UNIVERSIDAD DE LA REPÚBLICA
FACULTAD DE AGRONOMÍA

ASPECTOS MOLECULARES DE LOS MECANISMOS INVOLUCRADOS EN LA INTERACCIÓN NUTRICIÓN-REPRODUCCIÓN EN BOVINOS DE CARNE DE DIFERENTE TIPO GENÉTICO

por

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

MONTEVIDEO
URUGUAY
Agosto 2011

Tesis aprobada por el tribunal integrado por el Dr. Carlos Robello (Institut Pasteur Montevideo), Dra. Elsa Garófalo (Facultad de Veretinaria), Dra. Graciela García (Facultad de Ciencias), Dra. Laura Astigarraga (Facultad de Agronomía) y el Dr. Eduardo Casas (U.S. Meat Animal Research Center, USDA) el 11 de agosto de 2011. Autor: Jimena Laporta Sanchis. Directora Mariana Carriquiry, co-director Jorge Urioste.

AGRADECIMIENTOS

A mi tutora Ing. Agr. (PhD) Mariana Carriquiry quien me contagió su entusiasmo y dedicación por la ciencia y la investigación y quien hizo posible la realización de esta tesis. A mi familia, en especial mis padres Sylvia y Raúl, quienes con gran esfuerzo me apoyaron siempre desde mis comienzos. A las fuentes de financiación: 1. Proyecto INIA-FPTA#262. 2. Beca de Posgrados Nacionales (BE-POS-2009-851), Agencia Nacional de Investigación, ANII. 3. Fuentes de financiación para la asistencia a congresos; CSIC-congresos en el exterior y CSIC-I+D del grupo Mejoramiento Genético Animal.

TABLA DE CONTENIDO

Página

LISTA DE TABLAS Y FIGURAS

Tabla Nº (artículo) **Página**

FiguraNº (artículo) **Página**

RESUMEN

En vacas de cría, bajo pastoreo de campo nativo, ocurre un período de balance energético negativo (BEN) durante la gestación-invernal que afecta la performance productiva/reproductiva. Los mecanismos de adaptación hepática a cambios en el BEN en ganado de carne se desconocen. Esta tesis evaluó los cambios en PV, CC, parámetros endocrinos, metabólicos y de expresión hepática de genes candidatos (eje somatotrófico, gluconeogénesis y β-oxidación) y sus asociaciones con la respuesta productiva/reproductiva (*primer abordaje)* y, mediante la técnica de microarreglos, la expresión hepática de miles de genes a lo largo del año, en la búsqueda de genes co-expresados y vías metabólicas (*segundo abordaje)*. Ambos abordajes fueron realizados durante el período de gestación invernal, el periparto y etapa de lactancia (Mayo 2009-Enero 2010) en vacas (n=32) de cría de distintos grupo genético (Angus/Hereford-PU *vs.* sus cruzas F1-CR) sometidas a pastoreo de dos ofertas de campo nativo (alta y baja; 10 *vs.*6 kgMS/100kgPV/d). El abordaje 1 refleja un claro BEN durante la gestación invernal (disminución de PV, CC, y de concentraciones de glucosa, insulina e IGF-I y aumento de concentraciones de AGNE). Los cambios a lo largo del año en la expresión hepática de genes del eje somatotrófico estuvieron fuertemente modulados por la oferta de forraje de campo nativo. Las vacas en alta oferta, tuvieron un mejor estado nutricional (mayor insulina e IGF-I). La expresión hepática de enzimas gluconeogénicas y β-oxidación aumentó durante la gestación-invernal. Las vacas CR y las que pastorearon en alta OF produjeron mas leche y reiniciaron la ciclicidad ovárica antes en el tiempo, reflejando una partición de nutrientes diferencial. En el abordaje 2, al igual que en el 1, los mayores cambios en expresión de genes y actividad de vías metabólicas se dieron durante la gestación-invernal. Este abordaje revela nuevos posibles nuevos genes candidatos y novedosas vías metabólicas que se encienden o se apagan en respuesta al estado fisiológico y la oferta de forraje a lo largo del año, que pueden ser objeto de futuros estudios y podrian explicar las respuestas productivas y reproductivas de las vacas en pastoreo. *Palabras clave:* ganado, campo nativo, expresión génica, balance energético

Molecular aspects of the mechanisms involved in the interaction of nutrition reproduction in cattle meat of different genetic type

Beef cows on grazing conditions experience a period of negative energy balance (NEB) during pregnancy in winter, which affects the productive and reproductive performance. The mechanisms underlying liver adaptation to changes in the NEB in beef cattle remain unknown. This thesis assessed the changes in BW, BCS, endocrine, metabolic parameters and hepatic expression of candidate genes (somatotroph axis, gluconeogenesis and β-oxidation) and their associations to the productive/reproductive responses (*first approach*), and by microarray technology, the hepatic expression of thousands of genes, to sexplore patterns of co-regulation among genes and metabolic pathways (*second approach*). Both studies were conducted during winter-gestation, peripartum and early lactation period (May 2009-January 2010) in beef cows (n =32) of different genetic group (Angus/Hereford-PU *vs.* crossbreds, F1-CR) grazing two forage allowances of native pastures (*high* and *low*, 10 *vs.* 6 kgMS/100kgPV/d). The decreased BW, BCS, glucose, insulin and IGF-I, and increased NEFA concentrations, reflects a clear NEB in winter-gestation. Hepatic mRNA expression of somatotrophic axis genes throughout the year was strongly modulated by forage allowance. Cows grazing high forage, had a better nutritional status (increased insulin and IGF-I). The hepatic mRNA expression of gluconeogenic and β-oxidation enzymes increased during winter-pregnancy. Crossbred cows and cows grazing high forage produced more milk and commenced ovarian cyclicity earlier in time, reflecting differential nutrient partitioning. The second approach also showed that the greatest changes in gene expression and activity of metabolic pathways occurred during winter-pregnancy. This work reveals new candidate genes and novel metabolic pathways that switched on or off due to the changes that occur in cow's physiological state, as it transit throughout gestation-lactation period, and due to forage allowance, that may be the subject for future studies and could explain the productive and reproductive responses of the beef cow on grazing conditions. *Keywords*: cows, native pastures, gene expression, energy balance

1. INTRODUCCIÓN

1.1. PLANTEO DEL PROBLEMA

La cría de bovinos de carne en nuestro país involucra 6,7 millones de cabezas y 8,3 millones de hectáreas, que significan el 48% de las hectáreas de pastoreo con bovinos de carne y ovinos. La producción de carne basada en ecosistemas pastoriles (campo nativo), resulta en importantes aportes a la economía nacional y han tenido, en los últimos años, un fuerte incremento (Vidal, 2009). Particularmente, desde 1998 hasta la fecha, se observa un crecimiento sostenido del stock bovino. En el año 2009, el Producto Bruto Interno (PBI) total de nuestro país fue de 717.137 millones de dólares, del cual el 12.2 % correspondió al PBI agroindustrial, y dentro de este último el PBI Agropecuario aportó el 8.1% (57.922 millones de dólares; DIEA, 2010). Este incremento se explica fundamentalmente por el aumento de la faena, que llega a 2.771 miles de cabezas, siendo el mayor registro histórico. Los niveles récord se basan esencialmente en el descenso de la edad de faena de los novillos, aunque este proceso podría agotarse, por lo que es necesario aumentar la producción de terneros/vaca entorada para seguir aumentando la tasa de extracción (INIA, http://www.inia.org.uy/online/site/315838I1.php.). Sin embargo, el promedio nacional del porcentaje de preñez ha sido en los últimos años (2009-2010) de 64,3 % (DIEA, 2010), y se ha mantenido por debajo del 65% durante las 3 últimas décadas.

Uruguay cuenta con alternativas de bajo costo que combinan manejos del recurso forrajero, del estado corporal, del amamantamiento (destete temporario y precoz), de la suplementación pre y posparto en vacas primíparas y multíparas (Pérez-Clariget *et al.,* 2007; Quintans *et al.,* 2008; Simeone y Beretta, 2002; Rovira y Frachia, 2005**;** Soca *et al.,* 2008)**,** así como el manejo del genotipo de los animales (Espasandín *et al.,* 2006) que buscan incrementar los porcentajes de preñez y destete con el objetivo de incrementar así el ingreso económico del sistema criador. Sin embargo, la investigación sobre los mecanismos biológicos que explican esta respuesta es escasa. El aumento de las prácticas de forestación y agricultura ha provocado una mayor competencia entre rubros, trayendo como consecuencia una

elevada carga animal por hectárea y un aumento en el precio de la tierra y de las categorías de recría y cría (DIEA, 2010). En este marco, la investigación en producción animal debe incorporar el conocimiento de los procesos y mecanismos que operan a diversas escalas para mejor la eficiencia de la productividad sin deteriorar el ambiente. Es necesaria la generación de nuevos conocimientos sobre los mecanismos internos metabólicos, endócrinos y/o moleculares para intentar mejorar el comportamiento productivo y reproductivo, atenuar la variabilidad climática y/o efecto año, e incrementar la eficiencia global de utilización de vacas de cría pastoreando campo nativo.

Loor (2010) plantea que importantes avances podrían lograrse con la integración de datos generados a nivel del ARNm, proteínas, metabolitos y tejidos en diferentes estrategias de alimentación y con vacas de diferente mérito genético de manera de poder mejorar los modelos metabólicos existentes y proveer de herramientas para la manipulación de procesos complejos que puedan tener un impacto económico en el largo plazo. Es así que, este trabajo se enmarcará en el área de la comprensión de los mecanismos que regulan la partición de nutrientes y la homeostasis del metabolismo energético en vacas de carne en respuesta a diferentes estrategias de alimentación, relacionado estos mecanismos con las respuesta productivas y reproductivas.

1.2. VACA DE CRÍA Y BALANCE DE ENERGÍA

En nuestros sistemas de producción la cría se lleva a cabo principalmente en pastoreo sobre campo nativo, en el cual la producción estacional, las variaciones climáticas intra e inter anuales, y las diferencias en la calidad y cantidad de la pastura ofrecida (Berretta *et al.,* 2000), determinan que el aporte de nutrientes a la vaca resulte la principal limitante del proceso (Wright *et al.,* 1992). Esto provoca fluctuaciones en el estado nutricional de la vaca en momentos críticos del ciclo productivo que impactan negativamente sobre el comportamiento reproductivo de los rodeos de cría (Orcasberro, 1994). En particular, la baja producción invernal de forraje, coincide con el momento en el que las vacas se encuentran en gestación

avanzada o inicio de lactancia y determina un período de balance energético negativo (BEN), debido a que los requerimientos de gestación e inicio de lactancia no son satisfechos por el consumo de energía (Bell, 1995). Esta restricción alimenticia durante la gestación puede afectar el peso de los terneros al nacer (Spitzer *et al.,* 1995), y se refleja en un pobre estado nutricional de las vacas al momento del parto que se asocia a largos períodos de anestro posparto (92 días promedio en vacas adultas; Quintans *et al.,* 2004) y bajas tasas de preñez (Orcasberro, 1991), y afectan la producción de leche y por lo tanto el crecimiento postnatal-predestete del ternero (Quintans *et al.,* 2010; Astessiano*,* 2010) por lo tanto afectando así la eficiencia de uso del forraje del sistema de producción criador.

En condiciones pastoriles, a diferencia de la vaca de leche en estabulación donde el consumo de materia seca y energía no es limitante, las diferencias en la asignación de forraje de las pasturas nativas pueden afectar el consumo de energía de la vaca (Chapman *et al.,* 2007) y/o los requerimientos de mantenimiento (Brosh*,* 2007). Estas diferencias sumadas a la interacción con el genotipo de la vaca (por ejemplo: comportamiento diferencial de pastoreo entre vacas puras y cruza; Scarlato *et al.,* 2011; Provenza *et al.,* 2003) podría determinar mecanismos de adaptación diferenciales a los períodos de restricción alimenticia y/o BEN y por lo tanto modificar su comportamiento productivo y reproductivo (Jenkins y Ferrel, 1994). La capacidad del animal para adaptarse y sobrellevar períodos de BEN depende de la capacidad de los mecanismos endócrinos y metabólicos para mantener la homeostasis (equilibrio de las condiciones internas; Chilliard *et al.,* 1998). En este escenario, el hígado es la central metabólica del animal y el principal regulador e integrador del estatus metabólico, ya que sufre grandes cambios bioquímicos y fisiológicos (McCarthy *et al.,* 2010) dirigiendo la regulación de vías metabólicas y la expresión de genes clave en el metabolismo de carbohidratos, lípidos y proteínas (Loor, 2010).

El estado metabólico puede definirse como la cantidad de nutrientes y energía que están disponibles para el animal en un determinado momento y depende de la cantidad de alimento consumido, de la cantidad de reservas corporales y del ritmo de utilización de la energía (Blache *et al.,* 2006). En gestación avanzada, las demandas

de energía y proteína aumentan del 30 a 50% (dependiendo del peso del ternero al nacer; Bell, 1995) las cuales son satisfechas, en parte, por una serie de adaptaciones metabólicas maternas que incluyen no sólo cambios en el metabolismo de carbohidratos y proteínas, sino también en el metabolismo lipídico. Por otra parte, durante la transición desde el estado de preñez avanzada a no preñada-lactante y el inicio de lactación, las demandas de energía y proteína aumentan entre de 50 a 400% en la etapa de lactación (dependiendo del nivel de producción de leche; Bauman**,** 2000). En la medida que los requerimientos nutricionales para la producción de leche no son satisfechos por el consumo de alimentos se establece un BEN en el periparto que ha sido particularmente estudiado y caracterizado en vaca lechera de alta producción (Bauman, 2000).

Las adaptaciones a períodos de BEN implican importantes cambios internos para la vaca con el fin de promover la disponibilidad de glucosa y aminoácidos para el metabolismo del feto, y una creciente utilización de ácidos grasos no esterificados (AGNE) por los tejidos maternos (Bell, 1995) durante la gestación. Así mismo, el inicio de la lactación se caracteriza por una utilización prioritaria de la glucosa por parte de la glándula mamaria, de manera que el déficit energético en el resto de los tejidos, también provoca la movilización de los depósitos de grasa. En las primeras semanas posparto existe además proteólisis, con el fin de movilizar aminoácidos que contribuyan a la gluconeogénesis hepática y a la síntesis de proteína láctea en la glándula mamaria (Bauman 2000, Lucy 2008).

1.3. EJE SOMATRÓFICO Y PARTICIÓN DE NUTRIENTES

La hormona de crecimiento (GH) es la principal hormona responsable de la partición de nutrientes (homeorhesis: flujo constante; Bauman and Currie 1980). Alteraciones en las concentraciones sanguíneas de esta hormona, conjuntamente con los cambios en la síntesis hepática de glucosa, y en las concentraciones de insulina e IGF-I en la sangre, son indicativos de la disponibilidad de energía y del estatus metabólico de los animales. El hígado también puede considerarse como el principal regulador e integrador del estatus metabólico de los animales y es el sitio primario de

síntesis de IGF-I, en respuesta a la unión de la GH, con su receptor (GHR). En contraste con la típica asociación directa y positiva entre GH e IGF-I, en períodos de BEN mientras GH aumenta en sangre IGF-I disminuye, provocando un estado de resistencia a la insulina en tejidos periféricos redirigiendo los nutrientes (especialmente la glucosa) hacia el feto y la glándula mamaria para la producción de leche (Bell, 1995; Bauman, 2000). Este desacople del eje ha sido asociado a una disminución de GHR a nivel hepático, particularmente de la isoforma -1A (GHR-1A**,** Kobayashi *et al.,* 1999**;** Radcliff *et al.,* 2003; Kim *et al.,* 2004), a reducciones en las concentraciones de insulina (Mashek *et al.,* 2001**;** Butler *et al.,* 2003) y a cambios en las concentraciones circulantes o ARNm hepático de las proteínas de unión IGFBP2 e IGFBP3 (Roberts *et al.,* 1997; Mashek *et al.,* 2001; Loor *et al.,* 2005**;** Carriquiry *et al.,* 2009).

Sin embargo, la mayoría de la información científica relacionada con el mecanismo molecular hepático responsable del desacople del eje GH-IGF ha sido generada durante el periparto en vacas lecheras alimentadas *ad-libitum* y en condiciones de estabulación. Sin embargo, existen trabajos (Jiang *et al.,* 2005, Rhoads *et al.,* 2007**,** Lucy *et al.*, 2009), que demostrarían que los mecanismos que explican los cambios en las concentraciones de GH e IGF-I, el desacople de este eje y la movilización de reservas durante el periparto aún no están del todo claros y parecen estar relacionados con la selección por producción de leche más que con el BEN. La expresión de ARNm de GHR1A y/o IGF-I no se modificó en vacas lecheras en lactación tardía sometidas a una restricción alimenticia (Rhoads *et al.,* 2007), durante el periparto de vacas de carne alimentadas *ad libitum* (Jiang *et al.*, 2005; Schneider *et al.,* 2010; Astessiano, 2010), o en vacas de leche de baja producción (Lucy *et al.,* 2009). En contraste, Wang *et al.* (2003) reportaron en novillos con alimentación restringida, los niveles de IGF-I en sangre y la expresión hepática de ARNm de IGF-I se asociaron con menores niveles de ARNm de GHR (isoformas 1A y 1C). Schneider *et al.,* (2010) trabajando en vaca de carne reportó que las bajas concentraciones en sangre de IGF-I no se correlacionaban con la expresión de ARNm hepática de *GHR*, *GHR-1A* o *IGF-I,* lo que podría indicar y/o resaltar la participación en ganado de carne de otros componentes del eje somatotrófico, como

por ejemplo el papel de las proteínas de unión a la IGF-I (IGFBPs) las cuales modulan la actividad de IGF-I (Duan y Xu, 2005), así como otras isoformas del receptor de GH a nivel hepático (Wang *et al.,* 2003)

En períodos de BEN las altas concentraciones de GH y las bajas concentraciones de insulina, promueven la gluconeogénesis hepática (Drackley *et al.***,** 2001**;** Reynolds *et al.,* 2003) disminución de la utilización de glucosa en los tejidos periféricos, y movilización de AGNE del tejido adiposo (Etherton y Bauman, 1998), asociada a un aumento de su utilización periférica y de su oxidación hepática.

1.4. GLUCONEOGÉNESIS HEPÁTICA

El propionato es el principal precursor gluconeogénico en los rumiantes (Drakley *et al.,* 2001, Hungtington *et al.,* 2006) aportando entre el 55 y 70% de la glucosa formada en hígado, mientras que aminoácidos como alanina, glutamina, cisteína, glicina, serina y treonina, son otros importantes precursores para la gluconeogénesis hepática, aportando entre un 15 y un 20% (Reynolds *et al.,* 2003; Hungtington *et al.,* 2006). El glicerol, aunque de menor importancia en la síntesis de glucosa, puede ser un precursor de importancia en situaciones donde se de alta movilización grasa (Drakley *et al.,* 2001, Hungtington *et al.,* 2006). Cambios en el estatus nutricional del animal, en el consumo de energía, o en el perfil de nutrientes que aporta la dieta pueden determinar cambios en el nivel o tipo de precursores gluoconeogénicos y en el sistema homeostático de la glucosa (Velez y Donkin, 2005). De esta manera, la gluconeogénesis hepática es muy importante en rumiantes para satisfacer las demandas de glucosa durante períodos de BEN (Velez y Donkin, 2005) y está regulada por efectos alostéricos, efectos hormonales de corto plazo y por efectos adaptativos mediados por la activación o inactivación en la síntesis de enzimas claves (Coffee, 1998). La interacción y regulación de estos mecanismos permite el mantenimiento de los niveles de glucosa en sangre.

Las dos principales hormonas que regulan los niveles de glucosa en sangre son la insulina y el glucagón (Coffee, 1998; Drackley *et al.,* 2001). Concentraciones altas de insulina promueven la captación de glucosa por los tejidos, la síntesis de

glucógeno, la glucolisis y la síntesis de ácidos grasos, todos procesos anabólicos, a excepción de la glucolisis (Coffee, 1998). Si bien la insulina disminuye la gluconeogénesis hepática por disminuir la síntesis de glucosa desde sustratos que entran vía piruvato, ésta no altera la conversión de propionato a glucosa (Drakley *et al.,* 2001**,** Hungtington *et al.,* 2006). De manera inversa a la acción de la insulina, el glucagón promueve la liberación de glucosa a la sangre, estimula directamente la conversión de propionato en glucosa y el uso de aminoácidos para la neoglucogénesis (Drakley *et al.,* 2001). La regulación de la homeostasis de la glucosa resulta pues de la interacción de estas hormonas. Altos niveles de insulina contrarrestan los efectos del glucagón, es así que la variación en la relación insulina/glucagón juega un papel muy importante en la regulación de la neoglucogénesis (She *et al.,* 1999; Drakley *et al.,* 2001).

Dos enzimas clave en la vía metabólica de la gluconeogénesis hepática son la *piruvato carboxilasa*, PC y *piruvato carboxilasa kinasa*, PEPCK o PCK1 (isoenzima citosólica). La PC cataliza la reacción que carboxila el piruvato a oxaloacetato de esta forma priorizando el uso de alanina, glicerol y lactato como precursores gluconeogénicos (Coffee, 1998; Drackley *et al.,* 2001). La PCK1 cataliza la reacción que decarboxila y fosforila el oxaloacetato a fosfoenol piruvato, de esta forma priorizando la gluconeogénesis a partir de propionato y aminoácidos gluconeogénicos. Bobe *et al.,* (2009) plantean que la tasa de transcripción de estas enzimas estaría regulada por los niveles de insulina, glucagón y por la oferta de determinados sustratos neoglucogénicos al hígado. Durante el ayuno e inanición, cuando la glucosa endógena es necesaria para ciertos tejidos, la expresión hepática de estas enzimas aumenta, mientras que ante excesos de alimentación se da lo opuesto por una modulación negativa por parte de insulina (Jitrapakdee *et al.,* 2006).

