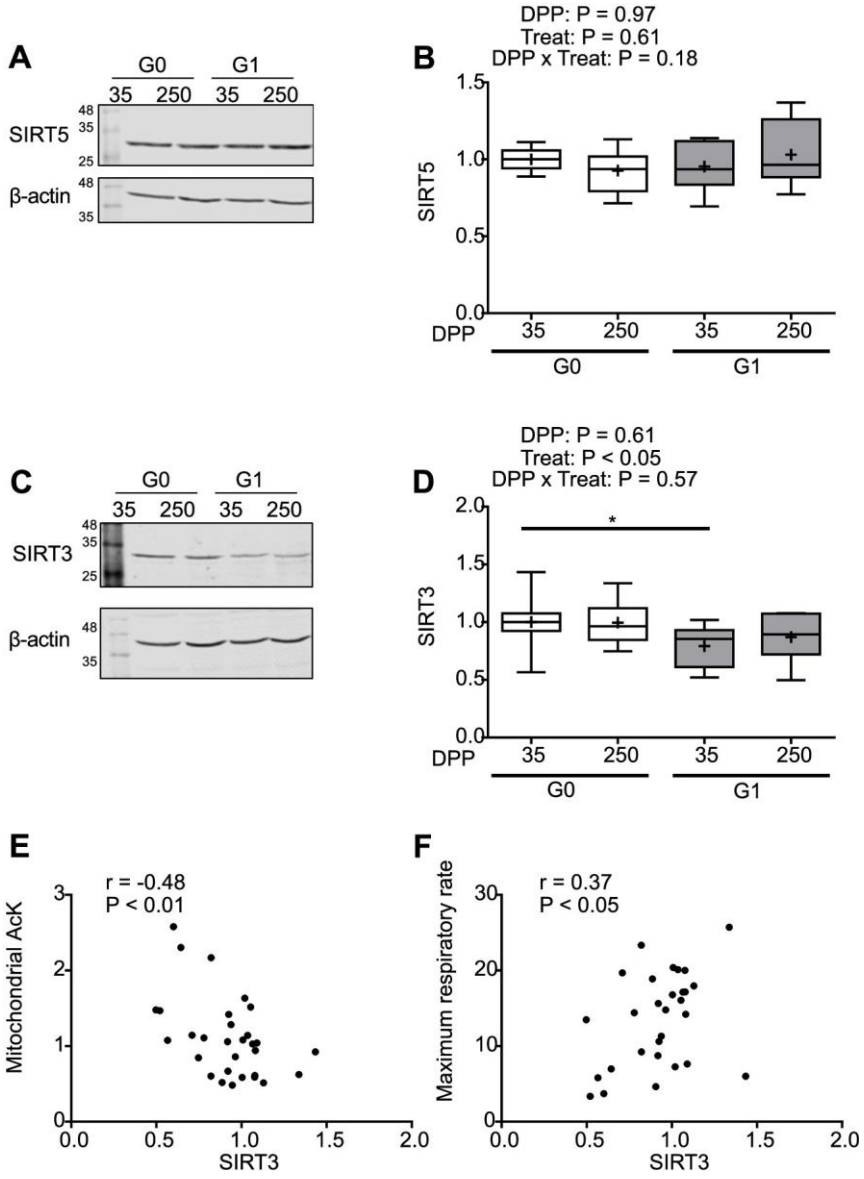


**Fig 6. Mitochondrial protein acetylation correlates with a decrease in sirtuin 3 levels. (A and C).** Representative western blots of sirtuin 5 (SIRT5) and sirtuin 3 (SIRT3) in liver homogenates from cows of both G0 and G1 groups at 35 and 250 DPP, β-actin was used as loading control. **(B and D)** Independent western blots of sirtuin 5 and sirtuin 3 were quantified by densitometry, normalized with the loading control and expressed in relation to the average value of the G0 group at 35 DPP. **(E)** Shows the correlation between mitochondrial AcK levels and sirtuin 3. **(F)** Shows the correlation between mitochondrial maximum respiratory rate and sirtuin 3. In box plots the box extends from the 25th to 75th percentile, the line in the middle of the box is the median, the cross is the mean and the whiskers represent the minimum and maximum values (N = 8). * $P < 0.05$. G0: Cows were fed TMR *ad libitum* from calving to 180 DPP. G1: Cows grazed *Festuca arundinacea* plus a commercial concentrate or *Medicago* supplemented with TMR (50% of G0 offer), depending on heat stress conditions, from calving to 180 DPP. From 180 to 250 DPP both groups grazed *Medicago sativa* and were supplemented with TMR (50% of G0 offer at 180 DPP).

model developed by Noziere et al., 2011 [60] we estimated the proportion of the different volatile fatty acids formed in the rumen with the different feeding strategies. These estimations suggested that in the pasture-based system (G1) the acetate:propionate ratio and the non-glucogenic:glucogenic ratio could be higher than in the TMR-system (G0) (2.80 vs. 2.04 and 3.50 vs. 2.65, respectively).

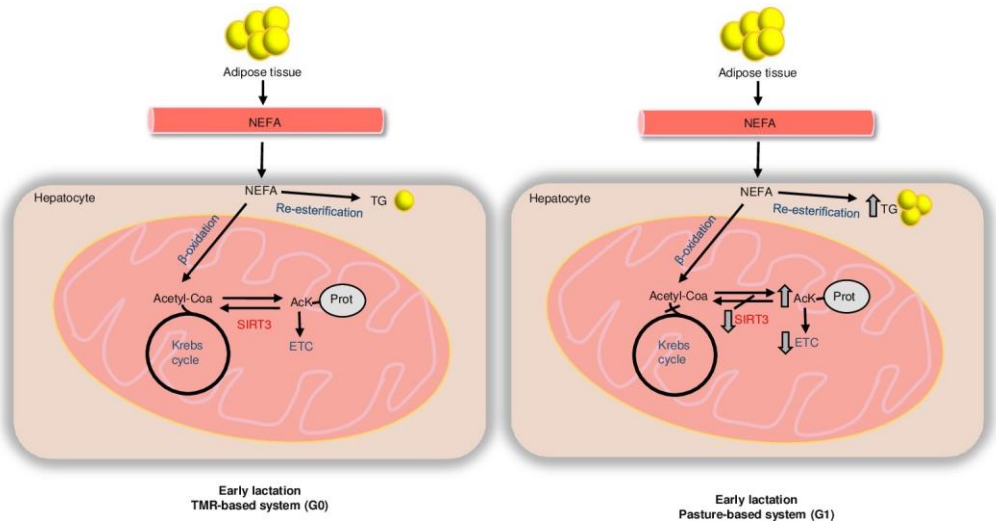


**Fig 7. Plausible mechanism behind metabolic changes during early lactation.** Lipid reserves are mobilized during early lactation, NEFA reach the blood stream and enter the hepatocyte where they are oxidized to acetyl-CoA in the β-oxidation pathway. The increase in Acetyl-CoA levels leads to acetylation of protein lysine residues. Lower levels of sirtuin 3 (SIRT3) in the liver of G1 cows may contribute to the increase in protein acetylation (AcK-Prot) in the G1 group with respect to the G0 group. Protein lysine acetylation impacts negatively on electron transport chain activity (ETC), Krebs cycle and β-oxidation resulting in an impaired oxidation of NEFA that are re-esterified to triglycerides (TG), giving rise to fatty liver.

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An increase in the supply of propionate might impact positively in oxaloacetate levels, favoring not only gluconeogenesis but also acetyl-CoA oxidation to $CO_2$ [10]. In agreement with this possibility mitochondrial acetylation was lower in TMR fed cows than in pasture fed cows in early lactation (Fig 7). Additionally, we observed ≀ 70% increase in acetylated protein lysine in isolated mitochondria from pasture fed cows during early lactation, compared to late lactation. Acetylation is a reversible covalent post-translation modification that can both inhibit or increase the activity and stability of key enzymes in β-oxidation, Krebs cycle, ketone body metabolism, electron transport chain and oxidative phosphorylation [49,50,61–63]. Increase in lysine acetylation occurs due to imbalances in acetylation and deacetylation reactions. Mitochondrial acetylation can occur non-enzymatically [56] or catalyzed by acetyltransferases (i.e. acetylase GCN5L1) [64]; while mitochondrial deacetylation is catalyzed by sirtuins 3 and 5 [65].

Previous studies in mice models have shown that sirtuin 3 regulates hepatic fatty acid metabolism [61]. Decreased sirtuin 3 activity and increased acetylation has been observed in mice that develop fatty liver under a high fat diet [20]. On the contrary, during fasting, increased sirtuin 3 levels promote the deacetylation of long-chain acyl coenzyme A dehydrogenase (LCAD) in the liver; which increases enzyme activity and fatty acid oxidation and prevents the accumulation of triglycerides [61]. Thus, different levels of sirtuin 3 could account, at least in part, for the differences in acetylation profiles between G0 and G1 cows during early lactation (Fig 7). Although the contribution to increased acetylation of other events, such as enzymatic or non-enzymatic acetylation cannot be discarded [56,66]. Since sirtuin 3 levels were higher in TMR-fed cows than pasture-fed cows, fatty acid oxidation rates might be higher in the former resulting in less accumulation of liver triglycerides. Our study suggests that higher sirtuin 3 levels in G0 dairy cows during early lactation might be responsible for a better adaptation to excessive energy requirements and that both acetylation and deacetylation reactions can be affected by diet.

Sirtuin 3 is considered a regulator of energy metabolism and homeostasis [20,65,67]. In particular, respiratory chain complexes I-V can be inhibited by acetylation [20,52,68,69] and sirtuin 3 catalyzes their deacetylation increasing electron transport flux and oxidative phosphorylation. Glutamate dehydrogenase is also a sirtuin 3 substrate [70]. Furthermore, Kendrick *et al*. observed that sirtuin 3 knockout results in decreased activity of mitochondrial respiratory complexes III and IV in mice under a high fat diet [20]. Thus, changes in sirtuin 3 and acetylation could underlie the decay in mitochondrial respiration observed during early lactation in pasture-fed cows. Further research is necessary to identify the molecular events behind the diet dependent differences in sirtuin 3 levels.

In human patients and mouse models oxidative stress markers, lipid peroxidation products [71–73] and protein 3-nitrotyrosine [74], correlate with the severity of liver damage and oxidative damage is considered the "second hit" required for the development of inflammation and cytotoxicity [19]. In ruminants, an increase in markers of oxidative stress has been reported in plasma of dairy cows in early lactation [75] and in hepatic biopsies of dairy cows with liver failure during the same period [76]. Nevertheless, the correlation between oxidative stress and liver damage has not yet been elucidated in dairy cows during lactation.

Herein we observed an increase in non-mitochondrial oxygen consumption in early lactation in both G0 and G1 cows compared to late lactation that could be due to an increase in ROS formation (e.g. superoxide or hydrogen peroxide formation by oxidases [27,45,46], or oxygen consumption in lipoperoxidation reactions [47]). Previous studies have shown that lactating cows have higher rates of peroxisomal fatty acid oxidation than non-lactating cows [1,77]. Peroxisomal fatty acid oxidases use oxygen as electron acceptor, reducing it to hydrogen peroxide, and could be responsible for the increase in non-mitochondrial oxygen consumption.

We did not find evidence of an increase in oxidative markers (i.e. 4-HNE-protein adducts or protein 3-nitrotyrosine) in liver homogenates or mitochondria from dairy cows during early lactation, in spite of evidence of triglyceride accumulation in the liver. However, when correlation analyses were performed we found that the cows with the highest 4-HNE-protein adduct levels also had the highest non-mitochondrial oxygen consumption rates, and a positive and significant correlation between these two parameters was obtained. These observations suggest that non-mitochondrial oxygen consumption and lipid peroxidation events might be related. Additionally, no significant correlation could be found between 4-HNE-protein adducts and the decrease in mitochondrial respiration. The fact that fatty liver was not observed in late lactation, that aspartate aminotransferase activity (marker of liver damage) was within normal ranges at all times for practically all the animals and that mitochondrial function was recovered in late lactation suggests that our cows might have not experienced relevant oxidative stress.

Although we cannot dismiss oxidative stress as a potential mediator of mitochondrial impairment, since oxidative damage is challenging to assess *in vivo* [48,78], the changes in mitochondrial function and fatty acid metabolism appear to be due to regulatory events, such as acetylation/deacetylation reactions, rather than irreversible oxidative damage.

## Conclusion

In this work we detected changes in respiratory parameters between early and late lactation and found an association between mitochondrial protein acetylation, respiration and fatty acid metabolism in dairy cows in early lactation. Our results show that cows in a pasture-based system, present impaired mitochondrial function during early lactation, increased acetylation of

mitochondrial proteins and decreased levels of sirtuin 3. Overall our results highlight the relevance of nutritional management in this crucial period.

During early lactation, an increase in acetyl-CoA, can promote acetylation of mitochondrial proteins. Higher levels of sirtuin 3 in cows in the TMR-based system versus pasture-based system can counter the increase in acetylation and help maintain mitochondrial homeostasis. However, lower sirtuin 3 levels in cows in the pasture-based system could result in increased acetylation of mitochondrial proteins affecting respiration, oxidative phosphorylation and fatty acid oxidation; potentially leading to accumulation of triglycerides in the liver.

## Supporting information

**S1 Fig. Subcellular fractionation of liver homogenates.** Liver biopsies were homogenized and subcellular fractions enriched in mitochondria, nuclei and cytosol were obtained as described previously [33]. Proteins from the different fractions were resolved by SDS/PAGE and Western blots performed with antibodies against proteins from mitochondria (SDHA), cytosol (β-actin) and nuclei (histone H3).
(TIF)

**S2 Fig. Protein tyrosine nitration in liver homogenates. (A)** Representative western blot of liver homogenates exposed to different concentrations of peroxynitrite ($ONOO^-$) in 100 mM phosphate buffer pH 7.4. **(B)** Representative western blot of 3-nitrotyrosine in liver homogenates of G0 and G1 cows at 35 and 250 DPP, and a positive control (C (+)). The positive control was obtained exposing the bovine serum albumin to 300 μM peroxynitrite in 100 mM phosphate buffer pH 7.4.
(TIF)

**S3 Fig. Evaluation of mitochondrial content in liver biopsies of dairy cows. (A and C)** Representative western blots of ATP synthase subunit α (ATP5A) and succinate dehydrogenase subunit A (SDHA) in liver homogenates of cows from both G0 and G1 groups at 35 and 250 DPP. β-actin and tubulin were used as loading controls. **(B and D)** Quantification by densitometry of ATP5A and SDHA levels normalized with the respective loading controls and expressed in relation to the average value of the G0 group at 35 DPP. **(E)** Citrate synthase specific activity was determined in liver homogenates of cows from the G0 and G1 group at 35 and 250 DPP. In box plots the box extends from the 25th to 75th percentile, the line in the middle of the box is the median, the cross is the mean and the whiskers represent the minimum and maximum values (N = 8–10).
(TIF)

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## 6. DIFFERENTIAL HEPATIC MITOCHONDRIAL FUNCTION AND GLUCONEOGENIC GENE EXPRESSION IN TWO HOLSTEIN STRAINS IN A PASTURE-BASED SYSTEM

García-Roche M, Talmón D., Cañibe G., Astessiano A. L., Mendoza A., Quijano C., Cassina A. and Carriquiry M.
Recientemente aceptado en el *Journal of Dairy Science*.

Este trabajo tuvo como objetivo estudiar el potencial de síntesis de ATP, a nivel mitocondrial, en vacas del modelo 2 (vacas Holstein NAH y NZH en la estrategia de máximo pastoreo) y su asociación con una vía altamente demandante energéticamente: la gluconeogénesis. Los estudios se realizaron durante la lactancia media tardía en primavera en tres años consecutivos. Nuestros resultados demostraron que las vacas NZH tuvieron mayores niveles de glucosa en plasma que las vacas NAH ($P < 0,0001$), como también mayor función mitocondrial que las vacas NAH ($P < 0,05$), mientras que la expresión génica hepática de enzimas claves de la gluconeogénesis estaba aumentada en vacas NAH con respecto a las vacas NZH ($P < 0,05$).

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# Differential hepatic mitochondrial function and gluconeogenic gene expression in 2 Holstein strains in a pasture-based system

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## ABSTRACT

The objective of this study was to assess hepatic ATP synthesis in Holstein cows of North American and New Zealand origins and the gluconeogenic pathway, one of the pathways with the highest ATP demands in the ruminant liver. Autumn-calving Holstein cows of New Zealand and North American origins were managed in a pasture-based system with supplementation of concentrate that represented approximately 33% of the predicted dry matter intake during 2017, 2018, and 2019, and hepatic biopsies were taken during mid-lactation at 174 ± 23 days in milk. Cows of both strains produced similar levels of solids-corrected milk, and no differences in body condition score were found. Plasma glucose concentrations were higher for cows of New Zealand versus North American origin. Hepatic mitochondrial function evaluated measuring oxygen consumption rates showed that mitochondrial parameters related to ATP synthesis and maximum respiratory rate were increased for cows of New Zealand compared with North American origin. However, hepatic gene expression of pyruvate carboxylase, phosphoenolpyruvate carboxykinase, and pyruvate dehydrogenase kinase was increased in North American compared with New Zealand cows. These results altogether suggest an increased activity of the tricarboxylic cycle in New Zealand cows, leading to increased ATP synthesis, whereas North American cows pull tricarboxylic cycle intermediates toward gluconeogenesis. The fact that this occurs during mid-lactation could account for the increased persistency of North American cows, especially in a pasture-based system. In addition, we observed an augmented mitochondrial density in New Zealand cows, which could be related to feed efficiency mechanisms. In sum, our results contribute to the elucidation of hepatic molecular mechanisms in dairy cows in production systems with higher inclusion of pastures.
**Key words:** intermediary metabolism, gluconeogenesis, dairy cows, grazing, Holstein-Friesian

## INTRODUCTION

In addition to the benefits in product quality (O'Callaghan et al., 2016), the inclusion of pastures in dairy cow systems results in lower feeding costs and, in consequence, a system more resilient to the volatility of markets, especially for exporting countries (Fariña and Chilibroste, 2019). Productive performance of pasture-based dairy systems is very much related to grazing management to ensure sufficient DM intake, which is generally reduced compared with confined systems, and appropriate quality of pastures (Chilibroste et al., 2012). Also, intrinsic to pasture-based systems is the greater energy expenditure due to walking and grazing activities (Jasinsky et al., 2019). Previous authors have shown that when nutrient concentration is insufficient, cows may show a poor metabolic status, as negative energy balance markers such as plasma nonesterified fatty acids and BHB and liver triglyceride concentrations are exacerbated (Meikle et al., 2013; García-Roche et al., 2021). Furthermore, hepatic energy metabolism has also been reported to be compromised in cows managed under a pasture-based system, as mitochondrial function is impaired during early lactation (García-Roche et al., 2019).

The genetic selection process of North American (**NAH**) and New Zealand (**NZH**) Holstein strains determined differences in their productive and reproductive performance in a pasture-based system. Although NAH produce more milk than NZH cows, the latter produce greater percentages of milk solids (Lucy et al., 2009; White et al., 2012). In addition, NAH cows are unable to maintain an acceptable body condition and

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weight during lactation and fail to maintain a 365-d calving interval, yielding poorer fertility and survival performance compared with NZH cows in grazing systems (Kolver et al., 2000; Harris and Kolver, 2001). Also, NZH cows have a lower energy requirement for maintenance than NAH cows (Talmón et al., 2020). Differences among strains have been associated with reduced uncoupling of the somatotropic axis and insulin resistance during the transition period in NZH compared with NAH cows (Chagas et al., 2006; Lucy et al., 2009). Additionally, previous research indicates differences between Holstein strains in hepatic energy metabolism; cows of NZH origin presented increased expression of pyruvate carboxylase (*PC*) mRNA, which could translate into a more active tricarboxylic acid (**TCA**) cycle (White et al., 2012). Altogether, these results could lead to higher intermediary metabolism activity for NZH cows and, thus, increased availability of oxidative phosphorylation precursors for NZH cows compared with NAH cows in a grazing system. Thus, we hypothesized that mitochondrial ATP synthesis would be increased in NZH cows relative to NAH cows. Therefore, in pasture-based systems, NZH cows would be better prepared to face the energy demands imposed by anabolic routes and other activities, as a result of increased ATP availability. Hence, our objective was to study the potential for hepatic ATP synthesis in diverging Holstein strains in a pasture-based system and its interplay with a highly energy-demanding pathway: gluconeogenesis.

## MATERIALS AND METHODS

The experiment was conducted at the Experimental Station of the Instituto Nacional de Investigación Agropecuaria (Colonia, Uruguay) during the spring of 2017, 2018, and 2019. Animal use and procedures were approved by the Animal Experimentation Committee of the Instituto Nacional de Investigación Agropecuaria, Uruguay (file number: INIA2017.2).

The experiment formed part of a larger study designed to evaluate the effect of cow genotype (NZH vs. NAH strains) in different feeding strategies on individual animal and whole-farm biophysical performance. More detailed descriptions of Holstein strains, animal and grazing management, as well as milk production, BW, and BCS from June 2017 through May 2019 were previously reported (Stirling et al., 2021).

### *Experimental Design*

Autumn-calving Holstein cows of NZH (512 ± 19 kg BW and 3.07 ± 0.12 BCS at calving; n = 12) and NAH strains (563 ± 29 kg BW, 3.10 ± 0.1 BCS at calving; n = 18) were used to evaluate 10 cows of each genotype for 3 yr. In the NZH group, 7 cows were evaluated for 3 yr, 4 for 2 yr, and 1 additional cow only in yr 3. In the NAH group, 3 cows were evaluated for 3 yr, 4 for 2 yr, and 11 only for 1 yr.

Both NZH and NAH strains had at least 75% of each cow's ancestors (2 generations: father and maternal grandfather) from New Zealand or from the United States or Canada, respectively (Stirling et al., 2021). In the present study, the NZH and NAH strains presented progeny of 7 sires in each group. The economic and productive selection index was 126 ± 11 and 105 ± 14 on average for NZH and NAH cows, respectively. Expected progeny differences were −187.6 ± 164 kg, +0.170 ± 0.111%, and +0.157 ± 0.145% for milk yield, milk fat, and milk protein content for NZH cows, and +44.9 ± 174 kg, +0.086 ± 0.064%, and +0.004 ± 0.068% for milk yield, milk fat, and milk protein content for NAH cows (Mejoramiento y Control Lechero Uruguayo; https://www.mu.org.uy).

Cows were paired in each strain group according to their calving date (May 4, 2017, ± 17 d; May 5, 2018, ± 23 d; May 2, 2019, ± 37 d) and lactation number (2.12 ± 0.8 lactations). Cows were managed in a mixed grazing system: during mid-lactation, cows grazed on daily strips of orchardgrass (*Dactylis glomerata*) and alfalfa (*Medicago sativa*) or fescue (*Festuca arundinacea*) in a rotational-grazing manner during 2 grazing sessions (a.m. and p.m. session: from 0500 to 1400 h and from 1500 to 0400 h, respectively). Average herbage mass was 1,713 ± 423 kg of DM/ha; 5 cm above ground level. Each strain group grazed on separate paddocks to ensure similar pasture allowance relative to their BW (11.2 ± 0.9 kg of DM/cow per day for NZH and 14.9 ± 1.5 kg of DM/cow per day for NAH, 5 cm above ground level, 3-yr average). Fresh water was offered in each paddock. Predicted DM intake was estimated according to the National Research Council model for dairy cattle (NRC, 2001). Hence, when forage allowance was considered restrictive, cows were supplemented with conserved forage (corn silage and pasture haylage mix in a 67:33 ratio, 3-yr average) to achieve predicted DM intake. Pasture forage allowance and haylage supplementation were adjusted weekly based on weekly pasture growth. Details of the grazing management were presented previously (Talmón et al., 2020; Stirling et al., 2021), and chemical composition and metabolizable energy concentration of feedstuffs are presented in Table 1. Briefly, pasture was offered in daily strips, which cows of each genotype grazed separately to keep similar herbage allowance relative to their BW and to ensure breeds behaved independently and avoid dominance. As cows grazed pastures in a rotational-grazing manner, they returned to defined

grazing areas once most of the grass tillers had between 2.5 and 3 leaves.

Cows were milked twice a day and supplemented with concentrate (33% of predicted daily DM intake, 6.8 ± 0.4 kg of DM/d for NZH, and 7.5 ± 0.9 kg of DM/d for NAH, 3-yr average). Milk yield was recorded daily, and milk samples were collected every 14 d. Solids-corrected milk (**SCM**) was calculated as SCM (kg) = 12.3 (F) + 6.56 (SNF) − 0.0752 (M), where F, SNF, and M are expressed as kilograms of fat, solids-not-fat, and milk, respectively (Tyrrell and Reid, 1965). As this work is part of a larger study, cow BCS (score 1 to 5; Edmonson et al., 1989) was determined every 14 d by one evaluator (Stirling et al., 2021).

### Plasma Collection and Liver Biopsies

Plasma samples and liver biopsies were collected in the spring during the months of October and November of 2017, 2018, and 2019 (at 187 ± 19, 176 ± 21, and 158 ± 29 DIM, respectively, in average 174 ± 23 DIM, mid-lactation). In this period, cows were in either the first or the second trimester of gestation (96 ± 38 d of gestation for NZH, and 78 ± 41 d of gestation for NAH). Indeed, this study is part of a larger study, which reported calving to conception days: 96.3 and 108.1 ± 4.9 (SEM), respectively, for NZH and NAH (Stirling et al., 2021).

Plasma samples were collected by venipuncture of the coccygeal vein using 10-mL heparinized Vacutest tubes (Vacutest Kima). Samples were centrifuged at 4,000 × *g* for 12 min and immediately stored at −20°C until analysis. Biopsies were taken using a 14-gauge biopsy needle (Tru-Core-II Automatic Biopsy Instrument; Angiotech) after local intramuscular administration of 3 mL of 2% lidocaine hydrochloride (Carriquiry et al., 2009) and either cryopreserved for mitochondrial oxygen consumption analyses (García-Roche et al., 2018) or immediately frozen in liquid nitrogen. All samples were stored at −80°C until analysis.

**Table 1.** Chemical composition and ME concentration (means ± SD) of feedstuffs offered during the experiment

| Item | Pasture[1] | Concentrate[2] | Conserved forage[3] |
|---|---|---|---|
| DM (%) | 23.0 ± 1.7 | 90.0 ± 1.3 | 45.1 ± 8.2 |
| CP (% DM) | 22.7 ± 3.8 | 19.6 ± 1.6 | 12.3 ± 2.8 |
| NDF (% DM) | 48.6 ± 3.4 | 35.7 ± 7.3 | 42.6 ± 9.5 |
| ADF (% DM) | 28.3 ± 4.1 | 19.1 ± 7.5 | 27.1 ± 7.3 |
| Ash (% DM) | 10.7 ± 0.5 | 7.9 ± 0.4 | 9.55 ± 4.3 |
| ME (Mcal/kg DM) | 2.48 ± 0.14 | 2.91 ± 0.03 | 2.48 ± 0.18 |

[1]*Dactylis glomerata* and *Medicago sativa* or *Festuca arundinacea*.

[2]Commercial concentrate (Prolacta 18, Prolesa S.A.) composed of corn grain, soybean meal, wheat bran, and vitamin and mineral mix. The SD represents the variation between feedstuff samples collected during the measurement period of the 3 yr of the experiment (2017, 2018, 2019).

[3]Corn silage and pasture haylage mix.

### Plasma and Hepatic Metabolites

Plasma glucose, urea, BHB, and nonesterified fatty acids (**NEFA**) were determined spectrophotometrically with commercial kits from Biosystems S.A. for glucose and urea and from Randox Laboratories Ltd. for BHB and NEFA, at λ = 505 nm, 600 nm, 340 nm, and 560 nm, respectively (Astessiano et al., 2015; García-Roche et al., 2021).

For hepatic free glucose and glycogen quantification, liver homogenates were performed using 500 µL of 2 *N* HCl and glass Dounce homogenizers. Homogenates were subjected to 100°C for an hour, for glycogen digestion to glucose by acid-heat hydrolysis (Bancroft and Fry, 1933). Free liver glucose and digested liver glycogen were determined using a kit from Biosystems S.A., following manufacturer instructions, after neutralizing acid samples with an equal amount of 2 *M* NaOH. Absorbance was measured at λ = 505 nm.

For liver triglyceride quantification, liver homogenates were obtained according to Armour et al. (2017). Briefly, liver tissue was homogenized in lysis buffer (140 m*M* NaCl, 50 m*M* Tris, and 1% Triton X-100, pH 8) and measured using a kit from Biosystems S.A., following manufacturer instructions at λ = 505 nm. For all metabolite assays intra- and interassay coefficient of variation (CV) were less than 10%. Liver triglyceride is expressed per unit of tissue protein.

### Mitochondrial Oxygen Consumption Rate

Mitochondrial function was studied measuring oxygen consumption rate in a high-resolution Oroboros Oxygraph 2k respirometer (Oroboros Instruments) at 37°C (García-Roche et al., 2018, 2019). Briefly, electrodes were calibrated in modified mitochondrial respiration medium (MIR05; 0.5 m*M* ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, 3 m*M* $MgCl_2 \cdot 6H_2O$, 60 m*M* 4-morpholinepropanesulfonic acid, 3-(N-morpholino)propanesulfonic acid, 20 m*M* taurine, 10 m*M* $KH_2PO_4$, 20 m*M* HEPES, 110 m*M* sucrose, 1 g/L BSA, pH 7.1) with a calculated saturated oxygen concentration of 191 µ*M* at 100 kPa barometric pressure at 37°C. Respiratory rates (pmol of $O_2$/min per mL) were calculated using DatLab 4 analysis software (Oroboros Instruments). Liver biopsies were weighed (2–10 mg) and added to the chamber, and oxygen consumption measurements were obtained before and after the sequential addition of specific substrates of the respiratory chain, 10 m*M* glutamate and 5 m*M* malate (complex I) or 20 m*M* succinate (complex II),

followed by 4 m*M* adenosine diphosphate (**ADP**), 2 μ*M* oligomycin (ATP synthase inhibitor), 2 to 4 μ*M* carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (**FCCP**, an uncoupler of oxidative phosphorylation). Maximum uncoupling was obtained by FCCP titration. It is important to emphasize that the maximum respiratory rate is obtained by maximum uncoupling by means of the titration of FCCP—an uncoupler—to dissipate the proton gradient completely without the regulation of the ADP/ATP ratio (García-Roche et al., 2018). This parameter is indicative of the activity of the electron transport chain complexes or the quantity of mitochondria in the tissue that could be drawn upon in situations of strenuous energy demands or damage (Brand and Nicholls, 2011). Respiration was inhibited with 0.5 μ*M* rotenone (complex I inhibitor) or 2.5 μ*M* antimycin A (complex III inhibitor). All respiratory parameters and indices were obtained as described in García-Roche et al. (2018). Briefly, non-mitochondrial oxygen consumption rate measured after the addition of specific inhibitors rotenone or antimycin A and subtracted from all other values before calculating the respiratory parameters. State 4 respiration was determined as the baseline measurement obtained with complex I and II substrates before the addition of ADP, and state 3 was determined after the addition of ADP. Oligomycin-resistant respiration (ATP-independent) was measured after addition of oligomycin, and oligomycin-sensitive respiration (ATP-dependent) was the difference between state 3 and oligomycin-resistant respiration. Finally, the maximum respiratory rate was determined after titration with FCCP. The leaking control ratio was calculated as oligomycin-resistant respiration divided by the maximum respiratory rate, as described previously by Gnaiger (2009) and Koliaki et al. (2015).