El aumento en la expresión de PC y PCK1 mejoraría (dependiendo de la eficiencia de la traducción) la síntesis de oxalacetato y fosfoenolpiruvato, respectivamente, probablemente para apoyar el aumento del uso de acetil-CoA (originado a partir de la β-oxidación de los AGNE) para obtener energía en el ciclo de de Krebs, o para apoyar a la síntesis de glucosa durante los períodos de BEN. De este modo, PC tiene un papel anaplerótico en el ciclo de Krebs, cuando los

intermediarios se destinan para diferentes propósitos, en particular para la gluconeogenesis durante BEN (Coffee, 1998). Estudios mostraron que la abundancia de ARNm de PC y actividad de la enzima se incrementó rápidamente en las vacas lecheras después del parto con la aparición del BEN (Greenfield *et al*., 2000**;** Loor, 2010) o en las vacas bajo restricción alimenticia (Baird *et al.,* 1980), mientras que el aumento de PCK1 es más lento (van Dorland *et al.,* 2009), esto probablemente se deba a que usan más lactato, glicerol y aminoácidos y menos propionato (asociado al menor consumo voluntario) como precursores gluconeogénicos (Huntington *et al.,* 2006; Baird *et al.,* 1980).

1.5. MOVILIZACIÓN DE RESERVAS Y OXIDACIÓN DE ÁCIDOS GRASOS

La tasa de movilización de reservas, principalmente del tejido adiposo, depende de factores como la severidad del BEN, del estado fisiológico de la vaca y de la CC. Los ácidos grasos son moléculas reducidas que se almacenan bajo la forma de tri-acil-glicéridos en el tejido adiposo. Un aspecto clave en la respuesta adaptativa a períodos de BEN es el uso de los AGNE como fuente de energía metabólica (ATP). Estos son movilizados del tejido adiposo por acción de la lipasa sensible a hormonas, y se transportan en sangre unidos a la albúmina. Una vez que los AGNE son liberados a la circulación, son captados principalmente por el hígado, donde son activados (acil-COA, intermediario activo) y oxidados a acetil-CoA por el sistema enzimático de la ß-oxidación en las mitocondrias de los hepatocitos, o por la glándula mamaria, para la síntesis de moléculas complejas (van Dorland *et al.,* 2011).

En períodos de BEN grandes cantidades de AGNE llegan al hígado para compensar el déficit energético, en este sentido, el hígado también juega un rol central a través de la interconversión de nutrientes (Reynolds *et al.,* 2003). Loor *et al.* (2005) y McCarthy *et al.,* (2010) encontraron que se daban cambios en la expresión de ARNm a nivel hepático de enzimas clave en el metabolismo de los lípidos. Entre ellos, el factor de transcripción *receptor activador de la proliferación de los peroxisomas-α*, PPAR-α, que actúa como sensor de niveles disponibles de AGNE y

regula la expresión de genes implicados en la oxidación de ácidos grasos **(**Forman *et al.,* 1997; Loor *et al.,* 2005). La enzima *carnitina palmitoil transferasa 1*, CPT-1 participa en la importación de AGNE en el interior de la mitocondria regulando la velocidad de oxidación de los ácidos grasos. Por otra parte, la *acil-CoA dehydrogenasa de cadena larga*, ACADVL participa en la oxidación mitocondrial de ácidos grasos de cadena larga y de cadena muy larga. La β-oxidación peroxisomal representa aprox. el 50% de la capacidad total de β-oxidación en el hígado de las vacas lecheras (Piot *et al.,* 1998), especialmente durante períodos de alta afluencia sostenida hepática de ácidos grasos. Siendo una vía alternativa para la oxidación de ácidos grasos de cadena larga (más de 20 átomos de carbono). La enzima *acil-CoA oxidasa-1 palmitoil*, ACOX cataliza la primera reacción y un paso regulatorio en este. Se ha sugerido que la dieta y el estado fisiológico serían factores determinantes para la inducción de β-oxidación peroxisomal. La actividad de estas enzimas mitocondriales y peroxisomales permite a los hepatocitos lidiar con el flujo excesivo de AGNE durante períodos de BEN (Loor, 2010). De esta manera, el acetil-CoA resultante de la oxidación puede incorporarse al Ciclo de Krebs para proveer energía en forma de ATP, o utilizarse para la síntesis de cuerpos cetónicos.

La insulina inhibe la síntesis de ARNm de CPT-1 mientras que la adición de glucagón o su segundo mensajero, AMP cíclico, induce la trascripción del gen CPT-1 en el hígado (Louet *et al.*, 2001; Park *et al.*, 1995). Se ha reportado que la actividad de CPT-1 en la vaca lechera se incrementa en el postparto (Drakley *et al.*, 2001), que no difiere en lactación temprana (0-110 días) (Mizutani *et al.*, 1999), o que decrece a medida que la lactación progresa de los 30 a los 180 días postparto (Aiello *et al.*, 1984). Las razones de estos resultados contrastantes, no están claras, mostrando la necesidad de información adicional. Por otro lado, la expresión hepática de CPT-1 y ACADVL aumentó (Loor *et al.*, 2005) o no cambió (van Dorland *et al.*, 2009) durante la lactancia temprana en vacas lecheras, y hemos encontrado un único caso de aumento de la expresión de ARNm hepático de ACOX durante cetosis clínica inducida por restricción alimenticia en el ganado lechero (Loor *et al.*, 2007).

Si bien las mayores concentraciones de AGNE en vacas lecheras se observa durante el periparto y lactancia temprana (Meikle *et al.,* 2004), resultados nacionales

de investigación en vacas de cría primíparas (Astessiano, 2010) y multíparas (Quintans *et al.*, 2010) en condiciones de pastoreo de campo nativo, mostraron mayores descensos de la CC y aumentos de los niveles de AGNE durante el pre que el posparto.

1.6. ESTADO METABÓLICO Y REPRODUCCIÓN

El estado metabólico de las vacas durante el último tercio de gestación y el posparto temprano contribuye a la eficiencia reproductiva de estos animales (Lucy, 2003). El éxito reproductivo en el posparto dependerá del correcto funcionamiento del eje hipotálamo-hipofisiario-gonadal, el cual tiene un rol dominante en la regulación de la reproducción. Este correcto funcionamiento requiere de la integración de señales periféricas que indican el estatus fisiológico y nutricional de la vaca e identifica a la misma como "pronta para concebir" y llevar adelante una gestación. Las señales propuestas incluyen: concentraciones circulantes de metabolitos (glucosa y AGNE) y hormonas (hormona de crecimiento, GH; insulina; IGF-I y sus proteínas de unión, IGFBP1-6; y leptina). Sin embargo, a pesar de considerables esfuerzos de la investigación, el mecanismo fisiológico específico responsable de ligar estos factores permanece sin comprenderse totalmente.

El eje GH-IGF-I-insulina tiene un importante papel en la eficiencia reproductiva ya que afecta la función del eje hipófisis-gonadal (Lucy, 2003) y la función uterina (Thatcher *et al.,* 2003). La duración del anestro ha sido asociada con menores concentraciones de IGF-I en vacas lecheras en condiciones pastoriles, vacas que ovulan en lactación temprana tiene mayores concentraciones de IGF-I que aquellas que fallan en ovular (Meikle *et al.,* 2004). Sinclair (2008) reportaron que los niveles circulantes de insulina eran responsables de una mayor proporción de la variación entre el intervalo parto-primera ovulación que la CC al parto o el consumo de energía posparto. Así mismo, Astessiano *et al.* (2011) han demostrado/confirmado que la duración del anestro posparto se encuentra negativamente asociada a las concentraciones de insulina y/o IGF-I en sangre en vacas de carne. Por otra parte, se ha demostrado que la duración del anestro posparto se encuentra asociada positivamente con la expresión hepática de ARNm de IGFBP2 y con sus

concentración en sangre (asociado a períodos de restricción nutricional; Straus, 1994) y negativamente con la relación de ARNm de IGFBP3 con respecto a IGFBP2 en vacas de cría (Astessiano *et al.,* 2011).

1.7. GENÓMICA FUNCIONAL: UN NUEVO ABORDAJE DE ESTUDIO

La tecnología de microarrays (microarreglos) se inserta dentro del campo de investigación de la genómica funcional, la cual se centra en el estudio de las funciones e interacciones entre genes centrándose en sus aspectos dinámicos (niveles de expresión génica, cantidad de mRNA). El concepto fundamental sobre el cual se basa esta tecnología, es la hibridación de sondas específicas que representan los genes (probes, sobre un sustrato sólido) con las moléculas diana (target, de la muestra). Cada sonda representa un gen y tiene una ubicación conocida en el array. El nivel de hibridación se indica mediante fluorescencia y se mide por análisis de imagen, el cual indica el nivel de expresión de cada gen.

Esta herramienta ofrece el potencial de llevar adelante miles de experimentos a la vez y de forma automatizada, obteniéndose grandes cantidades de información en poco tiempo permitiendo la evaluación de los niveles de expresión génica (ARNm) de miles de genes, incluso del genoma completo, de forma simultánea y en paralelo. Su gran utilidad en investigación radica en que los patrones de expresión conjunta pueden usarse para la caracterización molecular de fenómenos biológicos (Walsh y Henderson, 2004), siendo una herramienta útil para el estudio de redes regulatorias, vías metabólicas y el descubrimiento de genes candidatos que intervienen en los mecanismos que gobiernan la variación fenotípica observable. Información de la tecnología de microarreglos puede encontrarse en la revisión de Schulze y Downward (2001).

Pocos estudios han usado la tecnología de microarreglos para evaluar el transcriptoma hepático bovino y la mayoría han sido en vacas de leche en: diferentes momentos del ciclo gestación (Herath *et al.*, 2004**),** transición y lactación temprana (Loor *et al.,* 2005); en condiciones controladas de alimentación (Loor *et al.,* 2006; McCarthy *et al.,* 2010); y enfermedades metabólicas (ej. cetosis, Loor *et al.,* 2007). En vacas de carne, esta tecnología ha sido aplicada al estudio de perfiles de expresión

en glándula pituitaria en vacas en anestro y ciclando (Roberts and MacLean, 2011), y en endometrio durante la preñez temprana (Forde *et al.,* 2009), Connor *et al.* (2010) estudiaron la expresión hepática durante períodos de restricción alimentaria y re-alimentación en terneros. Según nuestro conocimiento, ningún trabajo ha utilizado esta técnica enfocada al estudio de como la nutrición y el grupo genético pueden afectar los mecanismos de regulación hepática durante la gestación y lactación temprana en vacas de carne en condiciones pastoriles.

Estudiar el efecto de distintos genotipos animales y el manejo de la oferta de forraje del campo natural a lo largo del año posibilita comprender estos mecanismos de adaptación en modelos animales y situaciones ambientales cambiantes de nuestros sistemas de producción. El explorar nuevas redes regulatorias (Anexo 6.3) y posibles nuevos genes candidatos junto a los cambios temporales en los niveles de hormonas y metabolitos en sangre y en la expresión hepática de genes del eje somatotrófico, oxidación de ácidos grasos y gluconeogénesis a lo largo del ciclo productivo, nos permitirá una mejor comprensión de los mecanismos fisiológicos y moleculares responsables de ligar el estatus endócrino y metabólico con la respuesta productiva y/o reproductiva en vacas de carne en condiciones de pastoreo de campo natural.

De esta manera, nuestra hipótesis de trabajo es que: los mecanismos endócrinos, metabólicos y moleculares de adaptación en vacas de cría cruzas y puras en sistemas pastoriles durante la gestación invernal, peri-parto y lactancia son distintos cuando son sometidas a dos ofertas de forraje de campo natural. Esto se asocia a una partición de nutrientes y un control metabólico diferencial que podría afectar la respuesta productiva y reproductiva de las vacas en el postparto.

ESTRUCTURA GENERAL DE LA TESIS

Consiste en dos artículos científicos, el primer, titulado ***"Effects of forage allowances and genetic group on metabolic and endocrine parameters and hepatic gene expression in beef cows on grazing conditions"*** constituye el segundo capítulo de esta tesis. En este artículo, a ser enviado a la revista *Domestic Animal Endocrinology*, se evaluaron los cambios en los perfiles de metabolitos (AGNE y

glucosa), hormonas (insulina e IGF-I) y de expresión hepática de genes candidatos involucrados en vías metabólicas clave (eje somtatrófico, oxidación de ácidos grasos y gluconeogénesis) asociadas con la coordinación homeorhética de la adaptación del hígado a cambios en el balance energético a lo largo de todo el año (gestación, periparto y lactancia) y su asociación con la respuesta productiva (peso del ternero al nacer, producción de leche) y reproductiva (reinicio de la actividad ovárica) en vacas de carne en condiciones pastoriles.

El segundo artículo, titulado ***"Functional genomics of liver metabolic adaptations during gestation and lactation: crossbred and purebred beef cows in two forage allowances of native pasture'',*** constituye el tercer capítulo de esta tesis, y será enviado a la revista *Journal of Animal Science*, se plantea un enfoque a la temática desde la visión de la genómica funcional. Mediante la técnica de microarreglos se evaluó el perfil de expresión hepática de miles de genes en forma simultánea en cuatro momentos a lo largo del ciclo productivo (gestación, periparto y lactancia) con el objetivo de identificaron posibles nuevos genes candidatos y nuevas vías metabólicas que podrían participar en el control y regulación de los grandes cambios energéticos que ocurren durante dichos períodos en vacas de carne en nuestras condiciones pastoriles, y que podrían explicar las diferentes respuestas fenotípicas que se observan entre grupos. Acalración: se realizó un único diseño experimental (n=32 animales), y sobre este se realizaron los dos abordajes: abordaje 1 (n=32; 8 animale spor tratamiento) y abordaje 2 (n=16 animales) seleccionados de los 32, n=4 por tratamiento, bloqueados por fecha de parto que se respetaron los dos bloques y todas las razas representadas. En el cuarto capítulo de esta tesis se presenta una discusión general y conclusiones globales de los dos artículos.

2. METABOLIC, ENDOCRINE AND MOLECULAR ADAPTATIONS TO NEGATIVE ENERGY BALANCE OF BEEF COWS ON GRAZING CONDITIONS

Effects of Forage Allowances and Genetic Group on Metabolic and Endocrine Parameters and Hepatic Gene Expression in Beef Cows on Grazing Conditions

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2.1 ABSTRACT

In spring-calving beef cows on grazing conditions, the liver undergoes physiological and biochemical changes in gene expression, accompanied by alterations in metabolite and hormones concentrations throught the year in an effort to re-establish metabolic homeostasis and counteract the adverse effects of negative energy balance (NEB) of winter-gestation. To gain new insights into these events, we evaluate metabolic (non-esterified fatty acids, NEFA and glucose), endocrine (insulin and insulin-like growth factor I, IGF-I) and hepatic mRNA profiles (somatotropic axis, gluconeogenesis and β-oxidation pathways) in multiparous beef cows (n=32) of different genetic group (purebred-PU: Angus and Hereford *vs*. their crossbred-CR) grazing native pastures at different forage allowances (6 *vs*. 10 kgDM/100kgBW/d, LO *vs*. HI). We also explore its associations with productive and reproductive performance. Body weight, BCS, and blood samples were collected every 28 days and liver biopsies at -165, -75, -45, -15 ± 10, +15 and +60 ± 3 days. Calf BW was determined at birth, milk yield at 31 and 55 ± 6 days and presence of corpus luteum in ten-day intervals from 60 to 180 ± 15 days. Results confirm a severe NEB during winter-gestation, evidenced by a decrease in BW, BCS, glucose and insulin concentrations, an increase in serum NEFA an in mRNA abundance of genes involved in β-oxidation and gluconeogenesis, while no drastic changes were observed during the peripartum period. The uncoupling of the somatotropic axis was only evident in HI cows, indicating a differential nutrient partitioning. The HI and CR cows had a better productive and reproductive performance, associated to different metabolic, endocrine, and liver gene expression profiles. The HI cows had a better metabolic status during the whole period that allowed to direct nutrients and energy towards this functions more efficiently trough the gestation-lactation cycle, showing a greater adaptive performance than LO cows. On the other hand, not drastic changes were observed trough the year due to the genetic group. However, CR cows had a better reproductive performance than PU cows associated to their better metab status in late fall. *Keywords*: Beef cattle, GH-IGF axis, Metabolites, Gene expression, Liver

2.2 INTRODUCTION

Beef cow-calf systems on grazing conditions are subject to large climate variations through the year which affect forage production, quantity and quality of native pastures, and therefore the amount and pattern of food intake, which is not constant during seasons [1]. In addition, in spring-calved cows, the increase in energy demands of the developing fetus and mammary gland of the last months of pregnancy [2] occurs during winter, period of limited forage production, leading to the onset of negative energy balance (NEB), and loss of BCS [3]. Differences in forage allowances of native pastures determine changes in herbage mass, height, and quality as well as in plant growth rate that can affect cow energy intake [4] and maintenance energy requirement through changes in grazing energy cost [5]. These changes, added to the interaction with cow genotype (i.e differential grazing and social behavior [6]) could determine differential adaptative mechanisms to periods of dietary restriction or NEB, and affect productive and reproductive performance [7]. Indeed, better productive and reproductive performances were reported in crossbred-F1 beef cows [8,9] grazing high forage allowances throughout the year [10].

The adaptability of ruminants to these periods of dietary restriction and/or NEB depends on the capacity of their endocrine and metabolic mechanisms to maintain homeostasis [11], and are likely to be mediated by the liver, which undergoes extensive physiological and biochemical changes [12], directing the transcription of gene networks, and synthesis of key enzymes of the metabolism of carbohydrates, lipids, and proteins [13]. These changes are characterized by an increased hepatic gluconeogenesis and decreased glucose utilization in peripheral tissues, as well as by an increased of mobilization of amino acids and non-sterified fatty acids (NEFA) from muscle and adipose tissue, respectively, associated with an increase in their use by the liver [2,14,15]. This has been illustrated by several authors [16,17,18,19] who have determined increased activity and/or hepatic mRNA expression of rate-limiting enzymes for gluconeogenesis and/or fatty acid oxidation during the NEB in early lactation in dairy cows.

Growth hormone (GH) plays a key role in endocrine regulation of nutrient partitioning. During periods of NEB, GH blood concentrations increase while the insulin-like growth factor-I (IGF-I) concentrations decrease, stimulating a state of insulin resistance in the peripheral tissues and redirecting nutrients (especially glucose) to the fetus and/or milk production [2, 14]. This uncoupling of GH-IGF axis has been associated with a state of GHR resistance in the liver [20,21] as a consequence of reduced mRNA expression of GH receptors (*GHR*), particularly of its isoform-1A (*GHR1A*) [22,20,21], reduced insulin concentrations [23,24], and changes in circulating or hepatic mRNA expression of IGF-I binding proteins 2 and 3 (*IGFBP2* and *IGFBP3*) [25,23,18,26].

Most scientific information related to the molecular mechanisms responsible for uncoupling of hepatic GH-IGF axis, as well as changes in gluconeogenesis and fatty acid oxidation, has been generated in dairy cows associated to their typical NEB during the transition period. Some studies have evaluated the molecular mechanisms related to the GH-IGF axis in response to undernutrition or feed restriction in other ruminants models (mid to late lactation pregnant dairy cows: [27], late lactation non-pregnant dairy cows: [28], periparturient beef cows: [29,30], steers: [31], pregnant and cyclic ewes: [32]), but there are no studies that address the hepatic mechanisms of adaptation to NEB during the last months of pregnancy, that typically occurs in spring-calved beef cows in rangeland conditions.We hypothesized that differences in productive and reproductive performances between genotypes and forage allowances can be associated to differential nutrient partitioning and metabolic, endocrine, and molecular control of metabolism. The objective of this study was to evaluate metabolic, endocrine, and hepatic mRNA profiles (involved in GH-IGF axis, gluconeogenesis and β-oxidation pathways) associated with liver homeorhetic adaptation to changes in energy balance through the year in beef cows of different genetic groups grazing native pastures at different forage allowances, and its associations with productive and reproductive performance.

2.3 MATERIALS AND METHODS

2.3.1 Location, animals and experimental design

The experiment was carried out at the Experimental Station Bernardo Rosengurtt (Facultad de Agronomía, Universidad de la República, Uruguay, 32°S 54°W) from May 2009 to March 2010. Animal procedures were performed according to protocols approved by the Animal Experimentation Committee of Universidad de la República. Thirty-two multiparous pregnant cows (5 to 6 years), were used in a complete randomized block design with a factorial arrangement of forage allowances (FA) of native pastures (High-HI and Low-LO; 10 *vs.* 6 kg DM/100kgBW/d in average, respectively, Table 1) and genetic group (GG, purebred: Angus-A and Hereford-H; PU and crossbred-F1: HA and AH; CR) which determined four treatments HI-CR, HI-PU, LO-CR and LO-PU.

After pregnancy diagnosis (April 2009), cows were selected (n = 8 per treatment) from a larger group (n = 61) and blocked according to their expected calving date. All cows belonged to a group of experimental animals generated as part of a diallel crossbreeding experiment carried out for 10 years at the Experimental Station [33]. Cows grazed the same forage allowance (HI or LO) since May 2007, and gestated and lactated one calf every year from 2007 to 2009. Cows were managed as a contemporary group and were in paddocks of 5 to 20 ha according to block and FA (block 1: 20 and 12 ha and block 2: 10 and 5 ha, for HI and LO, respectively) with good access to water.

Herbage allowance was adjusted monthly after measuring the amount of forage available in each plot [34] through the entry of animals ("put and take" grazing system [35]) of similar genetic group and physiological status. Forage availability and height, and environmental conditions throughout the experimental year are presented in Table 1. All cows had normal parturition and were free from health disorders during the experiment.

Table 1. Forage allowances, height, herbage forage mass availability and cow physiological stage (pregnant/lactating) for the different seasons through the experimental year.

	Fall [1]	Winter	Spring	Summer
Forage allowance (kg DM/100 kg BW/d)				
High	12.5	7.5	10	10
Low	7.5	7.5	5	5
Height (cm)				
High	2.84 ± 0.38	2.2 ± 0.3	6.1 ± 0.3	9.4 ± 0.38
Low	1.5 ± 0.38	1.6 ± 0.3	4.6 ± 0.3	7.9 ± 0.38
Herbage mass availabilitity (kg DM/ha)				
High	1591 ± 180	1171 ± 180	2634 ± 180	3549 ± 180
Low	920 ± 180	871 ± 180	1590 ± 180	2505 ± 180
Physiological stage of cows	pregnant	pregnant	pregnant-lactating	lactating
Days [2]	-165	-135, -105, -75 -45	-15 ,+15	+60

[1]Fall = March, April, May; Winter = June, July, August; Spring = September, October, November and Summer = December, January, February. [2] Days relative to partum.

2.3.2 Data and sample collection

Body weight (by electronic balance) and BCS (by visual assessment, scale of 1 = very thin to 8 = very fat [36]) were recorded, and blood samples were collected every 28 days during the pregnancy period (prepartum: May to October, 2009 – fall to spring) and every 15 days during the lactation period (postpartum: November 2009 to January 2010 - spring to summer). Thus, data and blood collection occurred at: -165, -135, -105, -75, -45, -15 ± 10 and +15, 30, 45 and 60 ± 3 (mean ± standard deviation) days relative to calving (days; average calving date: November 13, 2009).

Blood samples were collected in duplicate via venipuncture of the coccygeal vein in BD Vacutainer ® tubes (Becton Dickinson, NJ, USA), with (sodium fluoride and potassium oxalate) and without anticoagulant, and centrifuged at 2500 Xg for 20 min, either immediately or after 4 h of coagulation, for the collection of plasma and serum, respectively. Plasma and serum samples were stored at -20 °C until analysis.

Liver biopsies were collected at -165, -75, -45, and -15 ± 10 and 15 and 60 ± 3 days (corresponding to months of May, August, September, October, November and January). Samples of liver tissue (0.5 g of tissue approximately) were obtained using a biopsy needle (Tru-Core ®-II Automatic Biopsy Instrument, Angiotech, Lausanne, Switzerland) according to the procedure described by Carriquiry *et al.* [26]. Liver samples were immediately frozen in liquid nitrogen and stored at -80°C until total RNA was isolated.

Calf BW was determined at birth and average milk production, during the first 60 days of lactation, was estimated by two measurements at 31 and 55 ± 6 days using a milking machine according to Quintans *et al.* [3]. Commencement of luteal activity was defined as the day from calving to presence of corpus luteum. Presence of corpus luteum was determined by transrectal ultrasonography (Ambivision Digital AV-3018V Notebook, AMBISEA Technology Corp., Ltd., China bimodal probe of 5.0 and 7.5 MHz) every 10 days, from 60 to 180 ± 15 days, and confirmed with increased progesterone (P4) concentrations in serum above 1 ng/mL.

2.3.3 Metabolite and hormone analyses

The concentrations of blood metabolites (serum NEFA and plasma glucose) were determined by spectrophotometry, using commercial kits (NEFA, NEFA-HR (2) Wako, Chemicals, Richmond, USA; glucose: Oxidase / Peroxidase, BioSystems SA, Barcelona, Spain). The volume of samples and reagents were adjusted to a 96-well microplate and read in a Multiskan EX spectrophotometer (Thermo Scientific, Waltham, MA, USA). The intra and inter-assay CV for low, medium, and high controls did not exceed 13% for any of the metabolites analyzed.

Hormone concentrations were determined in serum samples. Concentrations of P4 were determined by radioimmunoassay (Coat-and-Count, Siemens Healthcare Diagnostics Inc, LA, USA). The assay detection limit was 0.2 ng/mL and intra-assay CV for low (0.5 ng/mL), medium (2.0 ng/mL) and high (10.0 ng/mL) controls were 7.4, 7.9, and 1.1%, respectively. Insulin concentrations were determined by an

immunoradiometric (IRMA) assay (INS-IRMA; Diasource, Brussels, Belgium). The assay detection limit was 3.2 μIU/mL, and intra-assay CV for control 1 (20.8 μIU/mL) and 2 (63.6 μIU/mL) were 3.4 and 7.4%, respectively. Concentrations of IGF-I were determined by IRMA assay (IGF1-RIACT; Cis Bio, Gif sur Yvette, France) described previously by [37] for bovines. The assay detection limit was 0.6 ng/mL, and intra-assay CV for control 1 (43.8 ng/mL) and control 2 (521.5 ng/mL) were 9.2 and 8.0%.

2.3.4 RNA isolation and reverse transcription

Total RNA form hepatic tissue was isolated using TRIzol (Invitrogen, Life Technologies, Carlsbad, CA, USA), followed by precipitation with lithium chloride and by DNase-treatment with a DNA-Free kit (Applied Biosystems/Ambion, Austin,TX, USA). Concentration of RNA was determined by measuring absorbance at 260 nm (NanoDrop ND-1000 Spectrophotometer; Nanodrop Technnologies Inc., Wilmington, DE, USA), and purity and integrity of all RNA isolates were assessed from 260/280 and 260/230 absorbance ratios and by electrophoresis in 1% agarose gel. Isolated RNA was stored at -80°C until analyzed. The SuperScript®III Transcriptase (Invitrogen), with random hexamers and 1 μg of total RNA as a template, was used to conduct the reverse transcription. The cDNA was stored at -20ºC until its use.

2.3.5 Quantitative real time RT-PCR

Primers (Table 2) were obtained from literature or specifically designed (Primer Express Software; Applied Biosystems, Foster City, CA, USA) to amplify cDNA from target genes: *GHR*, *GHR-1A*, *IGF-I*, *IGFBP3*, *IGFBP2*, *acyl-CoA oxidase-1* (*ACOX*), *acyl-CoA dehydrogenase very long chain* (*ACADVL*), *carnitine palmitoyltransferase-1*(*CPT-1*), *peroxisome proliferator-activated receptor-alpha* (*PPAR-α*), *pyruvate carboxylase* (*PC*), and *phosphoenolpyruvate carboxykinase-1* (*PCK1*) and from endogenous controls: *Hypoxanthine phosphoribosyltransferase* (*HPRT*) and *β-actin* (*ACTB*). Both *HPRT* and *ACTB* have been used before as endogenous control in tissues from ruminants [26, 18, 38] and their expression was

stable among treatments and across time points in this study (by microarrays analysis; Laporta *et al.*, unpublished results).

Table 2. Primers used for the quantification of target and endogenous control gene cDNA

Gene[a]	Accession no.[b]		Primer sequence	Lenght (bp)	Source
GHR	NM_176608	Sense	TCTGGGAATCCTAAATTCACCAA	91	Carriquiry et. al., 2009
		Antisense	CTGTAAACTGTGATTAGCCCCATCT		
GHR-1A	BTCN36539	Sense	AGCCTGGAGGAACCATACGA	94	Wu , 2007
		Antisense	GCTGCCAGAGATCCATTCTGTA		
IGF-I	XM_612412	Sense	CCAGACAGGAATCGTGGATG	89	Wu, 2007
		Antisense	ACTTGGCGGGCTTGAGAG		
IGFBP2	NM_174555	Sense	ATGCGCCTTCCGGATGA	83	Astessiano et. al., 2011
		Antisense	GTTGTACAGGCCATGCTTGTCA		
IGFBP3	NM_174556	Sense	AGCACAGACACCCAGAACTTCT	86	Carriquiry et. al., 2009
		Antisense	TTCAGCGTGTCTTCCATTTCC		
ACADVL	BTCN8936	Sense	CCAGCCCCTGTGGAAAATACTA	66	This paper
		Antisense	GCCCCCGTTACTGATCCAA		
ACOX1	NM_001035289.2	Sense	CCATTGCCGTCCGATACAGT	99	This paper
		Antisense	GTTTATATTGCTGGGTTTGATAATCCA		
CPT1	NM_001034349.2	Sense	TGCACGGCAACTGCTACAA	93	This paper
		Antisense	ACGCGTGCTCTCTGTTGAGT		
PC	NM_177946.3	Sense	CGTCTTTGCCCACTTCAAGG	70	This paper
		Antisense	GAAGAGGCGCGTATTGAGGC		
PCK1	NM_174537.2	Sense	TGGCCATGATGAACCCTACTC	77	This paper
		Antisense	GTCAAATTTCATCCAGGCATATC		
ACTB	BT030480	Sense	CGTGGC TACAGCTTCA CC	53	This paper
		Antisense	GAA ATCGTCCGTGACATCAA		
HPRT	XM_580802	Sense	TGGAGAAGGTGTTTATTCCTCATG	105	Carriquiry et al., 2009
		Antisense	CACAGAGGGCCACAATGTGA		

[a]GHR=growth hormone receptor, GHR-1A=GHR isoform-1A, IGF-I=insulin-like growth factor I, IGFBP2=IGF binding protein-2; IGFBP3=IGF binding protein-3, ACOX=acyl-CoA oxidase 1, ACADVL=acyl-CoA dehydrogenase very long chain, CPT1=carnitine palmitoyltransferase 1, PC=pyruvate carboxylase, PCK1=phosphoenolpyruvate carboxykinase 1, HPRT=hypoxanthine phosphoribosyltransferase and ACTB=ß-actin. [b] GeneBank.secuences.

Real time PCR reactions were performed using 7.5 µL SYBR® FAST One-Step qRT-PCR Kit mastermix (Biosystem, Massachussets, USA), equal amounts

(200 nM) of forward and reverse primers (Operon Biotechnologies GmbH; Cologne, Germany), and 3 μL diluted cDNA (1:7.5 in RNase/DNase free water) in a final volume of 15 μL. Samples were analyzed in duplicate in a 72-disk Rotor-GeneTM 6000 (Corbett Life Sciences, Sydney, Australia). Standard amplification conditions were 5 min at 95°C and 40 cycles of 10 s at 95°C, 45 s at 60°C, and 20 s at 72°C. Each disk included duplicate wells of water (non-template) for each set of primers and cDNA standard curves for the analyzed gene in the plate. Plasmids that encoded the target and endogenous control genes were diluted in yeast cDNA to achieve a starting concentration of 10^6 gene copies/μL for subsequent serial dilutions (n = 6 dilutions, from 10^6 to 10^1). Linear regressions were used to estimate the number of copies of target and control gene mRNA in the samples. The absolute expression of each target gene was normalized to the geometric mean of the expression of control genes (*HPRT* and *ACTB*). The efficiencies of amplification for all genes analyzed varied between 97 and 112%, and inter-and intra-assay CV between 4 and 6.5% and 8 and 21%, respectively.

2.3.6 Statistical analysis

Data were analyzed using the SAS Systems program (SAS 9.0V, SAS Institute Inc., Cary, NC). The univariate procedure was used to identify outliers and inconsistencies and to verify normality of residuals. Body weight, BCS, metabolite and blood hormone concentrations, and gene expression data were analyzed with a repeated measure mixed model analysis, using the MIXED procedure. The model included FA, GG, days (repeated measure) and their interactions as fixed effects, block as random effect and calving date as a covariate. The covariance structure SP(POW) (spatial power), and the Kenward-Rogers procedure to adjust the degrees of freedom of denominator were specified. Gene expression data of *CPT-1* with non-normal distribution was log transformed for analysis and re-transformed for presentation. Calves BW at birth and cow average milk production were analyzed with the same model without the effect of days. Commencement of luteal activity (days) was analyzed using the GENMOD procedure with Poisson distribution and the log transformation specified. The model included FA, GG, their interaction and

block as fixed effects, and calving date as a covariate. Mean separation was performed using the Tukey test, and differences were considered significant at $P \leq 0.05$ and trends when $0.05 \leq P \leq 0.10$. Pearson correlation coefficients to describe relationships between variables were estimated using the CORR procedure. Results are presented as least square means ± pooled standard error.

2.4. RESULTS

2.4.1 Body weight and body condition

Cow BW and BCS were greater in HI than LO cows (448 *vs*. 405 ± 11 kg and 4.3 *vs*. 3.7 ± 0.1 units; $P < 0.018$) and in CR than PU cows (443 *vs*. 410 ± 11 kg and 4.1 *vs*. 3.9 ± 0.1 units; $P < 0.059$) throughout all the period evaluated, and both were affected by days ($P < 0.001$). Cow BW decreased steadily during the first two months of winter (23 kg in average, from -165 to -105 days), increased markedly before calving (28 kg in average, from -45 to -15 days), decreased after calving (at 15 days), and then increased at 60 days reaching the initial values of late fall (Fig. 1A). Cow BCS decreased 0.5 units during the first two months of winter (up to -105 days), reached nadir at calving, remained low and stable until 30 days, and increased thereafter until 60 days without reaching the initial values of late fall (Fig. 1B). Although the nadir of BCS was at calving for all groups, it differed ($P < 0.050$) in magnitude, being greater in HI than LO cows and in CR than PU cows (4.2, 3.8, 4.1, and 3.5 ± 0.1 for HI-CR, LO-CR, HI-PU, and LO-PU, respectively).

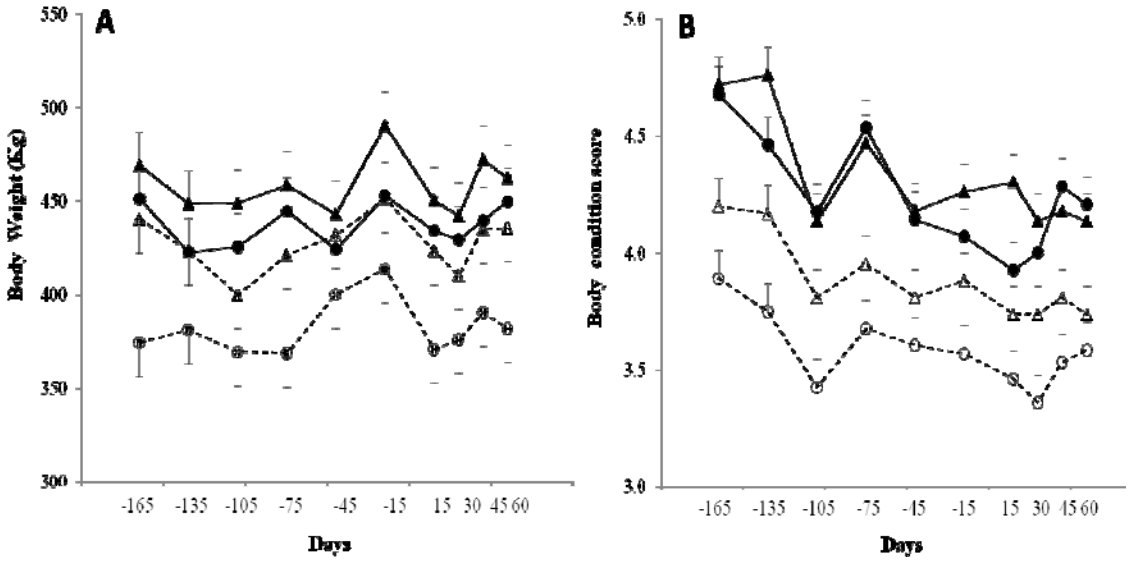

Fig. 1. Body weight (A) and body condition score (B) through the year (-165 to 60 days relative to partum, days) in purebred and crossbreed beef cows (n=32) grazing high and low (10 and 6 kgDM/100kgBW/d in average, respectively) forage allowances of native pastures (—●— High-Purebred, —▲— High-Crossbred, ···⊙··· Low-Purebred and···△···Low-Crossbred).

2.4.2 Calf body weight at birth and milk production during the first 60 days

Calf BW at birth did not differ due to FA, GG, or their interaction and averaged 35 ± 2 kg. Milk production during the first 60 days was greater ($P < 0.031$) in HI than LO cows (4.9 *vs.* 4.0 ± 0.3 kg/d) and in CR than PU cows (5.0 *vs.* 3.9 ± 0.3 kg/d).

2.4.3 Concentrations of metabolites and hormones

Serum NEFA concentrations were not affected by FA, GG, or their interaction but varied ($P < 0.001$) througout the year. Concentrations of NEFA increased markedly during winter (-165 to -45 days), decreased thereafter until 45 days, and increased slightly at 60 days (Fig. 2A, 2B). Plasma glucose concentrations were not affected by FA, GG, or their interaction but were affected by days ($P = 0.008$) and by the interaction between GG and days ($P = 0.037$) (Fig. 2C, 2D). Plasma glucose concentrations were maintained between -165 and -135 days, decreased thereafter

until -45 days, increased slightly at -15 days, remained stable until 45 days and then increased at 60 days recovering the initial concentrations of late fall. However, plasma glucose decreased markedly at -75 days in CR cows while the decline was detected at -45 days in PU cows. Plasma glucose was lower in CR than PU cows at -75 days but did not differ between GG in the rest of the period evaluated.

Serum insulin concentrations were greater ($P = 0.027$) for HI than LO cows (11 *vs.* 7.7 ± 1 μIU/mL) and were affected by days ($P < 0.001$) (Fig. 2E, 2F). Serum insulin was high at -165 days decreased markedly during winter gestation (-165 to -45 days), remained low at -15 days, and increased slightly at 15 days, without reaching at 60 days the initial concentrations of late fall. There was an interaction between GG and days ($P < 0.033$) on serum insulin as CR had greater concentrations than PU cows at -165 days. Serum concentrations of IGF-I were greater ($P = 0.005$) for HI than LO cows (78.7 *vs.* 44 ± 8 ng/mL), were affected by days ($P < 0.001$), and tended to be affected ($P = 0.08$) by the interaction between FA and days (Fig. 2G, 2H). Serum IGF-I concentrations were high at -165 days, decreased until -45 days, increased at -15 days, and then decreased again until 60 days, without reaching the initial concentrations of late fall. The decrease during winter, and the increase in serum IGF-I at -15 days was only observed in HI cows. Serum insulin and IGF-I concentrations were positively correlated ($P < 0.001$, $r = 0.39$, $n = 160$) and both were correlated with BCS ($P < 0.001$, $r > 0.47$, $n = 157$).

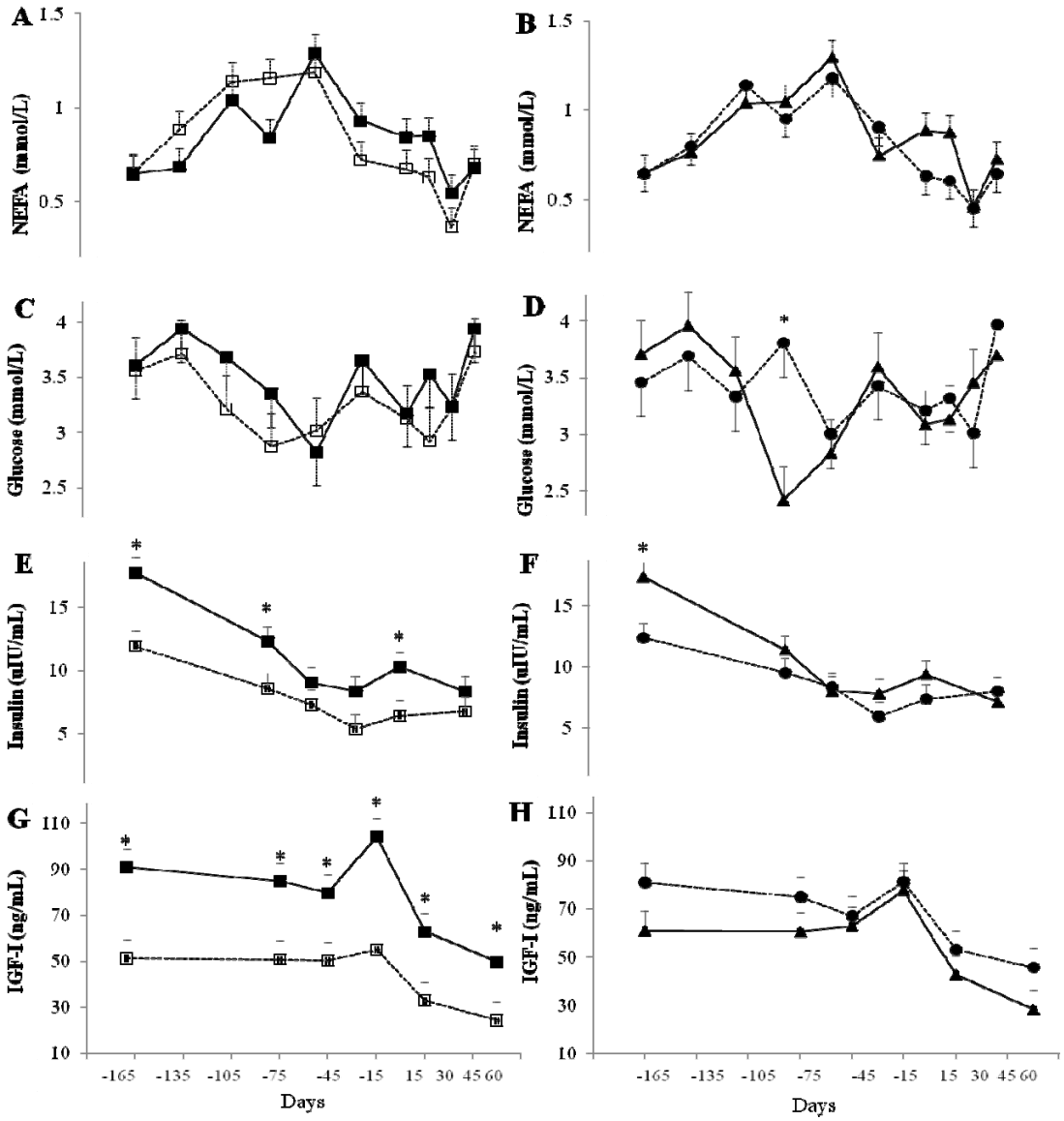

Fig. 2. Non-esterified fatty acids; (NEFA; A, B), glucose (C, D), insulin (E, F) and insulin-like growth factor I; IGF-I; G, H) concentrations throughout the year (-165 to 60 days relative to partum) in purebred and crossbreed (right panel) beef cows (n=32) grazing high and low forage allowances (10 and 6 kgDM/100kgBW/d in average; left panel) of native pastures through the year. Asterisks indicate differences ($P < 0.05$) between forage allowances (high *vs*. low) or genetic group (purebred *vs.*crossbred).