### *RNA Extraction and Quantitative PCR Analysis*

Total RNA extraction from liver tissue and cDNA synthesis by reverse transcription was performed (Carriquiry et al., 2009) using the Trizol reagent, followed by lithium chloride precipitation and DNase treatment using an Ambion DNA-Free DNA Removal Kit (Thermo Fisher Scientific). Concentration of RNA was determined by measuring absorbance at λ = 260 nm (NanoDrop ND-1000 Spectrophotometer; Nanodrop Technologies), and purity and integrity of RNA isolates were assessed from 260/280 and 260/230 absorbance ratios (greater than 1.9 and 1.8, respectively). Samples of RNA were stored at −80°C. A SuperScript III Reverse Transcriptase Kit (Invitrogen from Thermo Fisher Scientific) was used to perform retrotranscription along with random hexamers and 1 μg of total RNA as a template. The cDNA was stored at −20°C until its use in the real-time PCR. Primers (Supplemental Table S1, https://data.mendeley.com/datasets/gx5yh5t2bp/1) to specifically amplify cDNA of target genes: β-actin (*ACTB*), glucose-6-phosphatase (*G6PC*), hypoxanthine phosphoribosyl transferase (*HPRT1*), methylmalonyl-CoA mutase (*MMUT*), succinate dehydrogenase complex, subunit A, flavoprotein (*SDHA*), pyruvate carboxylase (*PC*), phosphoenolpyruvate carboxykinase (*PCK1*), pyruvate dehydrogenase E1 subunit α 1 (*PDHA1*), pyruvate dehydrogenase kinase (*PDK4*), peroxisome proliferator-activated receptor gamma coactivator 1-α (*PPARGC1A*), peroxisome proliferator-activated receptor α (*PPARA*), sirtuin 1 (*SIRT1*), NADH:ubiquinoneoxidoreductase core subunit V1 (*NDUFV1*, nuclear gene), and mitochondrially encoded cytochrome c oxidase I (*mt-CO1*, mitochondrial gene) were obtained from literature, or specifically designed using the Primer3 website (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and bovine nucleotide sequences available from NCBI (http://www.ncbi.nlm.nih.gov/).

Real-time PCR reactions were carried out in a total volume of 15 μL using Maxima SYBR Green/ROX qPCR Master Mix 2X (Thermo Fisher Scientific), using the following standard amplification conditions: 10 min at 95°C and 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30s at 72°C in a 48-well StepOne Real-Time PCR System (Applied Biosystems from Thermo Fisher Scientific). Melting curves were run on all samples to detect primer dimers, contamination, or presence of other amplicons. Each plate was designed including a pool of total RNA from bovine liver samples analyzed in triplicate to be used as the basis for the comparative expression results (exogenous control) and duplicate wells of non-template control (water). Gene expression was determined by relative quantification with respect to the exogenous control (Pfaffl, 2004) and normalized to the geometric mean expression of the endogenous control genes (*ACTB* and *HPRT*). Expression stability of 2 selected housekeeping genes was evaluated using the MS-Excel add-in Normfinder (MDL); values obtained with Normfinder were 0.004 for *ACTB* and 0.003 for *HPRT*. Amplification efficiencies or target and endogenous control genes were estimated by linear regression of a cDNA dilution curve (Supplemental Table S1, https://data.mendeley.com/datasets/gx5yh5t2bp/1). Intra- and interassay CV values were less than 1.4 and 2.6% (n = 5 dilutions, from 100 to 6.25 ng/well), respectively.

Determination of the ratio of mitochondrial DNA (**mtDNA**) to nuclear DNA (**nDNA**; mtDNA:nDNA) was performed according to Casal et al. (2018). Briefly, real-time PCR reactions prepared in separate tubes to

detect mitochondrial encoded cytochrome c oxidase I (*mt-CO1*, mitochondrial gene) and NADH:ubiquinone oxidoreductase core subunit V1 (*NDUFV1*, nuclear gene), respectively, and real-time PCR reactions were conducted as aforementioned. To calculate the mtDNA: nDNA ratio, the difference between cycle numbers was calculated (Ctn − Ctmt) and 2 was elevated to the power of said difference [$2^{(Ctn - Ctmt)}$], and results were expressed as fold change relative to NZH cows.

### Enzyme Activity

Citrate synthase (**CS**) activity was determined in 60 µg/mL of liver homogenates based on the formation of 5-thio-2-nitrobenzoic acid at λ = 412 nm ($\varepsilon_{412}$ = 13,700 L/mol.cm) in the presence of 20 m*M* Tris-HCl pH 8, 300 µ*M* acetyl-CoA, 500 µ*M* oxaloacetate, and 100 µ*M* 5,5′-dithio-bis (2-nitrobenzoic acid; García-Roche et al., 2019). Assays were performed in duplicate using a final volume of 200 µL in a Multiskan FC Microplate Photometer (Thermo Fisher Scientific).

Succinate dehydrogenase (**SDH**) activity was determined in 120 µg/mL of mitochondrial protein based on the reduction of 2,6-dichlorophenolindophenol (DCPIP) by decilubiquinone at λ = 600 nm ($\varepsilon_{600}$ = 21,000 L/mol.cm) in the presence of 50 µ*M* DCPIP, 1 m*M* potassium cyanide, 5 µ*M* rotenone, and 50 µ*M* decylubiquinone in a 25 m*M* phosphate buffer, pH 7.2. To quantify enzyme activity, baseline was subtracted from absorbance after addition of 10 m*M* succinate (Casal et al., 2018). Assays were performed in duplicate at 37°C using a final volume of 500 µL in a UV-2401 PC spectrophotometer (Shimadzu Corp.).

### Western Blots

Liver homogenates were prepared with a lysis buffer complemented with protease and phosphatase inhibitors: 1 m*M* phenylmethylsulfonyl fluoride, supplemented with SigmaFAST protease inhibitor cocktail and Calbiochem phosphatase inhibitor cocktail (Sigma-Aldrich; García-Roche et al., 2019). Protein content was determined with a Bradford assay using BSA as standard (Bradford, 1976), and samples were kept at −80°C until analysis. Liver homogenates were resolved (30 µg) in 12% Tris-glycine SDS/PAGE, along with protein ladders (SDS7B2, Sigma-Aldrich), and proteins were transferred overnight to nitrocellulose membranes. Membranes were blocked with blocking buffer (Tris-buffered saline with 0.1% Tween 20 and 0.5% skim milk) and incubated overnight at 4°C with primary antibodies against GAPDH (1:1,000, Abcam, ab9485), succinate dehydrogenase subunit A (SDHA, 1:2000, Abcam, ab14715), AMPKα (1:1,000, no. 2532, Cell Signaling Technology), and phosphoAMPKα (1:1,000, no. 2535, Cell Signaling Technology). For protein detection, membranes were washed and probed with secondary antibodies from LI-COR Biosciences: anti-mouse (1:10,000, IRDye 680, 926-68070) or anti-rabbit (1:20,000, IRDye 800, 926-32211). Immunoreactive proteins were detected with an infrared fluorescence detection system (Odyssey, LI-COR Biosciences), and bands were quantified with ImageStudio software (LI-COR Biosciences, version 2.0) by densitometry, and protein levels were normalized by protein levels of GAPDH the loading control.

### Statistical Analysis

Data were analyzed in a randomized block design using SAS Academic Edition (SAS OnDemand for Academics, SAS Institute Inc.), with cow as the experimental unit. Univariate and linear regression analyses were performed for all variables to identify outliers and inconsistencies and to verify normality of residuals. Variance homogeneity was tested using the Levene, Bartlett, and Brown-Forsythe tests. When data did not have normal distribution or variance was not homogeneous, logarithmic transformations were performed to more closely approximate normality and homogeneity requirements. Values were removed when the studentized residual was >3 and <−3; no more than 3 values per variable were excluded. Least squares means and pooled standard error values of all variables are presented as non-transformed data to aid in comparison among variables.

Data were analyzed using the MIXED procedure; the model included Holstein strain as a fixed effect and year and block as random effects. Compound symmetry was used as the covariance structure, and the Kenward-Roger procedure was used to adjust the denominator degrees of freedom. Least squares means tests were conducted to analyze differences between groups. Means were considered to differ when $P < 0.05$, and a trend was declared when $0.05 < P < 0.10$. Pearson correlations were calculated with the CORR procedure.

## RESULTS

### Body Condition Score, Milk Yield, and Milk Composition

In average, for the 3 years evaluated, mid-lactation (at 174 ± 23 d) milk yield was 20% higher for the NAH than the NZH strain ($P < 0.001$, Table 2). However, fat and protein percentages were 12 and 14% greater

**Table 2.** Milk yield and composition and BCS of mid-lactation New Zealand (NZH) and North American (NAH) cows in a pasture-based system, 3-yr average

| | Strain[1] | | | |
|---|---|---|---|---|
| Item | NZH | NAH | SEM[2] | *P*-value |
| Milk yield (kg/d) | 24.0 | 30.0 | 1.0 | <0.01 |
| Fat (kg/d) | 1.06 | 1.06 | 0.14 | 0.97 |
| Fat (%) | 4.58 | 4.01 | 0.15 | <0.01 |
| Protein (kg/d) | 0.86 | 0.84 | 0.10 | 0.66 |
| Protein (%) | 3.70 | 3.17 | 0.04 | <0.01 |
| Lactose (kg/d) | 1.10 | 1.24 | 0.14 | <0.05 |
| Lactose (%) | 4.76 | 4.72 | 0.04 | 0.23 |
| SCM[3] (kg/d) | 25.7 | 26.4 | 1.2 | 0.39 |
| Milk urea nitrogen (mg/dL) | 24.3 | 21.6 | 0.6 | <0.0001 |
| BCS (units) | 2.59 | 2.53 | 0.10 | 0.18 |

[1]NZH = New Zealand Holstein (n = 12); NAH = North American Holstein (n = 18).

[2]All data shown as LSM ± SEM.

[3]SCM (kg) = solids-corrected milk; calculated as 12.3(F) + 6.56 (SNF) — 0.0752(M), where F, SNF, and M are expressed as kg of fat, solids-not-fat, and milk, respectively (Tyrrell and Reid, 1965).

**Table 3.** Plasma and hepatic metabolites

| | Strain[1] | | | |
|---|---|---|---|---|
| Metabolite | NZH | NAH | SEM[2] | *P*-value |
| Plasma glucose (mmol/L) | 3.54 | 2.81 | 0.17 | 0.0001 |
| Plasma urea (mmol/L) | 6.00 | 5.42 | 0.38 | 0.14 |
| Plasma NEFA[3] (mmol/L) | 0.124 | 0.116 | 0.02 | 0.53 |
| Plasma BHB (mmol/L) | 0.42 | 0.39 | 0.04 | 0.57 |
| Liver triglyceride (mg/mg of liver protein) | 2.65 | 2.67 | 0.45 | 0.68 |
| Free liver glucose (mmol/g) | 0.011 | 0.010 | 0.004 | 0.17 |
| Hepatic glycogen (mg/mg of liver tissue) | 2.21 | 1.92 | 0.17 | 0.18 |
| Triglyceride/glycogen ratio[4] | 1.45 | 1.70 | 0.4 | 0.51 |
| Free glucose/glycogen ratio | 0.0049 | 0.0057 | 0.0021 | 0.94 |

[1]NZH = New Zealand Holstein (n = 11–12); NAH = North America Holstein (n = 13–14).

[2]All data shown as LSM ± SEM.

[3]NEFA = nonesterified fatty acids.

[4]Liver triglyceride was corrected for milligrams of liver tissue, for appropriate comparison.

for NZH than NAH cows ($P < 0.001$). This resulted in similar SCM yields between strains ($P = 0.39$). In line with the higher protein percentage for the NZH strain, milk urea nitrogen was approximately 11% greater for this strain ($P < 0.0001$). No differences were observed for BCS between strains either ($P = 0.18$).

### Plasma and Hepatic Metabolites

Plasma glucose was 25% higher for the NZH versus the NAH strain ($P < 0.001$, Table 3), although no differences were found between cow strains in plasma urea ($P = 0.14$), NEFA ($P = 0.53$), or BHB ($P = 0.57$) concentrations. Neither concentrations of hepatic free glucose ($P = 0.17$), glycogen ($P = 0.18$), or triglyceride ($P = 0.68$) nor the ratios of free glucose to glycogen ($P = 0.94$) or triglyceride to glycogen ($P = 0.51$) in liver differed between Holstein strains.

### Mitochondrial Function

Oxygen consumption rates were measured in liver biopsies after the addition of specific substrates for mitochondrial chain complexes I and II, and respiratory parameters were calculated (Table 4 and Figure 1A and 1B, respectively). Oligomycin-sensitive respiration was 1.3- to 1.4-fold greater for NZH than NAH cows ($P < 0.05$) when complex I and complex II substrates were used (Figure 1C and 1D, respectively). In addition, the maximum respiratory rate was calculated for both glutamate- and malate-driven (Figure 1E) and succinate-driven respiration (Figure 1F). For glutamate- and malate-driven respiration, the maximum respiratory rate tended to be 1.2-fold higher for NZH than NAH cows ($P = 0.05$, Figure 1E); for succinate-driven respiration, the maximum respiratory rate was almost 2-fold greater for NZH versus NAH cows ($P < 0.001$, Figure 1F). Also, state 3 respiration and oligomycin-resistant respiration were higher ($P < 0.01$) in NZH than NAH cows only when succinate was used as substrate. The leaking control ratio—an index of proton leak—was higher for NAH than for NZH cows when complex I substrates were used ($P < 0.05$). However, it did not differ among strains when assessing complex II (0.61 vs. 0.66 ± 0.04, NAH and NZH respectively, $P = 0.16$). Finally, non-mitochondrial respiration did not differ between strains.

### Mitochondrial Mass

New Zealand Holstein cows presented greater hepatic mitochondrial abundance than NAH cows (Figure 2), measured as both mtDNA:nDNA ratio (1.04 vs. 0.69 ± 0.10, $P < 0.05$, 2A) and CS activity (16.5 vs. 10.7 ± 1.9 mU/mg of protein, $P < 0.05$, Figure 2B) in liver homogenates.

### Succinate Dehydrogenase Enzyme Activity and Protein Levels

Hepatic SDH activity did not differ between strains; however, when normalized by mitochondrial content assessed by CS activity, it tended ($P = 0.08$) to be 1.3-fold greater in the NAH strain (Table 5). Nevertheless, hepatic SDHA protein levels, assessed by western blot, did not differ between groups (Supplemental Figure S1, https://data.mendeley.com/datasets/gx5yh5t2bp/1).

**Table 4.** Complex I- and II-dependent respiratory parameters of NZH and NAH cows in a pasture-based system during mid-lactation, 3-yr average

| Item | Strain[1] | | SEM[2] | *P*-value |
|---|---|---|---|---|
| | NZH | NAH | | |
| Complex I respiratory parameters[3] | | | | |
| State 3 respiration | 12.4 | 10.7 | 1.3 | 0.13 |
| State 4 respiration | 6.0 | 6.2 | 1.8 | 0.74 |
| Maximum respiratory rate | 14.3 | 11.5 | 1.9 | 0.05 |
| Oligomycin-resistant respiration | 6.7 | 6.3 | 1.1 | 0.42 |
| Oligomycin-sensitive respiration | 6.1 | 4.3 | 1.9 | <0.05 |
| Non-mitochondrial respiration | 6.6 | 6.1 | 1.0 | 0.38 |
| Leaking control ratio | 0.53 | 0.62 | 0.04 | <0.05 |
| Complex II respiratory parameters[3] | | | | |
| State 3 respiration | 35.7 | 21.3 | 5.8 | 0.001 |
| State 4 respiration | 22.7 | 16.3 | 2.9 | <0.05 |
| Maximum respiratory rate | 50.2 | 26.7 | 9.0 | <0.001 |
| Oligomycin-resistant respiration | 25.3 | 16.7 | 5.1 | <0.01 |
| Oligomycin-sensitive respiration | 9.3 | 5.2 | 1.1 | <0.05 |
| Non-mitochondrial respiration | 7.3 | 8.7 | 1.0 | 0.18 |
| Leaking control ratio | 0.61 | 0.66 | 0.04 | 0.16 |

[1]NZH = New Zealand Holstein strain (n = 12); NAH = North American Holstein strain (n = 18).

[2]All data shown as LSM ± SEM.

[3]Oxygen consumption rate measurements of liver biopsies were obtained after addition of 10 m*M* glutamate and 5 m*M* malate or succinate, 4 µ*M* ADP, 2 µ*M* oligomycin, up to 4 µ*M* carbonyl cyanide-p-trifluoromethoxyphenylhydrazone and 0.5 µ*M* rotenone, and 2.5 µ*M* antimycin. Oxygen consumption rates are expressed as pmol of $O_2$/min per mg of wet weight.

### Gene Expression of Gluconeogenic Enzymes

Hepatic expression of *PC*, *PCK1*, and *PDK4* mRNA was at least 2-fold greater ($P < 0.01$) for NAH than for NZH cows (Figure 3), whereas *SDHA*, *G6PC*, *MMUT*, *PDHA1*, *PPARGC1A*, and *SIRT1* mRNA did not differ between groups (Table 6). In addition, the *PC*:*PCK1* ratio tended to be more elevated for NAH than NZH cows (2.00 vs. 1.41, $P = 0.08$). Milk yield and glycogen reserves were negative correlated (r = −0.56 and $P < 0.05$), whereas positive correlations were found between *PC* and *PCK1* mRNA (r = 0.90 and $P < 0.0001$) and *PC* and *PDK* mRNA (r = 0.51 and $P < 0.05$). Additionally, milk yield and *PC* mRNA and milk yield and *PCK1* mRNA were positively correlated (r = 0.62 and $P < 0.01$ for both).

### The PPARGC1A, SIRT1, and AMPK Axis

No differences were found in hepatic gene expression of *PPARGC1A* (1.93 vs. 0.84 ± 0.31 relative mRNA abundance, NZH and NAH respectively, $P = 0.13$) or *SIRT1* (1.75 vs. 2.04 ± 0.26 relative mRNA abundance, NZH and NAH respectively, $P = 0.46$). Moreover, neither abundance of AMPK protein nor phosphorylated AMPK (pAMPK) protein differed between NAH and NZH cows (Table 7; Supplemental Figure S2, https://data.mendeley.com/datasets/gx5yh5t2bp/1).

**Table 5.** Succinate dehydrogenase enzyme activity and protein levels in NZH and NAH cows in a pasture-based system during mid-lactation

| Item[1] | Strain[2] | | SEM[3] | *P*-value |
|---|---|---|---|---|
| | NZH | NAH | | |
| SDH (mU/mg) | 28.5 | 29.4 | 4.1 | 0.89 |
| SDH:CS | 1.65 | 2.54 | 0.3 | 0.08 |
| SDHA (relative protein abundance) | 1.07 | 1.01 | 0.09 | 0.69 |

[1]SDH = succinate dehydrogenase; CS = citrate synthase; SDHA = succinate dehydrogenase subunit A.

[2]NZH = New Zealand Holstein (n = 11); NAH = North American Holstein (n = 11).

[3]All data shown as LSM ± SEM.

## DISCUSSION

Our findings indicated differences in hepatic energy metabolism between NZH and NAH cows in a pasture-based system during mid-lactation, observed in mitochondrial function, mitochondrial density, and mRNA expression of gluconeogenic genes. Production performance between NZH and NAH strains in pasture-based systems has been studied extensively; in fact, previous studies have found that although NAH cows produced more milk, the greater milk solids content of NZH cows accounted for no differences in milk solids yield between Holstein strains (Kolver et al., 2000; Lucy et al., 2009). Indeed, in the present study, during mid-lactation in a pasture-based system, with a supplementation of 33% of predicted intake, we found that despite milk yield being greater for NAH than NZH cows, fat and protein concentrations were greater for NZH than NAH, which resulted in similar SCM yields for both strains; this is consistent with previous studies (Kolver et al., 2000; White et al., 2012; Talmón et al., 2020). In line with greater protein concentration for NZH than NAH cows, milk urea nitrogen was also greater; however, plasma urea was not affected by strain and coincided with levels reported in other pasture-based systems (Bargo et al., 2002). Although a greater BCS for NZH than NAH cows could be expected (Kolver et al., 2002), Patton et al. (2009) reported, in agreement with our results, no differences between strains in BCS in a mid-lactation study. This could be a limitation of the present work, because a single moment of lactation and season is studied. Data from the larger study, where the herd

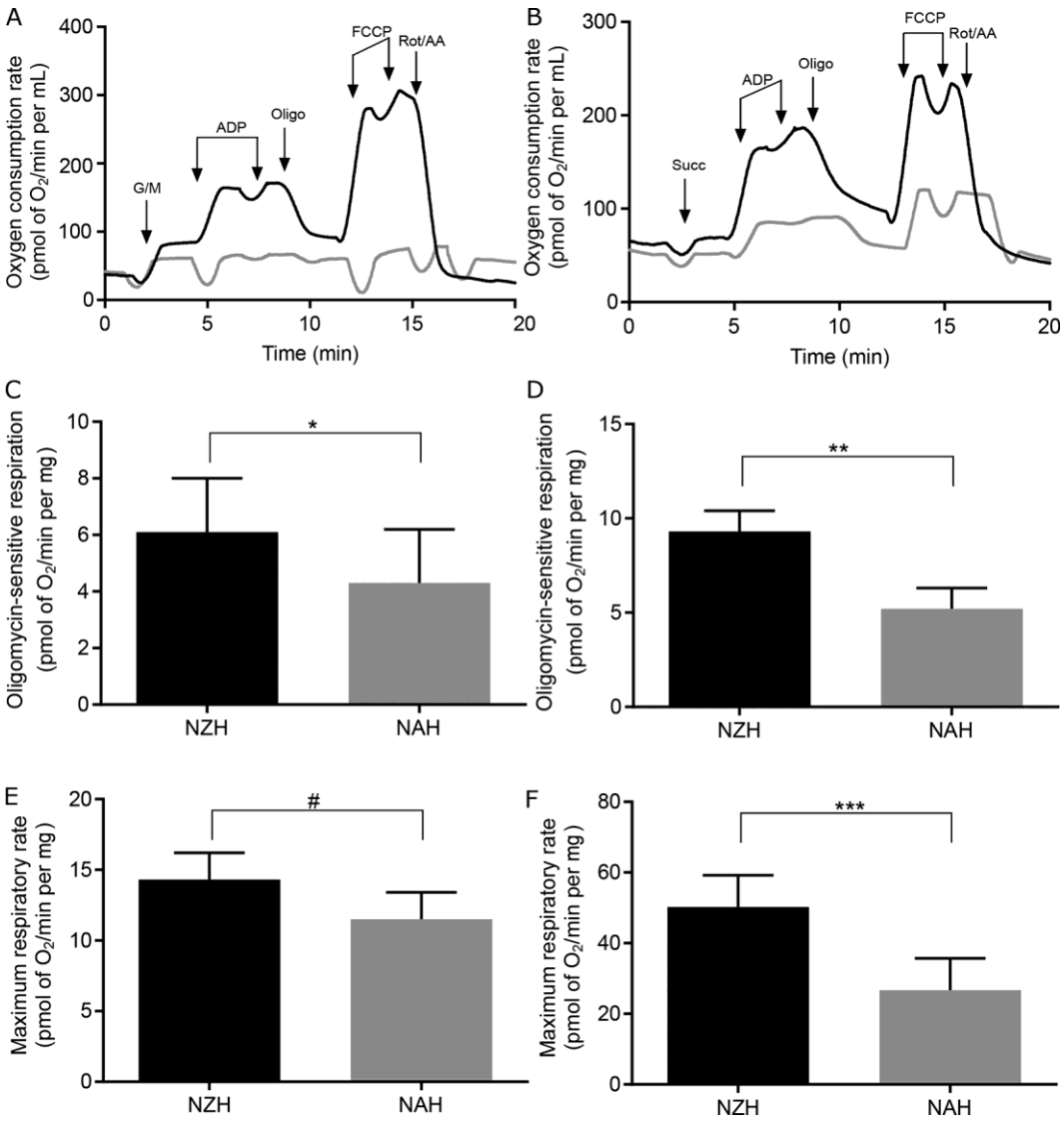


**Figure 1.** Mitochondrial function of New Zealand (NZH, n = 12) and North American (NAH, n = 18) cows in a pasture-based system during mid-lactation, 3-yr average. Oxygen consumption rates were measured in liver biopsies before and after the sequential addition of 10 m*M* glutamate and 5 m*M* malate (G/M; A, C, and E) or 20 m*M* succinate (Succ) (B, D, and F), 4 µ*M* ADP, 2 µ*M* oligomycin (Oligo), up to 4 µ*M* carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), and 0.5 µ*M* rotenone and 2.5 µ*M* antimycin (Rot/AA). Panels A and B show representative traces of oxygen consumption rates obtained for liver biopsies of NZH (black) and NAH (gray) cows. C and D show oligomycin-sensitive respiration, and E and F show maximum respiratory rate, obtained from oxygen consumption rate measurements performed as described in A and B. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and #0.05 < *P* < 0.10. Data represent LSM ± SEM.

is taken into consideration during 2 years, points out that mean BCS was lower for the NAH than for the NZH strain (Stirling et al., 2021).

Negative energy balance markers such as plasma NEFA, plasma BHB, and liver triglyceride did not indicate subclinical ketosis (>1.0 mmol/L of plasma BHB) or clinical fatty liver (>10% liver triglyceride wet weight; Bobe et al., 2004; Meikle et al., 2004) and were similar to previously reported values for mid- to late lactation in grazing conditions (García-Roche et al., 2021). However, the NZH cows had greater plasma glucose concentrations than the NAH cows, probably due to greater glucose uptake by the mammary gland (Bell and Bauman, 1997), as milk yield in NAH cows was greater.

Liver energy homeostasis is crucial to maintain 2 major pathways in dairy cows: gluconeogenesis and detoxification of ammonia via the urea cycle (White, 2020). Oxidative phosphorylation provides the main source for ATP in the cell, and this is the predominant function of mitochondria. Additionally, mitochondria are dynamically regulated to cater for a broad spectrum of energy-

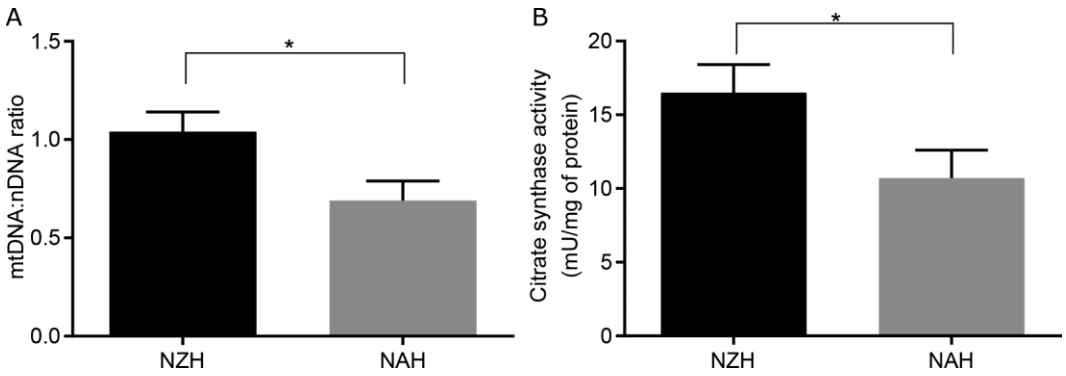


**Figure 2.** Mitochondrial abundance in New Zealand (NZH, n = 11) and North American (NAH, n = 11) cows in a pasture-based system during mid-lactation, 3-yr average. (A) Ratio of mitochondrial DNA to nuclear DNA (mtDNA:nDNA) assessed by real-time PCR in liver biopsies of NZH (black) and NAH (gray) cows. Results are expressed relative to NZH cows. (B) Citrate synthase activity measured in liver biopsies of NZH (black) and NAH (gray) cows. Data represent LSM ± SEM. *$P < 0.05$.

demanding situations, and their inability to respond adequately may be considered an impairment (Brand and Nicholls, 2011). Our previous work found that, in grazing cows, mitochondrial function was impaired during early lactation, which was correlated with increased liver triglyceride content as well as protein acetylation, but restored toward late lactation (García-Roche et al., 2019). However, this study indicated differences in mitochondrial function between Holstein strains during mid-lactation in a pasture-based system. Parameters related to ATP synthesis and maximum respiratory rate were increased for NZH compared with NAH cows; differences were more dramatic when complex II substrates were used. In spite of this, when evaluating the isolated activity of SDH, which evaluates only the catalytic oxidoreductase activity, it tended to be increased for NAH compared with NZH cows, suggesting that decreased respiratory parameters when complex II substrates were used in NAH cows may be related to overall oxidative phosphorylation capacity impairment (Bandara et al., 2021). Indeed, in human models, mitochondrial complex II is related to respiratory reserve capacity and is increased when PDK is inhibited (Pfleger et al., 2015); consistent with this, in our study hepatic *PDK4* was greater in NAH than NZH cows. Also, a recent study developed an SDH subunit D (SDHD) knockout mutant in human kidney cells and demonstrated that this decreased not only complex II-dependent respiration but also complex I-dependent respiration, given the repercussion of SDHD in the TCA cycle, which could have decreased the synthesis of reduction equivalents (Bandara et al., 2021).