(—■— High and - -□- - Low forage allowance; - -●- · Purebred and —▲— Crossbred).

2.4.4 Hepatic gene expression

The hepatic expression of *GHR* mRNA was not affected by FA, GG, or days. However, the interaction between the FA and days tended to affect (P = 0.082) (Fig. 3A, 3B), and the interaction among FA, GG, and days affected (P = 0.021) *GHR* mRNA (data not shown). The expression of this transcript decreased between -165 and -45 days, remaining stable thereafter until 60 days without reaching its initial expression of late fall. However, this decrease in the expression of *GHR* mRNA in winter (-45 days) was only evident in HI cows, as in LO cows its expression did not vary throughout the year. The expression of *GHR* mRNA was greatest at -165 days in HI-CR cow than in the other groups. Hepatic *GHR-1A* mRNA was not affected by FA, GG, or their interaction but was affected by days (P = 0.003) as it decreased from -165 to -45 days, maintaining its expression low and stable in the peri and postpartum (Fig. 3C, 3D).

The expression of *IGF-I* mRNA was not affected by the FA, GG, or their interaction, but there was an interaction between FA and days on the abundance of this transcript (P = 0.048) (Fig. 3E, 3F). In HI cows, hepatic *IGF-I* mRNA declined between -165 and -45 days, remained low and stable until 15 days, and tended to increase (P = 0.072) at 60 days. In contrast, in LO cows, the expression of *IGF-I* mRNA was stable, and tended to decrease (P = 0.075) only at -15 days (Fig. 3E). Expression of *IGFBP3* mRNA was not affected by FA, GG, or their interaction but was affected by days (P = 0.007) and by the interaction between GG and days (P = 0.021) (Fig. 3G, 3H). Expression of *IGFBP2* mRNA tended to be less (P = 0.084) in HI than LO cows (15.02 *vs.* 8.57 ± 3.4), and was affected by days (P < 0.001) (Fig. 3I, 3J). Hepatic *IGFBP2* and *IGFBP3* mRNA showed an opposite pattern during winter-gestation and had a similar pattern during the lactation period. While hepatic *IGFBP2* mRNA increased during pregnancy in winter (from -165 to -15 days), *IGFBP3* mRNA decreased at -75 and -45 days in PU cows while remained stable and increase at -45 in CR cows. Both transcripts decreased its expression at 15 days, and increased it again at 60 days.

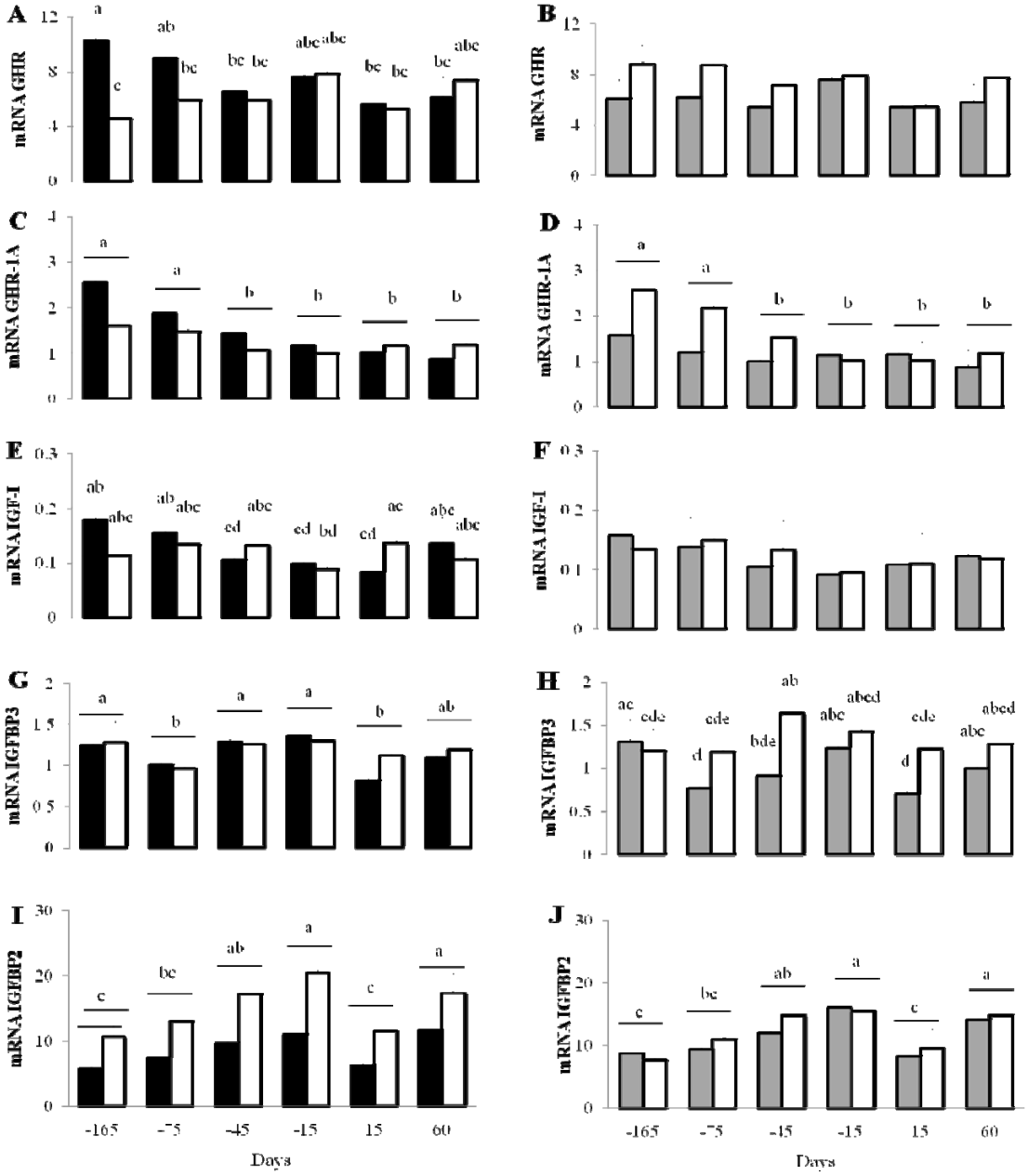

Fig. 3. Hepatic mRNA expression of *growth hormone receptor* (*GHR*, A,B), *GHR isoform-1A* (*GHR1A*; C,D), *insulin-like growth factor I* (*IGF-I*; E,F), IGF *binding protein 3*, (*IGFBP3*; G,H), IGF *binding protein 2* (*IGFBP2*; I,J), (*ß-actin* and *hypoxanthine phosphoribosyltransferase*, *HPRT* as endogenous controls), through the year (-165 to 60 days relative to partum) in purebred (■) and crossbreed (□; right panel) beef cows (n=32) grazing high (■) and low (□) forage allowances of native pastures (left panel; 10 and 6 kgDM/100kgBW/d in average). Letters above the

columns denote lsmeans differences ($P < 0.05$). Letters above the lines on colums indicate day effect ($P < 0.05$).

The hepatic expression of *GHR-1A* mRNA was correlated with the expression of *IGF-I* (P < 0.001, r = 0.29, n = 163) and *IGFBP3* (P < 0.001, r = 0.37, n = 163) mRNA. Serum insulin and IGF-I concentrations were negatively correlated with hepatic *IGFBP2* mRNA (P < 0.001, r = -0.37, n = 156 and P = 0.011, r = -0.26, n = 156, respectively). Surprisingly, milk yield during the first 60 days of lactation was positively correlated to *GHR* mRNA of fall (P < 0.001, r = 0.52, n = 28) and negatively correlated with *IGFBP2* mRNA (P = 0.017, r = -0.19, n = 163).

Hepatic mRNA abundance of genes involved in fatty acid oxidation *ACOX*, *ACADVL*, and *CPT-1*, were not affected by FA, GG, or their interaction but were affected by days (P < 0.027) (Fig. 4A-4F). The expression of these transcripts, increased during winter-gestation (-165 to -45 days), decreased during the peripartum (-15 and/or 15 days), and then increased again at 60 days. However, there was an interaction between the GG and days (P = 0.019) for *ACOX*, as at -75 days the expression of this transcript tended to be greater (P = 0.073) in CR than PU cows. The hepatic mRNA expression of *PPAR-α* tended to be affected by days (P = 0.056) as it decreased from -165 to -75 days, remained stable between -45 and 15 days, and increased again at 60 days (Fig. 4G, 4H).

Hepatic mRNA abundance of genes involved in gluconeogenesis, *PC* and *PCK1*, was not affected by FA, GG, or their interaction. Expression of *PC* mRNA was affected by days (P = 0.011), by the interactions between FA and days (P = 0.014), GG and days (P < 0.001) and among FA, GG, and days (P < 0.011). Expression of *PC* mRNA tended (P = 0.10) to increase from -165 to -75 days, remained high and stable until calving after when there was a slight decrease (P = 0.10) at 15 days to increase again at 60 days. However, *PC* mRNA tended (P = 0.083) to be less in HI than LO cows at -45 days, and the increase during winter (-165 to -75 days) was only evident in LO-CR cows, and the decrease at 15 days was

only evident in PU cows (Fig. 5A, 5B).

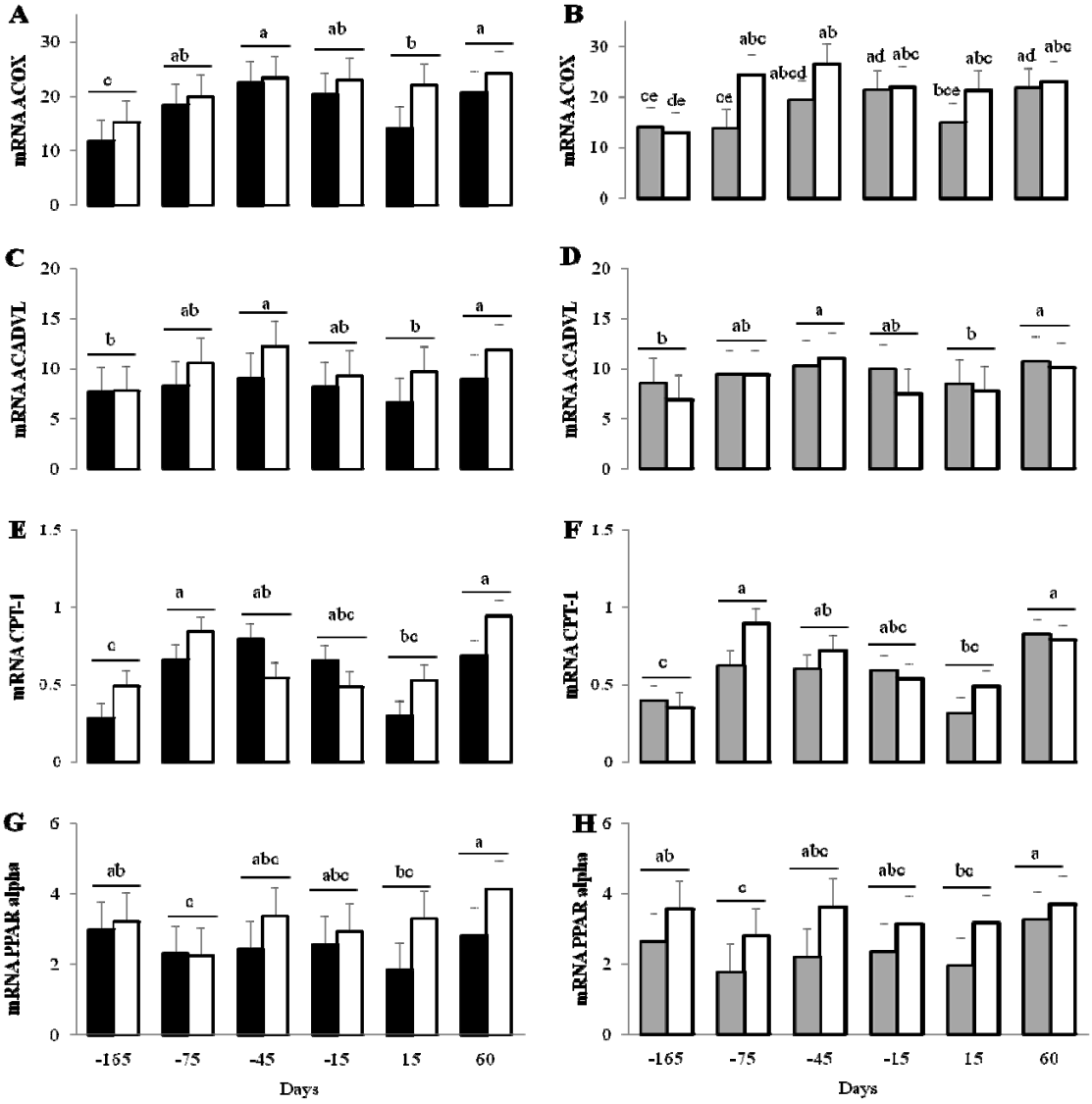

Fig. 4. Hepatic mRNA expression of genes involved in fatty acid β-oxidation *acyl-CoA oxidase-1* (*ACOX*), *acyl-CoA dehydrogenase very long chain* (*ACADVL*), *carnitine palmitoyltransferase 1* (*CPT1*), and *peroxisome proliferator-activated receptor-alpha* (*PPAR-α*) through the year (-165 to 60 days relative to partum) in purebred (■) and crossbreed (□right panel) beef cows (n=32) grazing high (■) and low (□) forage allowances (left panel; 10 and 6 kgDM/100kgBW/d in average) of native pastures through the year. Letters above the columns denote lsmeans

differences ($P < 0.05$). Letters above the lines on colums indicates day effect ($P < 0.05$).

Expression of *PCK1* mRNA was also affected by days ($P < 0.001$) and tended to be affected ($P = 0.056$) by the interaction among FA, GG, and days (Fig. 5C, 5D). Hepatic *PCK1* mRNA expression increased during gestation in winter (-165 to -45 days) and then remained high and stable until 60 days. However, the increased in winter was sharper in LO-CR cows than in the other groups.

The expressions of the transcripts involved in hepatic gluconeogenesis (*PC* and *PCK1*) were correlated with each other ($P < 0.001$; $r = 0.33$), and with the expression of transcripts involved in fatty acid metabolism *ACOX* and *ACADVL* ($P < 0.001$; $r > 0.45$). Also *PC* mRNA expression was negatively correlated with BCS and insulin concentrations ($P < 0.001$; $r < -0.31$).

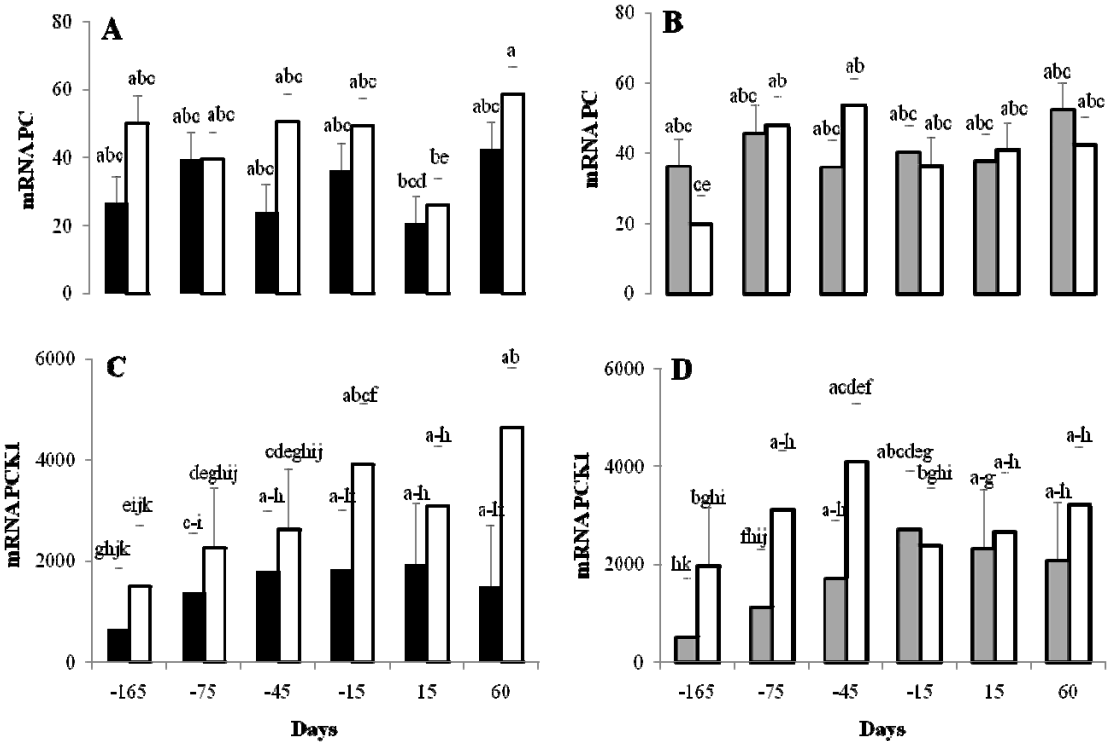

Fig 5. Hepatic mRNA expression of *pyruvate carboxylase* (*PC*; A, B) and *phosphoenolpyruvate carboxykinase 1, PCK1*; C, D) through the year (-165 to 60 days relative to partum) in purebred and crossbreed beef cows (n=32) grazing high and low forage allowances (6 and 10 kgDM/100kgBW/d, in average) of native pastures through the year. High-purebred (□) and low-purebred (■), left panel; high-crossbred (🔳) and low-crossbred (🔲) right panel. Letters above the columns denote lsmeans differences ($P < 0.05$).

2.4.5 Reproductive performance

Commencement of luteal activity was earlier ($P < 0.031$) for HI than LO cows and for CR that PU cows (82, 125, 150 and 175 ± 13 days for HI-CR, HI-PU, LO-CR and LO-PU, respectively). Commencement of luteal activity was negatively correlated with BCS ($P < 0.001$, $r = -0.44$, $n = 160$), serum IGF-I ($P < 0.001$, $r = -0.49$, $n = 157$), serum insulin ($P < 0.001$, $r = -0.38$, $n = 157$) and positively correlated with *IGFBP2* mRNA abundance ($P = 0.003$, $r = 0.29$, $n = 160$).

2.5 DISCUSION

In beef cows on rangeland conditions the more remarkable changes, in regard to the dynamics of metabolic and endocrine profiles and hepatic gene expression of the somatotropic axis and key enzymes of the metabolism of fatty acids and glucose, occurred during the last half of gestation in winter with the onset of NEB. These changes were highly modulated by FA of native pastures through the year. In the present study, both milk production and commencement of luteal activity were affected by FA and cow GG and these responses were associated with differential metabolic, endocrine and hepatic gene expression profiles through the gestation-lactation cycle.

2.5.1 Body condition score and metabolic and endocrine profiles

During winter gestation (-165 to calving), all cows, independent of the treatment, mobilized body reserves, evidenced by the decrease in BW and BCS and the elevated concentrations of NEFA, which are inversely related to the energy

balance and indicative of adipose tissue mobilization [39,23]. In agreement with the magnitude of BCS losses during winter, that did not differ drastically among groups, no differences were observed in serum NEFA suggesting similar lipolysis rates among cows. The increase in NEFA concentrations during winter gestation were accompanied by decreased concentrations of glucose and insulin as typically occur during NEB [40,41,42]. Insulin is a potent regulator of nutrient metabolism, playing a central role in the homeostasis of glucose, its secretion by the pancreas is glucose-stimulated, and decreased concentrations of this hormone favour gluconeogenesis and lipolysis [14] and is associated with reduced energy intake [11]. Thus, the changes observed in BCS, and NEFA, glucose, and insulin concentrations reflected the NEB period during winter as a consequence of decreased forage intake due to reduced herbage mass availability and height [4]; increased grazing energy requirements [5], and/or increased gestation requirements (fetal and maintenance of maternal non-uterine tissues), as last third of gestation is established.

It has been shown that a strategy developed in the ruminant to cope with feed restriction and NEB is the uncoupling of the GH-IGF-I axis, which results in increased concentrations of GH and decreased concentrations of IGF-I [11, 22]. This uncoupling has been related to decreased insulin concentrations, as this hormone stimulates hepatic IGF-I secretion by modifying hepatic GHR receptors and IGFBP secretion (see below, [23, 24, 41]). In addition, it has been demonstrated that IGF-I affected insulin secretion by pancreatic cells [44]. This association between insulin and IGF-I concentrations was reflected in the positive correlation between these hormones determined in the present study. Serum insulin and IGF-I concentrations were greater in HI cows during the whole period evaluated but although insulin decreased during winter for all cow groups, serum IGF-I concentrations decreased only in HI cows. This, together with changes in hepatic gene expression (see below), may reflect a better metabolic status and ability to adapt to changes in environmental conditions (i.e. nutrition) for HI than LO cows [11].