Along with the assessment of parameters related to ATP synthesis, we also assessed proton leak—a phenomenon that consumes part of the protonmotive force and thus compromises ATP turnover—by means of the leaking control ratio (Brand and Nicholls, 2011; Koliaki et al., 2015) and found it was higher for NAH than for NZH cows ($P < 0.05$) when complex I substrates were used, although this was not the case when complex II substrates were used ($P = 0.16$). It is important to

**Table 6.** Hepatic expression of gluconeogenic enzyme genes for NZH and NAH cows in a pasture-based system during mid-lactation

| | Strain[2] | | | |
|---|---|---|---|---|
| Gene[1] | NZH | NAH | SEM[3] | *P*-value |
| *SDHA* | 1.45 | 1.69 | 0.21 | 0.28 |
| *PC* | 0.76 | 2.23 | 0.33 | <0.01 |
| *PCK1* | 0.54 | 1.11 | 0.11 | <0.01 |
| *PDK4* | 0.48 | 0.95 | 0.21 | <0.05 |
| *G6PC* | 2.30 | 2.05 | 0.50 | 0.50 |
| *MMUT* | 0.43 | 0.50 | 0.11 | 0.37 |
| *PDHA1* | 2.02 | 1.82 | 0.29 | 0.62 |

[1]Genes: glucose-6-phosphatase (*G6PC*), methylmalonyl-CoA mutase (*MMUT*), succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (*SDHA*), pyruvate carboxylase (*PC*), phosphoenolpyruvate carboxykinase (*PCK1*), pyruvate dehydrogenase E1 subunit α 1 (*PDHA1*), pyruvate dehydrogenase kinase (*PDK4*).

[2]NZH = New Zealand Holstein (n = 11); NAH = North American Holstein (n = 11).

[3]All data shown as LSM ± SEM.

**Table 7.** Protein abundance of AMPK and pAMPK for NZH and NAH cows in a pasture-based system during mid-lactation

| | Strain[2] | | | |
|---|---|---|---|---|
| Protein[1] | NZH | NAH | SEM[3] | *P*-value |
| AMPK | 1.00 | 1.11 | 0.095 | 0.47 |
| pAMPK | 1.06 | 1.16 | 0.177 | 0.61 |
| pAMPK/AMPK | 1.07 | 0.99 | 0.200 | 0.54 |

[1]AMPK = AMP-activated protein kinase; pAMPK = phosphorylated AMP-activated protein kinase.

[2]NZH = New Zealand Holstein (n = 11); NAH = North American Holstein (n = 11).

[3]All data shown as LSM ± SEM.

bear in mind that studies assessing mitochondrial function by respiration measure the respiration rate, which represents the proton current generated by substrate oxidation, which then flows through the protonmotive force and is afterward divided between proton leak and ATP turnover (Brand and Nicholls, 2011). Hence, differences between 2 groups of animals can be observed at different levels of ATP turnover and steady levels of proton leak, or vice-versa, or even at different levels of both ATP turnover and proton leak.

In general terms, functional loss of any of the complex II subunits could account for the lower complex II and complex I respiration in NAH cows. It has been widely reported that functional loss or inhibition of complex II could lead to reactive oxygen species, which could further damage mitochondrial function (Dröse, 2013; Hadrava Vanova et al., 2020). An impairment of mitochondrial function can result in a deficient energy supply and hinder hepatic functions further progressing in disease, such as nonalcoholic fatty liver (Rector et al., 2010) and insulin resistance (Galgani et al., 2008; Peinado et al., 2014), as shown in mice and human models.

White et al. (2012) determined mRNA expression of gluconeogenic genes and hypothesized that NAH cows had decreased TCA cycling compared with NZH cows. Possible causes that explain impairment of the TCA cycle, a major contributor of reduction equivalents, are related to depletion of intermediates, yielding a drop in NADH/NAD+ ratios to the detriment of ATP synthesis (Burgess et al., 2004). Indeed, the TCA cycle in the dairy cow represents a major point of control, where oxaloacetate and acetyl-CoA are located in the crossroads; oxaloacetate levels may be depleted due

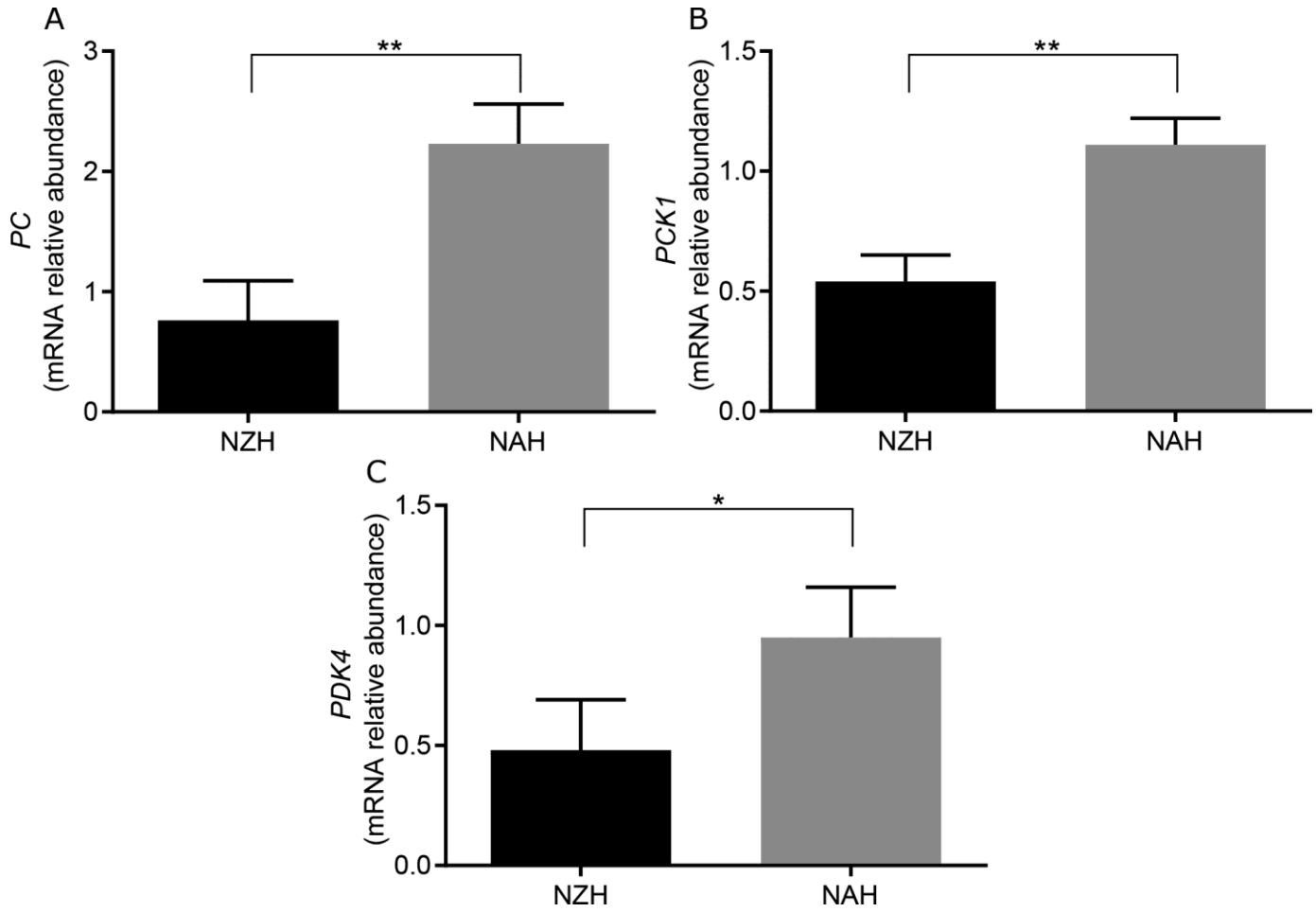


**Figure 3.** Hepatic gene expression of *PC*, *PCK1*, and *PDK4* for New Zealand Holstein (NZH) and North American Holstein (NAH) cows in a pasture-based system during mid-lactation. (A) Relative mRNA abundance of *PC* in liver biopsies of NZH (black, n = 11) and NAH (gray, n = 11) cows. (B) Relative mRNA abundance of *PCK1* in liver biopsies of NZH (black, n = 11) and NAH (gray, n = 11) cows. (C) Relative mRNA abundance of *PDK4* in liver biopsies of NZH (black, n = 11) and NAH (gray, n = 11) cows. **P* < 0.05, and ***P* < 0.01. Data represent LSM ± SEM.

to demanding levels of gluconeogenesis (Allen, 2014; White, 2015), and acetyl-CoA concentration may be overloaded due to high levels of fatty acid oxidation, especially during early lactation (Adewuyi et al., 2005), or acetyl-CoA levels may be decreased as pyruvate is used for gluconeogenesis (Akbar et al., 2013).

In our work, NAH cows showed increased mRNA expression of key enzymes of gluconeogenesis (*PC*, *PCK1*, *PDK4*) compared with NZH cows. In addition, as lactation progresses, NZH cows reduced metabolizable energy intake and milk yield compared with NAH cows (Talmón et al., 2020). Indeed, the positive correlation between milk yield and *PCK1* mRNA, the rate-limiting step of gluconeogenesis, confirmed that gluconeogenesis is the driver of milk production (Aschenbach et al., 2010). In addition, the *PC:PCK1* ratio tended to be higher for NAH than NZH cows, indicating that oxaloacetate for gluconeogenesis was derived from precursors other than propionate (Weld et al., 2019). Moreover, *MMUT* mRNA levels in this work were similar to those previously reported in pasture-fed cows, lower than those of cows fed TMR (García-Roche et al., 2021). This emphasizes the relevance of turning to mobilization of lipid and protein reserves for alternative gluconeogenic precursors in a pasture-based system and was in agreement with the increased *PC* mRNA abundance in NAH versus NZH cows. In fact, the positive correlation between *PC* and *PCK1* mRNA suggests that gluconeogenesis is mostly sustained by gluconeogenic precursors that enter the pathway at the pyruvate carboxylase step. Similar positive correlations are observed between milk yield and *PC* mRNA and milk yield and *PCK1* mRNA. Furthermore, studies performed in dairy cows with propionate infusions have pointed out that mitochondrial increases of the NADH/NAD+ ratio may cause malate to be shunted toward gluconeogenesis, because mitochondrial malate dehydrogenase—the enzyme that catalyzes the conversion of malate to oxaloacetate—requires NAD+ (Zhang et al., 2015; Kennedy and Allen, 2019). This is especially relevant in models with propionate infusions, where the contribution of pyruvate from alternative precursors—evidenced by *PC* mRNA abundance—in the gluconeogenic pathway is scarce (Zhang et al., 2015). In our study, in contrast, we did find a relevant contribution of pyruvate from alternative precursors in the gluconeogenic pathway, as the correlations between *PC* and *PCK1* mRNA and *PC* mRNA and milk yield were positive. By contrast, succinate dehydrogenase, an enzyme that has a role in electron transfer in the respiratory chain and also catalyzes a step in the TCA cycle in between propionate conversion to methylmalonyl-CoA and oxaloacetate formation, showed greater activity for NAH than for NZH cows, when corrected by mitochondrial mass. Hence, it is possible that gluconeogenesis from propionate and non-propionate precursors was increased in NAH cows. Neither abundance of mRNA and protein of SDHA nor abundance of *MMUT* mRNA differed between Holstein strains. Transcriptional or translational regulation of the steps that mediate mitochondrial propionate conversion into oxaloacetate to supply gluconeogenesis in bovine liver, which are little-known, could explain these results (Aschenbach et al., 2010).

In this work, we found that both *PCK1* mRNA and *PC* mRNA were increased in NAH cows; however, a previous work studying the expression of gluconeogenesis enzyme genes in different Holstein strains during early lactation while feeding less concentrate that the current study (White et al., 2012) found no differences in *PCK1* mRNA and that *PC* mRNA tended to be increased in NZH compared with NAH cows. These contrasting results could be due to differences in stage of lactation and level of supplementation, as both lactation stage and nutrition affect *PC* mRNA expression in dairy cattle (Greenfield et al., 2000; Velez and Donkin, 2005; García-Roche et al., 2021).

It is well established that glucose requirements of the gravid uterus increase appreciably during the latter half of pregnancy (Bell and Bauman, 1997). However, glucose demands of the gravid uterus might not have had a definitive effect in our results, because all the animals in this study were in the first trimester of pregnancy. Nonetheless, if the intrinsic differences in glucose metabolism observed among strains in this work during mid-lactation were also present during early lactation, this could in part explain the higher number of services per conception reported in the NAH versus the NZH strain in a maximum pasture treatment (Stirling et al., 2021), as low blood glucose concentrations after calving are associated with infertility in postpartum dairy cows (Green et al., 2012; Lucy et al., 2013).

Although we found no differences in hepatic *PDHA* expression, *PDK4* mRNA was greater for NAH than for NZH cows, further confirming that gluconeogenic precursors such as alanine and lactate would be channeled toward oxaloacetate instead of acetyl-CoA, as PDH activity is inhibited by PDK4 phosphorylation (Akbar et al., 2013). Studies in human and mice models have shown that downregulation of PDK4 increased ATP production, as it favors oxidative phosphorylation by yielding acetyl-CoA to the TCA cycle (Crewe et al., 2017; Liu et al., 2017). In line with our results, a previous study in mid-lactation feed-restricted cows found a downregulation of complex I proteins and upregulation of the gluconeogenic genes *PC* and *PDK4* (Akbar et al., 2013). Thus, impaired TCA cycling due to scarcity of the precursors oxaloacetate and acetyl-CoA could explain the reduced mitochondrial function related to

ATP synthesis in NAH cows relative to NZH cows. In sum, the hepatocyte of the lactating cow faces a rather controversial dilemma: gluconeogenesis versus energy production, sustained glucose output or healthy liver.

Mitochondrial function is crucial for cellular metabolism, and for this reason mitochondria have powerful feedback loops in order to maintain levels of ATP synthesis (Brand and Nicholls, 2011). One of the strategies used to face increasing energy demands is increase in mitochondrial density (Pesta et al., 2011). We studied mitochondrial abundance, measuring the maximum respiratory rate (García-Roche et al., 2018), CS activity, and mtDNA-to-nDNA ratio, and observed that they were increased for NZH compared with NAH cows. This could be a plausible mechanism to explain the ameliorated mitochondrial function in NZH cows. For instance, transition dairy cows with mild fatty liver have been found to present increased expression of oxidative phosphorylation complexes as well as mtDNA-to-nDNA ratio, probably as a response to impaired liver function due to lipid accumulation (Du et al., 2018). However, an increased ratio of mtDNA to nDNA, or any other proxy for mitochondrial abundance, does not necessarily reflect mitochondrial biogenesis and could also be a result of reduced degradation of damaged mitochondria, as observed previously in hepatic biopsies of human patients with nonalcoholic steatohepatitis (Koliaki et al., 2015). Increased mitochondrial abundance in cattle has been associated with increased adaptive capacity in several production systems. For instance, steers with low residual feed intake presented greater mitochondrial abundance and CS activity than steers with high residual feed intake; in other words, high-efficiency steers probably partitioned nutrients more efficiently toward energy metabolism (Casal et al., 2018). Moreover, a recent work carried out in high and low feed-efficient dairy cattle during weeks −4, 2, and 12 postpartum studied net fat oxidation, heat production, and mtDNA-to-nDNA ratio, and found that the high feed-efficient group had lower heat production (**HP**) per kilogram of metabolic BW ($BW^{0.75}$), as well as a higher number of relative mtDNA copies, pointing out that lower feed efficiency could result from fewer hepatic mitochondria and greater HP losses (Kennedy et al., 2021). Indeed, during yr 2 of the present study, our group studied energy partitioning and found that, at 192 DIM, residual HP per kilogram of $BW^{0.75}$ was lower in NZH than NAH cows. In particular, because HP is a function of maintenance and production, and no differences were found in retained energy among strains, differences in residual HP could be due to increased maintenance energy requirements of NAH cows (Talmón et al., 2020). Lower whole-body residual HP and higher numbers of relative mtDNA copies, combined with increased mitochondrial function favoring ATP synthesis, suggests that NZH cows could indeed be more feed efficient that NAH cows (Kennedy et al., 2021).

We explored whether increases in mitochondrial mass and activity in NZH cows could be due to mitochondrial biogenesis. However, mRNA expression of *PPARGC1A* and *SIRT1*, which encode PGC1α and SIRT1 master regulators of biogenesis (Cantó and Auwerx, 2009), did not differ between strains. Although gene expression was not altered in this study, protein expression and activity could be modulated by post-translational modifications, as protein expression of PGC1α has been reported to be increased in dairy cows with increased mitochondrial abundance (Du et al., 2018). Indeed, a mechanism of PGC1α activation is acetylation by SIRT1, and, in turn, SIRT1 is activated by AMPK by increasing levels of the oxidized electron carrier NAD+ (Cantó et al., 2009). Our recent work in dairy cattle in a TMR versus a pasture-based system showed that AMPK and its effector PGC1α could be upregulating gluconeogenic genes, alongside its positive association with SIRT1 (García-Roche et al., 2021). However, we did not observe differential activation of this pathway between the 2 strains.

The activation of AMPK has been studied in cell culture and mice models and has been shown to modulate energy metabolism in response to high AMP or low ATP levels, and its effects include switching to catabolic pathways, enhancing oxidative phosphorylation, and, as a result, inducing complete oxidation (Cantó et al., 2009; Jenkins et al., 2013). The present results did not show differences in phosphorylated AMPK for NZH versus NAH cows under pasture-based conditions during late mid-lactation. This could be explained, as previous authors have hypothesized, because the AMPK pathway is not an eligible indicator for whole-animal energy balance, as differences among cow groups with high and low liver fat contents were not elicited; however, its increase during early lactation is relevant (Schäff et al., 2012). To confirm this, we have previously shown that AMPK activation is a phenomenon that indeed takes place during early lactation but not during late lactation (García-Roche et al., 2021). Although it is possible that the AMPK signaling pathway does not play a significant role in cows of diverging strains during mid-lactation, the molecular mechanisms regulating hepatic nutrient partitioning still lack resolution of understanding. Hence, further studies emphasizing the activation of PGC1α by means of post-translation modifications, its activity, and its effect on hepatic cellular energy metabolism of dairy cows, would be useful.

## CONCLUSIONS

Our results showed that Holstein cows of New Zealand origin presented improved hepatic mitochondrial function compared with cows of North American origin during mid-lactation in a pasture-based system, as respiration parameters related to ATP synthesis were increased, while maintaining similar milk solids production. Furthermore, Holstein cows of North American origin have greater expression of gluconeogenic genes, which probably translates into decreased availability of reducing equivalents for oxidative phosphorylation, as neither pyruvate nor oxaloacetate are fueling the TCA cycle. In addition to improved mitochondrial function, Holstein cows of New Zealand origin had increased mitochondrial mass, which could be a plausible mechanism for augmenting the respiratory capacity. Although this work shows dramatic differences in hepatic cellular energy metabolism, further research is needed to elucidate the mechanistic regulation underlying this phenomenon. Moreover, information regarding cellular energy metabolism presented in this study could be useful for the selection of a genetic strain that is better adapted for a production system with a high inclusion of pastures.

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## 7. HEPATIC METABOLISM OF GRAZING HOLSTEIN COWS OF DIVERGING GENETIC ORIGIN SUPPLEMENTED WITH TOTAL MIXED RATION OR CONCENTRATE DURING LACTATION

García-Roche M, Talmón D., Cañibe G., Astessiano A. L., Mendoza A., Cassina A., Quijano C., and Carriquiry M.
Versión borrador.

Este trabajo tuvo como objetivo caracterizar las reservas energéticas hepáticas, la función mitocondrial y la acetilación de proteínas mitocondriales durante la lactancia. Además, mediante estudios de metabolómica y expresión génica, se intentó dilucidar las vías metabólicas diferenciales entre las distintas condiciones. Para ello se utilizaron vacas del modelo 2 (vacas Holstein NAH y NZH sometidas a dos estrategias de alimentación pastoriles, una con suplementación de concentrado (MaxP) y otra con suplementación con DTM (FixP) durante la lactancia. Nuestros resultados demostraron que durante lactancia temprana, los niveles de glucógeno en hígado ($P < 0.001$) disminuyeron mientras que los niveles de triglicéridos aumentaron ($P < 0.0001$), a su vez, la actividad carnitina palmitoiltransferasa (CPT) y la función mitocondrial se vieron disminuidas durante lactancia temprana ($P < 0.001$). El aumento de triglicéridos y disminución de la función mitocondrial y actividad CPT se vieron especialmente agravados en la estrategia MaxP ($P < 0.05$) sin efecto del genotipo. Por otra parte, los niveles de acetilación mitocondrial fueron más altos para el genotipo NAH en la estrategia MaxP, confirmando su asociación con la disfunción mitocondrial. Por otra parte, la expresión génica hepática de enzimas relacionadas con el metabolismo de los ácidos grasos: acil-CoA deshidrogenasa de cadena muy larga ($P < 0.05$) y acetil-CoA acetiltransferasa ($P = 0.08$) junto con reguladores transcripcionales: receptor activado por proliferador de peroxisomas alfa ($P = 0.08$), receptor de ácido retinoico alfa ($P < 0.05$) y receptor X retinoico beta ($P < 0.05$) aumentó en vacas NZH vs. vacas NAH durante la lactancia media tardía, mientras que la

expresión génica de la molécula CD36 fue menor en la estrategia MaxP que la estrategia FixP ($P < 0.05$). El análisis metabolómico de las vacas NAH en ambas estrategias de alimentación durante lactancia media tardía mostró que el ciclo de la urea se enriqueció en la estrategia MaxP y que la vía de señalización del fosfatidilinositol, la gluconeogénesis, el metabolismo de las purinas y las vías de las pentosas fosfato se enriquecieron en la estrategia FixP, lo que sugiere que las vacas FixP se volcaron hacia vías anabólicas durante la lactancia media tardía.

## Hepatic metabolism of grazing Holstein cows of diverging genetic origin supplemented with total mixed ration or concentrate during lactation

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**Abstract**

The objective of the study was to characterize hepatic metabolic adaptations of grazing Holstein cows of diverging origin supplemented with a total mixed ration or an energy-protein concentrate. Multiparous autumn calving Holstein cows of New Zealand and North American genetic origin grazed in one or two sessions and received supplementation of concentrate in the form of a total mixed ration or pelleted in the milking parlor from May through November of 2018. Hepatic biopsies were taken at - 45 ± 17, 21 ± 7, 100 ± 23 and 180 ± 23 days in milk. Solids corrected milk peaked at 21 DIM, especially for the strategy with two grazing sessions and supplementation of concentrate and solids corrected milk was greater for the New Zealand Holstein than the North American Holstein strain during late mid-lactation. During early lactation, liver glycogen decreased and liver triglyceride levels increased, followed by a decrease in mitochondrial function and carnitine palmitoyltransferase activity.

The increase in liver triglyceride levels and decrease in hepatic mitochondrial function and carnitine palmitoyltransferase activity was exacerbated in the strategy that combined maximum grazing and supplementation with a commercial energy-protein concentrate. Both mitochondrial function and carnitine palmitoyltransferase activity correlated negatively with liver triglyceride levels. Mitochondrial acetylation in liver biopsies was increased in North American Holstein cows under the maximum grazing strategy and was positively correlated with liver triglyceride levels. Hepatic gene expression of enzymes related to fatty acid metabolism: very long-chain acyl-CoA dehydrogenase and acetyl-CoA acetyltransferase alongside transcriptional regulators: peroxisome proliferator-activated receptor alpha, retinoic acid receptor alpha and retinoic X receptor beta was increased in New Zealand vs. North American cows during late mid-lactation, while gene expression of CD36 molecule was decreased in the strategy with maximum grazing. Metabolomic analysis of North American Holstein cows in both feeding strategies during late mid-lactation showed that the urea cycle was enriched in the strategy with maximum grazing and that the phosphatidylinositol signaling system, gluconeogenesis, purine metabolism and pentose phosphate pathways were enriched in the strategy with one grazing session and supplementation of total mixed ration, suggesting that cows in the latter grazing system shifted their hepatic metabolome toward anabolic pathways during late mid-lactation.
**Keywords: Mitochondrial function, acetylation, metabolomics, pasture, dairy cattle, Holstein-Friesian**

## Introduction

High-yielding dairy cows undergo extreme metabolic adaptions during lactation. The transition from gestating to lactating represents a period of excessive nutrient demands which requires the coordination of multiple tissues and will be crucial for the success of the oncoming lactation (Bauman, 2000; Roche et al., 2013). Particularly, during the transition period, dairy cows are especially susceptible to transition period disorders which may be caused in

part by the mobilization of energy, protein, vitamin and mineral body reserves (Drackley, 1999).

In addition to this, the inclusion of pastures in dairy systems represents lower feeding costs (Bargo et al., 2003) turning mixed grazing systems into a competitive alternative for the intensification of dairy systems, especially in exporting countries (Fariña and Chilibroste, 2019; Stirling et al., 2021). Furthermore, the efficiency of dairy systems depends on productive and reproductive characteristics and in turn, productive performance is closely tied to ensuring sufficient dry matter (DM) intake (Bargo et al., 2002b; Chilibroste et al., 2012) and good quality pastures (Bargo et al., 2003). When nutrient concentration in the diet is insufficient, excessive mobilization of body reserves during early lactation may present higher levels of negative energy balance markers such as non-esterified fatty acids, beta-hydroxybutyrate and liver triglyceride (Meikle et al., 2013a; García-Roche et al., 2021). The liver, in turn, plays a key role in the metabolism of carbohydrates, proteins and lipids (Wei et al., 2008) and is key in the regulation of cholesterol and triglyceride metabolism alongside the maintenance of homeostasis and blood glucose levels (Knebel et al., 2015). However, hepatic mitochondrial function -key in energy metabolism- is decreased in grazing cows early lactation when compared to cows fed total mixed ration (TMR) and is associated to increased mitochondrial acetylation levels (García-Roche et al., 2019). Also, retained energy in milk and tissue are lower in primiparous cows grazing system with supplementation of TMR vs. cows in a TMR system this is probably due to the higher maintenance requirements associated with grazing and walking activities (Jasinsky et al., 2019).

In line with this, the suitability of the Holstein strain is also a crucial matter, since the New Zealand Holstein (NZH) strain has lower maintenance costs than the North American Holstein (NAH) strain during late lactation when managed in a grazing system with supplementation of a commercial

concentrate (Talmón et al., 2020). Indeed, the New Zealand Holstein (NZH) strain has shown to be more suitable for grazing systems than the North American Holstein (NAH) strain since it maintains higher average body condition score, body weight and energy and feeding efficiency (Kolver et al., 2000; Stirling et al., 2021; Talmón et al., 2022). Alongside, from a metabolic standpoint, the NAH strain presents uncoupling of the somatotropic axis during early lactation while the NZH strain (Lucy et al., 2009). Herein, we hypothesize that cows subjected to the system with higher inclusion of grazing will mobilize body reserved at a higher level than cows supplemented with TMR during the transition period which will translate into mitochondrial impairment and higher acetylation levels; furthermore, cows of the NZH strain will present an improved metabolic adaptation that cows on the NAH strain.

## Materials and Methods

The experiment was conducted at the Experimental Station of the Instituto Nacional de Investigación Agropecuaria (Colonia, Uruguay) during the 2018. Animal use and procedures were approved by the Animal Experimentation Committee (CEUA) of the Instituto Nacional de Investigación Agropecuaria, Uruguay (file number: INIA2017.2).

The experiment formed part of a larger study designed to evaluate the effect of cow strain (NZH vs. NAH) in different feeding strategies on individual animal and whole-farm biophysical performance. A more detailed description of Holstein strains, animal and grazing management, as well as milk production, body weight (BW) and body condition score (BCS) from June 2017 through May 2019 were previously reported (Stirling et al., 2021).

### *Experimental Design*

Multiparous autumn calving Holstein cows of NZH (538 ± 63 kg BW and 3.23 ± 0.19 BCS at calving); N = 24 and NAH (582 ± 59 kg BW, 3.03 ± 0.28 BCS at calving; N = 24) genetic origin were used in order to evaluate the effect of

supplementing with a total mixed ration (TMR) or energy-protein concentrate on hepatic metabolism.

Both NZH and NAH strains had at least 75% of each cow's ancestors (2 generations: father and maternal grandfather) from New Zealand, or from the United States or Canada respectively (Stirling et al., 2021). In the present study, the NZH strain presented a progeny of eleven sires and the NAH strain presented a progeny of twelve sires. The economic and productive selection index was 125 ± 10 and 106 ± 14 on average for NZH and NAH cows, respectively. The expected progeny difference was -141 ± 179 kg, +0.134 ± 0.148 % and +0.142 ± 0.07 % for milk yield, milk fat and milk protein content for NZH cows, while it was +45 ± 177 kg, +0.06 ± 0.16 % and +0.01 ± 0.07 % for milk yield, milk fat and milk protein content for NAH cows (Mejoramiento y Control Lechero Uruguayo; https://www.mu.org.uy).

In the prepartum period cows were managed as single groups within each strain, cows grazed on *Dactylis glomerata* and *Medicago sativa* (7.8 ± 1.13 vs. 9 ± 0.85 kg DM/d/cow for NZH and NAH) and were offered a TMR (10.7 ± 1.13 vs. 13.05 ± 0.92 kg DM/d/cow for NZH and NAH) with a 65:35 forage to concentrate ratio DM basis with water *ad libitum*. Cows were paired in each strain group according to their calving date (May 6, 2018 ± 20 days) and lactation number (3.1 ± 1.0 lactations). Cows of both strains were milked twice a day and at calving were allocated in two types of mixed grazing systems, either pasture DM intake was fixed to 33% of total DM intake (FixP) and cows had access to one grazing session during the daytime after the AM milking session (0500 to 1400 h) and were supplemented with TMR in feedpads after the PM milking session (1500 to 0400 h) or maximum grazed pasture with concentrate supplementation (MaxP). Predicted DM intake was estimated weekly according to the National Research Council (NRC) model for Dairy Cattle (2001) in order to adjust pasture forage allowance and supplementation. Concentrate was offered as 33% of total DM intake as annual average for both

treatments. In the FixP strategy concentrate was offered in the TMR composed of high-moisture corn grain (47% on a DM basis), soybean meal (37%), soy hulls or wheat bran (16%), urea, sodium bicarbonate, bicalcium phosphate, magnesium oxide, yeast, and a mineral-vitamin and in the MaxP strategy a pelleted commercial concentrate was offered in the milking parlor. Cows grazed on daily strips of *Dactylis glomerata* and *Medicago sativa* or *Festuca arundinacea* in a rotational-grazing manner, each strain group grazed on separate paddocks to assure similar pasture allowance (5 cm above ground level) relative to their BW. In the case of the MaxP strategy, when two grazing sessions were not possible due to pasture growth, cows had one a.m. grazing session and after p.m. milking remained in the loafing pad with access conserved forage composed of corn silage and pasture haylage. Water was offered *ad libitum* in each paddock or feedpad. Details of the grazing management were presented previously (Stirling et al., 2021) and offered DM intake and chemical composition and metabolizable energy concentration of feedstuffs are presented in Tables 1 and 2, respectively. Briefly, pasture was offered in daily strips which cows of each genotype grazed separately to keep similar herbage allowance relative to their BW and to ensure breeds behaved independently and avoid dominance. As cows grazed pastures in a rotational-grazing manner they returned to defined grazing areas once most the grass tillers had between 2.5 and 3 leaves.