In contrast with the dairy cow model, in this study, moderate changes were

observed in metabolic and endocrine profiles during the peripartum period, especially when compared to winter-gestation. Even though serum insulin and IGF-I concentrations decreased with the onset of lactation, favouring nutrients partitioning towards the mammary gland [14] we did not find a negative association between insulin and milk yield as reported previously for beef cows [45, 3]. In addition, serum concentrations of NEFA decreased and plasma glucose increased during early lactation which would probably indicate a partial recovery of energy intake (and therefore energy balance) with the increase in herbage mass availability, height, and quality as temperature rise in spring [46].

2.5.2 Hepatic expression of somatotropic axis genes

In the present study, and in agreement with circulating IGF-I concentrations, hepatic *GHR* and *IGF-I* mRNA decreased during winter gestation only for HI cows, while their expressions remained unchanged for LO cows. The decrease during winter was associated to greater expression of these transcripts in HI cows at -165 days that together with their greater BCS, and greater serum concentrations of insulin and IGF-I, would indicate a better metabolic status of the cows at late fall (-165 days).

The changes in *GHR* and *IGF-I* mRNA may be important endocrine events that control metabolic process [27] which would suggest as stated before a differential ability to adapt to changes in energy intake/balance through the year, and therefore in nutrient partitioning between HI and LO cows. During the NEB of early lactation in dairy cows, the decrease in *GHR* and *IGF-I* mRNA has been explained by a reduction of the liver-specific *GHR-1A* mRNA [22]. This is consistent with our results for HI cows during NEB of winter gestation (reduction of *GHR*, *GHR-1A*, and *IGF-I* mRNA, reduction of serum IGF-I) as well as with the positive correlation between *GHR-1A* and *IGF-I* mRNA determined here. However, *GHR-1A* mRNA decreased also for LO cows, while *GHR* and *IGF-I* mRNA and serum IGF-I remained constant. These results would suggest an increased expression of other transcripts variants of *GHR* mRNA (1B or 1C) as a compensatory mechanism or

mechanisms independent of *GHR-1A* mRNA transcription for LO cows [27, 31].

The IGF circulate in plasma complexes to a family of structurally related IGFBPs (1 to 6) involved in the conversion, transport and modulation of the activity of IGF-I [47, 48]. The *IGFBP-3* has an important functional role in the somatotropic axis, since circulating IGF-I forms a ternary complex with this binding protein (IGFBP3/IGF-I) that longer IGF-I half-life and potentiate its role [49]**.** It has been proposed that IGFBP3 expression can represents one plausible mechanism whereby insulin regulates plasma IGF-I concentrations [18,23,26]. Indeed, in this study the decrease in *IGFBP3* mRNA abundance during gestation coincided with the greater drop in insulin concentrations (at -75 days). On the contrary, IGFBP2 inhibits IGF-I action by preventing the binding to its receptors [49]. Control of *IGFBP2* mRNA changes dramatically in response to changes in nutritional status [48], in accordance with our results that showed greater hepatic *IGFBP2* mRNA expression in LO cows through all the period evaluated. Moreover, increased *IGFBP2* mRNA was associated with periods of nutritional restriction [50], as reflected in the increase in this transcript expression during winter-gestation and the negative correlation between circulating insulin and *IGFBP2* mRNA determined in this study. In addition, the greater *IGFBP2* mRNA and reduced *IGFBP3* to *IGFBP2* mRNA ratio determined in LO cows, could explain the reduced circulating concentrations of IGF-I as previously described [48,23] and is in agreement with the negative correlation between serum IGF-I and *IGFBP2* mRNA reported here.

The decrease in serum insulin and IGF-I concentrations during early lactation, were accompanied by lower levels of hepatic *GHR-1A* and *IGF-I* mRNA and *IGFBP3* to *IGFBP2* mRNA ratio, associated with the increase in milk production as reported previously in dairy cows [26, 18]. In agreement with our results, the expression of *GHR*, *GHR-1A*, and *IGF-I* mRNA did not change during the peripartum or early lactation in beef cows [29, 30] or in a strain of low-producing dairy cattle [51] suggesting that the onset of lactation does not mean an important NEB in beef cows on grazing conditions and that the uncoupling of GH-IGF axis

mechanism of early lactation is probably related to genetic selection for milk production [29, 51]. Similar to our results, Schneider *et al.* [30] did determine decreased circulating IGF-I concentrations after calving. Interestingly, as previously reported in beef cows [30], but in contrast with reports in dairy cows [22, 26], serum IGF-I concentrations were not correlated with hepatic expression of *GHR*, *GHR-1A* or *IGF-1* mRNA. These results indicate that other components of the somatotropic axis, as the decreased ratio *IGFBP3* to *IGFBP2* mRNA reported here, could explained the reduction in IGF-I concentrations in beef cows postpartum as previously suggested [25]

2.5.3 Fatty acid oxidation gene hepatic expression

The rate of mobilization of reserves, mainly from adipose tissue, depends on factors like the severity of BEN and cow physiological status. Once NEFA are released from adipose tissue into the circulation, are taken up mainly by the liver as substrates for mitochondrial or peroxisomal β-oxidation, or by the mammary gland for the synthesis of complex molecules [19]. The activity of mitochondrial (CPT-1 and ACADVL) and peroxisomal (ACOX) enzymes allows hepatocytes to deal with the overflow of NEFA during periods of NEB [13]. Expression of hepatic *CPT-1* and *ACADVL* mRNA has increased [18] or not changed [19] during the NEB of early lactation in dairy cows, suggesting that the influxes of NEFA are determinant in the up-regulation of these genes. In this study, the increased hepatic *CPT-1* and *ACADVL* mRNA expression during the NEB of winter gestation, and at 60 days during lactation period, may reflect the energy need for maternal metabolism during gestation or milk production, respectively, and coincided with the observed increases in serum NEFA and decreases in BCS and serum insulin and IGF-I concentrations. In agreement with the similar BCS losses and serum NEFA concentrations, we did not observed changes in *CPT-1* or *ACADVL* mRNA expression among cows groups (FA and GG).

We have found only one report of increased *ACOX* mRNA expression in feed restricted cattle, during induced clinical ketosis in dairy cattle [52]. However,

peroxisomal β-oxidation represented approx. 50% of the total capacity of β-oxidation in the liver of dairy cows during NEB [53, 54]. In the present study, similar to the increased expression in genes encoding for key enzymes of the mitochondrial β-oxidation, hepatic A*COX* mRNA increased during winter and early lactation as it has been proposed that peroxisomal β-oxidation plays a role in relation to the metabolism of fatty acids during periods of sustained high hepatic influx of fatty acids [53, 54]. Expression of *ACOX* mRNA increased more during winter in CR than PU cows, this and previous results [55] would suggested that interactions of diet, physiological stage and genotype were important for induction of peroxisomal β-oxidation.

The transcription factor PPAR-α, is a nuclear receptor that regulates the expression of genes involved in fatty acid metabolism, acts as a sensor of available levels of NEFA [18], and has a central role in the molecular adaptations necessary to cope with feed restriction [52]. Contrary to what we expected, even though *PPAR-α* mRNA was correlated with *CPT-1* and *ACOX* mRNA, hepatic *PPAR-α* mRNA showed a different pattern of expression than fatty acid oxidation genes as its abundance did not increase during winter-gestation but only at 60 days. In agreement with this result, Loor [13] reported that activation of this nuclear receptor via known pharmaceutical ligands or long-chain fatty acids did not alter *PPAR-α* mRNA, but caused marked upregulation of its target genes in liver (*ACOX1*, *CPT1A* and *ACADV;* [56]) and suggested that PPAR-α activity is not only regulated at a transcriptional level, but also, through post-transcriptional changes or mediated by the action of coativators (i.e *peroxisome proliferative activated receptor, gamma coactivator-1 alpha*, PPARGC1a; [52]). Moreover, Sanderson et. al. [57] suggested a potential role of PPAR-δ in hepatic adaptations to fasting and/or elevated blood NEFA. Thus, activity and mRNA data available for enzymes of mitochondrial and peroxisomal oxidation point at a concerted action at two levels of regulation in allowing hepatocytes to cope with the sudden influx of NEFA [13].

2.5.4 Gluconeogenic enzymes gene hepatic expression

Interestingly, the expressions of the transcripts involved in fatty acid metabolism (*ACOX* and *ACADVL*) were correlated with the expression of transcripts involved in hepatic gluconeogenesis, thus, reflecting the coordinated regulation of these two metabolic pathways [58]. Ruminants rely largely on hepatic gluconeogenesis to support glucose metabolism and to supply glucose for lactose synthesis [59]. Although plasma glucose concentrations decreased during winter-gestation in this study, the hepatic mRNA expression of two enzymes responsible for key steps in hepatic gluconeogenesis pathway, PC (conversion of pyruvate to oxaloacetate) and PCK1 (phosphoenolpyruvate formation from oxaloacetate) increased markedly. The increase in *PC* and *PCK1* mRNA would enhance (depending on translation efficiency) the synthesis of oxaloacetate and phosphoenolpyruvate, respectively, probably to support the increased use of acetil-CoA (originated from NEFA β-oxidation) to obtain energy in tricarboxylic acid cycle (TCA), or to support glucose synthesis during periods of NEB. Thus, PC has an anaplerotic role for the TCA, when intermediates are removed for different purposes, particularly during NEB for gluconegenesis [60].

The rate of gluconeogenesis is responsive to substrate availability and relative concentrations of gluconeogenic precursors [61]. Propionate is the most important products of starch digestion and a key carbon sources for gluconeogenesis, followed by L-lactate and glucogenic amino acids [62]. Studies showed that *PC* mRNA abundance and /or enzyme activity increased rapidly in dairy cows after calving with the onset of NEB [16] or in cows under feed restriction [63] as cows in NEB likely use relatively more lactate, glycerol and amino acids and less propionate as gluconeogenic precursors [62,63]. The increase in *PC* and *PCK1* mRNA was sharper in LO-CR cows which could indicate a differential glucose metabolism homeostasis mechanism for cow of different GG grazing different FA. The decreased in the mRNA expression of *PC* but not in *PCK1* mRNA observed at 15 days in the present study (more evident in HI than LO cows), would indicate an adaptation in the profile of precursors, probably from gluconeogenic amino acids and/or propionate,

associated with an increase in food intake when herbage mass availability, height, and quality increased as temperature rise in spring [46].

As lactation progresses and milk production reaches the peak of lactation (60 days for beef cows [64]), although it appeared to be a recovery in energy balance during the postpartum (reflected in maintenance of BCS, reduced serum NEFA and increased plasma glucose as discussed above), expression of mRNA of these enzymes remained elevated. The elevated hepatic *PC* and specially of *PCK1* mRNA expression during lactation were associated to the decreased insulin concentrations as previously reported [65] and would reflect an adaptation to the greater glucose requirement for lactose synthesis in the mammary gland [16].

2.5.5 Reproductive performance

Blood concentrations of glucose, NEFA, insulin, and IGF-I are indicative of the availability of energy and provide short- or long-term signals that mediate the effects of nutrition on reproduction [66]. In agreement with previous reports, commencement of luteal activity was negatively correlated with BCS [67], circulating IGF-I and insulin concentrations [68,69] and positively correlated with hepatic expression of *IGFBP2* mRNA [25]. Indeed, shorter commencement of luteal activity determined for HI cows, was associated with the greater BCS and serum insulin and IGF-I concentrations and with less *IGFBP2* mRNA in these cows during the whole period. Cows grazing HI had greater metabolic status during the whole period which would indicate that were able to adapt better to changes in nutrient and energy supply through the gestation-lactation cycle without diminishing their status and their productive (i.e milk yield) and reproductive (i.e commencement of luteal activity) performance [70]. Shorter commencement of luteal activity in CR than PU cows were associated to a greater BCS and a better metabolic status (greater BCS, serum insulin concentration and hepatic *GHR* mRNA abundance) particularly in late of fall (-165 days) and with greater hepatic *IGFBP*3 mRNA expression in late winter (-75 and -45 days) and in early lactation (15 days). These results would support the concept of a ‘metabolic memory’ that allows for nutritional information to be carried

forward beyond the life of the original metabolic stimulus [71].

2.6 CONCLUSIONS

We found differential metabolic, endocrine and hepatic gene expression profiles in beef cows of different GG grazing different FA that varied in a coordinated fashion with changes in herbage mass availability of native pastures through the year and with the physiological status of the cow. A period of NEB (decreased BCS, increased serum NEFA and plasma glucose, decreased serum insulin and IGF-I) was established during winter-gestation (-165 to -15 days) in beef cows on grazing conditions, while no drastic changes in metabolite, endocrine and hepatic gene expression profiles were observed during the peripartum and lactation period. In addition, more significant differences among time points regarding endocrine and the hepatic mRNA expression were observed in HI than LO cows, suggesting a greater adaptive performance to changes in nutrient and energy supply through the year in these cows. Finally, greater milk production did not affect reproduction performance, as greater milk yield and shorter commencement of luteal activity were observed in HI and CR cows.

2.7. ACKNOWLEDGMENTS

The authors thank Bernardo Rosengurtt Experimental Unit staff, for animal care and assistance throughout the study, Dr. V. Gutierrez, Bach's. F. Pereyra and P. Machado, for their help during the experimental phase, and Prof. J. Urioste and A. Meikle for the review of this manuscript. This study was supported by research project INIA-FPTA # 262 awarded to M. Carriquiry.

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3. FUNCTIONAL GENOMICS OF LIVER IN BEEF CATTLE DURING GESTATION AND LACTATION

Functional Genomics of Liver Metabolic Adaptations During Gestation and Lactation: Crossbred and Purebred Beef Cows in Two Forage Alowances of Native Pastures

Functional genomics of liver in beef cattle during gestation and lactation

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3.1 SUMMARY

Genomic approaches provide a powerful tool for studying the genetic mechanisms governing phenotypic variation. We explored the temporal changes in hepatic gene expression and the metabolic adaptations during the gestation-lactation cycle in multiparous (n=16) purebred (PU-Angus and Hereford) and crossbred (F1-CR) beef cows, grazing high and low forage allowances (10 *vs*.6 kgDM/100kgBW/d; LO *vs*. HI) of native pastures throughout the year. Cow body condition score (BCS) was recorded, and serum samples were collected at -165, -75, -45, -15±10, +15 and +60 ±3 days relative to partum, to measure NEFA by spectrophotometry, and insulin and IGF-I by immunoradiometric assay (IRMA). Liver tissue was collected at -165, -15 and +15 and +60 ±3 days, to measure gene expression, using an Agilent 4*44k gene expression bovine array. We found a total of 4,663 genes to be altered by days (272 with at least a 2.5-fold difference), one gene by forage allowance, and 148 by the interaction between forage allowance and genetic group (47>2 fold-change). Large genome-wide changes in genes expression patterns were observed particularly during winter-pregnancy period (-165 *vs*. -15 days) when beef cows experience some degree of negative energy balance. Network analysis using GSEA revealed extensive changes in gene expression through time, which classified into 66 gene networks including glucose and lipid metabolism and cell cycle. It appears to be an enhanced gluconeogenesis and fatty acids oxidation, and an inhibitory impact on cell growth, a decrease in DNA replication and transcription, with a tendency toward un-regulated cell cycle progression. We demonstrate that higher forage allowance of native pastures was reflected in a better cow metabolic status that promotes hepatic gene expression, particularly associated with energy glucose metabolism. This genomic approach contributes to our knowledge of the biology of energy balance in beef cow. We highlighted new candidate genes, entire metabolic pathways, and regulatory mechanisms in the liver during the gestation-lactation cycle of the beef cow. This genes and pathways can be target of future studies, as they may explain, in part, cow's productive and reproductive performance in the postpartum period.

***Key words*:** beef cattle, functional genomics, liver, gene expression, microarray

3.2 INTRODUCTION

Energy balance (EB) is one of the most important factors affecting reproductive performance (i.e. length of postpartum anoestrus) in beef cows, since the reproductive axis integrates various nutritionally related signals that, directly or indirectly, affect follicular growth and/or ovulation, oocyte quality, and embryo mortality (Webb *et al.*, 2004; Hess *et al.*, 2005). In spring-calving beef cows in rangeland conditions, last months of gestation and calving occur during winter or early spring when quantity and quality of pastures limit energy intake (Chapman *et al.*, 2007) and increase the energy cost of grazing (Brosh, 2007). This determines the onset of negative energy balance (NEB) and the loss of body condition score (BCS) before calving (Quintans *et al.*, 2010) to sustain the increasing energy demands of the developing fetus and mammary gland (Bell, 1995). This nutritional and metabolic stress can contribute to the poor reproductive performance (McCarthy *et al.*, 2010).

The liver is a metabolically active major organ in mammals. Correct function of the liver, as well as the performance of essential body functions, are controlled through the coordinated expression of a large number of genes (Columbano and Ledda-Columbano, 2003). Metabolic and endocrine regulation depends, partly, on transcriptional control of gene networks, which affect the synthesis of plasma proteins, nuclear factors, and enzymes involved in gluconeogenesis, glycogen synthesis, glucose, lipid, and cholesterol metabolism (Jungermann and Katz, 1989). Environmental (including nutrition), autocrine, endocrine, or paracrine signals ultimately lead to changes in hepatic gene expression and can govern the rates at which genes are transcribed into mRNA (Columbano and Ledda-Columbano, 2003; Loor, 2010). Particularly, pregnancy and lactation are dominant physiological stages during which alterations in hepatic metabolism could be expected because of a greater demand for nutrients by the fetus and mammary gland (Bell, 1995; Wallace, 2000; Drackley *et al.*, 2001). In this way, liver is a logical tissue for assessing transcript profiling to study short and long-term adaptations in hepatic gene expression associated with changes in energy balance throughout the year, and to identify key regulatory pathways involved in these processes.

Genomic approaches, such as microarray technology, provide a powerful tool for studying the genetic mechanisms governing phenotypic variation, inferring regulatory networks, and discovering candidate genes (for a review see Schulze and Downward, 2001). Most of the studies that have used this technology to evaluate the ruminant hepatic transcriptome have been performed in dairy cows during pregnancy (Herath *et al.*, 2004), peripartum and early lactation period (Loor *et al.*, 2005), adaptations to severe negative EB or dietary restriction (Loor *et al.*, 2006; McCarthy *et al.*, 2010), and metabolic diseases (Loor *et al.*, 2007). In beef cattle, microarrays technology has been used to study expression profiles of muscle in beef quality research (Hocquette *et al.*, 2007), anterior pituitary glands of anestrous and cycling postpartum cows (Roberts and MacLean, 2011), and endometrial gene expression in early pregnancy (Forde *et al.*, 2009). Connor *et al.* (2010) studied hepatic gene expression profile during periods of feed restriction and re-alimentation in Angus steers. Timperio *et al.* (2009) found significant differences in liver metabolism between two breeds selected for milk and meat traits, respectively, despite being closely genetically related, suggesting that genetic selection affects gene expression profile. However, no studies have focus on understanding how nutrition and genetic group may affect hepatic regulatory mechanisms during gestation and early lactation in beef cows grazing native pastures.

We hypothesize that temporal patterns of hepatic gene expression vary due to cow physiological stage, and that may differ depending on the forage allowance of native pastures (i.e. nutrition) and animal genetic background. This work focuses on assessing temporal changes in hepatic gene expression in purebred and crossbred cows, grazing high and low forage allowances of native pastures throughout the year. To understand the architecture of genetic regulatory networks, pathway analysis of this high-throughput transcriptomics data, was also explored.

3.3 MATERIALS AND METHODS

3.3.1 Location, animals, experimental design and treatments

Animal procedures were performed according to protocols approved by the

Animal Experimentation Committee of Universidad de la República. Detailed descriptions of the native pastures, animal management, data and sample collection and analyses, and production responses have been reported elsewhere (Laporta *et al.*, 2011). Briefly, sixteen multiparous (5 to 6 years) pregnant cows, eight purebred (PU; Angus-A and Hereford-H) and eight crossbred (CR; HA and AH), were randomly selected from a larger group (n = 32), and used to evaluate the effect of genetic group (CR *vs.* PU) and forage allowance of native pastures (High, HI *vs.* Low**,** LO; 10 *vs.* 6 kgDM/100kg BW/d, in average throughout the year, respectively), on liver gene expression from gestation to early lactation period (May 2009 to February 2010). Thus, we evaluated 4 treatments HI-CR, HI-PU, LO-CR and LO-PU (n = 4 biological replicates per treatment).

All cows belonged to or were descendants of experimental animals generated as part of a diallel crossbreeding experiment carried out for 10 years at the Experimental Station (Espasandín *et al.*, 2006). Cows were assigned to the same forage allowance (HI or LO) since May 2007 and gestated and lactated one calf every year from 2007 to 2009. The experiment was conducted in a complete randomized block design with two spatial replicates. Forage allowances were adjusted monthly, after measuring the amount of forage available in each of the plots (Haydock and Shaw, 1975) through the entry of animals ("put and take" grazing system; Mott and Lucas, 1952) of similar genetic group and physiological status. All cows had normal parturition and were free from health disorders during the experimental period.

3.3.2 Data and sample collection and analyses

Body condition score (by visual assessment, scale of 1 = very thin to 8 = very fat; Vizcarra *et al.*,1986) were recorded, and blood samples were collected at -165, -75, -45 and -15 ± 8, and +15 and, +60 ± 3 (standard deviation) days relative to calving (days; average calving date: November 7, 2009). Liver tissue was collected at -165 and -15 ± 8, and +15 and, +60 ± 3 days, corresponding to the end of fall and initiation of winter (May), end of winter and pregnancy (October), early (November)

and close to peak of lactation period (January), respectively. Blood samples were collected in duplicate via venipuncture of the coccygeal vein in BD Vacutainer ® tubes (Becton Dickinson, NJ, USA), without anticoagulant and centrifuged, after 4 h of coagulation, at 2500 Xg for 20 min for the collection of serum. Samples were analyzed for NEFA, insulin, and *insulin-like growth factor-I* (IGF-I) concentrations, as described by Laporta *et al.* (2011).

Liver biopsies were collected using a 14-gauge biopsy needle (Tru-Core®-II Automatic Biopsy Instrument; Angiotech, Lausanne, Suitzerland) as described by Carriquiry *et al.* (2009). Samples were immediately frozen in liquid nitrogen and stored at - 80°C until total RNA extraction. Total RNA was isolated using TRIZOL (Invitrogen, Life Technologies, Carlsbad, CA, USA), as described by Laporta *et al.* (2011). Integrity and quality were evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, ON, USA). The RNA integrity number was determined using the algorithm of the Agilent 2100 expert software and RNA LabChip® kits. The average RIN for the 64 samples was 6.7±0.4.

3.3.3 Microarray target preparation and hybridization

A single-channel microarray analysis was performed using Agilent 4x44K Bovine (v2) Gene Expression Microarray (Part Number G2519F, design ID 023647) which contains 43,803 probes representing 17,123 unique genes. Amplification, probe labelling, and hybridization were carried out following the manufacturer's specified protocol ‘Agilent One-Color Microarray-Based Gene Expression Analysis’ (Quick Amp Labeling, G4140-9004, Agilent Technologies Santa Clara, CA). Amplification of 600 ng of total RNA was performed for single stranded cDNA synthesis after spikes (known mixtures of RNA used as internal controls) were added. Complimentary RNA (cRNA) synthesis and amplification was performed using reverse transcriptase and T7 Oligo (dT) promoter primer. All the samples were labeled with a green dye (cyanine 3-labeled Cy3). Labeled cRNA was purified, fragmented, and then hybridized to the corresponding slides. Slides were incubated overnight (17 h) at 65°C for the dye-labeled cRNA to hybridize to their

complementary sequences on the array. Chips were washed, to remove the un-hybridized cRNA, and scanned using Agilent Scanner (G2505B, Agilent Technologies). Images were processed using Agilent Feature Extraction software (9.5 v.; Agilent Technologies). To assess the quality of the data all spike-in were checked for reproducibility and linearity. All microarray images and quality control measurements were within recommended limits.

3.3.4 Analysis of microarray data

Statistical analysis was performed using GeneSpring Multi-Omic Analysis Software (11.5X v.; Agilent Technologies). Normalization of the raw data (gprocessedsignal thresholded to 1.0 and Log2 transformed) were performed per-chip and per-gene using the percentile shift (75^{th} percentile) and baseline to median of all samples transformation, respectively. Three criteria were implemented to filter unreliable intensity values based on flags values (D-detected; ND-not detected and C-compromised) derived from the Feature Extraction Software. Features not positive and significant and not above background were classified as ND, and features saturated or population outliers were classified as C. Only probes containing 75% of D flags were accepted. All probes that did not meet these criteria were eliminated and all further analyses were performed on the remaining 29.715 probes.

The effect of forage allowances, genetic group, time (days), and their interactions were evaluated using a three-way ANOVA test. Significance levels were adjusted for the number of comparisons using Benjamini and Hochberg's false discovery rate (FDR, Benjamini and Hochberg, 1995). Differences in relative expression were considered significant at a FDR=0.10. Gene Ontology (GO) analysis was performed on differentially expressed genes. Enrichments for GO terms were tested by a hypergeometric test and probability values were corrected using the Benjamini-Yekutieli method (Benjamini and Yekutieli, 2000).

To further understand transcriptomic changes that occurred at a systemic level, Gene Set Enrichment Analysis (GSEA; Subramanian *et al.*, 2005) was carried

out in GeneSpring software. It required the download (http://www.broadinstitute.org/gsea/msigdb/) of 'canonical-pathways' (C2,v3.0) in the MsigDB database a collection of 880 gene sets curated from various sources including KEGG, Reactome and Biocarta. Gene sets were permuted 1000 times to estimate p-values for enrichments. Gene sets with fewer than 15 genes represented in the list were dropped as an additional filter to minimize false positives (FDR=0.25).

Differentially expressed genes throughout time were hierarchically clustered and visualized graphically, to identify biologically relevant groups of genes. To design clusters for HI and LO forage allowance, and PU and CR cows, t-tests (FDR = 0.10) were performed to search for differentially expressed genes.

3.3.5 Quantitative real-time RT-PCR

To validate results obtained by microarray, absolute mRNA quantification of 10 differentially expressed genes throughout time [*growth hormone receptor* (*GHR*), *IGF-1*, *IGF-1 binding protein 2 and 3* (*IGFBP2* and *IGFBP3*), *acyl-CoA oxidase-1* (*ACOX*), *acyl-CoA dehydrogenase very long chain* (*ACADVL*), *carnitine palmitoyltransferase 1* (*CPT-1*), *pyruvate carboxylase* (*PC*), *phosphoenolpyruvate carboxykinase 1* (*PCK1*), and *fibroblast growth factor-21* (*FGF-21*)] was performed by real time RT-PCR. Total RNA, previously extracted for the microarray study, was converted to cDNA with SuperScript®III Transcriptase (Invitrogen), all procedures and RT-PCR reactions and conditions used were described by Laporta *et al.* (2011). Primers used for the quantification of target and endogenous control (*hypoxanthine phosphoribosyltransferase* and *ß-actin*) cDNA are shown in Table 1.

To achieve sufficient biological replication, all genes were tested separately using a different source of RNA from 12 different cows (n=3 per treatment). Data of BCS, metabolites and hormone concentrations, and normalized gene expression values were analyzed using a linear model with repeated measures. The model included the fixed effects of forage allowance, genetic group, days (repeated

measure), and their interactions, using the MIXED procedure of SAS (SAS Institute Inc; Cary N.C, 2001).

Table 1. Primers used for the quantification by real time RT-PCR of target and endogenous control genes cDNA for microarray validation.

Gene[1]	Accession no.[2]		Primer sequence	Lenght (bp)
GHR	NM_176608	Sense	TCTGGGAATCCTAAATTCACCAA	91
		Antisense	CTGTAAACTGTGATTAGCCCCATCT	
IGF-I	XM_612412	Sense	CCAGACAGGAATCGTGGATG	89
		Antisense	ACTTGGCGGGCTTGAGAG	
IGFBP2	NM_174555	Sense	ATGCGCCTTCCGGATGA	83
		Antisense	GTTGTACAGGCCATGCTTGTCA	
IGFBP3	NM_174556	Sense	AGCACAGACACCCAGAACTTCT	86
		Antisense	TTCAGCGTGTCTTCCATTTCC	
CPT1	NM_001034349.2	Sense	TGCACGGCAACTGCTACAA	93
		Antisense	ACGCGTGCTCTCTGTTGAGT	
ACADVL	BTCN8936	Sense	CCAGCCCCTGTGGAAAATACTA	66
		Antisense	GCCCCCGTTACTGATCCAA	
ACOX1	NM_001035289.2	Sense	CCATTGCCGTCCGATACAGT	99
		Antisense	GTTTATATTGCTGGGTTTGATAATCCA	
PC	NM_177946.3	Sense	CGTCTTTGCCCACTTCAAGG	70
		Antisense	GAAGAGGCGCGTATTGAGGC	
PCK1	NM_174537.2	Sense	TGGCCATGATGAACCCTACTC	77
		Antisense	GTCAAATTTCATCCAGGCATATC	
FGF21	XM_602991.2	Sense	CGGATCGCTGCACTTTGAC	76
		Antisense	CTGGTAGACGTTGTATCCATCTTCA	
ACTB	BT030480	Sense	CGTGGCTACAGCTTCACC	53
		Antisense	GAAATCGTCCGTGACATCAA	
HPRT	XM_580802	Sense	TGGAGAAGGTGTTTATTCCTCATG	105
		Antisense	CACAGAGGGCCACAATGTGA	

[1]GHR = growth hormone receptor, IGF-I = insulin-like growth factor-I, IGFBP2 = IGF-binding protein-2; IGFBP3 = IGF-binding protein-3, CPT1=carnitin palmitoyl transferase 1, ACADVL=acyl-CoA dehydrogenase, very long chain, ACOX1=acyl-CoA oxidase 1, palmitoyl, PC = pyruvate carboxilase, PCK1 = phosphoenolpyruvate carboxykinase 1, FGF-21= fibroblast growth factor-21, ACTB=ß-actin (endogenous control gene), HPRT=hypoxanthine phosphoribosyltransferase. [2]GeneBank sequences. Primer references see Laporta *et al.*, 2011, Carriquiry *et al.*, 2009.

3.4 RESULTS

3.4.1 Body condition score and metabolic and hormone parameters

Body condition score, and concentrations of NEFA, insulin and IGF-I were affected by days ($P < 0.002$). Cow BCS and insulin concentrations decreased markedly during winter-gestation period (-165 to -45 days), and remained low and

stable without reaching the initial values of end of fall (-165 days) at +60 days postpartum. In contrast, serum NEFA concentrations increased markedly during winter-gestation (from -165 to -75 days), reaching NEFA peak at -75 in LO and -45 days in HI, and decreased thereafter until +60 days (Figure 1 A, B and C). Even though IGF-I concentrations were stable during winter-gestation, there was an increase at -15 days, only for HI cows, followed by a sharp decrease during the postpartum period (Figure 1 D). Cows grazing HI forage allowances had greater ($P < 0.039$) BCS and IGF-I concentrations during the whole period evaluated.

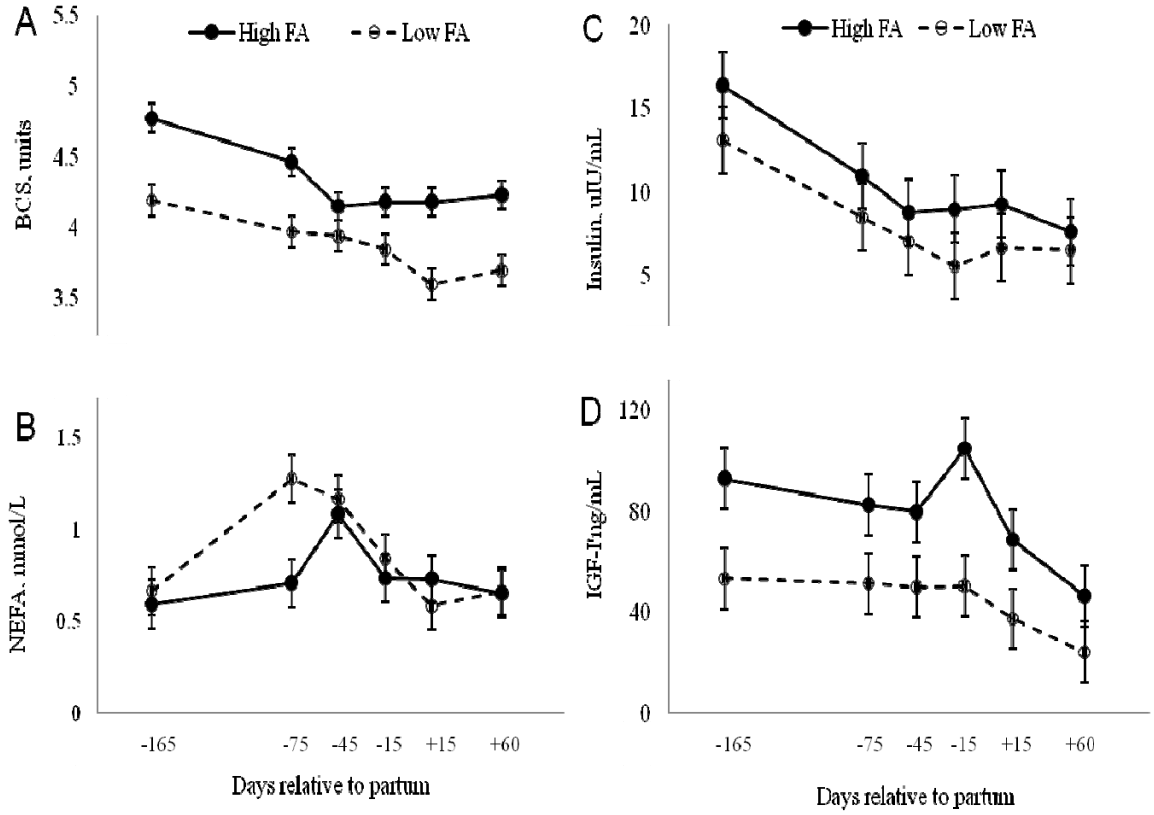

Figure 1. Body condition score (BCS, A) and non-esterified fatty acids (NEFA, B), insulin (C) and insulin-like growth factor-I (IGF-I, D) concentrations throughout the gestation-lactation period (-165 to +60 days relative to partum) in high and low, 10 and 6 kgDM/100kgBW/d forage allowances (FA), respectively, of purebred (Hereford/Angus) and their crosses (F1-Angus/Hereford and Hereford/Angus) cows (n=16).

3.4.2 Global transcriptomic in liver

A total of 29,715 transcripts were detected as expressed in liver, of those, 4,784 differentially expressed genes from the three-way ANOVA test were found. The correlation between averaged gene expressions for all arrays was 0.92 ± 0.04 (standard deviation). The four biological replicates in each treatment also showed, as expected, high similarity between patterns of expression.

3.4.3 Temporal changes in hepatic gene expression

Out of the 4,784 differentially expressed genes, 4,663 changed through days (272 with at least a 2.5-fold difference in at least one time point comparison). A list of the fifty, out of the 272, most significant differentially expressed genes for this effect is shown in Table 2. A complete list of the 4,663 differentially expressed genes across time can be seen in **supp. mat. A**. Several patterns of gene expression over time were observed in the differentially expressed genes. Some of them decreased or increased their relative level of expression throughout the whole period (-165 to +60 days), while others changed their level of expression markedly at specific time points. Gene ontology analysis revealed 108 genes involved in *molecular functions* such as catalytic activity (38 genes, particularly hydrolase, oxidoreductase, and transferase activities), binding (89 genes, particularly protein binding) and signal transducer activities (9 genes). Eighty-nine genes were involved in *cellular components,* particularly intracellular (51 genes) and membrane (39 genes) components, and 93 in *biological processes* such as immune response (10 genes), metabolic (61 genes) and signalling processes (21 genes), and response to stimulus (22 genes).

In order to explore patterns of co-regulation, expression profiles of the 272 genes that were differentially expressed through days (2.5-fold change), were hierarchically clustered and visualized in a heat map (Figure 2). Large contiguous patches of colors representing roughly three groups of genes that share similar expression patterns could be identified: *group A*, containing genes lowly expressed from -165 to +15 days and strongly down-regulated at +60 days;

Table 2. Subset of fifty most significant, out of 272 transcripts, differentially expressed (FDR=0.1) across time with 2.5-fold difference or more in expression level in at least one time point.

			Fold change		
GeneName	Symbol	Raw p-value [1]	-165 *vs.* -15[2]	-15 *vs.* −15	−15 *vs.* −60
myomesin 1, 185kDa	MYOM1	2.72E-11	-3.946[3]	-1.479	-1.141
histidine ammonia-lyase	HAL	2.19E-10	-4.632	1.406	-1.119
dynein, cytoplasmic 1, intermediate chain 1	DYNC1I1	3.55E-10	-3.651	1.018	-1.379
cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	4.73E-10	3.059	-1.073	2.341
inhibin, beta A	INHBA	9.07E-10	5.083	-1.143	1.363
stromal cell-derived factor 2-like 1	SDF2L1	2.22E-09	3.621	-1.299	1.183
cyclin D1	CCND1	2.39E-09	-3.773	1.113	1.055
protein phosphatase 1, regulatory (inhibitor) subunit 3C	PPP1R3C	3.83E-09	4.803	-1.421	1.114
pyruvate dehydrogenase kinase, isozyme 4	PDK4	7.03E-09	-6.378	1.713	-1.110
Uncharacterized protein LOC285141 homolog	MGC152346	1.00E-08	-3.588	1.320	-1.183
adrenergic, beta-2-, receptor, surface	ADRB2	2.96E-08	-3.305	1.379	1.093
glucose-6-phosphatase, catalytic subunit	G6PC	4.88E-08	-3.193	1.639	1.196
poly(A) binding protein, cytoplasmic 1	PABPC1	5.39E-08	-1.341	1.175	3.771
claudin 15	CLDN15	6.29E-08	-3.802	1.035	-1.059
cysteine-rich with EGF-like domains 1	CRELD1	6.81E-08	-1.144	1.602	3.240
DnaJ (Hsp40) homolog, subfamily B, member 11	DNAJB11	7.30E-08	3.320	-1.147	1.384
similar to ubiquitin and ribosomal protein S27a	LOC100139732	9.21E-08	1.264	1.421	7.872
similar to Chromosome 14 open reading frame 73	LOC526547	1.40E-07	-3.377	1.110	-1.479
mesencephalic astrocyte-derived neurotrophic factor	MANF	1.47E-07	3.671	-1.315	1.300
STEAP family member 4	STEAP4	2.21E-07	4.724	-1.732	1.212
heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	HSPA5	4.00E-07	3.194	-1.212	1.791
fibrinogen beta chain	FGB	5.76E-07	2.142	1.056	13.358
chemokine (C-C motif) ligand 26	CCL26	1.10E-06	3.169	1.443	2.841
adenylate kinase 3-like 1	AK3L1	1.69E-06	5.362	-1.205	2.292

chemokine (C-C motif) ligand 24	CCL24	1.73E-06	5.499	1.369	1.116
nucleolar protein 10	NOL10	2.95E-06	2.082	1.678	4.381
similar to TAFA5	FAM19A5	5.60E-06	-3.075	1.364	1.094
reticulon 1	RTN1	8.64E-06	1.214	1.043	4.230
inhibitor of DNA binding 1, dominant (-)helix-loop-helix protein	ID1	1.06E-05	3.030	1.087	1.127
histidine decarboxylase	HDC	1.20E-05	-3.401	2.316	-1.261
similar to mKIAA1394 protein	LOC783525	1.20E-05	-3.272	1.198	-1.338
ring finger protein 125	RNF125	1.29E-05	5.942	-1.953	1.653
similar to hCG1647286	LOC617386	1.47E-05	1.301	1.329	3.184
cell division cycle 16 homolog (S. cerevisiae)	CDC16	1.79E-05	1.322	-1.001	3.085
similar to cholesterol 7alpha-hydroxylase	LOC510507	2.01E-05	-5.437	1.709	-1.395
phosphoenolpyruvate carboxykinase 1 (soluble)	PCK1	2.31E-05	-3.170	1.408	-1.063
solute carrier family 25, member 30	SLC25A30	2.557E-05	-3.487	1.567	-1.666
stearoyl-CoA desaturase (delta-9-desaturase)	SCD	4.637E-05	1.076	-2.233	4.230
S100 calcium binding protein G	S100G	5.374E-05	-7.948	1.096	-1.092
chemokine (C-X-C motif) ligand 16	CXCL16	6.878E-05	1.032	-1.140	3.457
ribosomal protein L13	RPL13	8.957E-05	1.305	1.103	4.653
malic enzyme 3, NADP(+)-dependent, mitochondrial	ME3	1.094E-04	1.142	-1.215	3.036
enolase-phosphatase 1	ENOPH1	1.592E-04	-1.304	1.028	3.121
p21 protein (Cdc42/Rac)-activated kinase 4	PAK4	4.503E-04	-1.175	1.413	3.086
chemokine (C-X-C motif) ligand 2	CXCL2	4.629E-04	3.059	-1.647	1.191
apolipoprotein A-IV	APOA4	4.978E-04	-3.150	1.930	-1.823
fibroblast growth factor 21	FGF21	2.030E-03	4.062	1.208	1.166
tumor necrosis factor (ligand) superfamily, member 9	TNFSF9	2.168E-03	-3.164	-1.263	-1.039

[1]Raw p-values from 3 way ANOVA analysis, transcripts were ordered according to their p-values. [2] -165, -15, +15 and +60 = days relative to parturition. [3] i.e. negative fold change means that the transcript is down-regulated at -165 days and up-regulated at -15 days.

group B, containing genes strongly up-regulated at -165 days and lowly expressed the rest of the period, and *group C* containing genes strongly down-regulated at -165 days, then some of them turned on at -15 days and remained highly expressed, while others decreased its expression to increase it again at +60 days, or remained lowly expressed the rest of the period. Even though groups of genes within the same group show very similar patterns of expression, in general, expression of individual genes can be distinguished indicating subtle differences in their regulation.

Some genes involved in translation and gene expression (*ribosomal protein L13 and S6,* RPL13, RSPS6; *eukaryotic translation initiation factor subunit J*, EIF3J; *poly (A) binding protein cytoplasmatic 1*, PABPC1) clustered together in *group A*. Genes involved in chemokine signalling pathway (*chemokine C-C motif ligand 24 and 26,* CCL26 and CCL24; *chemokine C-X-C motif ligand 2 and 16*, CXCL2 and CXCL16), cytokine receptor binding (*inhibin A and E*, INHBA, INHBE), regulation of cell cycle progression and growth (*cyclin-dependent kinase inhibitor 1A*, CDKN1A and *growth arrest and DNA-damage inducible β*, GADD45B), and heat shock proteins (*heat shock 70kDa protein 5, glucose-induced*, HSPA5 and *DnaJ-Hsp40 subfamily B member 11,* DNAJB11) were co-expressed and cluster together in *group B*. Finally, genes involved in glycolysis and gluconeogenesis (PCK1; *aldolase B fructose-biphosphate,* ALDOB; *glucose-6-phosphatase, catalytic subunit*, G6PC, *acyl-CoA synthetase short-chain family member 2,* ACSS2) and trasmembrane transport (*similar to sodium dependent glycine transporter 2*, SLC6A5; *nucleoporin 54 and 62 kDa*, NUP54 and 62) were clustered together in *group C*.