Table 1. Offered dry matter intake of pasture, concentrate and conserved forage (mean ± SD) grazing cows of NAH and NZH genetic origin supplemented with total mixed ration (FixP) or an energy-protein concentrate (MaxP).

| | Stage of lactation | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 21 DIM | | | | 100 DIM | | | | 180 DIM | | | |
| | NZH | | NAH | | NZH | | NAH | | NZH | | NAH | |
| | FixP | MaxP | FixP | MaxP | FixP | MaxP | FixP | MaxP | FixP | MaxP | FixP | MaxP |
| Offered DM intake (kg/d/cow) | | | | | | | | | | | | |
| Pasture | 5.8 ± 2.4 | 7.7 ± 3.6 | 7.3 ± 3.0 | 8.8 ± 4.2 | 6.1 ± 1.1 | 9.5 ± 4.7 | 7.7 ± 0.5 | 11.2 ± 4.8 | 6.7 ± 0.5 | 10.1 ± 1.9 | 7.7 ± 0.7 | 11.9 ± 2.4 |
| Concentrate | - | 6.7 ± 0.1 | - | 7.7 ± 0.2 | - | 7.3 ± 0.2 | - | 8.3 ± 0.3 | - | 6.3 ± 0.4 | - | 7.2 ± 0.4 |
| Conserved forage[1] | - | 4.5 ± 3.8 | - | 5.2 ± 4.0 | - | 4.5 ± 4.3 | - | 4.6 ± 4.4 | - | 4.1 ± 1.7 | - | 4.4 ± 0.8 |
| Total mixed ration[2] | 13.1 ± 2.6 | - | 14.8 ± 2.6 | - | 15.1 ± 0.9 | - | 16.5 ± 0.4 | - | 13.7 ± 0.8 | - | 15.8 ± 0.7 | - |
| Total DM intake | 18.9 ± 0.5 | 18.9 ± 0.5 | 22.0 ± 0.5 | 21.7 ± 0.6 | 21.2 ± 0.3 | 21.2 ± 0.3 | 24.2 ± 0.3 | 24.2 ± 0.3 | 20.5 ± 0.2 | 20.5 ± 0.1 | 23.5 ± 0.1 | 23.5 ± 0.1 |

DIM = Days in milk, NZH = New Zealand Holstein; NAH = North American Holstein.

[1.] Composed of corn silage and pasture haylage in a 75:25 ratio in DM basis in average throughout the experimental period. 2. 55:45 forage:concentrate ratio in DM basis. SD represents the variation between within the experimental period.

Table 2. Chemical composition and metabolizable energy concentration (means ± SD) of feedstuffs offered during the experiment.

DIM = Days in milk, NZH = New Zealand Holstein; NAH = North American Holstein; DM = dry matter; CP = crude protein; NDF = neutral detergent fiber; ADF = acid detergent fiber; ME = metabolizable energy.
SD represents the variation between feedstuff samples.

| | -45 DIM | | 21 DIM | | | | 100 DIM | | | | 180 DIM | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Pasture | TMR | Pasture | Concentrate | Conserved forage | TMR | Pasture | Concentrate | Conserved forage | TMR | Pasture | Concentrate | Conserved forage | TMR |
| DM (%) | 30.1 ± 5.5 | 54.3 ± 8.6 | 17.0 ± 5.5 | 90.1 ± 0.8 | 40.5 ± 7.9 | 50.9 ± 3.8 | 20.8 ± 4.1 | 88.8 ± 0.3 | 43.5 ± 10.0 | 54.3 ± 8.6 | 23.7 ± 2.1 | 88.7 ± 0.5 | 50.1 ± 13.0 | 54.3 ± 8.6 |
| CP (% DM) | 19.8 ± 2.3 | 12.8 ± 2.9 | 24.6 ± 1.9 | 20.6 ± 1.4 | 11.8 ± 4.0 | 16.2 ± 2.5 | 24.4 ± 1.6 | 22.4 ± 0.6 | 13.2 ± 4.6 | 16.3 ± 1.3 | 24.8 ± 2.8 | 21.6 ± 0.4 | 12.7 ± 5.5 | 14.7 ± 0.2 |
| NDF (% DM) | 45.2 ± 5.2 | 41.6 ± 3.6 | 48.4 ± 1.6 | 30.1 ± 4.3 | 47.4 ± 10.0 | 34.8 ± 0.8 | 51.5 ± 3.5 | 30.3 ± 4.8 | 45.2 ± 7.0 | 33.9 ± 1.9 | 50.7 ± 3.6 | 26.8 ± 2.0 | 45.5 ± 9.3 | 37.9 ± 3.2 |
| ADF (% DM) | 28.5 ± 2.1 | 25.7 ± 3.3 | 27.8 ± 1.0 | 14.0 ± 4.9 | 31.8 ± 8.3 | 21.8 ± 1.0 | 31.5± 3.4 | 12.4 ± 2.1 | 29.8 ± 4.5 | 20.6 ± 0.4 | 29.7± 2.4 | 11.4 ± 0.8 | 29.2 ± 7.7 | 26.0 ± 1.4 |
| ME (MJ/kg DM) | 10.1 ± 0.3 | 10.4 ± 0.4 | 10.1 ± 0.1 | 11.75 ± 0.6 | 9.7 ± 1.0 | 10.9 ± 0.1 | 9.7 ± 0.4 | 11.9 ± 0.2 | 9.9 ± 0.5 | 11.0 ± 0.04 | 9.9 ± 0.3 | 12.06 ± 0.1 | 10.0 ± 0.9 | 10.4 ± 0.17 |

Average herbage mass was 1,147 ± 659, 1,353 ± 809, 1,105 ± 681 and 1,604 ± 760 kgDM per hectare for NZH in FixP, NAH in FixP, NZH in MaxP and NAH in MaxP, respectively at 21 ± 7 DIM, 1,515 ± 449, 1,545 ± 495, 1,482 ± 404 and 1,471 ± 472 kgDM per hectare for NZH in FixP, NAH in FixP, NZH in MaxP and NAH in MaxP, respectively at 100 ± 23 DIM and 1,859 ± 519, 2,028 ± 534, 1,507 ± 386 and 1,573 ± 163 kgDM per hectare for NZH in FixP, NAH in FixP, NZH in MaxP and NAH in MaxP, respectively at 180 ± 23 DIM.

Milk yield was recorded daily and milk samples were collected every 14 d. Solid corrected milk was calculated as: SCM (kg) = 12.3(F) + 6.56 (SNF) – 0.0752 (M); where F, SNF and M are expressed as kg of fat, solids-not-fat and milk, respectively (Tyrrell and Reid, 1965). As this work is part of a larger study, cow BCS (score 1 to 5; Edmonson et al., 1989) was determined every 14 d by one evaluator (Stirling et al., 2021).

***Plasma Samples and Liver Biopsies***

Plasma samples and liver biopsies were collected at -45 ± 17, 21 ± 7, 100 ± 23 and 180 ± 23 DIM. Biopsies were taken using a 14-gauge biopsy needle (Tru-Core-II Automatic Biopsy Instrument) after local intramuscular administration of 3 mL of 2% lidocaine hydrochloride (Carriquiry et al., 2009) and either cryopreserved for mitochondrial oxygen consumption analyses (García-Roche et al., 2018) or immediately frozen in liquid nitrogen. All samples were stored at -80 °C until analysis.

***Plasma and Hepatic Metabolites***

Plasma aspartate aminotransferase and gamma-glutamyl transferase catalytic activity were determined with a commercial kit from Biosystems, following manufacturer instructions as previously described

Quantification of free liver glucose and glycogen was performed in liver biopsies homogenized in 500 µL 2N HCl as previously described by García-Roche et al., (2021) . Briefly, homogenates were subjected to 100°C during an hour for glycogen digestion to glucose by acid-heat hydrolysis (Bancroft and Fry, 1933). Free liver glucose and digested liver glycogen were determined using a kit from

Biosystems S.A. (Costa Brava, Barcelona, Spain), following manufacturer instructions after neutralizing acid samples with an equal amount of 2M NaOH. Absorbance was measured at λ = 505 nm.

For liver triglyceride quantification, liver homogenates were performed according to Armour et al. 2017. In brief, liver biopsies were homogenized in lysis buffer (140 mM NaCl, 50 mM Tris and 1% Triton X-100, pH 8) and measured using a kit from Biosystems S.A. (Costa Brava, Barcelona, Spain), following manufacturer instructions at λ = 505 nm. For all metabolite assays intra and inter-assay coefficient of variation (CV) were less than 10%.

***Mitochondrial Oxygen Consumption Rate***

Mitochondrial respiration was studied measuring oxygen consumption rate in a high-resolution respirometer OROBOROS Oxygraph - 2k (Oroboros Instruments, Innsbruck, Austria) at 37°C (García-Roche et al., 2018, 2019). Briefly, electrodes were calibrated in modified MIR05 respiration medium (0.5 mM EGTA, 3mM $MgCl_2 \bullet 6H_2O$, 60 mM MOPS, 20 mM taurine, 10 mM $KH_2PO_4$, 20 mM HEPES, 110 mM sucrose, 1 $g.L^{-1}$ BSA, pH 7.1) with a saturated oxygen concentration of 191 µM at 100 kPa barometric pressure at 37 °C. Respiratory rates (pmol $O_2.min^{-1}.mL^{-1}$) were calculated using the DatLab 4 analysis software (Oroboros Instruments). Liver biopsies were weighed (2-10 mg), added to the chamber and oxygen consumption measurements were obtained before and after the sequential addition of specific substrates of the respiratory chain, 10 mM glutamate and 5 mM malate (complex I) or 20 mM succinate (complex II), followed by 4 mM adenosine diphosphate (ADP), 2 µM oligomycin (ATP synthase inhibitor), 2-4 µM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, an uncoupler of oxidative phosphorylation). Maximum uncoupling was obtained by FCCP titration. Respiration was inhibited with 0.5 µM rotenone (complex I inhibitor) or 2.5 µM antimycin A (complex III inhibitor). All respiratory parameters and indices were obtained as described in García-Roche et al. (2018). Briefly, non-mitochondrial oxygen consumption rate measured after the addition of specific inhibitors rotenone or antimycin A and subtracted from all other values before calculating the respiratory parameters. State 4 respiration was determined as the baseline measurement obtained with complex I and II substrates before

the addition of ADP and state 3 was determined after the addition of ADP. Oligomycin-resistant respiration (ATP-independent) was measured after addition of oligomycin and oligomycin sensitive respiration (ATP-dependent) was the difference between state 3 and oligomycin-resistant respiration. Finally, the maximum respiratory rate was determined after titration with FCCP.

### *Carnitine Palmitoyltransferase Activity*

Specific activity of carnitine palmitoyltransferase (CPT) was measured by following the release of CoA-SH from palmitoyl-CoA at 412 nm using 5,5'-dithio-bis-(2-nitrobenzoic acid) and a molar extinction coefficient of 13600 $M^{-1}.cm^{-1}$ according to Bieber et al., (1972). To isolate mitochondrial fractions liver biopsies were homogenized in 0.25 M sucrose and 0.2 mM EDTA adjusted to pH 7.5 with Tris-HCl buffer with protease inhibitors (SigmaFast Protease Inhibitor Cocktail and 1 mM phenylmethylsulfonyl fluoride), homogenates were centrifuged for 13 min at 750 g and 4°C. Then, the supernatant was collected and centrifuged at 6700 g and 4°C for 12 minutes. Finally, the pellet was resuspended and washed in homogenization buffer repeating the 6700 g centrifugation. The remaining pellet was resuspended in 70 mM sucrose, 2 mM HEPES buffer at pH 7.4 and 1 mM EDTA with protease inhibitors (SigmaFast Protease Inhibitor Cocktail and 1 mM phenylmethylsulfonyl fluoride). Protein content was determined with the Bradford assay using bovine serum albumin as standard (Bradford, 1976). For the assay, 116 mM Tris-HCl, pH 8.0, 0.09% Triton X-100, 1.1 mM NaEDTA, 0.035 palmitoyl-CoA, 0.12 mM DTNB, 1.1 mM L-carnitine and the mitochondrial suspension were used. For each sample, an assay was run without the addition of L-carnitine and palmitoyl-CoA to correct for endogenous L-carnitine and palmitoyl-CoA in the sample. Assays were performed in duplicate using a final volume of 200 µL in a Multiskan$^{TM}$ FC Microplate Photometer (Thermo Fisher). Data is presented per unit of tissue protein.

### *Western Blots*

Mitochondrial fractions were obtained as previously described by García-Roche et al., (2019) using a lysis buffer (250 mM sucrose, 50 mM Tris-HCl, 5 mM $MgCl_2$) with protease inhibitors (SigmaFast Protease Inhibitor Cocktail and 1 mM phenylmethylsulfonyl fluoride) and deacetylase inhibitors, (1 µM trichostatin A

and 5 mM nicotinamide, pH 7.4) and a mitochondrial resuspension buffer composed of 50 mM Tris-HCl, 1 mM EDTA, 0.5% Triton-X-100 with protease and deacetylase inhibitors, pH 6.8. Protein content was determined with the Bradford assay using bovine serum albumin as standard (Bradford, 1976) and samples were kept at -80˚C until analyzed. Liver homogenates were resolved (30 µg) in 12% Tris-Glycine-SDS polyacrylamide gels (SDS/PAGE), along with protein ladder (#P7712, New England Biolabs) and proteins were transferred overnight to nitrocellulose membranes. Membranes were blocked with blocking buffer (Tris buffered saline with 0.1% Tween 20 and 0.5% skimmed milk) and incubated overnight at 4˚C with a primary antibody against acetylated lysine (1:1000, Cell Signaling Technology, 9441). For protein detection, membranes were washed and probed with secondary antibodies from LI-COR Biosciences: anti-rabbit (1:15,000, IRDye 680, 926–68071). Immunoreactive proteins were detected with an infrared fluorescence detection system (Odyssey, LI-COR Biosciences) and bands were quantified with ImageJ software by densitometry and protein levels were normalized by Ponceau staining.

***RNA Isolation and qRT-PCR***

Extraction of total RNA from liver tissue and cDNA synthesis by reverse transcription was performed (Carriquiry et al., 2009) using the Trizol reagent followed by lithium chloride precipitation and DNase treatment using Ambion™ DNA-free™ DNA Removal Kit (ThermoFisher Scientific, Waltham, USA). Concentration of RNA was determined measuring absorbance at λ = 260 nm (NanoDrop ND-1000 Spectrophotometer; Nanodrop Technologies, Wilmington, DE, USA), and purity and integrity of RNA isolates were assessed from 260/280 and 260/230 absorbance ratios (greater than 1.9 and 1.8, respectively). Samples of RNA were stored at −80°C. The SuperScript™ III Reverse Transcriptase kit (Invitrogen™ from ThermoFischer Scientific, Waltham, USA) was used to perform retrotranscription along with random hexamers and 1 µg of total RNA as a template. The cDNA was stored at −20°C until its use in the real-time PCR. Primers (Supplementary information 1) to specifically amplify cDNA of target genes: very long-chain acyl-CoA dehydrogenase (*ACADVL),* acetyl-CoA acetyltransferase 1 *(ACAT1),* ß-actin *(ACTB),* acyl-CoA oxidase 2 *(ACOX2),* apolipoprotein A4 *(APOA4),* apolipoprotein A5 *(APOA5),* apolipoprotein C2

*(APOC2),* CD36 molecule *(CD36),* CD40 *molecule (CD40),* carnitine palmitoyl-transferase 1 *(CPT1A),* liver fatty acid binding protein *(FABP1),* fibroblast growth factor 21 *(FGF21),* hydroxymethylglutaryl-CoA synthase 2 *(HMGCS2),* hypoxanthine phosphoribosyl transferase *(HPRT1);* nuclear receptor subfamily 1 group H member 3 *(LXRA),* nuclear factor kappa B subunit 1 *(NFKB1),* nuclear factor kappa B inhibitor alpha *(NFKB1A),* peroxisome proliferator-activated receptor alpha *(PPARA),* peroxisome proliferator-activated receptor gamma coactivator 1-alpha *(PPARGC1A),* retinoic acid receptor alpha *RARA),* retinoic X receptor alpha *(RXRA),* retinoic X receptor beta *(RXRB),* retinoic X receptor gamma *(RXRG),* sterol regulatory element binding transcription factor 1 *(SREBP-1),* tumor necrosis factor alpha *(TNFA),* tumor necrosis factor receptor superfamily member 1A *(TNFRSF1A)* were obtained from literature or specifically designed using the Primer3 website (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and bovine nucleotide sequences available from NCBI (http://www.ncbi.nlm.nih.gov/) .

Real time PCR reactions were carried out in a total volume of 15 µl using Maxima SYBR Green/ROX qPCR Master Mix 2X (Thermo Fisher Scientific, Waltham, USA), using the following standard amplification conditions: 10 min at 95°C and 40 cycles of 15 s at 95 °C, 30 s at 60°C, and 30s at 72°C in a 48-well StepOne™ Real-Time PCR System (Applied Biosystems™ from Thermo Fischer Scientific, Waltham, USA). Melting curves were run on all samples to detect primer dimers, contamination, or presence of other amplicons. Each plate was designed including a pool of total RNA from bovine liver samples analyzed in triplicate to be used as the basis for the comparative expression results (exogenous control) and duplicate wells of non-template control (water). Gene expression was determined by relative quantification with respect to the exogenous control (Pfaffl, 2004) and normalized to the geometric mean expression of the endogenous control genes (*ACTB* and *HPRT*). Expression stability of two selected housekeeping genes was evaluated using MS-Excel add-in Normfinder (MDL, Aarhus, Denmark), values obtained with Normfinder were for 0.004 for *ACTB* and 0.003 for *HPRT*. Amplification efficiencies or target and endogenous control genes were estimated by linear regression of a cDNA dilution curve

(Supplementary information 1). Intra and inter-assay CV values were less than 2.3 and 3.1%, (n = 5 dilutions, from 100 to 6.25 ng/well), respectively.

***Targeted Metabolomics***

Metabolomic analysis was performed in a subset FixP cows of NAH genetic origin (N = 7) and MaxP cows of NAH genetic origin (N = 5) at 180 DIM using gas chromatography time-of-flight mass spectrometry (GCToF/MS) as previously described by Fiehn et al., (2008) at the Core Facility West Metabolomics Center (UC Davis Genome Center, Davis, CA, USA). Selected cows had all previous variables analyzed during all the experimental period and tissue was available for metabolomic analysis. Briefly, analyses were performed in a Leco®Pegasus IV mass spectrometer with a Rtx-5Sil MS, Restek®Corporation column (30 m length × 0.25 mm internal diameter with 0.25 µm film made of 95% dimethyl/5%diphenylpolysiloxane). Mass spectrometry parameters were unit mass resolution set at 17 spectra/s from 80 to 500 Da and ionization energy set in -70 eV and equipped with an 1800 V detector voltage, 230 °C transfer line, and a 250°C ion source. The ChromaTOF 2.32 was used for data preprocessing without smoothing and apex masses were reported for use in the BinBase algorithm. Ion annotation was performed as described by Fiehn et al., (2008). Data is reported as peak height normalized by the sum of all peak heights of each sample. Metabolomic data analysis were performed using MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/, accessed on 4 January 2022). Data were normalized using log transformations and data quality was assessed comparing pooled samples to individual samples in multivariate analysis. For statistical analyses, *p* values (false discovery rate (FDR), α level = 0.10) and fold changes were calculated for comparisons between groups for each molecular feature. A principal component analysis (PCA) was performed on the metabolomic dataset to analyze the overall variance among cows. The partial least squares discriminant analysis (PLS-DA) was performed and variable importance in projection (VIP) scores were obtained. Metabolites were considered to differ when VIP > 1.0 and pathway enrichment analysis was performed with the Kyoto Encyclopedia of Genes and Genomes pathway database. Significant enrichment of metabolic pathway was set at FDR < 0.10. Statistical evaluation of group

differences by t-test was performed and visualized with volcano plots setting the FDR at < 0.10.

### *Statistical Analysis*

Data were analyzed using the SAS System program (SAS Academic Edition; SAS Institute Inc., Cary, NC, USA), with cow as the experimental unit. Univariate and linear regression analyses were performed for all variables to identify outliers and inconsistencies and to verify normality of residuals. When data did not have normal distribution, logarithmic transformations were performed to approximate more closely to normality and homogeneity requirements. Values were removed when the studentized residual was >3 and < -3, no more than three values per variable were excluded. Least square means and pooled standard error values of all variables are presented as non-transformed data to aid in the comparison among variables.

Data were analyzed as repeated measures using the MIXED procedure, the model included Holstein strain, DIM and feeding strategy and their interactions as fixed effects. Compound symmetry was used as the covariance structure and the Kenward-Rogers procedure was used to adjust the denominator degree of freedom. Least square means tests were conducted to analyze differences between groups. For gene expression data, contrasts were performed with Tukey tests. Means were considered to differ when $P \leq 0.05$ and a trend was declared when $0.05 < P \leq 0.10$. The CORR procedure was used to perform Pearson correlations and Spearman rankings.

## Results

### *Milk Yield and Milk Composition*

The interaction between milk yield and treatment (P < 0.01, Table 3) shows that milk yield was higher for the MaxP than the FixP treatment at 21 and 180 DIM. In addition, NAH cows had higher milk yield than NZH cows (P < 0.001, Table 3). Fat content in milk peaked at 21 DIM and decreased from 100 to 180 DIM for MaxP, while it increased for FixP (P < 0.01, Table 3). Protein content in milk was greater in NZH than NAH cows during 21 DIM (P < 0.01, Table 3). Solids corrected milk peaked at 21 DIM and was greater for the MaxP treatment (P <

0.001, Table 3). In addition, it was greater for the NZH strain than the NAH strain at 180 DIM ($P < 0.001$, Table 3).

Table 3. Milk yield and composition of grazing cows of NAH and NZH genetic origin supplemented with total mixed ration (FixP) or an energy-protein concentrate (MaxP) during early and mid-lactation.

| | | Strains | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | NZH | | NAH | | | *P*-value[1] | | | | |
| | DIM | FixP | MaxP | FixP | MaxP | SEM | DIM | Gen | Treat | DIM*Gen | DIM*Treat |
| Milk yield | 21 | 25.6bc | 29.8b | 30.7a | 33.7a | 1.3 | <0.0001 | <0.001 | <0.05 | 0.72 | <0.01 |
| (kg/d) | 100 | 27.0b | 28.1b | 32.2a | 32.3a | | | | | | |
| | 180 | 19.8d | 22.7cd | 24.4c | 28.6b | | | | | | |
| Fat (%) | 21 | 5.11a | 5.23a | 5.11a | 5.09a | 0.21 | <0.0001 | 0.17 | 0.24 | 0.06 | <0.0001 |
| | 100 | 3.96bc | 4.74ab | 3.87c | 4.95a | | | | | | |
| | 180 | 5.06a | 4.49b | 4.34bc | 4.00bc | | | | | | |
| Protein (%) | 21 | 4.00ax | 3.99ax | 3.71aby | 3.44b | 0.11 | <0.0001 | <0.01 | 0.83 | <0.01 | <0.01 |
| | 100 | 3.38c | 3.41bc | 3.17c | 3.75ab | | | | | | |
| | 180 | 3.76ab | 3.73ab | 3.47b | 3.23c | | | | | | |
| Solids | 21 | 30.6b | 35.8ay | 36.0a | 39.0ax | 1.1 | <0.0001 | 0.47 | <0.01 | <0.001 | <0.001 |
| corrected milk | 100 | 19.1d | 20.9c | 18.6d | 21.9c | | | | | | |
| (kg/d) | 180 | 22.0cx | 20.6cd | 20.0dy | 18.9d | | | | | | |

Data are shown as least square means ± standard error. ab denote differences between values ($P < 0.05$) while xy denote tendencies ($0.05 < P < 0.1$). [1]The interaction Gen*Treat is not shown because it was not significant for any variable and the interaction DIM*Gen*Treat is not shown because it was not significant for most variables, except protein content ($P < 0.05$). DIM: days in milk; Treat: treatment; Gen: genetic origin. N = 10-12.

***Hepatic Metabolites***

Hepatic free glucose ($P < 0.001$, Fig. 1A) was only affected by moment of lactation as it peaked during pre-partum. Liver glycogen was affected by the interaction between treatment and strain as it was the highest for NZH in the MaxP treatment (P = 0.05, Table 4), liver glycogen was also affected by moment of lactation, plummeting during early lactation, and increasing toward early mid-lactation ($P < 0.0001$, Fig. 1B). The liver glycogen to glucose ratio suggests that glycogen was preferentially stored during mid-lactation as opposed to the pre-partum and early lactation ($P < 0.001$, Fig. 1C). Liver triglyceride levels increased three-fold from pre-partum to early lactation and decreased four-fold from early to mid-lactation ($P < 0.0001$, Fig. 1D), liver triglyceride levels were 50% higher for the MaxP vs. FixP treatment ($P < 0.05$, Fig. 1D and Table 4). In addition, when DIM 21 and 180 were analyzed separately, the interaction between stage of lactation and treatment was significant (P < 0.01, Fig. 1E) since the MaxP treatment showed higher levels of liver triglyceride than the FixP treatment at 21 DIM. Liver triglyceride to glycogen ratio showed that there was a preference for triglyceride rather than glycogen reserves at 21 DIM (P < 0.0001, Fig. 1F). In addition, the MaxP treatment had a preference for triglyceride rather than glycogen reserves (P < 0.05, Fig. 1F and Table 4) and NAH cows in the FixP treatment tended to have the lowest mean liver triglyceride to glycogen ratio value (P = 0.06, Table 4). Mean plasma levels of aspartate aminotransferase were < 39, 69 and 71 ± 5 IU/L during -45, 21 and 180 DIM and mean plasma gamma-glutamyl transferase levels were < 26, 27 and 27 ± 2 IU/L during -45, 21 and 180 DIM.

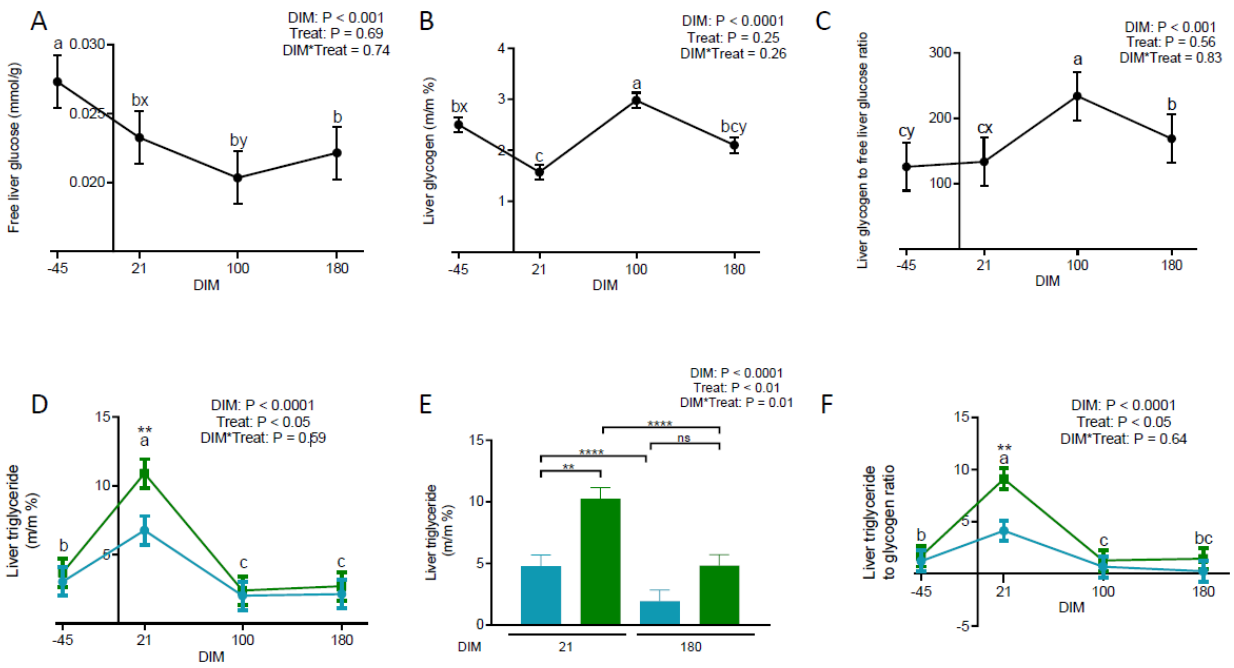


Figure 1. Hepatic energy reserves in cows of both NZH and NAH genetic origin in the FixP (blue) and MaxP (green) treatment at 21 and 180 DIM (N = 9-11). (A) Free liver glucose, (B) liver glycogen, (C) liver glycogen to free liver glucose levels, (D) liver triglyceride expressed per unit of protein during lactation, (E) liver triglyceride at 21 and 180 DIM and (F) liver triglyceride to glycogen ration. Different letters (a and b for P ≤ 0.05, x and y for P ≤ 0.10) and asterisks (* for P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001, ns for non significant) depict differences between means; letters for DIM effect and asterisks for treatment effect. Treat: treatment; Gen: genetic origin.

Table 4. Hepatic energy reserves of grazing cows of NAH and NZH genetic origin supplemented with total mixed ration (FixP) or an energy-protein concentrate (MaxP).

| | Strains | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | NZH | | NAH | | | *P*-value | | |
| | FixP | MaxP | FixP | MaxP | SEM | Gen | Treat | Treat*Gen |
| Free liver glucose (mmol/g) | 0.025 | 0.023 | 0.020 | 0.024 | 0.003 | 0.52 | 0.69 | 0.34 |
| Liver glycogen (m/m %) | 2.20b | 2.78a | 2.15b | 1.99b | 0.42 | 0.03 | 0.25 | 0.05 |
| Liver glycogen to free liver glucose ratio | 202 | 178 | 140 | 143 | 48 | 0.32 | 0.56 | 0.21 |
| Liver triglyceride (m/m %) | 3.79 | 4.91 | 3.31 | 5.66 | 0.80 | 0.28 | 0.04 | 0.50 |
| Liver triglyceride to glycogen ratio | 2.31ab | 2.63a | 0.84b | 4.13a | 0.80 | 0.71 | 0.01 | 0.06 |

Data are shown as least square means ± standard error. ab denote differences between values ($P \leq 0.05$). N = 36-44.Treat: treatment; gen: genetic origin.

***Mitochondrial Function and CPT Activity***

Oxygen consumption rates were measured in liver biopsies after the addition of specific substrates for mitochondrial chain complexes I and II and respiratory parameters were calculated (Table 5 and Fig. 2A and 2B and 2C and 2D, respectively). Oligomycin-sensitive respiration driven by glutamate and malate was lower at 21 DIM for the MaxP treatment ($P < 0.05$, Fig. 2A and 2B) and it tended to be lower for the MaxP treatment when succinate was used as substrate ($P = 0.08$). However, when only 21 and 180 DIM were analyzed separately, succinate driven oligomycin-sensitive respiration was affected by DIM as it was lower at 21 DIM ($P < 0.05$). With respect to overall means, the interaction treatment by strain was observed for the maximum respiratory rate and respiratory control ratio ($P < 0.05$, Table 5, Fig. 3) in the case of complex-I respiratory parameters and the highest values were observed in cows of NZH origin in the MaxP treatment. On the other hand, the interaction treatment by

strain was observed in all complex-II respiratory parameters (state 3 respiration, state 4 respiration, maximum respiratory rate, oligomycin-resistant respiration, oligomycin-sensitive respiration and respiratory control rate, $P < 0.05$, Table 5, Fig. 3) except non-mitochondrial respiration and the highest values were observed in cows of NZH origin in the MaxP treatment and cows of NAH origin in the FixP treatment.

Specific activity of CPT was affected by moment of lactation and treatment fixed effects since it was lower during 21 DIM ($P < 0.001$, Fig. 2E) and was lower for the MaxP than the FixP treatment ($P < 0.05$, Fig. 2E). In the case of overall means, activity of CPT was affected by treatment ($P < 0.05$) and tended to be affected by genotype ($P = 0.09$) as it was greater for the FixP than MaxP treatment and tended to be greater for cows of NAH origin vs. cows of NZH in the MaxP treatment (4.03, 3.23, 4.49 and 3.89 ± 0.36, for NZH cows in the FixP and MaxP treatment and for NAH cows in the FixP and MaxP treatment, respectively). Correlations were performed complex-I oligomycin-sensitive respiration and hepatic energy reserves at 21 and 180 DIM (Fig. 2F and 2G) and negative association was found between oligomycin-sensitive respiration and liver triglyceride ($r = -0.40$, $P < 0.01$ for Pearson correlation and $r = -0.50$, $P < 0.001$ for Spearman ranking, Fig. 2F), while no association was found between oligomycin-sensitive respiration and liver glycogen ($r = 0.032$, $P = 0.86$ for Pearson correlation and $r = 0.034$, $P = 0.85$ for Spearman ranking, Fig. 2G). Alongside, the correlation between CPT activity and liver triglyceride was also negative ($r = -0.25$, $P < 0.05$ for Pearson correlation and $r = -0.33$, $P < 0.01$ for Spearman ranking, Fig. 2H).

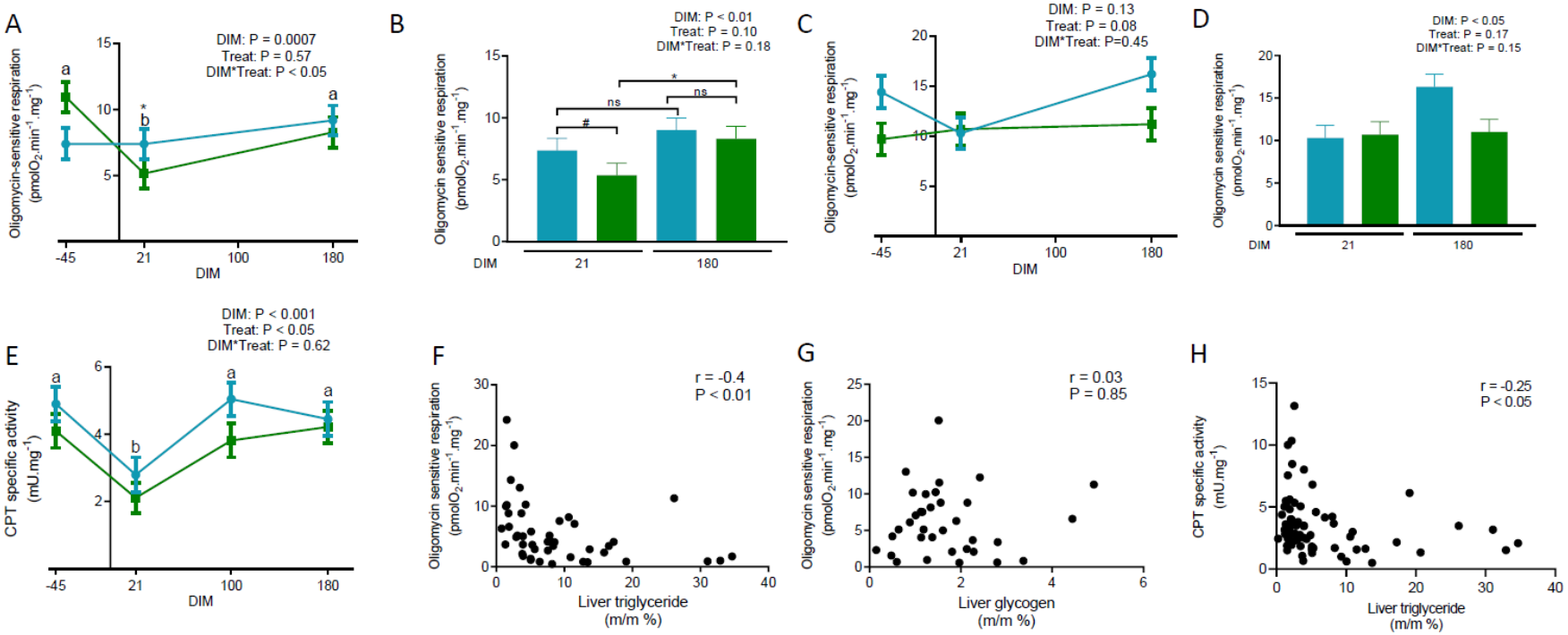


Figure 2. Mitochondrial function and CPT activity in cows of both NZH and NAH genetic origin in the FixP (blue) and MaxP (green) treatment at 21 and 180 DIM (N = 9-11). (A) Complex-I oligomycin sensitive respiration during lactation, (B) complex-I oligomycin sensitive respiration at 21 and 180 DIM, (C) complex-II oligomycin sensitive respiration during lactation, (D) complex-II oligomycin sensitive respiration at 21 and 180 DIM, (E) specific activity of CPT, (F) correlation between complex-I oligomycin-sensitive respiration and liver triglyceride, (G) correlation between complex-I oligomycin-sensitive respiration and liver glycogen and (H) correlation between CPT activity and liver triglyceride. Different letters (a and b for $P \leq 0.05$, x and y for $P \leq 0.10$) and asterisks (* for $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$, ns for non significant) depict differences between means; letters for DIM effect and asterisks for treatment effect. Treat: treatment; Gen: genetic origin.

Table 5. Hepatic mitochondrial function of grazing cows of NAH and NZH genetic origin supplemented with total mixed ration (FixP) or an energy-protein concentrate (MaxP).

| | Strains | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | NZH | | NAH | | | *P*-value | | |
| *Complex-I respiratory parameters*[3] | FixP | MaxP | FixP | MaxP | SEM | Gen | Treat | Treat*Gen |
| State 3 respiration | 12.2 | 14.4 | 14.0 | 12.3 | 1.3 | 0.80 | 0.95 | 0.19 |
| State 4 respiration | 3.93 | 3.96 | 4.15 | 3.72 | 0.42 | 0.83 | 0.95 | 0.45 |
| Maximum respiratory rate | 12.4ab | 15.5ax | 14.3ab | 12.4aby | 1.2 | 0.62 | 0.97 | 0.04 |
| Oligomycin-resistant respiration | 4.90 | 5.55 | 5.16 | 4.95 | 0.37 | 0.84 | 0.58 | 0.18 |
| Oligomycin-sensitive respiration | 7.15 | 8.84 | 8.83 | 7.42 | 1.03 | 0.93 | 0.57 | 0.29 |
| Non-mitocondrial respiration | 5.90 | 6.56 | 6.19 | 6.18 | 0.25 | 0.84 | 0.19 | 0.18 |
| Respiratory control ratio | 3.43aby | 4.52ax | 4.22a | 3.29b | 0.37 | 0.61 | 0.90 | 0.0072 |
| *Complex-II respiratory parameters*[3] | | | | | | | | |
| State 3 respiration | 35.5a | 40.6a | 39.8a | 27.3b | 3.2 | 0.04 | 0.07 | 0.0006 |
| State 4 respiration | 22.55a | 25.59a | 23.93a | 17.41b | 1.79 | 0.05 | 0.16 | 0.01 |
| Maximum respiratory rate | 46.8a | 54.8a | 54.3a | 35.0b | 5.1 | 0.15 | 0.20 | 0.0023 |
| Oligomycin-resistant respiration | 23.3a | 28.1a | 24.7a | 16.1b | 1.9 | 0.0043 | 0.12 | 0.0009 |
| Oligomycin-sensitive respiration | 12.24abx | 12.44a | 15.04a | 8.59by | 1.42 | 0.54 | 0.08 | 0.01 |
| Non-mitocondrial respiration | 6.46 | 6.74 | 6.22 | 6.88 | 0.51 | 0.76 | 0.15 | 0.76 |
| Respiratory control ratio | 1.61ab | 1.71a | 1.73a | 1.54b | 0.05 | 0.38 | 0.18 | 0.0021 |

Data are shown as least square means ± standard error. ab denote differences between values ($P < 0.05$) while xy denote tendencies ($0.05 < P < 0.1$). N = 40 – 44. Treat: treatment; gen: genetic origin.

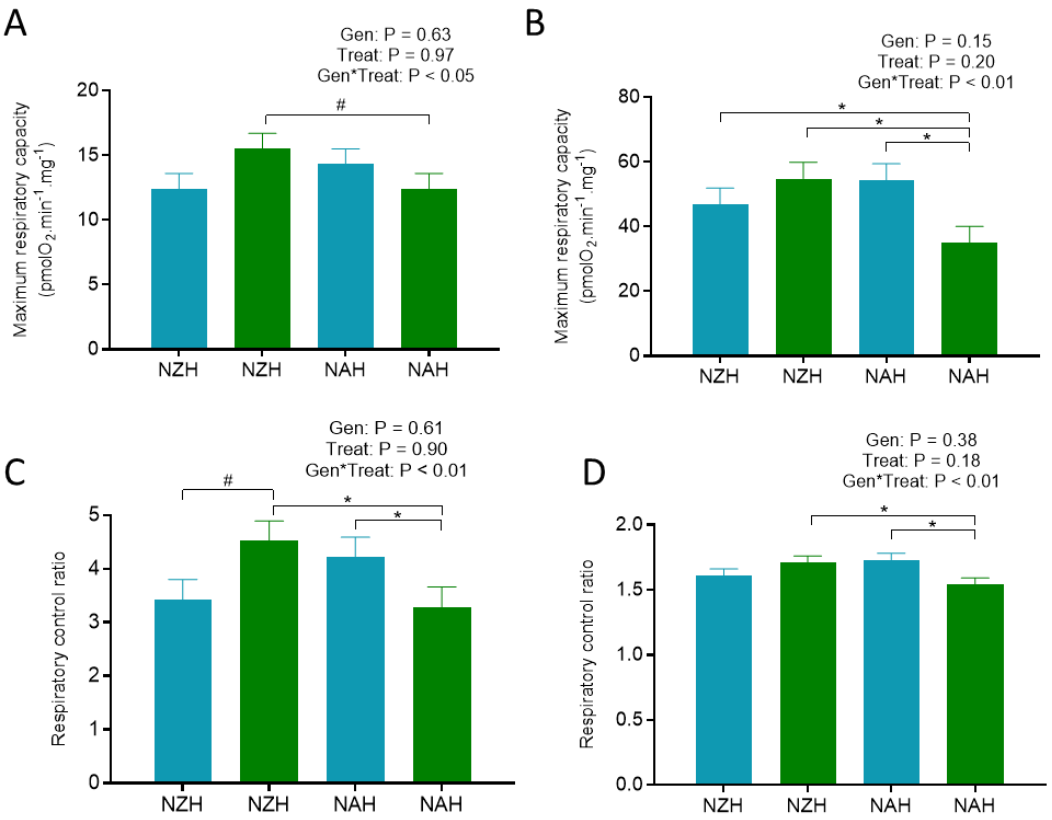


Figure 3. Hepatic mitochondrial function of grazing cows of NAH and NZH genetic origin supplemented with total mixed ration (FixP, blue) or an energy-protein concentrate (MaxP, green), (N = 40-44). (A) Complex-I maximum respiratory rate, (B) complex-II I maximum respiratory rate, (C) complex-I respiratory control ratio, (D) complex-II respiratory control ratio. * for $P < 0.05$, # for $0.05 < P < 0.1$. Treat: treatment; Gen: genetic origin.

### *Mitochondrial Protein Acetylation*

Levels of acetylated lysine in mitochondrial fractions of liver biopsies was affected by the interaction between genetic origin and treatment and the group with the highest levels of acetylated lysine were cows of NAH origin in the MaxP treatment ($P < 0.05$, Fig. 4A and B). The correlation between mitochondrial acetylated lysine levels and liver triglyceride was negative ($r = -0.30$, $P < 0.05$ for Pearson correlation and $r = -0.30$, $P < 0.05$ for Spearman ranking, Fig. 4C).

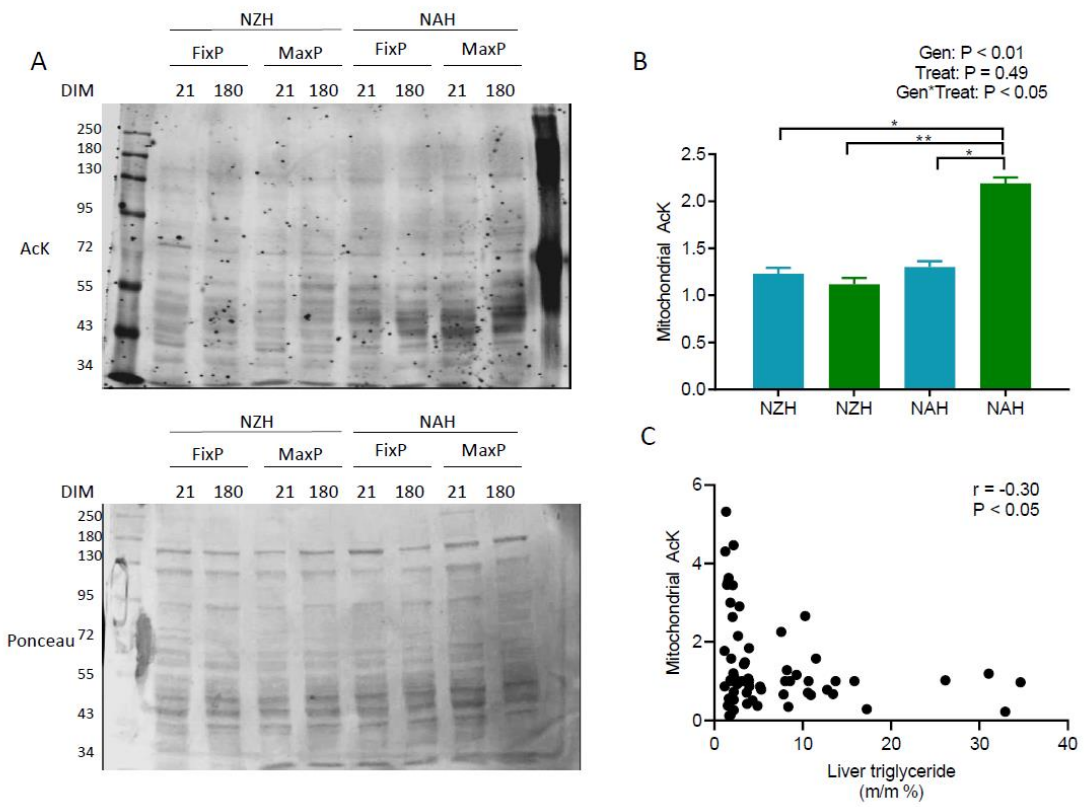


Figure 4. Mitochondrial protein acetylation correlates with liver triglyceride levels. (A) Representative western blots of mitochondrial AcK and in mitochondrial fractions from cows of both NZH and NAH genetic origin in the FixP (blue) and MaxP (green) treatment at 21 and 180 DIM (N = 8), bottom panel shows ponceau S staining for protein normalization. (B) Western blots were quantified by densitometry, normalized with the loading control and expressed in relation to the average value of the NZH-FixP group at 21 DPP. (C) Shows the correlation between mitochondrial acetylated lysine levels in mitochondrial fractions and liver triglyceride in liver biopsies. * for P ≤ 0.05 and ** for P < 0.001, * depict least square means differences between means. Treat: treatment; Gen: genetic origin.

### *Hepatic Gene Expression of Fatty Acid Metabolism and Transcription Factors*

Hepatic expression of genes related to fatty acid metabolism and relevant transcription factors was assessed at 180 DIM in cows of NZH and NAH origin in the MaxP treatment and in cows of NAH origin in the FixP and MaxP treatment. Hepatic gene expression of *ACADVL, RARA* and *RXRB* was at least 80% greater ($P < 0.05$) for NZH than NAH cows (Fig. 5A, 4E and 4F, Table 6) and expression of *ACAT1, CD40* and *PPARA* tended ($P < 0.10$) to be greater for NZH than NAH cows (Fig. 5B, 5C and 5D, Table 6). Hepatic expression of *CD36* was almost 3-fold greater ($P < 0.05$) for FixP than MaxP cows (Fig. 5G, Table 6). Abundance

of mRNA of *ACOX2, APOA4, APOA5, APOC2, CPT1A, FABP1, FGF21, HMGCS2, LXRA, NFKB1, NFKB1A, PPARGC1A, RXRA, RXRG, SREBP-1, TNFA,* and *TNFRSF1A* did not differ among strains or treatments.

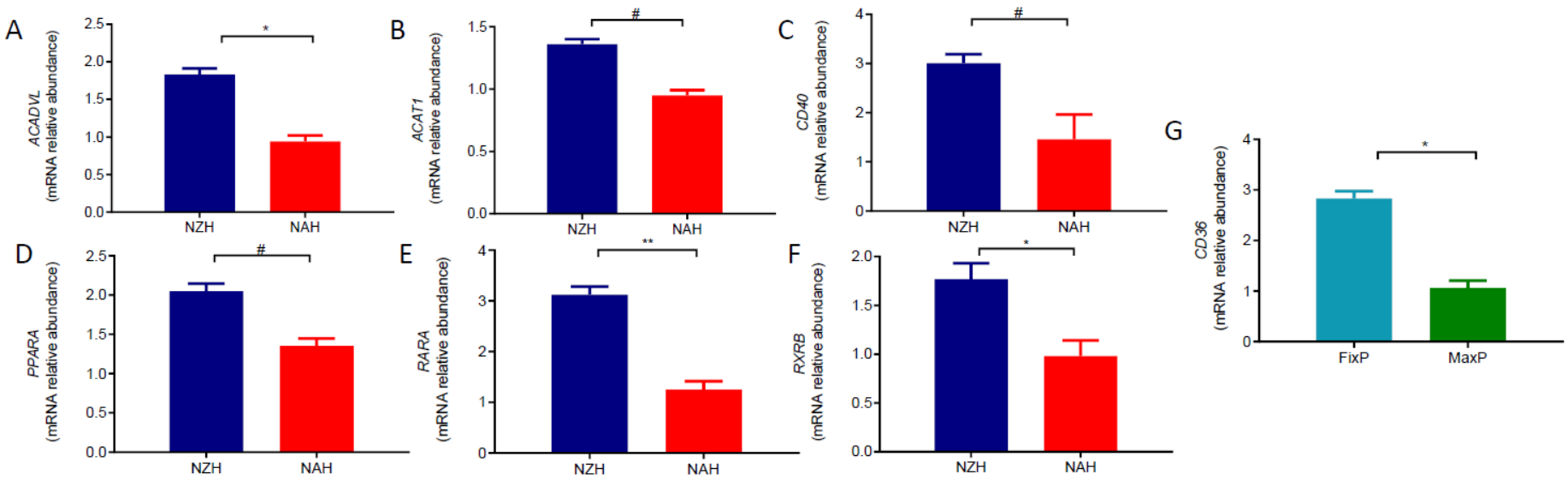


Figure 5. Hepatic gene expression of fatty acid metabolism and transcription factors. (A) Relative mRNA abundance of *ACADVL* in liver biopsies from cows of both NZH (blue) and NAH (red) genetic origin in the MaxP treatment at 180 DIM (N = 8). (B) Relative mRNA abundance of *ACAT1* in liver biopsies from cows of both NZH (blue) and NAH (red) genetic origin in the MaxP treatment at 180 DIM (N = 8). (C) Relative mRNA abundance of *CD40* in liver biopsies from cows of both NZH (blue) and NAH (red) genetic origin in the MaxP treatment at 180 DIM (N = 8). (D) Relative mRNA abundance of *PPARA* in liver biopsies from cows of both NZH (blue) and NAH (red) genetic origin in the MaxP treatment at 180 DIM (N = 8). (E) Relative mRNA abundance of *RARA* in liver biopsies from cows of both NZH (blue) and NAH (red) genetic origin in the MaxP treatment at 180 DIM (N = 8). (F) Relative mRNA abundance of *RXRB* in liver biopsies from cows of both NZH (blue) and NAH (red) genetic origin in the MaxP treatment at 180 DIM (N = 8). (G) Relative mRNA abundance of *CD36* in liver biopsies from cows of NAH origin in the FixP (blue) or MaxP (green) treatment at 180 DIM (N = 8). Asterisks and numerals depict differences in means from Tukey tests * for $P < 0.05$, ** for $P < 0.001$, and # for $0.05 < P \leq 0.10$.

Table 6. Hepatic gene expression of grazing cows of NAH and NZH genetic origin supplemented with total mixed ration (FixP) or an energy-protein concentrate (MaxP) at 180 DIM.

| Gene[1] | Treatments | | | SEM | *P*-value[2] | |
|---|---|---|---|---|---|---|
| | FixP | MaxP | | | NZH vs. | FixP vs. |
| | NAH | NZH | NAH | | NAH | MaxP |
| *ACADVL* | 0.89 | 1.83 | 0.94 | 0.23 | 0.04 | 0.99 |
| *ACAT1* | 1.07 | 1.36 | 0.95 | 0.12 | 0.08 | 0.79 |
| *ACOX2* | 1.80 | 2.17 | 1.15 | 0.70 | 0.48 | 0.89 |
| *APOA4* | 1.15 | 1.91 | 1.62 | 0.28 | 0.78 | 0.50 |
| *APOA5* | 1.56 | 1.71 | 1.65 | 0.73 | 0.99 | 0.98 |
| *APOC2* | 2.45 | 2.41 | 2.36 | 0.72 | 0.87 | 0.96 |
| *CD36* | 2.83 | 1.07 | 1.06 | 0.46 | 0.99 | 0.03 |
| *CD40* | 1.42 | 3.01 | 1.46 | 0.50 | 0.098 | 0.99 |
| *CPT1A* | 0.69 | 0.87 | 0.91 | 0.28 | 0.99 | 0.78 |
| *FABP1* | 2.17 | 1.27 | 1.82 | 0.70 | 0.63 | 0.83 |
| *FGF21* | 2.06 | 2.35 | 2.76 | 0.70 | 0.71 | 0.96 |
| *HMGCS2* | 1.27 | 1.68 | 0.94 | 0.28 | 0.17 | 0.68 |
| *LXRA* | 2.50 | 1.52 | 1.77 | 0.61 | 0.88 | 0.36 |

| | | | | | | |
|---|---|---|---|---|---|---|
| *NFKB1* | 1.87 | 2.10 | 1.77 | 0.46 | 0.59 | 0.97 |
| *NFKBIA* | 1.52 | 2.21 | 1.57 | 0.51 | 0.17 | 0.99 |
| *PPARA* | 1.08 | 2.05 | 1.35 | 0.27 | 0.08 | 0.76 |
| *PPARGC1A* | 0.27 | 0.44 | 0.42 | 0.08 | 0.98 | 0.49 |
| *RARA* | 1.64 | 3.12 | 1.25 | 0.47 | 0.01 | 0.54 |
| *RXRA* | 2.16 | 2.23 | 1.96 | 0.70 | 0.92 | 0.96 |
| *RXRB* | 1.71 | 1.77 | 0.98 | 0.46 | 0.04 | 0.18 |
| *RXRG* | 1.28 | 2.03 | 2.28 | 0.44 | 0.93 | 0.15 |
| *SREBP-1* | 2.12 | 1.63 | 1.30 | 0.56 | 0.86 | 0.40 |
| *TNFA* | 1.53 | 1.38 | 1.31 | 0.38 | 0.99 | 0.91 |
| *TNFRSF1A* | 1.48 | 0.99 | 1.47 | 0.39 | 0.40 | 0.99 |

[1]Genes: Very long-chain acyl-CoA dehydrogenase (*ACADVL)*, acetyl-CoA acetyltransferase 1 *(ACAT1)*, ß-actin *(ACTB)*, acyl-CoA oxidase 2 *(ACOX2)*, apolipoprotein A4 *(APOA4)*, apolipoprotein A5 *(APOA5)*, apolipoprotein C2 *(APOC2)*, CD36 molecule *(CD36)*, CD40 *molecule (CD40)*, carnitine palmitoyl-transferase 1 *(CPT1A)*, liver fatty acid binding protein *(FABP1)*, fibroblast growth factor 21 *(FGF21)*, hydroxymethylglutaryl-CoA synthase 2 *(HMGCS2)*, hypoxanthine phosphoribosyl transferase *(HPRT1);* nuclear receptor subfamily 1 group H member 3 *(LXRA)*, nuclear factor kappa B subunit 1 *(NFKB1)*, nuclear factor kappa B inhibitor alpha *(NFKB1A)*, peroxisome proliferator-activated receptor alpha *(PPARA)*, peroxisome proliferator-activated receptor gamma coactivator 1-alpha *(PPARGC1A)*, retinoic acid receptor alpha *RARA)*, retinoic X receptor alpha *(RXRA)*, retinoic X receptor beta *(RXRB)*, retinoic X receptor gamma *(RXRG)*, sterol regulatory element binding transcription factor 1 *(SREBP-1)*,

tumor necrosis factor alpha *(TNFA),* tumor necrosis factor receptor superfamily member 1A *(TNFRSF1A).*

[2]NZH = New Zealand Holstein (N = 8); NAH = North American Holstein (N = 8). All data is shown as least square means ± standard error. Data are shown as least square means ± standard error.

### *Targeted Metabolomics*

Liver biopsies of cows of NAH genetic origin in the FixP and MaxP treatments at 180 DIM were assayed by the GCToF/MS method and the final metabolomic dataset consisted of 170 metabolites. Principal component analysis (PCA) clustering showed that the principal components 1 and 2 accounted for 42% of the variation (Fig. 6A) and partial least squares-discriminant analysis (PLS-DA) score plots clearly differentiated cows in the FixP and MaxP treatments with the first two components accounting for 39.8% of the variation (Fig. 6B). Thirty-nine differential metabolites were selected for having variable importance in projection (VIP) scores > 1 (Fig. 5C), these included amino acids and peptides, bile acids, disaccharides and monosaccharides. Among them, fructan (KEGG C01355), sucrose (KEGG C00089), taurodeoxycholate (KEGG C05463), citrate (KEGG C00158), taurine (KEGG C00245), deoxycholic acid (KEGG C04483) and phosphoenolpyruvate (KEGG C00074) were higher in FixP than MaxP (VIP > 1.5), while 2-hydroxyglutarate (KEGG C02630), creatinine (KEGG C00791), alpha-linoleic acid (KEGG C06437) and 2-methyl-L-alanine (KEGG C02721) were higher in MaxP than FixP (VIP > 1.5). Differentially abundant metabolites were determined using a volcano plot (Fig. 5D) yielding creatinine (KEGG C00791, FDR < 0.01, FC = 0.5) downregulated for FixP vs. MaxP, inositol (KEGG C00137, FDR < 0.05, FC = 1.5 upregulated for FixP vs. MaxP and phosphoenolpyruvate (KEGG C00074, FDR = 0.08, FC = 1.7) upregulated for FixP vs. MaxP.