3.4.4. Forage allowance and genotype effect

Only one gene (*ATP-binding cassette, sub-family A*, ABCA4) changed due to forage allowance (down-regulated 1.6-fold in HI, compared to LO cows), but gene expression was not affected by genetic group. However, there was an interaction between forage allowance and genetic group, reflected in 148 differentially expressed genes (**supp. mat. B**). Out of the 148 genes, 74 presented more than 2-fold

change difference. Gene ontology analysis of the 74 differentially expressed genes identified 12 genes that play important roles in molecular functions and biological processes such as biosynthetic and catabolic processes, and cell signalling and communication (Table 3).

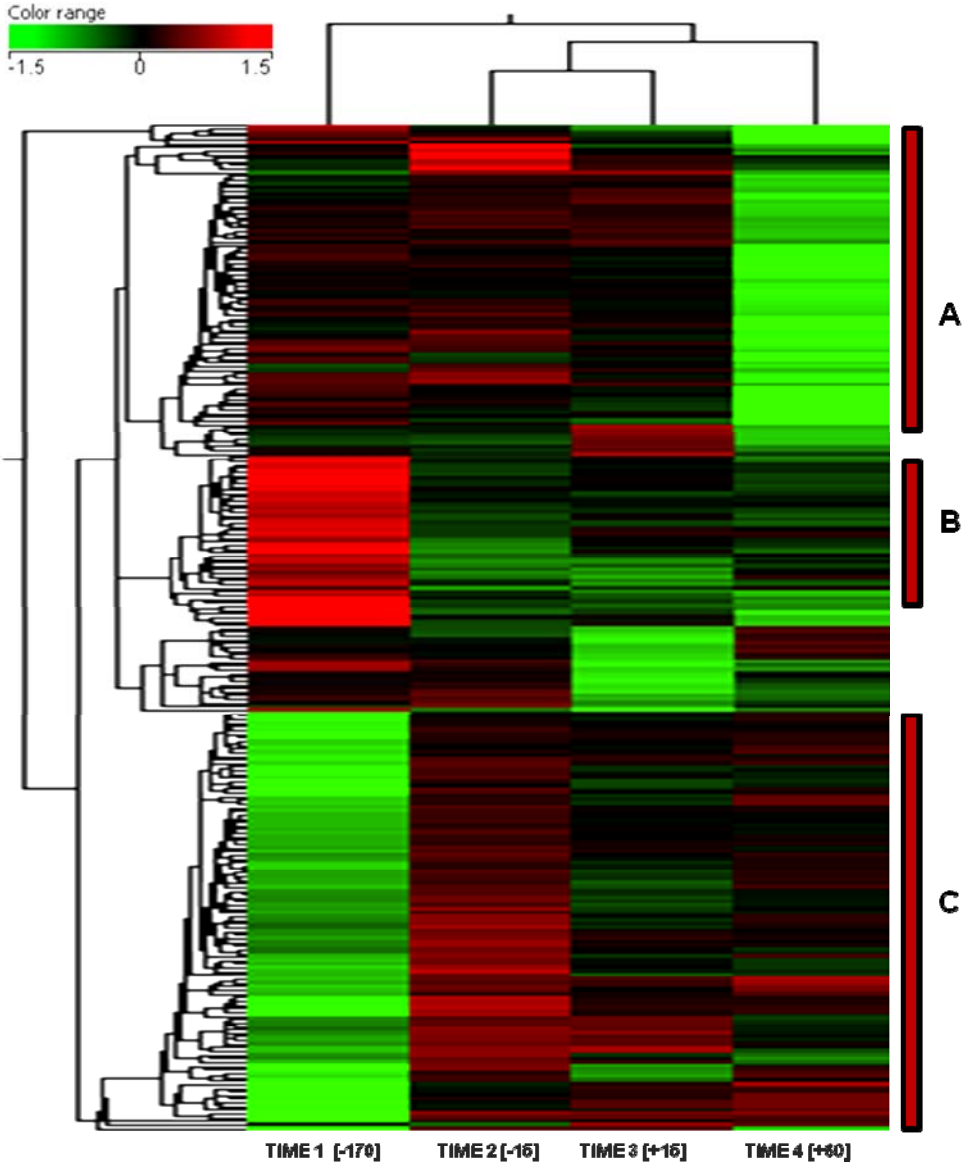

Figure 2. Expression levels of differentially expressed genes through time (-165, -15 ± 8 and +15, + 60 ± 3 days relative to partum) from 3 way ANOVA test (FDR=0.10) were hierarchically clustered and shown in a heat map. Level of expression is represented by color scale from green (low) to red (high) as indicated by a scale bar

in the upper left corner. Dendograms for distances are also shown for the genes (left) and samples (top).

Interestingly, 10 of those genes had the opposite pattern of expression when the different genetic groups were compared, in high or low forage allowance (CR-HI *vs.* PU-HI and CR-LO *vs.* PU-LO). These genes were up-regulated in HI and down-regulated in LO forage allowance for CR cows (compared to PU cows), and two genes behaved in the opposite way.

Table 3. Fold changes of twelve differentially expressed genes out of the 148 genes from 3 way ANOVA interaction between genetic group and forage allowance.

		Fold Change [1]			
Gene name	Symbol	HI-CR *vs.* HI-PU [2]	LO-CR *vs.* LO-PU	HI-CR *vs.* LO-CR	HI-PU *vs.* LO-PU
Aldolase C, fructose-bisphosphate	ALDOC	2.38	-1.65	2.39	-1.64
Amyloid P component, serum	APCS	-1.10	5.22	-1.14	5.06
Cadherin 2, type 1, N-cadherin (neuronal)	CDH2	5.31	-2.95	3.31	-4.73
Enolase-phosphatase 1	ENOPH1	2.77	-1.68	2.06	-2.27
Fatty acid synthase	FASN	2.34	-1.42	1.95	-1.71
Fermitin family homolog 3	FERMT3	2.20	-2.00	2.21	-1.99
Glycoprotein (transmembrane) nmb	GPNMB	-2.86	2.69	-4.70	1.64
Transforming growth factor beta regulator 1	TBRG1	2.22	-1.62	1.95	-1.84
Transforming growth factor beta 1 induced	TGFB1I1	4.17	-1.27	1.74	-3.04
PREDICTED: similar to KIAA1453 protein	USP36	2.36	-1.60	2.02	-1.88
Calcium channel, voltage-dependent	CACNA1A	4.20	-1.23	2.42	-2.14
Diacylglycerol lipase, beta	DAGLB	2.51	-1.82	2.36	-1.93

[1] Positive fold changes means that the transcript is up-regulated. [2] PU = purebred cows (Hereford/Angus), CR = crossbred cows (F1), LO = low forage allowance, 6 kgDM/100kgBW/d, HI = high forage allowance, 10 kgDM/100kgBW/d**.** [3]Negative fold change means that the transcript is down-regulated.

The t-test analysis showed 225 differentially expressed genes between HI and LO cows (**supp. mat. C**). Forty-seven out of the 225 differentially expressed genes showed a 2-fold change difference. Cluster analysis of these 47 genes identified patches of colours representing roughly two groups of genes that share similar expression patterns: *group A*, containing 28 genes strongly up-regulated in HI cows

and down-regulated in LO cows (including *enolase phosphatase*, ENOPH1 and *aldolase C*, ALDOC, involved in gluconeogenesis and glucose metabolism), and *group B,* containing 19 genes with the opposite pattern of expression, being down-regulated in HI and up-regulated in LO cows (Figure 3).

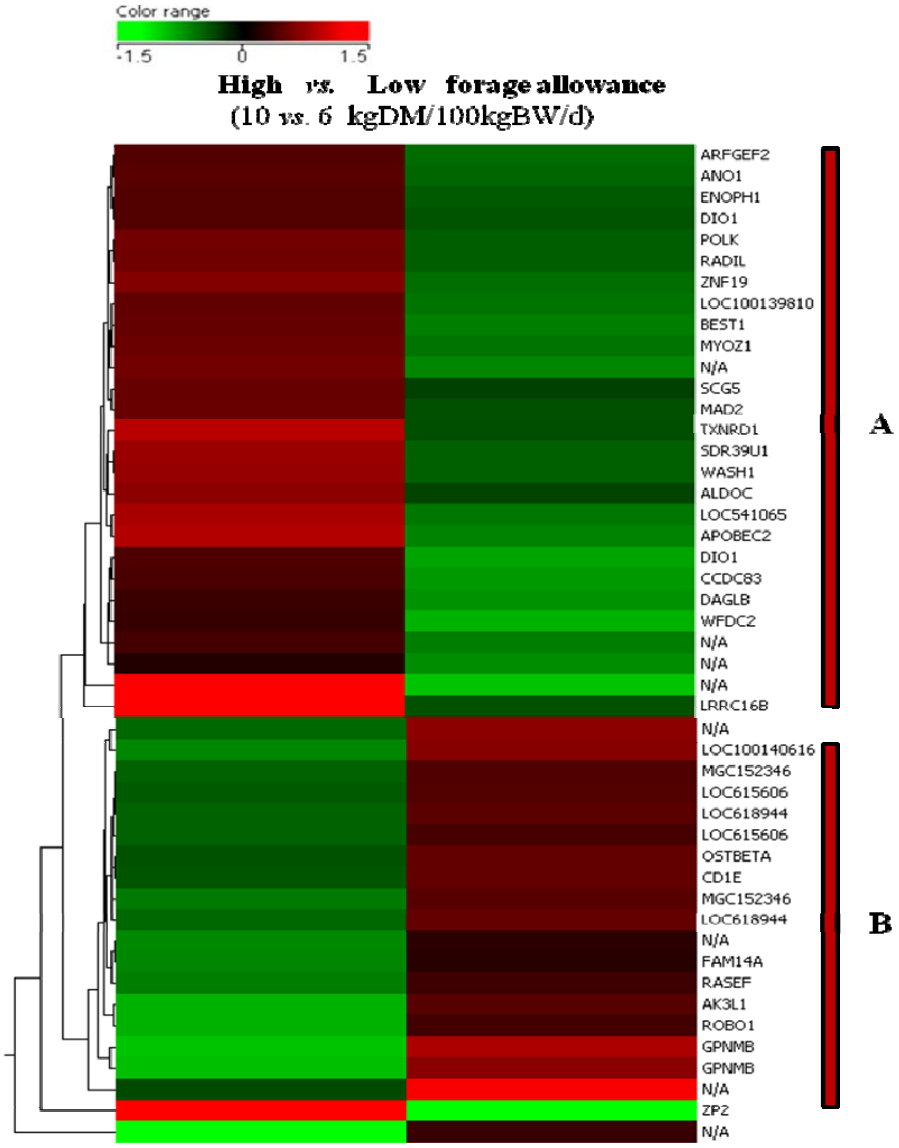

Figure 3. Differentially expressed genes from T-test (FDR=0.10) analysis between *high* and *low f*orage allowances (10 *vs.* 6 kgDM/100kgBW/d) of native pastures. Genes were hierarchically clustered and shown in a heat map. Level of expression is represented by color scale from green (low) to red (high) as indicated by a scale bar in the upper left corner. Dendograms for distances are also shown for the genes (left).

Only 2 genes were differentially expressed between CR and PU cows, (*fatness obesity associated gene*, FTO *and transmembrane protein 149*, TMEM149; 1.4 and 3.8-fold up-regulated in CR cows, respectively), therefore, no cluster was designed.

3.4.5 Pathway analysis

The previous analyses were complemented using the GSEA approach, to identify and characterize pathways that were up and down regulated between forage allowances (HI *vs.* LO), genetic groups (CR *vs.* PU), and throughout days (as cow physiological stage changed from pregnancy to lactation). Forty-eight highly significant pathways with positive and negative enrichment scores (ES) across days were identified. Interestingly, more than 80 % of the genes (mRNA) in the original gene set, for each one of the pathways identified in this study, were detected as expressed in the analyzed dataset (Table 4).

The greater differences in the GSEA analysis were identified during the gestation period (-165 *vs.* -15 days), on the contrary, little changes were observed during the peripartum (-15 *vs.* +15 days) or lactation (+15 *vs.* +60 days) periods. Eighteen pathways with negative and 28 with positive ES were identified for the -165 *vs.* -15 days comparison, meaning that the entire pathway either increased or decreased its expression, respectively. Only five and twelve pathways were positively enriched for the -15 *vs.* +15 and +15 *vs.* +60 days comparison, respectively (Table 4). On the other hand, three pathways (ES > 0.55) were identified between HI and LO cows, corresponding to gluconeogenesis, glucose and pyruvate metabolism (31/31, 54/56 and 15/18 genes detected as expressed in the analyzed dataset/total count in original gene set, respectively; Table 4). No significant pathways between CR and PU cows were found.

Table 4. Gene Set Enrichment Analysis (GSEA) results for the parameter time, FDR=0.25.

		-165 *vs.* -15		-15 *vs.* +15		+15 *vs.* +60		
GSEA pathway	**Size (n/m)**	**p-value**	**ES**	**p-value**	**ES**	**p-value**	**ES**	**database**
Glycolysis and Gluconeogenesis	52/62	0.002	-0.593	0.670	0.238	0.066	0.489	KEGG
Fatty acid metabolism	38/42	0.004	-0.716	0.606	0.284	0.661	0.252	KEGG
Steroid hormone biosynthesis	33/55	0.031	-0.573	0.259	0.463	0.463	0.328	KEGG
Glycine - Serine and Threonine metabolism	28/31	0.008	-0.617	0.054	0.549	0.616	0.308	KEGG
N-glycan biosynthesis	40/46	0.002	0.632	0.704	0.250	0.575	0.306	KEGG
Glycerolipid metabolism	38/49	0.022	-0.486	0.120	0.414	0.297	-0.343	KEGG
Propanoate metabolism	28/33	0.041	-0.635	0.735	0.278	0.556	0.351	KEGG
mRNA degradation	49/59	0.044	0.489	0.473	-0.276	0.928	0.199	KEGG
RNA polymerase	25/29	0.112	0.515	0.026	0.505	0.799	0.242	KEGG
Proteasome	41/48	0.020	0.635	0.135	-0.462	0.016	0.621	KEGG
PPAR signaling pathway	61/69	0.002	-0.633	0.111	0.364	0.026	0.425	KEGG
TGF Beta signaling pathway	63/86	0.012	0.405	0.819	0.197	0.206	0.292	KEGG
Maturity onset diabetes	15/25	0.029	0.594	0.375	0.373	0.346	-0.384	KEGG
Proteasome pathway	19/19	0.014	0.711	0.131	-0.546	0.091	0.583	Biocarta
IGF1-MTOR pathway	19/20	0.024	0.554	0.227	0.414	0.126	0.505	Biocarta
Aminoacid and oligopeptide SLC transporters	30/48	0.012	-0.470	0.006	0.594	0.393	-0.347	Reactome
Apoptosis	111/129	0.058	0.390	0.971	0.156	0.060	0.398	Reactome
Chaperon mediated protein folding	41/50	0.091	0.531	0.180	-0.397	0.400	0.379	Reactome
Cyclin E associated in G1 and S transition	53/58	0.012	0.602	0.314	-0.354	0.014	0.581	Reactome
Cytochrome P450 arranged by substrate type	26/49	0.019	-0.605	0.308	0.369	0.448	0.307	Reactome
Cytosolic TRNA aminoacylation	22/23	0.039	0.671	0.511	0.363	0.297	0.431	Reactome
Elongation and processing of capped transcripts	126/134	0.006	0.496	0.734	-0.199	0.563	0.269	Reactome
Gene expression	382/425	0.038	0.386	0.383	0.241	0.232	0.309	Reactome

M-G1 transition	54/61	0.039	0.517	0.809	-0.216	0.049	0.503	Reactome
Membrane trafficking	74/78	0.024	0.485	0.526	0.272	0.886	0.205	Reactome
Metabolism of lipids and lipoproteins	193/228	0.033	-0.432	0.128	0.347	0.135	0.360	Reactome
mRNA splicing	101/107	0.008	0.533	0.725	-0.205	0.586	0.262	Reactome
ORC1 removal from chromatine	57/63	0.044	0.514	0.541	-0.266	0.016	0.515	Reactome
P53 independent DNA damage response	40/43	0.018	0.648	0.303	-0.387	0.018	0.630	Reactome
Peptide ligand binding receptors	91/173	0.008	-0.447	0.008	0.455	0.234	-0.293	Reactome
Peroxisomal lipid metabolism	19/20	0.041	-0.651	0.376	0.404	0.992	0.151	Reactome
Processing of capped introns - pre mRNA	130/138	0.006	0.520	0.694	-0.209	0.377	0.301	Reactome
Pyruvate metabolism	15/18	0.010	-0.620	0.158	0.437	0.390	0.469	Reactome
Regulation of lipid metabolism by PPAR alfa	53/61	0.024	-0.453	0.094	0.326	0.130	0.394	Reactome
Rrgulation of ornithine decarboxylase	44/47	0.020	0.625	0.591	-0.289	0.033	0.602	Reactome
RNA polymerase II transcription	87/92	0.024	0.483	0.805	0.201	0.778	0.226	Reactome
RNA polymerase III transcription	28/34	0.022	0.538	0.556	0.280	0.949	-0.167	Reactome
RNA_Polymerase_III_transcrition iniciation	23/29	0.014	0.584	0.523	0.306	0.983	-0.151	Reactome
SCF beta TRCP mediated degradation of EMI1	46/48	0.042	0.564	0.346	-0.353	0.006	0.633	Reactome
Signaling by BMP	15/23	0.004	0.736	0.245	-0.454	0.503	-0.395	Reactome
Steroid metabolism	49/62	0.025	-0.585	0.487	0.319	0.016	0.589	Reactome
Synthesis of bile acids and salts	16/19	0.009	-0.767	0.176	0.483	0.852	0.252	Reactome
Synthesis of GPI anchored proteins	22/26	0.018	0.531	0.057	0.491	0.895	0.234	Reactome
Transcription	135/197	0.025	0.427	0.594	0.221	0.907	0.171	Reactome
VIF mediated degradation of APOBEC3G	44/47	0.010	0.636	0.424	-0.334	0.010	0.638	Reactome
Stabilization of P53	44/46	0.043	0.601	0.207	-0.404	0.020	0.591	Reactome
T-cell signal transduction	35/44	0.015	-0.512	0.107	0.448	0.610	0.251	Reactome
SIG_PIP3 signaling in B lymphocytes	26/33	0.016	-0.523	0.142	0.387	0.823	0.229	Reactome

[1] n = number of genes in the analyzed dataset; m = number of total genes in the original gene set. [2]T1, T2, T3 and T4 = -165, -15, +15 and +60 days relative to parturition, respectively. [3] Enrichment Score (ES).

3.4.6 Microarray validation by RT-PCR

The expression patterns for 10 of the ten examined genes detected by microarray analysis were very similar, in terms of both direction of fold-change and magnitude, when assessed by RT-PCR underscoring the validity and high degree of fidelity of our experimental procedures and data analysis. There were minor discrepancies in expression patterns only in 1 or 2 time points (Figure 4).

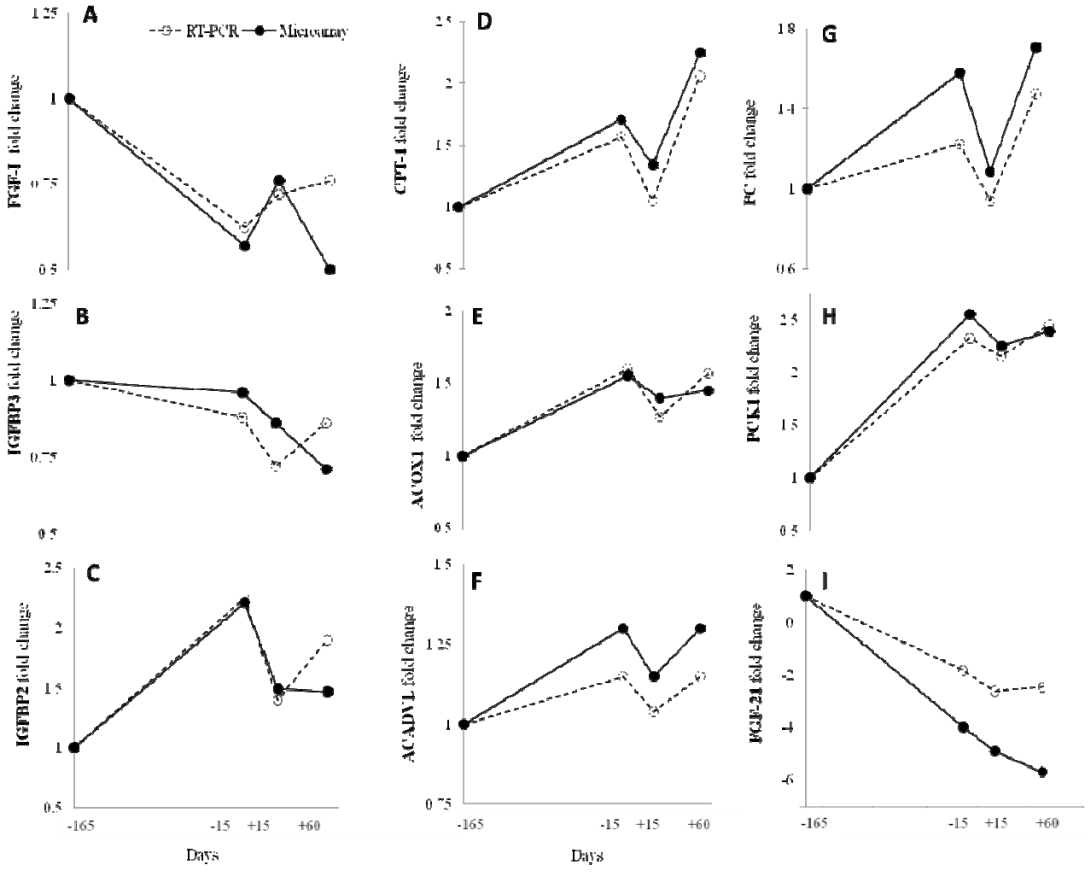

Figure 4. Comparison of expression patterns (fold change relative to -165 days) observed with microarrays *vs.* real time RT-PCR for 9 genes: *insulin-like growth factor-I* (IGF-I, A), *IGF binding protein 3* (IGFBP3, B) and 2 (IGFBP2, C), *carnitin palymityil transferase–I* (CPT-1, D)*, acyl-CoA oxidase 1, palmitoyl* (ACOX1, E), *acyl-CoA dehydrogenase,very long chain* (ACADVL, F), *piruvate carboxylase* (PC, G), *pyruvate carboxykinase-I* (PCK1, H), and *fibroblast growth factor-21* (FGF-21, I). Microarray and real-time RT-PCR mRNA expression are indicated in full line and black circles, and dot lines and empty circles, respectively.