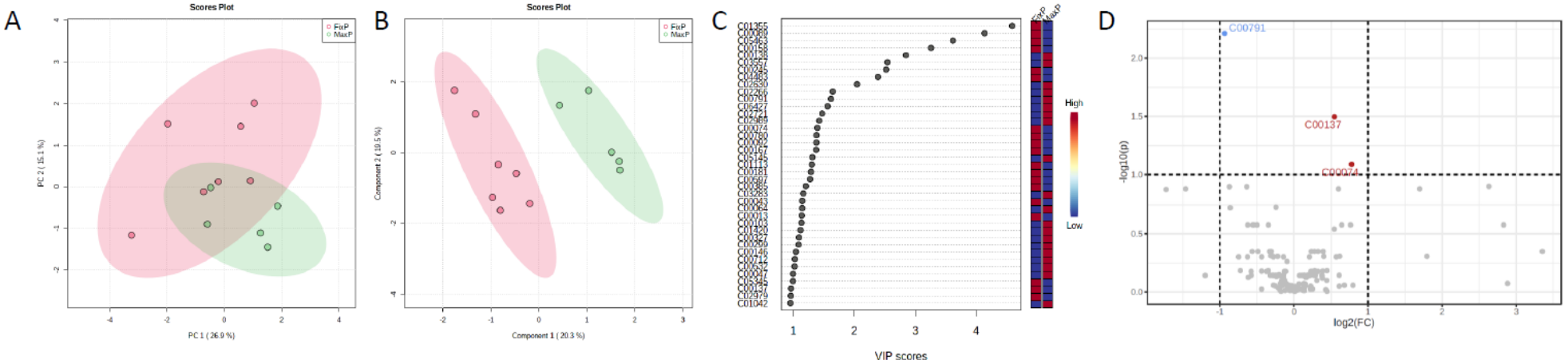


Figure 6. Targeted metabolomics in liver biopsies of cows of NAH genetic origin in the FixP (N = 7) and MaxP (N = 5) treatments at 180 DIM. (A) Principal component analysis scores plot for FixP (red) and MaxP (green). (B) Partial least squares-discriminant analysis (PLS-DA) score plot for FixP (red) and MaxP (green). (C) Variable importance in projection (VIP) scores plot based on the top 39 metabolites with the highest VIP values for the 1$^{st}$ component of PLS-DA for FixP vs. MaxP (red: upregulated; blue: downregulated). (D) Differentially abundant metabolites determined using a volcano plot for cows of NAH genetic origin in the FixP vs. MaxP treatments; KEGG C00791: creatinine, KEGG C00137: inositol, KEGG C00074: phosphoenolpyruvate (red: upregulated; blue: downregulated).

Pathway enrichment analysis of differential metabolites yielded eleven pathways with FDR < 0.10 (Fig. 7, Supplementary table 2). Phosphatidylinositol signaling system and pyruvate metabolism had FDR < 0.05. Moreover, as seen in Figure 8, metabolites related to carbohydrate metabolism such as phosphoenolpyruvate, fructose-6-phosphate, glucose-6-phosphate and sucrose (P < 0.05) were upregulated in FixP, as well as myo-inositol (P < 0.05) from the phosphatidylinositol signaling system, citrate (P < 0.10) from the TCA cycle and xanthine (P < 0.05) from purine metabolism. On the other hand, metabolites related to the urea cycle such as citrulline (P < 0.05), ornithine (P < 0.10) and glutamine (P < 0.10) were upregulated in MaxP, as well as the spontaneous reaction yielding creatinine (P < 0.05).

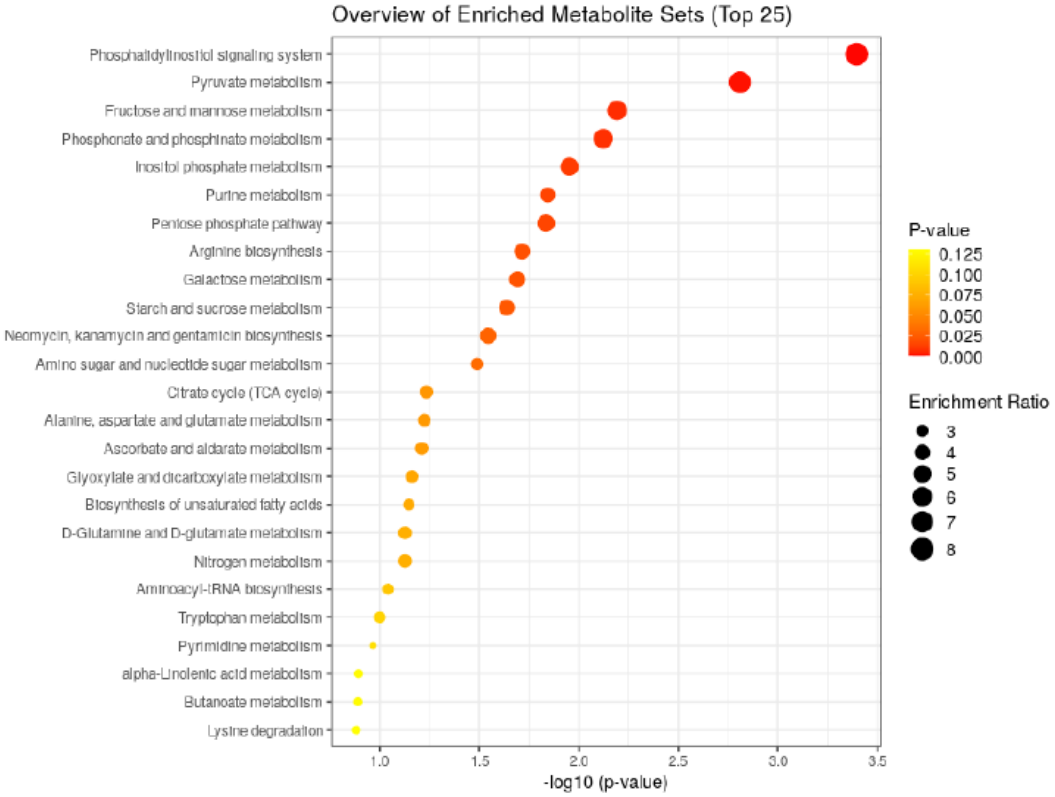


Figure 7. Enrichment ratio and *p*-value from enrichment analysis of pathways within the 39 differential hepatic metabolites between cows of NAH genetic origin in the FixP (N = 7) and MaxP (N = 5) treatments at 180 DIM. Phosphatidylinositol signaling system FDR = 0.01, pyruvate metabolism FDR = 0.03, fructose and mannose metabolism FDR = 0.06, phosphonate and phosphinate metabolism FDR = 0.06, inositol phosphate metabolism FDR = 0.07, purine metabolism FDR = 0.07, pentose phosphate pathway FDR = 0.07, arginine biosynthesis FDR = 0.07, galactose metabolism FDR = 0.07, starch and sucrose metabolism FDR = 0.08, amino sugar and nucleotide sugar metabolism FDR = 0.09.

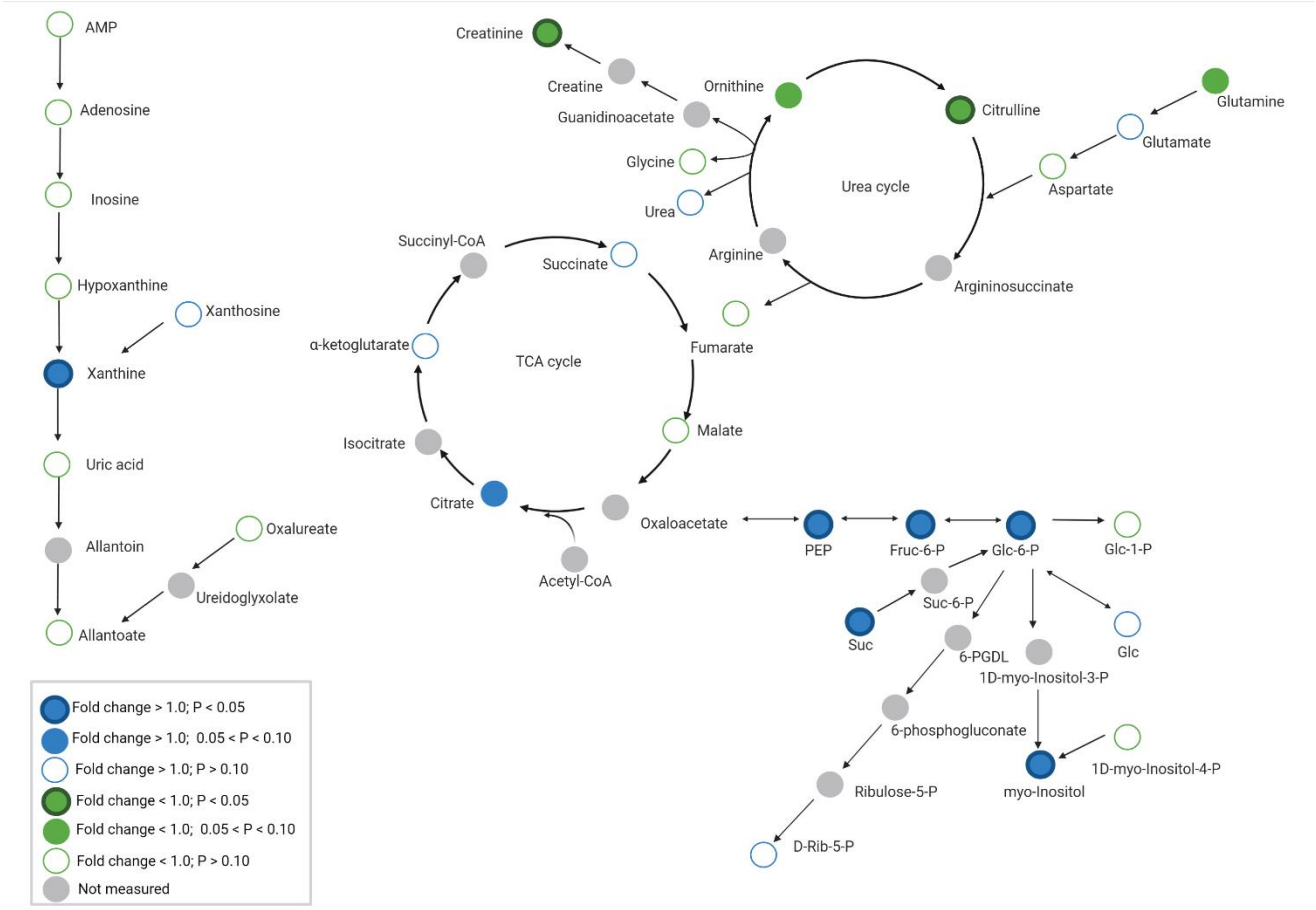


Figure 8. Main metabolic pathways identified to be differentially regulated for cows of NAH genetic origin in the FixP (blue circles, positive fold change) vs. MaxP (green circles, negative fold change) treatments at 180 DIM according to metabolic pathway analysis performed in liver biopsies. Colored circles depict measured compounds and gray circles correspond to undetected metabolites, concentric circles correspond to $P < 0.05$, filled circles correspond to $0.05 < P < 0.10$ and empty circles correspond to $P > 0.10$.

## Discussion

Our results showed hepatic mitochondrial function was impaired during early lactation and CPT activity was decreased, while liver triglyceride levels were increased, this was especially exacerbated in MaxP vs. FixP cows. In addition, acetylation levels were greater for NAH cows in the MaxP feeding strategy. These results pointed out to dramatic changes in fatty acid and energy metabolism during early lactation, hence, to further explore metabolic adaptations of the NZH and NAH strains and the NAH strain in the MaxP and FixP treatment we studied hepatic gene expression of MaxP NZH, MaxP NAH and FixP NAH cows during mid lactation. Gene expression of key enzymes in fatty acid metabolism carried out in liver biopsies of mid-late lactation showed that NZH cows had greater

abundance of *ACADVL* mRNA and tended to have greater abundance of *ACAT1* and transcription factors associated to mitochondrial and peroxisomal fatty acid oxidation *RARA*, *RXRB* and tended to have greater mRNA abundance of *PPARA*. According to metabolomic the phosphatidylinositol signaling system, gluconeogenesis, purine metabolism and pentose phosphate pathways were enriched in FixP cows, while the urea cycle was enriched in MaxP cows.

Related to production performance, the most significant differences were between cows of different genetic origin and cows with different feeding strategy at 21 DIM as NAH cows had greater SCM than NZH cows and MaxP cows had greater SCM than FixP cows. In addition, at 180 DIM NZH cows had greater SCM than NAH cows. Although we found differences in the interaction between strain and DIM and treatment and DIM, total annual means of milk solids across three years did not show differences in treatment although they did show differences among strains, probably accounted for the greater milk yield of NAH cows (Kolver et al., 2002; Stirling et al., 2021), which is in accordance with our work.

Hepatic liver triglyceride levels indicated that during early lactation cows in the MaxP treatment reached levels close to clinical fatty liver (> 10% liver triglyceride wet weight) (Bobe et al., 2004), independent of Holstein strain. Consistent with this, previous authors have demonstrated that plasma non-esterified fatty acids and beta-hydroxybutyrate are similar among strains (Lucy et al., 2009; Grala et al., 2011), suggesting mobilization is similar among strains. Herein we found hepatic mitochondrial function measured as oligomycin-sensitive respiration was impaired during early lactation and it was negatively associated with liver triglyceride, in addition, FixP vs. MaxP cows maintained higher levels of oligomycin-sensitive respiration. Differences were more significant in Complex-I driven respiration, our results are consistent with a previous work that found mitochondrial function was impaired during early lactation when cows were fed TMR or in a pasture-based system (García-Roche et al., 2019). In addition, our present study also shows a strain by treatment interaction when annual means are assessed, in this case Complex-I maximum respiratory rate and the respiratory control ratio are lower for NAH cows in the MaxP treatment and Complex-II state 3, oligomycin-sensitive respiration and the I maximum

respiratory rate are lower for NAH cows in the MaxP treatment. Indeed, recent proteomic evidence have confirmed that oxidative phosphorylation and mitochondrial dysfunction are relevant pathways in the pathogenesis of NEB (Swartz et al., 2021; Zhang et al., 2021). Mitochondria are at the crossroads of energy metabolism as they convert energy from nutrients to utilizable energy for cellular functions, hence an impairment would not only account for inefficient energy conversion but also a propensity to lipid, protein and DNA damage due to increased production of reactive oxygen species (Mavangira and Sordillo, 2017). It is important to note that the maintenance of cellular functions such as protein and lipid resynthesis and ion transport represents between 40 and 55% of basal energy expenditure and are essential functions for tissue maintenance; in fact hepatic, cardiac and gastrointestinal tissues can increase their metabolic rate without resorting to hypertrophy which would represent a higher energy expenditure (Baldwin et al., 1980). Indeed, these characteristics make liver tissue an attractive target for improving energy efficiency.

Alongside, although we did not find an effect of moment of lactation in mitochondrial acetylation levels we did find a strain by treatment interaction where NAH cows in the MaxP treatment had the highest levels of mitochondrial protein acetylation, consistent with the mitochondrial impairment found in NAH cows in the MaxP treatment. We have previously reported an association between mitochondrial early lactation impairment and mitochondrial acetylation, especially aggravated in cows in a pasture-based system vs. cows fed TMR, as well as a negative association between mitochondrial deacetylase sirtuin 3 protein levels and mitochondrial acetylation (García-Roche et al., 2019). Even though we did not confirm an effect of moment of lactation we did confirm that plane of nutrition may affect mitochondrial function and mitochondrial protein acetylation as feeding strategies with a higher contribution of grazed forage are detrimental to mitochondrial function. Acetylation has been reported to inactivate mitochondrial enzymes related to fatty acid oxidation such as 3-hydroxy-3-methylglutaryl-CoA synthase 2 and very long-chain specific acyl-CoA dehydrogenase (ACADVL) (Grabacka et al., 2016) and sirtuin 3 deacetylates these enzymes enhancing lipid metabolism (Newman et al., 2012).

A rate limiting enzyme in fatty acid oxidation is CPT and in the present study, we demonstrated that CPT activity -activity of both CPT1 and *CPT2* since assays were performed in frozen biopsies- was decreased during early lactation and in the MaxP vs. FixP treatment. Although mRNA abundance of *CPT1A* is responsive to elevated levels of hepatic lipid accumulation in calf hepatocytes (Du et al., 2018b) and *CPT1B* is upregulated in severe NEB cows (McCarthy et al., 2010); Li et al., (2012) have shown that abundance of CPT1 mRNA and abundance of *CPT2* mRNA and its protein levels are decreased in hepatic biopsies of ketotic vs. non-ketotic cows. In addition, a study performed comparing early lactation fed-restricted cows and control cows showed that CPT1 activity was not affected by treatment (Dann and Drackley, 2005), indeed, our results are representative of the lactation curve and shed some light on metabolic adaptations of two pasture-based treatments across lactation. The fact that CPT activity is impaired during early lactation when liver triglyceride levels are the highest further aggravates NEB.

Gene expression of key fatty acid oxidation enzymes and transcription factors was studied at 180 DIM comparing NZH and NAH cows in MaxP and NAH cows in MaxP and FixP, in order to focus on strain and treatment differences to elucidate underlying molecular mechanisms that could explain metabolic adaptations during the lactation curve and were not intrinsic to early lactation negative energy balance. The increase in mRNA of *ACADVL*, *RARA* and *RXRB* in NZH vs. NAH and trend of increased mRNA abundance of *ACAT1*, *CD40* and *PPARA* suggests both mitochondrial and peroxisomal fatty acid oxidation pathways are upregulated in NZH cows (Keller et al., 1993; Song et al., 2010). Recently, a previous study suggested that inhibition of retinoid X receptor function is the most prominently enriched pathway in liver of NEB cows accounting for impaired anti-inflammatory cytokine transcription (Swartz et al., 2021). Since acetylation levels were higher in NAH cows in the MaxP treatment and *RARA* and *RXRB* hepatic mRNA abundance was lower and *CD40* mRNA abundance tended to be lower than in NZH cows, it is possible that a pro-inflammatory profile could have negatively impacted in mitochondrial function. From the 24 genes studied only of CD36 was differentially expressed in liver biopsies of NAH cows in the FixP vs. NAH cows in the MaxP treatment, although CD36 has been

demonstrated to be upregulated in severe vs. mild NEB cows (McCarthy et al., 2010); recent evidence in mice overexpressing CD36 has suggested that CD36 functions as a protective metabolic sensor in the liver under lipid overload and metabolic stress (Garbacz et al., 2016) since it promoted glycogen synthesis. Indeed, annual mean of liver glycogen levels showed a strain by treatment interaction indicating that liver glycogen levels were lower for NAH cows in the MaxP treatment. Consistent with these findings, metabolomic analyses showed gluconeogenesis was enriched in NAH cows in the FixP treatment. In addition, recently a novel role has been elucidated for CD36 in mice fed a low-fat diet where CD36 deficiency led to decreased insulin signaling and disordered glucose metabolism (Yang et al., 2020). Interestingly, in a model of hypoxia inhibition of the phosphatidylinositol 3-kinase pathway block the hypoxia-dependent induction of CD36 expression and promoter activity (Mwaikambo et al., 2009). Herein, the phosphatidylinositol signaling system appeared to be enriched in metabolomic analyses of FixP vs. MaxP NAH cows at 180 DIM, the phosphatidylinositol 3-kinase signaling pathway has been studied in mice and cell models and is suggested to modulate lipid metabolism inhibiting catabolism (DeBerardinis et al., 2006) and is downregulated in a mouse model of fatty liver (Yang et al., 2018); to participate of insulin signaling (Kanai et al., 1993). This is consistent with our data as liver triglyceride was augmented in MaxP cows and liver triglyceride to glycogen ratio tended ($P = 0.06$) to be increased in NAH MaxP cows, although liver triglyceride content alone has been widely used as a clinical fatty liver diagnostic test, liver triglyceride to glycogen ratio has been more recommended by Kirovski & Sladojevic, (2017). Cows in the FixP treatment when compared to cows in the MaxP treatment enriched other anabolic pathways such as purine metabolism and pentose phosphate pathways, the latter contributes to fueling the TCA cycle as it yields NADPH and pyruvate and has been found to be upregulated in adipose tissue of over-fed cows (Minuti et al., 2020). With regards to metabolomic analyses of MaxP vs. FixP cows the only identified enriched pathway was the urea cycle. This is in accordance with previous reports that have shown that limiting the supply of rumen degradable nitrogen can reduce milk (Bargo et al., 2002a) and blood urea (Aschemann et al., 2012; Zhang et al., 2019).

## Conclusion

In this work we confirmed hepatic mitochondrial function impairment in early lactation cows in a pasture-based system with concentrate supplementation vs. TMR supplementation and found that carnitine palmitoyltransferase activity is also impaired during early lactation, both phenomena are negatively correlated with liver triglyceride content. A strain by treatment interaction was found evidencing Holstein cows of North American origin in the treatment with maximum grazed pasture had the lowest values in respiratory parameters and highest levels of mitochondrial protein acetylation; suggesting that high levels of grazed pasture is detrimental to hepatic energy metabolism. In addition, when hepatic gene expression was analyzed during late mid-lactation, results yielded that Holstein cows of New Zealand origin had higher mRNA abundance of enzymes and transcription factors involved in mitochondrial and peroxisomal fatty acid oxidation. Finally, metabolomic analyses showed that Holstein cows of North American origin in the treatment with TMR supplementation compared to Holstein cows of North American origin in the treatment with concentrate supplementation shifted their hepatic metabolome during late mid-lactation toward anabolic pathways while the latter enriched the urea cycle. Overall, our results show that cows of both Holstein strains mobilize body reserves during early lactation; however, Holstein cows of New Zealand origin in the maximum pasture treatment maintained a more functional hepatic energy and fatty acid metabolism suggesting a better adaptation. Our work highlights the relevance of selecting a suitable Holstein strain for pasture-based systems.

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**Author Contributions**

MGR conceived and designed lab experiments, performed field and lab experiments, analyzed the results, and wrote the original draft and revised versions of the manuscript. MC conceptualized and designed field experiment, conceived and designed lab experiments, supervised field and lab experiments and results analyses, obtained funding and administrated the project, and reviewed and edited the manuscript. CQ conceived, designed and supervised lab experiments, and reviewed and edited the manuscript. AC conceived, design and supervised lab experiments. AM conceptualized and designed field experiment and obtained funding. DT, ALA and GC performed field and lab experiments. All authors reviewed the manuscript and approved the final version of the manuscript.

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# 8. DISCUSIÓN GENERAL

El presente trabajo se centra en la caracterización de las adaptaciones metabólicas del metabolismo energético hepático y de los componentes asociados a la regulación transcripcional y postraduccional de vacas con o sin inclusión de pasturas (artículos 1 y 2) y la adecuación del genotipo Holstein en sistemas de alimentación de base pastoril con suplementación de concentrado o DTM (artículos 3 y 4).

Actualmente, se estima que la productividad de la vaca lechera está sostenida por el consumo de forraje producido en el establecimiento. En este aspecto, se identifica una clara oportunidad para Uruguay, ya que sistemas de producción en crecimiento sostenido como Nueva Zelanda, Australia, Estados Unidos y Holanda duplican la cosecha de forraje en comparación con los sistemas lecheros de Uruguay (Fariña y Chilibroste, 2019). Sin embargo, el consumo de MS en sistemas pastoriles puede ser insuficiente para sostener la alta producción de leche y es posible que no se alcance el potencial genético (Chilibroste et al., 2012; Kolver y Muller, 1998). Por otra parte, cuando se han comparado sistemas de base pastoril con sistemas que incorporan DTM se pudo demostrar que las vacas en los sistemas con mayores niveles de inclusión pastoril mostraron un pobre estado metabólico, ya que marcadores del BEN se ven incrementados frente a vacas en una DTM (Meikle et al., 2013) y además presentan una menor expresión de genes relacionados con el crecimiento embrionario y la función uterina en vacas bajo un sistema pastoril (Astessiano et al., 2017), así como la ciclicidad ovárica atrasada (Meikle et al., 2013), indicativos de un pobre desempeño reproductivo.

Las vacas lecheras de alta producción sufren adaptaciones metabólicas extremas para sostener niveles de producción de leche que superan en hasta cuatro veces los requerimientos de energía, proteínas y minerales (Drackley, 1999; Bauman, 2000). El hígado juega un rol clave en la coordinación de las adaptaciones metabólicas, ya que participa del metabolismo de los glúcidos, proteínas y lípidos (Wei et al., 2008), en la regulación del metabolismo del colesterol y triglicéridos, así como también en el mantenimiento de la homeostasis y de los niveles de glucosa en sangre (Knebel et al., 2015).

En relación con el metabolismo de los glúcidos, hallamos que los animales sometidos a sistemas con inclusión de pasturas recurren a la movilización de glucógeno durante la lactancia temprana, ya que el catabolismo del glucógeno es mayor en vacas en base pastoril (G1) que en vacas alimentadas con DTM (G0, García-Roche et al., 2021). Asimismo, no hallamos efecto del tratamiento en los niveles de glucógeno en vacas de distinto genotipo Holstein en un sistema de base pastoril con suplementación de concentrado (MaxP) en sala o suplementación de DTM (FixP, artículo 4).

En relación a la gluconeogénes observamos que la abundancia de ARNm de *PCK1*, que codifica fosfoenolpiruvato carboxiquinasa, una enzima clave de la gluconeogénesis (Greenfield et al., 2000b), fue similar entre G0 y G1. Sin embargo, encontramos evidencia de que las vacas G0 y G1 probablemente utilizaron precursores gluconeogénicos distintos, ya que encontramos una mayor abundancia de ARNm de metil-malonil mutasa (*MMUT)* en el caso de G0, y una mayor abundancia de piruvato carboxilasa (*PC)* en el caso de G1 (García-Roche et al., 2021) en lactancia temprana. La enzima *MMUT* cataliza la reacción de entrada del propionato a la gluconeogénesis (Aschenbach et al., 2010) a partir del cual se sintetiza la mayoría de la glucosa cuando el propionato dietario es suficiente. Sin embargo, cuando el propionato es insuficiente se recurre a precursores alternativos como lactato, glicerol y aminoácidos (Kristensen, 2005; Larsen y Kristensen, 2013). Estos precursores son convertidos a piruvato e ingresan a la vía gluconeogénica; consecuentemente, la conversión de piruvato a oxalacetato es catalizada por la enzima piruvato carboxilasa (PC) (Greenfield et al., 2000a). Por otra parte, cuando estudiamos vacas del genotipo Norte Americano (NAH) y neozelandés (NZH) en lactancia media y en la estrategia MaxP hallamos que las vacas NAH tenían mayor abundancia de ARNm de genes de las enzimas de la gluconeogéneis *PC,* fosfoenolpiruvato carboxiquinasa *(PCK1)* y de piruvato deshidrogenasa quinasa que inhibe la enzima piruvato deshidrogenasa (*PDK4)* que las vacas NZH (artículo 3); esto indica que la vía gluconeogénica a partir de piruvato es relevante en vacas NAH durante lactancia media, lo cual podría confirmar su mayor persistencia (Lucy et al., 2009). Además, la abundancia de ARNm de *MMUT* es baja enfatizando en la utilización de reservas corporales como fuente de precursores gluconeogénicos

alternativos en vacas en pastoreo. En suma, nuestros resultados sugieren que la movilización de reservas entre vacas alimentadas con DTM y vacas con inclusión de pasturas es diferencial y especialmente agravada en vacas con mayores niveles de pasturas (García-Roche et al., 2019); además, los efectos de esta movilización se observan de forma más pronunciada en vacas del genotipo NAH en base pastoril cuando se compara con vacas del genotipo NZH en base pastoril (artículo 4).

En relación con el metabolismo lipídico, los niveles de marcadores de movilización como BHB y AGNEs en plasma, así como también los niveles de triglicéridos hepáticos, fueron mayores en vacas G1 vs. vacas G0. Asimismo, la abundancia de ARNm de *HMGCS2* en hígado tendió a ser mayor en el grupo G1 (García-Roche et al., 2021), lo cual podría ser indicativo de una mayor movilización de reservas lipídicas (Astessiano et al., 2015). La expresión génica hepática de *FABP1* —enzima involucrada en el transporte de los ácidos grasos— y *CPT1A* —enzima que cataliza la entrada de ácidos grasos de cadena larga para su oxidación mitocondrial— se vieron aumentadas durante lactancia temprana en comparación con lactancia media solo para las vacas G0, lo que sugiere que las vacas G1 no logran adaptar su metabolismo a niveles más altos de oxidación de ácidos grasos y, por lo tanto, acumulan triglicéridos en el hígado (García-Roche et al., 2021). Otros autores han indicado que la expresión génica de *FABP1* está disminuida en vacas con balance energético severo, lo cual podría limitar la oxidación de ácidos largos de cadena larga y resultar en la acumulación hepática de triglicéridos (Loor, 2010; McCarthy et al., 2010; Guzmán et al., 2013; Albrecht et al., 2019). En paralelo, las vacas en la estrategia MaxP tuvieron mayor acumulación de triglicéridos en hígado que las vacas FixP, alcanzando un pico en lactancia temprana, y, además, la actividad específica de CPT se vio disminuida en las vacas MaxP alcanzando los menores niveles en lactancia temprana (artículo 4). Esto está de acuerdo con nuestro trabajo anterior donde hallamos menores niveles de ARNm de *CPT1A* en vacas con inclusión de pasturas frente a vacas alimentadas con DTM, así como también con trabajos anteriores que han demostrado que niveles de ARNm de *CPT1* y niveles de ARNm y proteína de *CPT2* se ven disminuidos en vacas con cetosis en comparación con vacas control (Li et al., 2012). Sin embargo, cuando se

compararon los distintos genotipos Holstein (NZH vs. NAH) en las estrategias MaxP y FixP no se encontraron diferencias significativas entre genotipos en la acumulación de triglicéridos en hígado (artículo 4); esta evidencia sugiere que ambos genotipos tienen una movilización similar. Estos hallazgos son consistentes con trabajos anteriores que han demostrado que los niveles en plasma de BHB y AGNEs son similares entre genotipos (Lucy et al., 2009; Grala et al., 2011). En suma, nuestros resultados sugieren que la estrategia nutricional tiene un impacto mayor al genotipo en la movilización de lípidos y su depósito a nivel del hígado, jerarquizando el rol de las intervenciones a nivel nutricional a fin de evitar el BEN.

De hecho, en este trabajo encontramos que la disfunción mitocondrial hepática se instaura durante el BEN de lactancia temprana —período de mayor movilización de reservas corporales— por su asociación negativa con marcadores como BHB en plasma y triglicéridos en hígado, y está especialmente agravada en los tratamientos con mayor inclusión de pastoreo y en el genotipo NAH en la estrategia MaxP (García-Roche et al., 2019, artículo 3, artículo 4). En detalle, en el caso del modelo 1, las mayores diferencias se observaron en la respiración sostenida por sustratos del complejo I mitocondrial en la máxima capacidad respiratoria y en la respiración asociada a la síntesis de ATP (respiración en estado 3 y respiración sensible a oligomicina), donde estos valores se vieron aumentados en las vacas G0 frente a las vacas G1 a los 35 DEL (García-Roche et al., 2019). Esto apoya la relevancia de la estrategia nutricional a la hora de evitar la disfunción mitocondrial a nivel hepático. Recordemos que en el hígado se llevan a cabo diversos procesos con alta demanda energética, entre ellos, la gluconeogénesis, siendo clave la preservación de la síntesis de ATP a nivel mitocondrial.

En paralelo, en el caso del modelo 2, también se observaron diferencias en los parámetros anteriormente mencionados y en la respiración sostenida por sustratos del complejo I en las vacas FixP frente a las vacas MaxP a los 21 DEL (artículo 4). Sin embargo, en contraste con las vacas G0 que fueron alimentadas con una DTM, en el segundo modelo las vacas FixP pastorearon el equivalente anual al 33% del consumo de MS ofrecido. Por tanto, aunque las vacas no se

hayan mantenido en un sistema confinado, la suplementación con DTM y el menor tiempo de pastoreo contribuyeron a evitar la disfunción mitocondrial.
En relación con los parámetros respiratorios de los genotipos NAH vs. NZH, las mayores diferencias se observaron en la interacción genotipo por estrategia de alimentación, donde las vacas NAH MaxP mostraron los menores valores de la máxima capacidad respiratoria, la respiración en estado 3, la respiración sensible a oligomicina y en el índice del control respiratorio cuando se utilizaron sustratos del complejo II mitocondrial (artículo 4). En particular, esto está dado en los 180 DEL, fecha que coincide con primavera, la estación donde la producción de forraje para cosecha directa se maximiza y momento en el que los genotipos Holstein de origen divergente pueden diferenciarse (artículo 3). Estos resultados indican que en un sistema con fuerte base pastoril el genotipo NZH mantuvo una mejor funcionalidad mitocondrial hepática, lo cual podría traducirse en mayor aporte de ATP para la función hepática.

Como ya mencionamos, la homeostasis energética hepática es clave para mantener dos vías metabólicas de crucial importancia para la vaca lechera: la gluconeogénesis y el ciclo de la urea (White, 2020). La fosforilación oxidativa representa la principal fuente de ATP en la célula y es la función predominante de las mitocondrias. Además, las mitocondrias se regulan dinámicamente para atender un amplio espectro de situaciones que demandan energía y su incapacidad para responder adecuadamente puede considerarse una disfunción (Brand y Nicholls, 2011). Una de las estrategias utilizadas para enfrentar una creciente demanda de energía es aumentar la abundancia mitocondrial (Pesta et al., 2011). Un estudio reciente realizado en vacas lecheras de alta y baja eficiencia desarrollado durante el período de transición demostró que las vacas de alta eficiencia tenían una menor producción de calor por kg de peso metabólico y mayor abundancia mitocondrialn sugiriendo que una baja eficiencia alimenticia podría resultar de un menor número de mitocondrias y mayores pérdidas de calor (Kennedy et al., 2021). En esta línea, en el modelo 2 encontramos una mayor abundancia mitocondrial hepática de las vacas NZH en la estrategia MaxP comparadas con las vacas NAH en la misma estrategia (artículo 3). Además, las vacas NZH durante lactancia media tardía tuvieron una menor producción de calor residual por kg de peso metabólico que las vacas

NAH, lo cual podría estar asociado con menores costos de mantenimiento (Talmón et al., 2020). Menores tasas de producción de calor y mayor abundancia mitocondrial hepática, combinado con una función mitocondrial aumentada (artículo 3), sugieren que las vacas NZH pueden ser más eficientes que las vacas NAH (Kennedy et al., 2021). Es importante destacar que el mantenimiento de las funciones celulares, como la resíntesis de proteína y lípidos y el transporte de iones, representa entre el 40 y el 55% del gasto energético basal. Tales funciones son esenciales para el mantenimiento de los tejidos. En especial los tejidos hepático, cardíaco y gastrointestinal pueden aumentar su tasa metabólica sin recurrir a la hipertrofia, lo cual representaría un gasto energético mayor (Baldwin et al., 1980). En efecto, estas características tornan al tejido hepático en un blanco atractivo para mejorar la eficiencia energética.

Por otra parte, en este trabajo observamos que la acetilación presenta un mecanismo postraduccional plausible en la disfunción mitocondrial, ya que los tratamientos que presentaron disfunción mitocondrial como el grupo de pastoreo G1 (García-Roche et al., 2019) y las vacas NAH en la estrategia MaxP también presentaron los mayores niveles de acetilación mitocondrial (artículo 4). La acetilación es una modificación postraduccional reversible que puede modificar la actividad y estabilidad de enzimas clave de la beta-oxidación, el ciclo de Krebs, el metabolismo de los cuerpos cetónicos, la cadena de transporte de electrones y la fosforilación oxidativa (Hirschey et al., 2010; Wang et al., 2010; Wagner y Payne, 2011; Anderson y Hirschey, 2012; Alrob et al., 2014). Los aumentos en la acetilación se dan por un desbalance en las reacciones de acetilación y desacetilación. La acetilación de proteínas mitocondriales puede ser no enzimática (Wagner y Payne, 2013) o catalizada por acetiltransferasas como la acetilasa GCN5L1 (Drazic et al., 2016), mientras que la desacetilación mitocondrial está catalizada por las sirtuinas 3 y 5 (Osborne et al., 2014). En este sentido, nosotros observamos que los niveles de proteína de la desacetilasa sirtuina 3 estaban aumentados en homogeneizados de biopsias hepáticas de animales en el tratamiento G0 y que la disfunción mitocondrial se correlacionaba negativamente con los niveles de sirtuina 3 (García-Roche et al., 2019). Es posible que, por un lado, sirtuina 3 tenga como sustrato proteínas mitocondriales acetiladas relacionadas a vías catabólicas que ceden su potencial reductor a la

cadena respiratoria y esté expresada en mayor abundancia en vacas alimentadas con una DTM mitigando los efectos adversos de la movilización de reservas que resulta del balance energético negativo de lactancia temprana —para ahondar en este mecanismo se adjunta el Informe de Avance del Proyecto CSIC Iniciación (ANEXO)—. Por otro lado, los niveles de ARNm de la sirtuina 1 estaban aumentados en el tratamiento G0, especialmente durante lactancia temprana (García-Roche et al., 2021). Es posible que sirtuina 1 actúe a nivel transcripcional favoreciendo la expresión de genes relacionados al catabolismo (Purushotham et al., 2009). Estos resultados sugieren que la acetilación de proteínas puede cumplir un rol relevante en la regulación de las vías catabólicas mitocondriales hepáticas, encargadas de la obtención de energía, y apuntan a las sirtuinas como blancos farmacológicos interesantes en este contexto.

En esta línea, también estudiamos la activación de la proteína quinasa activada por AMP (AMPK) por medio de la fosforilación en el modelo 1 y encontramos que se veía aumentada durante la lactancia temprana. Esta proteína es un regulador de la homeostasis energética capaz de coordinar múltiples vías metabólicas para adaptar los procesos celulares al estado energético y actúa, en primera instancia, de forma rápida y directa por medio de la fosforilación de enzimas clave. Sin embargo, se ha demostrado que tiene un efecto sobre la expresión de genes con el fin de aumentar la actividad de vías catabólicas (Foretz y Viollet, 2011). Está reportado que tanto AMPK y la sirtuina 1 (*SIRT1*) regulan la actividad de PGC-1α, un factor de transcripción que regula la expresión de genes relacionados a la oxidación de lípidos y el metabolismo mitocondrial (Cantó y Auwerx, 2009). En nuestro trabajo vimos que la expresión génica hepática de *SIRT1* estaba aumentada durante la lactancia temprana únicamente para el grupo G0, mientras que la expresión de PGC-1α estaba aumentada durante la lactancia temprana, sin diferencia entre tratamientos. La *SIRT1* es capaz de aumentar la actividad de transcripcional de PGC-1α por medio de la acetilación (Cantó y Auwerx, 2009) y, por su parte, PGC-1α induce la transcripción de genes gluconeogénicos (Herzig et al., 2001; Yoon et al., 2001; Puigserver et al., 2003; Rodgers et al., 2005; Rowe y Arany, 2014). La *SIRT1* también regula positivamente al receptor activado por proliferador de peroxisomas alfa (PPAR$\alpha$), promoviendo la transcripción de genes de la $\beta$-oxidación de ácidos grasos mitocondrial (Purushotham et al.,

2009). En esta línea, también observamos que la expresión génica hepática de *PPARA* (PPARα) está aumentada durante la lactancia temprana frente al inicio de la lactancia media para las vacas G0 y G1, sin diferencias entre los tratamientos (García-Roche et al., 2021). Esto está de acuerdo con un importante aumento a nivel hepático en la gluconeogénesis y en las vías relacionadas al transporte y oxidación de los ácidos grasos durante la lactancia temprana, que obedece a la adaptación del metabolismo de la vaca lechera (Loor, 2010).

En paralelo, estudiamos la expresión génica de enzimas claves de vías relacionadas al transporte y oxidación de los ácidos grasos en vacas NZH vs. vacas NAH durante lactancia media tardía y vimos que la expresión génica de *PPARA* tendió a estar aumentada y la expresión de componentes de la vía de señalización de receptores de retinoides RAR-RXR también estuvieron aumentados. Tanto PPARα como los receptores de retinoides pueden actuar en conjunto y activar la transcripción de genes asociados a la oxidación de ácidos grasos (Keller et al., 1993; Song et al., 2010; Li et al., 2021). La importancia de PPARα en la coordinación de la adaptación metabólica en vacas lecheras ha sido resaltada extensamente por otros autores, ya que es un factor ampliamente expresado en el hígado y es activado por ácidos grasos no esterificados (Roche et al., 2013). El hecho de que estos factores de transcripción estén aumentados en las vacas NZH vs. vacas NAH apunta a una mejor adaptación metabólica.

# 9. CONCLUSIONES

En este trabajo demostramos que la movilización de reservas lipídicas es mayor en las estrategias con mayor proporción de forraje de cosecha directa y que, a diferencia de las vacas alimentadas con una dieta totalmente mezclada, las vacas de pastoreo recurren a la movilización de reservas para sostener la gluconeogénesis. La mayor movilización de reservas lipídicas tiene como consecuencia la disfunción mitocondrial hepática, que, además, está relacionada con la acetilación mitocondrial y negativamente relacionada a la desacetilasa mitocondrial sirtuina 3 y la desacetilasa nuclear sirtuina 1; ambas se encuentran en mayores niveles (de proteína y ARNm, respectivamente) en las vacas alimentadas con una dieta totalmente mezclada. Hipotetizamos que el eje proteína quinasa activada por AMP (AMPK), sirtuina 1 (*SIRT1*) y coactivador del receptor gamma 1-alfa activado por el proliferador de peroxisomas (PGC-1α) plantea un posible mecanismo por el cual dos sensores metabólicos del metabolismo energético (AMPK y *SIRT1*) actúan de forma coordinada para regular la transcripción génica de genes relacionados al metabolismo de la glucosa, de los ácidos grasos y de la biogénesis mitocondrial por medio del coactivador transcripcional PGC-1α.

A pesar de que vacas Holstein del genotipo norteamericano y vacas Holstein del genotipo neozelandés tengan una movilización de reservas similar, la mejor adaptación del metabolismo energético hepático de las vacas del genotipo neozelandés a los sistemas pastoriles, demostrado por su mayor función y abundancia mitocondrial, le confiere a este genotipo un mejorado estado metabólico durante lactancia media-tardía. Por otra parte, el genotipo Holstein norteamericano muestra tener una mayor persistencia de la lactancia por su actividad gluconeogénica aumentada durante lactancia media-tardía. Los mecanismos moleculares subyacentes sugieren que las vacas del genotipo neozelandés tienen mayor expresión de las vías de oxidación de los ácidos grasos tanto mitocondrial como peroxisomal.

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# 11. ANEXOS

MATERIAL SUPLEMENTARIO

# GLUCOSE AND FATTY ACID METABOLISM OF DAIRY COWS IN A TOTAL MIXED RATION OR PASTURE-BASED SYSTEM DURING LACTATION

García-Roche M, Cañibe G, Casal A, Mattiauda DA, Ceriani M, Jasinsky A, Cassina A, Quijano C and Carriquiry M (2021)
Frontiers in Animal Science 2:622500. doi: 10.3389/fanim.2021.622500

**Table S1.** Primers used for real time qPCR quantification.

| Gene[1,2] | Accession#[3] | | Primer sequence[4] | Length(bp) | Efficiency |
|---|---|---|---|---|---|
| ACACA | NM_174224 | F | GGAGGAGGAGGGAAGGGAAT | 264 | 0.88 |
| | | R | CGAGCAGCAATAACATGGCC | | |
| ACTB | NM_173979 | F | CTCTTCCAGCCTTCCTTCCT | 178 | 1.09 |
| | | R | GGGCAGTGATCTCTTTCTGC | | |
| ADIPOR1 | NM_001034055 | F | GGCTCTACTACTCCTTCTAC | 154 | 1.07 |
| | | R | ACACCCCTGCTCTTGTCTG | | |
| CPT1A | NM_001304989 | F | CAAAACCATGTTGTACAGCTTCCA | 93 | 1.17 |
| | | R | GCTTCCTTCATCAGAGGCTTCA | | |
| FABP1 | NM_175817 | F | GTTCATCATCACCGCTGGCT | 101 | 0.91 |
| | | R | CCACTGCCTTGATCTTCTCCC | | |
| *G6PC* | NM_001076124 | F | TGAGGATGGAGAAGGGAATG | 203 | 1.18 |
| | | R | TGAACCAATCCTGGGAGTTC | | |
| HMGCS2 | NM_001045883 | F | AGAACGTCTGCCCTCTTTCA | 81 | 1.00 |
| | | R | TACAAGGCTGCTGTGTCCAG | | |
| HPRT1 | NM_001034035 | F | TGGAGAAGGTGTTTATTCCTC | 105 | 1.00 |
| | | R | CACAGAGGGCCACAATGTGA | | |
| *MMUT* | NM_173939 | F | GCAGAACTGGACTCCAAGCT | 202 | 1.21 |
| | | R | TTGGCCATTCCACCCATCTC | | |
| LEPR | NM_173928 | F | ACCACACCTTCCGTTCTCAG | 164 | 0.91 |
| | | R | GGGACAACACTCTTGACTC | | |
| PC | NM_177946.4 | F | CGTCTTTGCCCACTTCAAGG | 70 | 1.05 |
| | | R | GAAGAGGCGCGTATTGAGGC | | |
| PCK1 | NM_174737 | F | TGGCCATGATGAACCCTACTC | 77 | 1.36 |
| | | R | GTCAAATTTCATCCAGGCATATC | | |
| *PPARGC1A* | NM_177945 | F | TGAACCCAGCTGCTGAAGAG | 216 | 1.20 |
| | | R | AGAACCTGCGGTGTCTTCAG | | |
| PPARA | NM_001034036 | F | CGGTGTCCACGCATGTGA | 56 | 1.36 |
| | | R | TCAGCCGAATCGTTCTCCTAAA | | |
| *SIRT1* | NM_001192980 | F | TACCCCATGAAGTGCCTCAG | 238 | 1.29 |
| | | R | ATATTGAGGGGTGTGGGTGG | | |

[1] Acetyl-CoA carboxylase (ACACA), ß-actin (ACTB), adiponectin receptor 1 (ADIPOR1), carnitine palmitoyl-transferase 1 (CPT1A), liver fatty acid binding proten (FABP1), glucose-6-phosphatase (*G6PC*), hydroxymethylglutaryl-CoA synthase 2 (HMGCS2), hypoxanthine phosphoribosyl transferase (HPRT1); methylmalonyl-CoA mutase (*MMUT*), leptin receptor (LEPR), pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PCK1), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PPARGC1A*), peroxisome proliferator-activated receptor alpha (PPARA), sirtuin 1 (*SIRT1*).

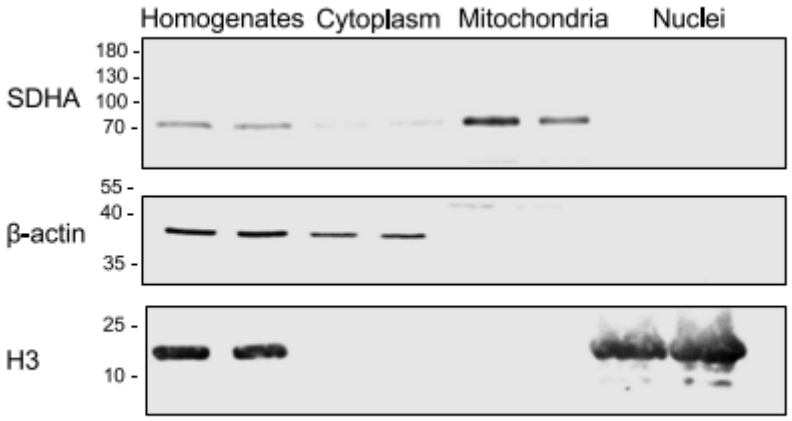
[2] Uniprot abbreviations: Acetyl-CoA carboxylase (ACC1), ß-actin (ACTB), adiponectin receptor 1 (ADIPOR1), carnitine palmitoyl-transferase 1 (CPT1A), liver fatty acid binding proten (FABP1), glucose-6-phosphatase (*G6PC*), hydroxymethylglutaryl-CoA synthase 2 (HMGCS2), hypoxanthine phosphoribosyl transferase (HPRT1); methylmalonyl-CoA mutase (*MMUT*), leptin receptor (LEPR), pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PCK1), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), peroxisome proliferator-activated receptor alpha (PPARA), sirtuin 1 (*SIRT1*).

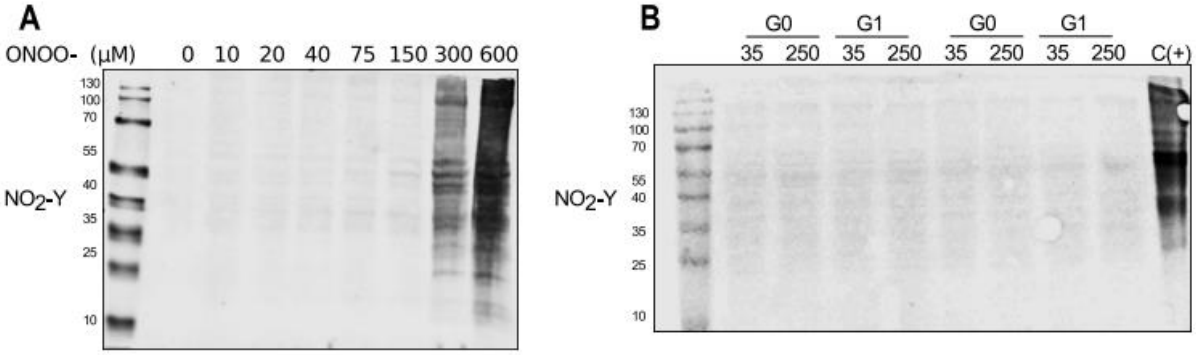
[3] Gene bank sequences and links: ACACA – https://www.ncbi.nlm.nih.gov/nuccore/NM_174224.2; ACTB: https://www.ncbi.nlm.nih.gov/nuccore/NM_173979.3;
ADIPOR1: https://www.ncbi.nlm.nih.gov/nuccore/NM_001034055;
CPT1A: https://www.ncbi.nlm.nih.gov/nuccore/NM_001304989.2;
FABP1: https://www.ncbi.nlm.nih.gov/nuccore/NM_175817;
*G6PC*: https://www.ncbi.nlm.nih.gov/nuccore/NM_001076124.2;
HMGCS2: https://www.ncbi.nlm.nih.gov/nuccore/NM_001045883;
HPRT1: https://www.ncbi.nlm.nih.gov/nuccore/NM_001034035;
*MMUT*: https://www.ncbi.nlm.nih.gov/nuccore/NM_173939.2
LEPR: https://www.ncbi.nlm.nih.gov/nuccore/NM_173928.2
PC: https://www.ncbi.nlm.nih.gov/nuccore/NM_177946.4
PCK1: https://www.ncbi.nlm.nih.gov/nuccore/NM_174737.2
*PPARGC1A*: https://www.ncbi.nlm.nih.gov/nuccore/NM_177945
PPARA: https://www.ncbi.nlm.nih.gov/nuccore/77404270
*SIRT1*: https://www.ncbi.nlm.nih.gov/nuccore/NM_001192980.1

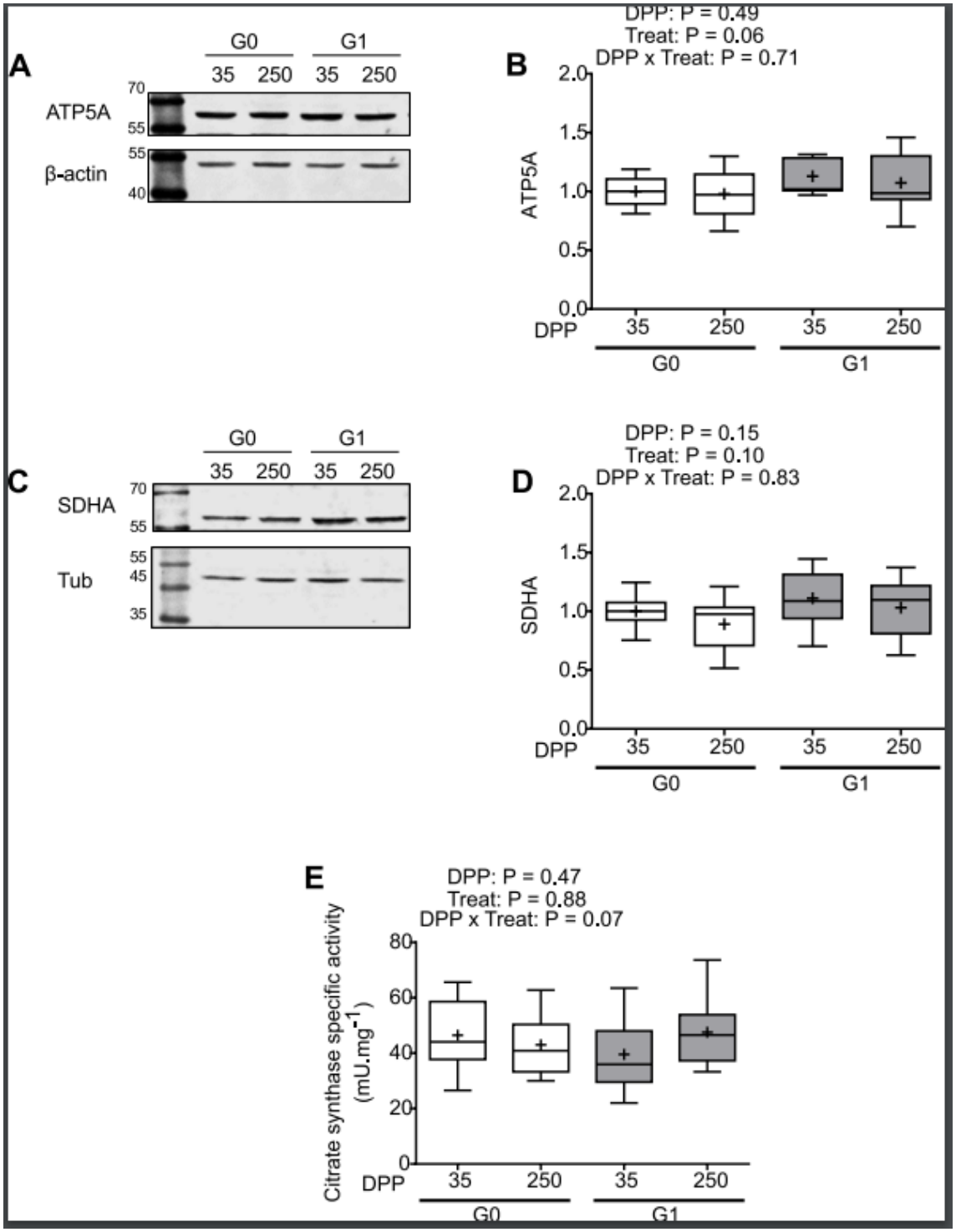
[4]F = foreword; R = reverse

# IMPAIRED HEPATIC MITOCHONDRIAL FUNCTION DURING EARLY LACTATION IN DAIRY COWS: ASSOCIATION WITH PROTEIN LYSINE ACETYLATION

García-Roche M, Casal A, Mattiauda DA, Ceriani M, Jasinsky A, Mastrogiovanni M., Trostchanshy A., Carriquiry M Cassina A and Quijano C (2019) PLoS ONE 14(3): e0213780. https://doi.org/10.1371/journal. pone.0213780


Homogenates
Cytoplasm
Mitochondria
Nuclei
SDHA
180
130
100
70
β-actin
55
40
35
H3
25
10


A
ONOO- (μM) 0 10 20 40 75 150 300 600
130
100
70
55
40
35
25
10
NO2-Y
B
G0 G1 G0 G1
35 250 35 250 35 250 35 250 C(+)
130
100
70
55
40
35
25
10
NO2-Y


A
G0
G1
35
250
35
250
70
55
ATP5A
55
40
β-actin
B
DPP: P = 0.49
Treat: P = 0.06
DPP x Treat: P = 0.71
ATP5A
2.0
1.5
1.0
0.5
0.0
DPP
35
250
35
250
G0
G1
C
G0
G1
35
250
35
250
70
55
SDHA
55
45
35
Tub
D
DPP: P = 0.15
Treat: P = 0.10
DPP x Treat: P = 0.83
SDHA
2.0
1.5
1.0
0.5
0.0
DPP
35
250
35
250
G0
G1
E
DPP: P = 0.47
Treat: P = 0.88
DPP x Treat: P = 0.07
Citrate synthase specific activity
(mU.mg-1)
80
60
40
20
0
DPP
35
250
35
250
G0
G1

# DIFFERENTIAL HEPATIC MITOCHONDRIAL FUNCTION AND GLUCONEOGENIC GENE EXPRESSION IN TWO HOLSTEIN STRAINS IN A PASTURE-BASED SYSTEM

García-Roche M, Talmón D., Cañibe G., Astessiano A. L., Mendoza A., Quijano C., Cassina A. and Carriquiry M.

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**Table S1.** Primers used for real time qPCR quantification.

| Gene[1,2] | Accession#[3] | | Primer sequence[4] | Length(bp) | Efficiency |
|---|---|---|---|---|---|
| ACTB | NM_173979 | F | CTCTTCCAGCCTTCCTTCCT | 178 | 1.00 |
| | | R | GGGCAGTGATCTCTTTCTGC | | |
| *G6PC* | NM_001076124 | F | TGAGGATGGAGAAGGGAATG | 203 | 1.00 |
| | | R | TGAACCAATCCTGGGAGTTC | | |
| HPRT1 | NM_001034035 | F | TGGAGAAGGTGTTTATTCCTC | 105 | 1.39 |
| | | R | CACAGAGGGCCACAATGTGA | | |
| *MMUT* | NM_173939 | F | GCAGAACTGGACTCCAAGCT | 202 | 1.16 |
| | | R | TTGGCCATTCCACCCATCTC | | |
| *SDHA* | NM_174178.2 | F | ACATGCAGAAGTCGATGCAG | 155 | 1.23 |
| | | R | GGTCTCCACCAGGTCAGTGT | | |
| PC | NM_177946.4 | F | AGGGAAGCTCCTATTTGCTCC | 234 | 1.16 |
| | | R | CGGTGGATGTGGTCCTTCTCT | | |
| PCK1 | NM_174737 | F | TGGCCATGATGAACCCTACTC | 76 | 1.00 |
| | | R | GTCAAATTTCATCCAGGCATATC | | |
| *PDHA1* | NM_001101046.2 | F | ATCCTCTGTCGTCCCCTTCT | 86 | 1.12 |
| | | R | CACCTCATGCGAAGAGTTGA | | |
| *PDK4* | NM_001101883.1 | F | TCGTATTTCTACCCGGATGC | 113 | 1.04 |
| | | R | CTGCTGCCACATCACAGTT | | |
| *PPARGC1A* | NM_177945 | F | TGAACCCAGCTGCTGAAGAG | 216 | 1.21 |
| | | R | AGAACCTGCGGTGTCTTCAG | | |
| PPARA | NM_001034036 | F | CGGTGTCCACGCATGTGA | 56 | 1.36 |
| | | R | TCAGCCGAATCGTTCTCCTAAA | | |
| *SIRT1* | NM_001192980 | F | TACCCCATGAAGTGCCTCAG | 238 | 1.00 |
| | | R | ATATTGAGGGGTGTGGGTGG | | |
| NDUFV1 | XM_010821048.2 | F | GTTCTTCTTAGGTTCTCACGTGG | 251 | - |
| | | R | TGAGAATTACTGACGTGACCTCT | | |
| *mt-CO1* | NC_006853.1 | F | TCTTCCCACAACACTTTCTAGGA | 198 | - |
| | | R | TGTCGTGGTTAAGTCTACAGTCA | | |

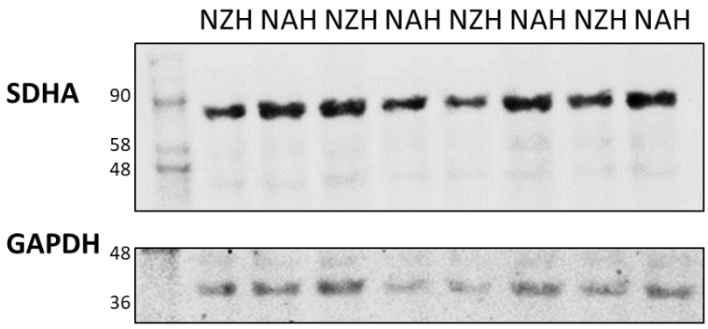
[1] ß-actin *(ACTB),* glucose-6-phosphatase *(G6PC),* hypoxanthine phosphoribosyl transferase *(HPRT1);* methylmalonyl-CoA mutase *(MMUT),* succinate dehydrogenase complex, subunit A, flavoprotein (Fp) *(SDHA),* pyruvate carboxylase *(PC),*

phosphoenolpyruvate carboxykinase *(PCK1*), pyruvate dehydrogenase E1 subunit alpha 1 *(PDHA1),* pyruvate dehydrogenase kinase *(PDK4),* peroxisome proliferator-activated receptor gamma coactivator 1-alpha *(PPARGC1A),* peroxisome proliferator-activated receptor alpha *(PPARA),* sirtuin 1 *(SIRT1).* NADH:ubiquinoneoxidoreductase core subunit V1 (*NDUFV1*, nuclear gene) and mitochondrially encoded cytochrome c oxidase I (*mt-CO1*, mitochondrial gene).

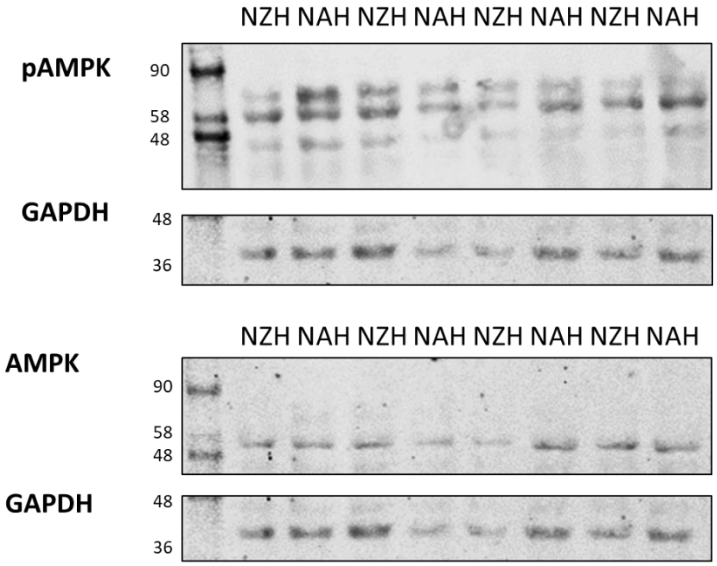
[2] Uniprot abbreviations: ß-actin (ACTB), glucose-6-phosphatase (*G6PC*), hypoxanthine phosphoribosyl transferase (HPRT1); methylmalonyl-CoA mutase (*MMUT*), succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (*SDHA*), pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PCK1), pyruvate dehydrogenase E1 subunit alpha 1 (PDH-A1), pyruvate dehydrogenase kinase 4 (*PDK*4), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), peroxisome proliferator-activated receptor alpha (PPARA), sirtuin 1 (*SIRT1*), NADH:ubiquinoneoxidoreductase core subunit V1 (NDUFV1). mitocondrially encoded cytochrome c oxidase I (MT-CO1),

[3] Gene bank sequences and links: ACTB:
https://www.ncbi.nlm.nih.gov/nuccore/NM_173979.3;
*G6PC*: https://www.ncbi.nlm.nih.gov/nuccore/NM_001076124.2;
HPRT1: https://www.ncbi.nlm.nih.gov/nuccore/NM_001034035;
*MMUT*: https://www.ncbi.nlm.nih.gov/nuccore/NM_173939.2
*SDHA*: https://www.ncbi.nlm.nih.gov/nuccore/NM_174178
PC: https://www.ncbi.nlm.nih.gov/nuccore/NM_177946.4
PCK1: https://www.ncbi.nlm.nih.gov/nuccore/NM_174737.2
*PDHA1*: https://www.ncbi.nlm.nih.gov/nucleotide/402691728
*PDK*4: https://www.ncbi.nlm.nih.gov/nucleotide/156120414
*PPARGC1A*: https://www.ncbi.nlm.nih.gov/nuccore/NM_177945
PPARA: https://www.ncbi.nlm.nih.gov/nuccore/77404270
*SIRT1*: https://www.ncbi.nlm.nih.gov/nuccore/NM_001192980.1
NDUFV1: https://www.ncbi.nlm.nih.gov/nuccore/XM_010821048.2
Mt-CO1: https://www.ncbi.nlm.nih.gov/nuccore/NC_006853.1

[3]F = foreword; R = reverse




Supplementary Figure 1. Succinate dehydrogenase subunit A (*SDHA*) levels in liver homogenates of cows from both NZH and NAH groups in a pasture based system during mid-lactation. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control.




Supplementary Figure 2. Phosphorylated AMPK and total AMPK levels in liver homogenates of cows from both NZH and NAH groups in a pasture based system during mid-lactation. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control.

# HEPATIC METABOLISM OF GRAZING HOLSTEIN COWS OF DIVERGING GENETIC ORIGIN SUPPLEMENTED WITH TOTAL MIXED RATION OR CONCENTRATE DURING LACTATION

García-Roche M, Talmón D., Cañibe G., Astessiano A. L., Mendoza A., Cassina A., Quijano C., and Carriquiry M.

Versión borrador.

**Table S1.** Primers used for real time qPCR quantification.

| Gene[1,2] | Accession#[3] | | Primer sequence[4] | Length(bp) | Efficiency |
|---|---|---|---|---|---|
| *ACADVL* | NM_174494.2 | F | CCAGCCCCTGTGGAAAATACTA | 62 | 0.72 |
| | | R | GCCCCCGTTACTGATCCAA | | |
| *ACAT1* | NM_001046075.1 | F | AGAGCATGTCCAATGTCCCC | 70 | 0.94 |
| | | R | TCTTCAAGCTTTACCCCACCA | | |
| *ACOX2* | NM_001102015.2 | F | CCCTACATGGCATCCTGACT | 186 | 1.44 |
| | | R | CATAACAGCCGAGTGCTGAA | | |
| *APOA4* | NM_001037480.1 | F | TGAATCCAGGAAGGATCTGG | 249 | 0.89 |
| | | R | CTTGGAAGAGGGTGTTGAGC | | |
| *APOA5* | NM_001083492.2 | F | GACGACCTGTGGGAAGACAT | 218 | 1.15 |
| | | R | CTGAGGCCTAGGATGACAGC | | |
| *APOC2* | NM_001102380.2 | F | CAGAGTCTGCCACCTCAGTG | 197 | 1.21 |
| | | R | GCCTTGGCTGTATCCCAGTA | | |
| *ACTB* | NM_173979 | F | CTCTTCCAGCCTTCCTTCCT | 178 | 1.00 |
| | | R | GGGCAGTGATCTCTTTCTGC | | |
| *CD36* | NM_001278621.1 | F | ATTTGACCCAGCACTTGAGG | 181 | 1.20 |
| | | R | CGGGTCTGATGAAAGTGGTT | | |
| *CD40* | NM_001105611.2 | F | AGGGCTTTTGGATACCGTCT | 210 | 1.20 |
| | | R | AACAGGACTCCCATCGTGAC | | |
| *CPT1A* | NM_001304989 | F | CAAAACCATGTTGTACAGCTTCCA | 140 | 0.77 |
| | | R | GCTTCCTTCATCAGAGGCTTCA | | |
| *FABP1* | NM_175817 | F | GTTCATCATCACCGCTGGCT | 101 | 1.00 |
| | | R | CCACTGCCTTGATCTTCTCCC | | |
| *FGF21* | XM_024979245.1 | F | CGGATCGCTGCACTTTGAC | 76 | 0.97 |
| | | R | CTGGTAGACGTTGTATCCATCTTCA | | |
| *HMGCS2* | NM_001045883 | F | AGAACGTCTGCCCTCTTTCA | 81 | 0.71 |
| | | R | TACAAGGCTGCTGTGTCCAG | | |
| *HPRT1* | NM_001034035 | F | TGGAGAAGGTGTTTATTCCTC | 105 | 1.00 |
| | | R | CACAGAGGGCCACAATGTGA | | |
| *LXRA* | NM_001014861.1 | F | CATCAACCCCATCTTCGAGT | 235 | 1.37 |
| | | R | GCTCACCAGTTTCATCAGCA | | |
| *NFKB1* | NM_001076409.1 | F | CTGGAAGCACGAATGACAGA | 215 | 1.12 |

| | | | | | |
|---|---|---|---|---|---|
| | | R | GTGCTGTCTGGAAGGAAAGC | | |
| *NFKB1A* | NM_001045868.1 | F | CTGCACTTAGCCATCATCCA | 230 | 1.17 |
| | | R | TGCTCACAGGCAAGGTGTAG | | |
| *PPARA* | NM_001034036 | F | CGGTGTCCACGCATGTGA | 56 | 1.20 |
| | | R | TCAGCCGAATCGTTCTCCTAAA | | |
| *PPARGC1A* | NM_177945 | F | TGAACCCAGCTGCTGAAGAG | 216 | 1.28 |
| | | R | AGAACCTGCGGTGTCTTCAG | | |
| *RARA* | NM_001014942.4 | F | CAAGACAAATCCTCCGGCTA | 217 | 0.85 |
| | | R | TGTTCCGGTCATTTCTCACA | | |
| *RXRA* | NM_001304343.1 | F | TCCAAAGATGGCTTTCAACC | 150 | 1.45 |
| | | R | AGGAGCTGAAACCAGGACAA | | |
| *RXRB* | NM_001083640.1 | F | GGCAAACACTACGGGGTTTA | 181 | 1.56 |
| | | R | CCTCCCTCTTCATGCCAGTA | | |
| *RXRG* | NM_001075408.1 | F | ATGAAGATATGCCCGTGGAG | 161 | 0.81 |
| | | R | GGCCCATTCAACGAGAGTAA | | |
| *SREBP-1* | NM_001113302.1 | F | CTACATCCGCTTCCTTCAGC | 93 | 1.12 |
| | | R | TCCTTCAGCGATTTGCTTTT | | |
| *TNFA* | NM_174197.2 | F | AACTCTCCCTTCCTGCCAAT | 169 | 1.24 |
| | | R | GGACACCTTGACCTCCTGAA | | |
| *TNFRSF1A* | NM_174674.2 | F | TCCAGTCCTGTCTCCATTCC | 236 | 1.24 |
| | | R | CTGGCTTCCCACTTCTGAAC | | |

[1] Genes: Very long-chain acyl-CoA dehydrogenase (*ACADVL),* acetyl-CoA acetyltransferase 1 *(ACAT1),* ß-actin *(ACTB),* acyl-CoA oxidase 2 *(ACOX2),* apolipoprotein A4 *(APOA4),* apolipoprotein A5 *(APOA5),* apolipoprotein C2 *(APOC2),* CD36 molecule *(CD36),* CD40 *molecule (CD40),* carnitine palmitoyl-transferase 1 *(CPT1A),* liver fatty acid binding protein *(FABP1),* fibroblast growth factor 21 *(FGF21),* hydroxymethylglutaryl-CoA synthase 2 *(HMGCS2),* hypoxanthine phosphoribosyl transferase *(HPRT1);* nuclear receptor subfamily 1 group H member 3 *(LXRA),* nuclear factor kappa B subunit 1 *(NFKB1),* nuclear factor kappa B inhibitor alpha *(NFKB1A),* peroxisome proliferator-activated receptor alpha *(PPARA),* peroxisome proliferator-activated receptor gamma coactivator 1-alpha *(PPARGC1A),* retinoic acid receptor alpha (*RARA),* retinoic X receptor alpha *(RXRA),* retinoic X receptor beta *(RXRB),* retinoic X receptor

gamma *(RXRG),* sterol regulatory element binding transcription factor 1 *(SREBP-1),* tumor necrosis factor alpha *(TNFA),* tumor necrosis factor receptor superfamily member 1A *(TNFRSF1A).*

**Table S2** – Quantitative enrichment analisis

| Pathway | Total Cmpd | Hits | Statistic Q | Expected Q | Raw *p* | Holm *p* | FDR |
|---|---|---|---|---|---|---|---|
| Phosphatidylinositol signaling system | 28 | 1 | 72.988 | 9.0909 | 0.0004024 | 0.013279 | 0.013279 |
| Pyruvate metabolism | 22 | 1 | 64.957 | 9.0909 | 0.0015482 | 0.049542 | 0.025545 |
| Fructose and mannose metabolism | 20 | 1 | 54.074 | 9.0909 | 0.0064239 | 0.19914 | 0.062125 |
| Phosphonate and phosphinate metabolism | 6 | 1 | 52.684 | 9.0909 | 0.0075303 | 0.22591 | 0.062125 |
| Inositol phosphate metabolism | 30 | 2 | 46.534 | 9.0909 | 0.011138 | 0.323 | 0.068862 |
| Purine metabolism | 65 | 2 | 32.804 | 9.0909 | 0.014314 | 0.40079 | 0.068862 |
| Pentose phosphate pathway | 22 | 2 | 43.553 | 9.0909 | 0.014607 | 0.40079 | 0.068862 |
| Arginine biosynthesis | 14 | 2 | 37.404 | 9.0909 | 0.019259 | 0.50073 | 0.074545 |
| Galactose metabolism | 27 | 4 | 37.708 | 9.0909 | 0.020331 | 0.50826 | 0.074545 |
| Starch and sucrose metabolism | 18 | 3 | 36.899 | 9.0909 | 0.023001 | 0.55203 | 0.075905 |
| Neomycin kanamycin and gentamicin biosynthesis | 2 | 1 | 39.549 | 9.0909 | 0.02848 | 0.65503 | 0.085439 |
| Amino sugar and nucleotide sugar metabolism | 37 | 4 | 25.659 | 9.0909 | 0.032425 | 0.71334 | 0.089168 |
| Citrate cycle (TCA cycle) | 20 | 2 | 29.891 | 9.0909 | 0.058276 | 1 | 0.12942 |
| Alanine aspartate and glutamate metabolism | 28 | 3 | 27.414 | 9.0909 | 0.059698 | 1 | 0.12942 |
| Ascorbate and aldarate metabolism | 8 | 2 | 28.163 | 9.0909 | 0.061477 | 1 | 0.12942 |
| Glyoxylate and dicarboxylate metabolism | 32 | 2 | 27.342 | 9.0909 | 0.068723 | 1 | 0.12942 |
| Biosynthesis of unsaturated fatty acids | 36 | 2 | 24.953 | 9.0909 | 0.071335 | 1 | 0.12942 |
| D-Glutamine and D-glutamate metabolism | 6 | 2 | 28.385 | 9.0909 | 0.074513 | 1 | 0.12942 |
| Nitrogen metabolism | 6 | 1 | 28.385 | 9.0909 | 0.074513 | 1 | 0.12942 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Aminoacyl-tRNA biosynthesis | 48 | 2 | 24.826 | 9.0909 | 0.090643 | 1 | 0.14956 |
| Tryptophan metabolism | 41 | 1 | 24.756 | 9.0909 | 0.099768 | 1 | 0.15678 |
| Pyrimidine metabolism | 39 | 2 | 20.731 | 9.0909 | 0.10784 | 1 | 0.16176 |
| alpha-Linolenic acid metabolism | 13 | 1 | 21.609 | 9.0909 | 0.12784 | 1 | 0.16626 |
| Butanoate metabolism | 15 | 1 | 21.539 | 9.0909 | 0.12854 | 1 | 0.16626 |
| Lysine degradation | 25 | 1 | 21.297 | 9.0909 | 0.13099 | 1 | 0.16626 |
| Biotin metabolism | 10 | 1 | 21.297 | 9.0909 | 0.13099 | 1 | 0.16626 |
| Glycolysis / Gluconeogenesis | 26 | 5 | 15.875 | 9.0909 | 0.15712 | 1 | 0.19203 |
| Pentose and glucuronate interconversions | 18 | 5 | 12.756 | 9.0909 | 0.21065 | 1 | 0.24826 |
| Glycosylphosphatidylinositol (GPI)-anchor biosynthesis | 14 | 1 | 9.703 | 9.0909 | 0.32433 | 1 | 0.36907 |
| Glycine serine and threonine metabolism | 33 | 1 | 5.2617 | 9.0909 | 0.47328 | 1 | 0.50381 |
| Cysteine and methionine metabolism | 33 | 1 | 5.2617 | 9.0909 | 0.47328 | 1 | 0.50381 |
| Primary bile acid biosynthesis | 46 | 1 | 3.9378 | 9.0909 | 0.5364 | 1 | 0.5364 |
| Taurine and hypotaurine metabolism | 8 | 1 | 3.9378 | 9.0909 | 0.5364 | 1 | 0.5364 |

**Principales actividades realizadas y avances obtenidos hasta la fecha:**

Para llevar a cabo la primera parte de este proyecto se utilizaron datos y muestras colectadas en un experimento llevado adelante en la Estación Experimental Mario Cassinoni (Paysandú, Fagro) de agosto 2015 a abril 2016, de acuerdo con el protocolo CHEA aprobado por el CEUA de Facultad de Agronomía, Udelar (número de expediente: 021130-001914-15). En este experimento 24 vacas Holstein multíparas de parto en agosto bloqueadas por fecha de parto, PV, BCS, producción previa y número de lactancias fueron utilizadas en un diseño de bloques completos al azar con dos tratamientos durante los primeros 180 días posparto (DPP):

a) una DTM ofrecida *ad libitum* (G0) y
b) pastoreo (dos turnos) con suplementación (G1) de un concentrado comercial hasta 113 DPP.

Como se puede observar en la tabla 1, la expresión génica de la desacetilasa nuclear sirtuina 1 (*SIRT1*) en biopsias de hígado de vacas de los tratamientos G0 y G1 durante lactancia temprana fue mayor para para el grupo G0 que para el grupo G1 ($P < 0.05$) mientras que se mantuvo similar tanto para la acetilasas *GCN5L1* como para las demás sirtuinas 3, 4 y 5 ($P > 0.1$).

**Tabla 1** – Expresión génica de acetilasas y desacetilasas en biopsias de hígado de vacas de los tratamientos G0 y G1 durante lactancia temprana.

| Genes[1] | Tratamientos | | SEM | *P*-valor |
|---|---|---|---|---|
| | G0 | G1 | | |
| *GCN5L1* | 0,45 | 0,44 | 0,09 | 0,96 |
| *SIRT1* | 0,90 | 0,55 | 0,11 | 0,04 |
| *SIRT3* | 1,76 | 0,89 | 0,60 | 0,20 |

| | | | | |
|---|---|---|---|---|
| *SIRT4* | 0,13 | 0,14 | 0,07 | 0,70 |
| *SIRT5* | 0,62 | 1,11 | 0,22 | 0,11 |

*[1]Control general de la síntesis de aminoácidos 5 tipo-1 (GCN5L1), sirtuina 1 (SIRT1), sirtuina 3 (SIRT3), sirtuina 4 (SIRT4), sirtuina 5 (SIRT5).*

Por otra parte, en trabajos anteriores los niveles de proteína SIRT3 y SIRT5 en biopsias de hígado también fueron estudiados mediante la técnica de *western blot* e indicaron que si bien los niveles de SIRT5 no fueron diferentes entre tratamientos, los niveles de SIRT3 fueron mayores para G0 que para G1 (García-Roche et al., 2019). Las diferencias presentadas en este proyecto pueden deberse al hecho de que ambas desacetilasas no sean controladas transcripcionalmente.

En este primer bloque de resultados fue posible cumplir con el objetivo de profundizar en los mecanismos que subyacen a la acetilación (equilibrio entre desacetilación y acetilación). Es decir que se estudió la expresión génica de proteínas involucradas en el equilibrio entre desacetilación y acetilación.

Por otra parte, en vista de la emergencia sanitaria y las complicaciones presentadas tanto para la importación de reactivos como para realizar trabajo de laboratorio en distintas instituciones, en lugar de realizar las inmunoprecipitaciones y poner a punto la espectrometría de masas para la identificación de proteínas acetiladas se decidió enviar las muestras a un laboratorio de referencia Creative Proteomics en Shirley, Nueva York, EE. UU. Por ende, el segundo bloque de resultados pretende realizar una aproximación a la identificación de proteínas acetiladas en biopsias de hígado.

Los datos del acetiloma mostraron que se identificaron 500 residuos acetilados. A partir de los 500 residuos acetilados se pudieron identificar 183 proteínas en total (Figura 1), de las cuales 17 pertenecieron únicamente al tratamiento G0 y 12 únicamente al tratamiento G1.

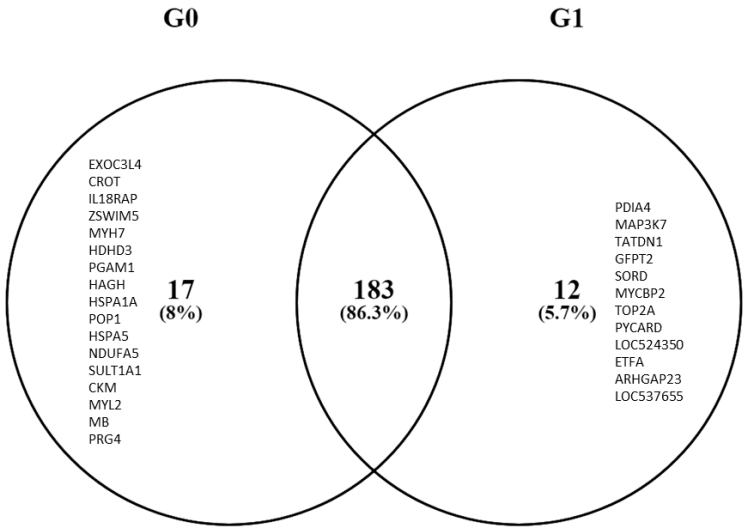



**Figura 1** – Diagrama de Venn construido a partir de las proteínas identificadas en el acetiloma de biopsias hepáticas de los tratamientos G0 y G1 durante lactancia temprana.

Por otra parte, se llevó a cabo el enriquecimiento de vías incluyendo los residuos diferencialmente acetilados entre el grupo G0 y G1. Para el caso del grupo G1 se observó que la (FDR < 0,05, Figura 2) tanto la vía de síntesis de ácidos biliares como el metabolismo de los ácidos grasos son vías diferencialmente acetiladas para el tratamiento G1.

| | Bos taurus (REF) | Client Text Box Input ( Hierarchy ) NEW! | | | | | |
|---|---|---|---|---|---|---|---|
| Reactome pathways | # | # | expected | Fold Enrichment | +/- | raw P value | ▲ FDR |
| Metabolism | 763 | 16 | 3.18 | 5.03 | + | 1.25E-07 | 1.87E-04 |
| Synthesis of bile acids and bile salts via 7alpha-hydroxycholesterol | 16 | 3 | .07 | 45.00 | + | 6.39E-05 | 4.79E-02 |
| Fatty acid metabolism | 62 | 4 | .26 | 15.48 | + | 1.65E-04 | 4.93E-02 |
| Bile acid and bile salt metabolism | 25 | 3 | .10 | 28.80 | + | 2.10E-04 | 5.25E-02 |
| Metabolism of lipids | 269 | 7 | 1.12 | 6.24 | + | 1.54E-04 | 5.75E-02 |
| Synthesis of bile acids and bile salts | 20 | 3 | .08 | 36.00 | + | 1.15E-04 | 5.76E-02 |

**Figura 2** – Enriquecimiento de vías del grupo G1 vs. G0 en el acetiloma de biopsias hepáticas durante lactancia temprana.

Asimismo, para el caso del grupo G0 no se pudo realizar el enriquecimiento de vías, ya que el FDR > 0,1 (Figura 3). Este dato confirma resultados anteriores que destacan que la acetilación es una modificación postraduccional relevante para el tratamiento con mayor inclusión de pasturas-G1 (García-Roche et al., 2019).

| | Bos taurus (REF) | Client Text Box Input ( Hierarchy ) NEW! | | | | | |
|---|---|---|---|---|---|---|---|
| Reactome pathways | # | # | expected | Fold Enrichment | +/- | raw P value | ▲ FDR |
| Metabolism | 763 | 16 | 3.05 | 5.25 | + | 6.69E-08 | 1.00E-04 |
| Gluconeogenesis | 11 | 2 | .04 | 45.55 | + | 1.19E-03 | 1.97E-01 |
| Regulation of TP53 Activity through Acetylation | 14 | 2 | .06 | 35.79 | + | 1.81E-03 | 2.08E-01 |
| HSF1-dependent transactivation | 10 | 2 | .04 | 50.11 | + | 1.01E-03 | 2.15E-01 |

**Figura 3** – Enriquecimiento de vías del grupo G0 vs. G1 en el acetiloma de biopsias hepáticas durante lactancia temprana.

En la Figura 4 se esquematizan los sitios acetilados en proteínas del metabolismo de los ácidos grasos, con énfasis en su oxidación por medio de las vías de beta-oxidación, ciclo de Krebs y cadena respiratoria para el tratamiento G1 durante lactancia temprana. El detalle de dichos sitios y las referencias bibliográficas se encuentran en la Tabla 2.

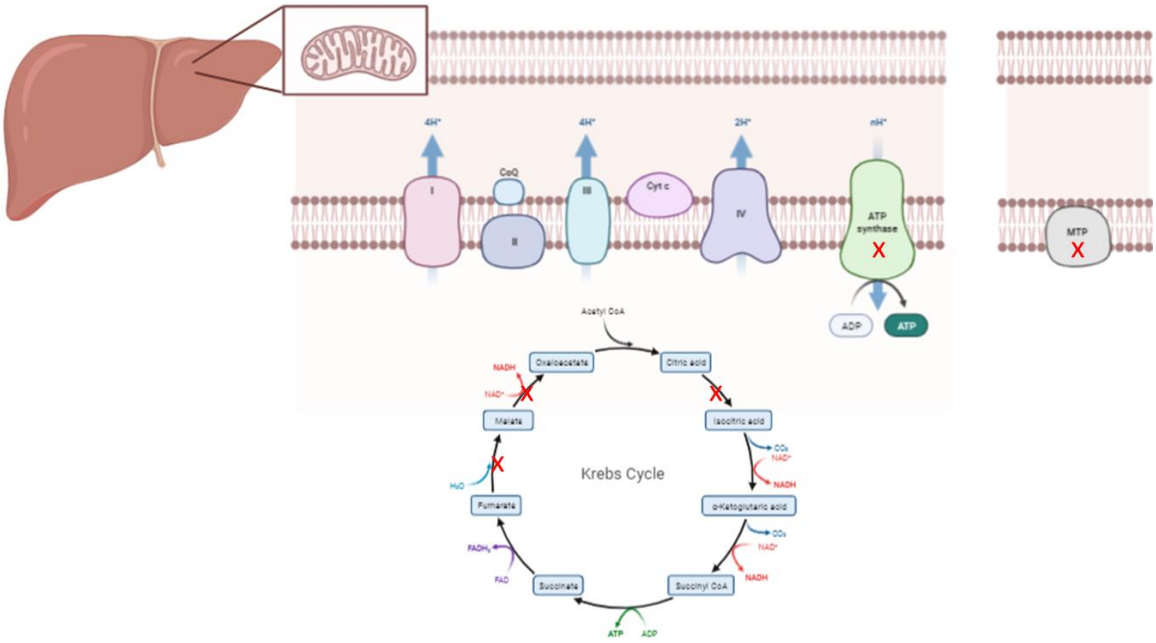



**Figura 4** – Esquema representativo de sitios acetilados en biopsias hepáticas del tratamiento G0 durante lactancia temprana.

**Tabla 2** – Proteínas acetiladas en biopsias hepáticas del tratamiento G1 durante lactancia temprana.

| Proteína | Uniprot | FC | Residuo | Ref. | Sustrato SIRT3 |
|---|---|---|---|---|---|
| ATP synthase subunit gamma | ATP5F1C | 1,7 | Lys197 | Choudhary et al., 2009 | ? |
| ATP synthase subunit alpha | ATP5A1 | 1,7 | Lys261 | Vassilopoulos et al., 2014 | Sí |

| | | | | | |
|---|---|---|---|---|---|
| Aconitate hydratase | ACO2 | 1,8 | Lys685 | ? | ? |
| Fumrarate hydratase | FH | No está en G0 | Lys66 | ? | ? |
| Malate dehydrogenase | MDH2 | 1,7 | Lys335 | ? | ? |
| Citramalyl-CoA lyase | CLYBL | 1,5 | Lys58 | ? | ? |
| Hydroxyacyl-CoA Dehydrogenase Trifunctional Multienzyme Complex Subunit Alpha | HADHA | 1,9 | Lys644 | Still et al., 2013 | Sí |
| Hydroxyacyl-CoA Dehydrogenase | HADH | 2,7 | Lys81 | ? | ? |
| Hydroxyacyl-CoA Dehydrogenase | HADH | 2,4 | Lys87 | ? | ? |
| Hydroxyacyl-CoA Dehydrogenase | HADH | 1,7 | Lys206 | ? | ? |
| Hydroxyacyl-CoA Dehydrogenase | HADH | 1,5 | Lys132 | ? | ? |

Finalmente, a pesar de que el objetivo haya sido identificar proteínas mitocondriales acetiladas, se realizaron mapas de calor y se halló que las regiones con mayores intensidades correspondían a la acetilación de histonas (Figura 5). Es importante destacar que la acetilación de histonas es dependiente de la disponibilidad del acetil-CoA y, a su vez, controla la expresión de genes clave del metabolismo (Martínez-Reyes y Chandel, 2020). Esto representa un punto relevante de la regulación del metabolismo.

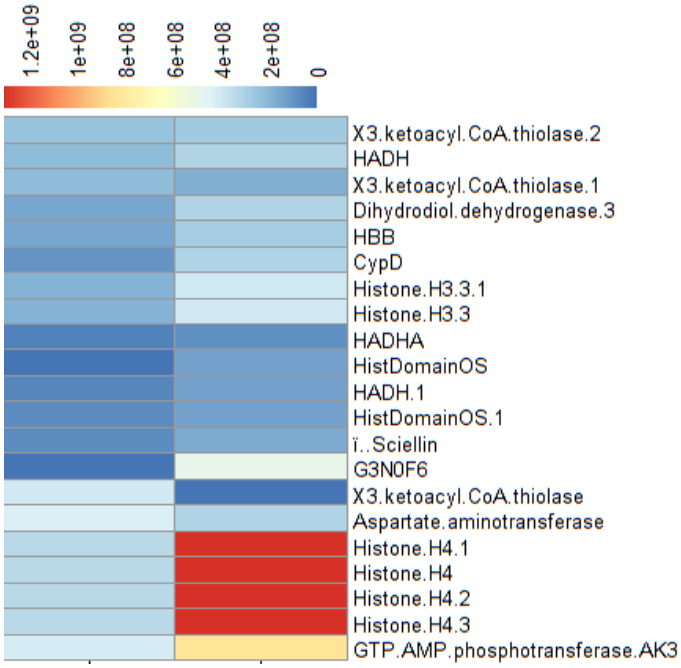



**Figura 5** – Mapa de calor de las intensidades de residuos acetiladas en biopsias hepáticas de G0 (izquierda) y G1 (derecha).

En conclusión, nuestros resultados confirman el hallazgo de que la acetilación es una modificación postraduccional relevante en el hígado de vacas lecheras sometidas a una estrategia de alimentación con mayor inclusión de pasturas. Por otra parte, consideramos relevante que se lleven a cabo más estudios para determinar cómo se regula el equilibrio desacetilasas-acetilasas, por ejemplo: determinar la actividad específica de estas enzimas.

**Vínculo con el equipo de investigación**

En relación con el desarrollo del vínculo con el equipo de investigación, fue posible intercambiar puntos de vista y realizar una discusión de los resultados tanto con el grupo de Facultad de Agronomía como con el grupo de Facultad de Medicina. Dichas instancias fueron útiles para la puesta a punto de metodologías, así como también para el análisis bioinformático de los resultados.

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