The exception to microarray confirmation by RT-PCR was *GHR,* since it decreased it level of expression by microarrays but remained stable when assessed by RT-PCR (data not shown). Nonetheless, different sensitivity and bias were expected for these different quantification methods. Moreover, the housekeeping genes HPRT, and ACTB mRNA expression used as endogenous controls in RT-PCR procedure, were also stable through time by microarrays. Gene expression pattern of the genes evaluated using a different source of RNA can be seen in **supp. mat. D**.

3.5 DISCUSSION

To our knowledge, this is the first time-course microarray experiment aimed at studying liver molecular control of energy balance through the gestation-lactation cycle in beef cows in rangeland conditions at a genomic level. In agreement with other authors (Quintans *et al.*, 2010), beef cows in rangeland conditions experienced some degree of NEB, particularly during winter-gestation (here, -165 to -15 days), as the increased gestation energy requirements, were accompanied by a decreased forage availability and dry-matter intake in winter (Berretta, 2000; Chapman *et al.*, 2007). This was reflected in the 0.5 units loss of BCS, increased NEFA concentrations and decreased serum insulin concentrations during winter without a recovering (except for serum NEFA) of initial values of end of fall (-165 days) during the lactation period. As expected, metabolic status (BCS and IGF-I) was better in HI than LO cows. A more detailed description of metabolic and endocrine parameters during the gestation-lactation period was reported by Laporta *et al.* (2011).

The factor that contributed most to altered hepatic gene expression during the gestation-lactation cycle of beef cows in rangeland conditions was time (days effect), evidenced by more than 4000 differentially expressed genes. This was probably associated to the dramatic changes that occur in quantity and quality of native pastures throughout the year (energy intake) and cow physiological stage, as they transit from pregnancy (autumn-winter) to calving (spring) and lactation (spring-summer). Various authors (Meikle *et al.*, 2004; Loor *et al.*, 2005; McCarthy *et al.*,

2010) had described and characterized the metabolic, hormonal and molecular changes in high-yielding dairy cows during the peripartum (transition from late pregnancy to early lactation), and showed that the greatest changes occur typically during this period, as cows experience a short but severe NEB, since energy demands for milk production exceed energy intake. Our findings in beef cows revealed that liver metabolic upload, is not essentially during this period (-15 to +15 days), since no drastic changes were observed, neither in terms of differentially expressed genes, nor in metabolic pathways. On the contrary, the greatest liver activity (differential genes and metabolic pathways) in our study was observed during the winter-gestation period (-165 to -15 days) characterizing an adaptation of beef cow hepatic metabolism to moderate nutrient restriction during an extended period of time. In addition, the early lactation period (+15 and +60 days), as peak milk yield approaches, did not imply a metabolic challenge to the beef cow probably associated to the low milk production (Laporta *et al.* 2011).

Using the GSEA approach to evaluate microarray data at the level of gene sets, groups of genes that share common biological function (Subramanian *et al.*, 2005), we identified various pathways involved in intermediate metabolism (fatty acid and glucose metabolism) and cell turnover (proliferation, growth and apoptosis) that were up-regulated and down-regulated during winter-gestation period, respectively. We will focus our attention on these pathways and will discuss about some differentially expressed genes through time that are part of these metabolic pathways.

3.5.1 Hepatic gene expression related to intermediate metabolism

Glucose is a primary energy source for certain animal tissues and a precursor for lactose synthesis in the mammary gland. The liver has been estimated to supply up to 90% of glucose requirements in ruminants through hepatic gluconeogenesis (Nafikov *et al.*, 2007) to support glucose metabolism for the fetus and lactose synthesis (Bell, 1995; Velez and Donkin, 2004). Oxidative metabolism of glucose in the mitochondria is regulated by *pyruvate dehydrogenase* (PDH). *Pyruvate*

dehydrogenase kinase isozyme 4 (PDK4) protein inactivates the PDH complex by phosphorylating one of its subunits, reducing the conversion of pyruvate, produced from the oxidation of glucose and amino acids, to acetyl-CoA contributing to the regulation of glucose metabolism. In this study, *PDK4* mRNA expression increased 6-fold during winter-gestation period, suggesting that greater PDK4, if translated to more protein, would contribute, by decreasing the conversion to acetyl-CoA, to conserve three-carbon compounds (lactate, alanine, and pyruvate) that serve as primary substrates for gluconeogenesis (Harris *et al.*, 2002). Moreover, and in accordance with our results, Kwon and Harris (2004) reported that expression of this gene was negatively regulated by insulin and feed intake. Also, PDK4 is thought to act as a marker of lipid status (through acetyl-CoA production; Holness and Sugden, 2003), thus, regulation of PDH activity is therefore important for both glucose and lipid metabolism.

Hepatic mRNA expression of rate-limiting gluconeogenic enzymes, PCK1 (also known as PEPCKc-cytosolic isoform), *glucose-6-phosphatase, catalytic* (G6PC) and PC showed a concerted up-regulation (3-fold for *PCK1* and *G6PC*, and 1.6-fold for *PC* mRNA) during winter-gestation. Up-regulation of gluconeogenic pathways, greater *PC*, *PCK1*, and *G6PC* mRNA, could enhance gluconeogenic capacity during periods of NEB in beef cattle associated with increased glucose demands and the need to maintain blood glucose concentrations (Greenfield *et al.*, 2000; Bradford and Allen, 2005; Velez and Donkin, 2005; Loor *et al.*, 2006). In this study, the decreased influx of propionate during winter, as energy intake decreased with herbage mass availability (Chapman *et al.,* 2007) and resulting hypoinsulinemia, may explain the raise in the mRNA expression of these enzymes (Drackley *et al.*, 2001). In addition, expression of *PC* mRNA is part of the adaptive response to feed intake restriction and it has been shown that increased with NEFA concentrations (White *et al.*, 2011). The activity of PC increases the synthesis of oxaloacetate from pyruvate during feed restriction and since oxaloacetate deficiency is likely to be limiting the citric acid (TCA) cycle in periods of NEB, the activation of PC may provide an alternative mechanism of oxaloacetate production for energy

generation, particularly NEFA oxidation in the TCA cycle (Velez and Donkin, 2005; McCarthy *et al.*, 2010). These results are therefore consistent with an environment of reduced glucose availability coupled with raised of fatty acid mobilization and oxidation in the liver.

A prominent feature of the energy/metabolic response to the NEB state involves reliance on fatty acids as a source of energy (McCarthy *et al.,* 2010). In this study, various pathways related with lipid and fatty acid metabolism were identified as differentially regulated through days. Reduced insulin concentrations, as described here during winter-gestation, stimulates the hormone-sensitive lipase in adipose tissue increasing the rate of lipolysis, and therefore increasing the influx of NEFA for hepatic mitochondrial β-oxidation. Increased transcription of hepatic key enzymes involved in this process has been reported by several authors in dairy cows in NEB (Loor *et al.*, 2005; 2006; 2007; van Dorland *et al.*, 2009; McCarthy *et al.*, 2010).

The mitochondria ß-oxidation of long-chain fatty acids is regulated by CPT enzyme system that is involved in transferring fatty acids from the cytosol to the mitochondrial matrix (Britton *et al.*, 1995). In this study, the hepatic mRNA expression of *CPT1* isoform A (*CPT1-A*, liver-specific) was represented on the array but not detected as differentially expressed through time. Hepatic *CPT1-A* mRNA has increased (Loor *et al.*, 2005) or no changed (Selberg *et al.*, 2005; van Dorland *et al.*, 2009) during early lactation NEB of dairy cows. Surprisingly, two other isoforms *CPT1-B* (muscle-specific) and *CPT1-C* (brain-specific), increased by 1.7 and 2-fold during winter-gestation, respectively. The hepatic expression of *CPT1-B* mRNA has been previously reported by McCarthy *et al.* (2010) that in agreement with our results, showed an increased expression in dairy cows in severe negative energy balance during early lactation. However, this is the first study that reported *CPT1-C* mRNA expression in the cattle liver. These results would indicate that hepatic metabolism adaptation to fatty acid oxidation in response to chronic nutrient restriction would involve up-regulation of *CPT1B* and *CPT1C* mRNA isoforms, being their transcriptional regulation at least partially independent of transcription of *CPT1A*. There was also a coordinated increased mRNA expression of other genes

involved in mitochondria fatty acid ß-oxidation. We identified increased expression of *acyl-CoA deshydrogenase* (*ACAD*), transcripts which enzyme catalyzes the initial step in each cycle of fatty acid β-oxidation in the mitochondria. Thus, we report the expression not only of the transcript involved in very long-chain fatty acid oxidation (*ACADVL*, with 1.3-fold increase) as reported by Loor *et al.* (2005; 2006) and McCarthy *et al.* (2010) during the transition period in dairy cows, but also of the transcripts of enzymes involved in medium- (*C-4 to C-12 straight chain, ACADM*, 1.6-fold increase), and short- (*C-2 to C-3 short chain, ACADS*, 1.6-fold increase) chain fatty acid oxidation.

Moreover, when cytosolic fatty acids accumulate due to impairment of oxidative capacity in mitochondria, alternative pathways in the peroxisomes (β-oxidation) and in microsomes (ω-oxidation) are activated (Kohjima *et al.*, 2007). The hepatic expression of *ACOX1* and *acyl-Coenzyme A oxidase 2, branched chain* (*ACOX2*) mRNA increased 1.5 and 2-fold, respectively during the winter-gestation period, decreasing or remained stable the rest of the period. In addition to this, the *enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase (EHHADH*), that catalyzes the second and third reactions of the peroxisome β-oxidation cycle, increased 2.8-fold during winter-gestation. The increase in these enzymes that participate in peroxisomal β-oxidation of very long-chain and branched-chain fatty acids agreed with the important role of this metabolic pathway in ruminants in NEB (Grum *et al.*, 1994; 1996). In addition, long-chain and very-long-chain fatty acids are also metabolized by the cytochrome P450 CYP4A ω-oxidation system to dicarboxylic acids to serve as substrates for β-oxidation (Reddy and Hashimoto, 2001; Kohjima *et al.*, 2007). In this study, the *cytochrome P450, family 4, subfamily A, polypeptide 11* (*CYP4A11*) increased 2.7-fold in winter-gestation. These mRNA expression data of mitochondrial and peroxisomal, and also microsomal, fatty acid oxidation enzymes point out a orchestrated action at three levels of regulation with the ultimate goal of allowing hepatocytes to cope with the increased influx of NEFA during winter-gestation in rangeland beef cows.

During high rates of fatty acid oxidation, the liver generates large amounts of acetyl-CoA that exceed the capacity of the TCA cycle, resulting in the synthesis of ketone bodies (β-hydroxybutyrate and acetoacetate) in the mitochondria, which are then oxidized in other tissues to produce energy. The mRNA expression of *3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2 (HMGCS2*), key enzyme of this process (Voet and Voet, 2004), increased 2.5-fold during winter-gestation, showing that cows also relied in ketone bodies as source of energy during this period. van Dorland *et al.* (2009) did not find changes in *HMGCS2* mRNA during the NEB of peripartum and early lactation in dairy cows but Hegardt (1999) observed that HMGCS2 activity is increased by fasting in rats.

In addition to changes in mRNA expression of key enzymes of the intermediate metabolism, various transporters solute carrier (SLC) were expressed in liver and *amino acid and polypeptide SLC transporters* pathway increased its expression significantly during winter-gestation. While it is not well characterized in ruminants, the SLC superfamily encodes membrane-bound transporters, comprises 55 gene families having at least 362 putatively functional protein-coding genes in humans (He *et al*., 2009). The gene products include passive transporters, symporters and antiporters, located in all cellular and organelle membranes. Particulatly, when we explore differentially expressed genes through time, we found: fatty acid (*SLC27A1, A2*; Stahl, 2004), glucose (*SLC2A6, A8, SLC5A8*; Zhao and Keating, 2007), amino acid (*SLC38A10, SLC43A2*; Sundberg *et al.*, 2008), and monocarboxilate (*SLC16A1, A5, A8, and A11*; Meredith and Christian, 2008) transporters, most of them involved in the partitioning of energy supply in several tissues. All these transcripts that are involved in the influx and efflux of many vital substrates increased their expression from -165 to -15 days, thus suggesting not only a greater metabolism (as described above) but also greater activity in fatty acid, glucose, and amino acid transport into the cell and mitochondria during this period.

3.5.2. Hepatic gene expression of peroxisome proliferator-activated receptors (PPARs) and FGF-21

Hepatic fatty acid oxidation is regulated by the nuclear receptor PPARα (Hu, *et al.*, 2005) that is activated by long-chain fatty acids or their derivatives. In addition, recent work (Im *et al.*, 2011) has shown that this nuclear receptor can also up-regulate transcription of gluconeogeneic enzymes (i.e *GP6C*). Although various known PPARα target genes (*ACADVL*, *ACOX1*, *CP4A11*, liver-specific adiponectin receptor *ADIPOR2*, and angiopoietin-like 4 *ANGPTL4*) were up-regulated in winter-gestation we did not find *PPARα* mRNA as differentially expressed across days. Reports on hepatic *PPARα* mRNA expression during NEB in the periparturient dairy cow have been contradictory, showing up-regulation (Loor *et al.*, 2005), or no changes (van Dorland *et al.*, 2009; Carriquiry *et al.*, 2009). However, as discussed by Loor (2010) activation of this nuclear receptor does not always alter *PPARα* mRNA. In addition, we did not find any other PPAR isoforms, *PPARδ* or *PPARγ* mRNA differentially expressed across days, although they have been involved in hepatic energy substrate homeostasis (Sanderson *et al.*, 2009; Liu *et al.*, 2011; Rogue *et al.*, 2011) or in ketosis development in dairy cows (Loor *et al.*, 2007). However, our results showed PPARγ *coactivator 1 alpha* (*PGC-1α*) expression increased winter-gestation. The PGC-1α, coactivates both PPARα and PPARγ and is involved in several aspects of the hepatic fasting response activating the entire program of gluconeogenesis and lipid oxidation (Rhee *et al.*, 2003; Estall *et al.*, 2009) and Loor *et al.* (2007) determined up-regulation of *PGC-1α* mRNA expression in nutritional-induced ketosis in dairy cows during the early postpartum. Recent studies in mice showed that expression of *PGC-1α* regulated hepatic expression of *FGF-21* mRNA (Estall *et al.*, 2009), a novel member of the FGF family that is preferentially expressed in the liver (Nishimura *et al.*, 2000).

The FGF-21 is a hormone induced during fasting and starvation, in a PPAR-α dependent manner, that play important endocrine roles in the regulation of lipid and glucose metabolism (stimulates lipolysis from adipose tissue during feeding but

inhibits it during fasting, favor glucose uptake in peripheral tissues and stimulate hepatic ketogenesis; Badman *et al.*, 2007; Inagaki *et al.*, 2007). In this study, *FGF-21* mRNA showed a 4-fold decreased during winter-gestation and remained low during the lactation period. Contrary to our findings, Carriquiry *et al.* (2009) reported that *FGF-21* mRNA increased 4-fold after calving in dairy cows. Indeed, other studies reported that hepatic *FGF-21* mRNA increased when mice are in NEB (Inagaki *et al.*, 2007; Badman *et al.*, 2007). However, Estall *et al.* (2009) reported that PGC-1α negatively regulates hepatic *FGF-21* mRNA expression by modulating the intracellular heme concentrations through mRNA expression of the rate-limiting enzyme in heme biosynthesis, delta-aminolevulinate synthase-1 (*ALAS-1*). In agreement with their results, we found the decrease in hepatic *FGF-21* mRNA was associated with 1.7–fold increase in both *PGC-1α* and *ALAS-1* mRNA. Estall *et al.* (2009) emphasized the difference between the liver metabolic demands during fasting *vs.* starvation and discussed that while PGC-1α function is essential during a physiological need to boost hepatic metabolism (to promote glycogenolysis, gluconeogenesis, and fatty acid oxidation) in fasting (Spiegelman and Heinrich, 2004), *FGF-21* mRNA expression and circulating concentrations increased only after long-term fasting (starvation) when it is important for the regulation of adipose tissue catabolism and torpor (Inagaki *et al.*, 2007; Badman *et al.*, 2007), a state of decreased activity and metabolism.

Thus, under the conditions of this study, if translated to protein, the increased *PGC-1α* mRNA expression would promote glycogenolysis, gluconeogenesis, and fatty acid oxidation and would regulate negatively *FGF-21* mRNA expression that would stimulate lipolysis and decreased glucose uptake from adipose tissue. This mechanism could reflect adaptation of rangeland beef cows in winter-gestation to a moderate chronic NEB, while acute NEB of dairy cows in early lactation and prolonged fasted experimental mice, are in a more profound starvation/energy-sparing state. In addition, although it has been suggested that FGF-21 plays an important role in hepatic ketogenesis (Inagaki *et al.*, 2007; Badman *et al.*, 2007), but in agreement with Hotta *et al.* (2009), it seems not to be required for ketogenesis as

in the present study decreased *FGF-21* mRNA was associated with increased *HMGCS1* mRNA (see above).

3.5.3. Hepatic gene expression related with cell, growth, proliferation, and apoptosis

Cell growth and proliferation requires an intricate coordination between stimulatory signals, arising from nutrients and growth factors, and inhibitory signals arising from intracellular and extracellular stresses. The mTOR pathway responds to various signals, including nutrients (glucose and amino acids), energy (ATP and AMP), and growth factors, and is co-regulated by IGF-1/AKT pathway to ensure both a reasonable level of nutrients and a positive signal for cell growth and division, and protein synthesis (Feng, 2010). The IGF-1/mTOR pathway was down-regulated during winter-gestation period (-165 to -15 days), in accordance with decreased insulin and glucose concentrations during this period. Particularly, it has been observed that mTOR pathway regulates protein synthesis through modulating mRNA transcription, ribosomal biosynthesis, and translational control. In the present study, with down-regulation of IGF-1/mTOR pathway several pathways involved in transcription and translation were down-regulated during winter gestation (i.e. RNA polymerase, mRNA degradation, processing of capped introns-pre mRNA, chaperone-mediated protein folding). Also, we identified various down-regulated eukaryotic translation factors (EIFs, *EIF2B5*, *EIF2S2* and *EIF4E*) included in IGF/mTOR pathway.

According to Feng (2010), nutritional stress not only signals to shut down mTOR, but also results in the transient phosphorylation (activation) of the p53 protein that negatively regulate cell growth and division in response to stress (Stewart and Pietenpol, 2001; Lavin and Gueven, 2006; Feng 2010) and inhibits mTOR/IGF-I pathways. Contrary to this, *p53 stabilization pathway* was also down-regulated, indicating that p53 action would seem to be inactivated. Thus, p53 and mTOR/IGF-I cross-talk seems to be disabled in winter-gestation in rangeland beef cows. Decreased p53 stabilization is consistent with decreased pathways related with

cycle arrest (cyclin E G1/S transition of the cell cycle, M/G1 transition) and apoptosis (Stewart and Pietenpol, 2001; Lavin and Gueven, 2006), increasing cellular proliferative capacity by moving forward the passage of the cells through the cell cycle and by preventing apoptosis or senescence (Lavin and Gueven, 2006). During pregnancy, enhanced hepatic metabolism is essential to accommodate the increased demand for energy from the developing fetus and the detoxification of fetal metabolites and increased liver mass has been reported in rodents (Hollister *et al.*, 1987; Milona *et al.*, 2010) and in ruminants (Scheaffer *et al.*, 2001; Scheaffer *et al.*, 2004; Meyer *et al.*, 2010). The molecular mechanisms that explains liver enlargement during pregnancy has not been completely elucidated, and it has been proposed that would depend on hypertrophy (Hollister *et al.*, 1987; Milona *et al.*, 2010) and hyperplasia (Milona *et al.*, 2010). Gielchinsky *et al.* (2010) working in the effect of pregnancy in the regenerative capacity of the aged liver in mice, showed that pregnancy switch from proliferation-based liver regeneration (with up-regulation of p-53) to a regenerative process mediated by cell growth and determined that a key mediator of this switch was the Akt/mTORC pathway; its inhibition blocked hypertrophy, while increasing proliferation.

In the context of these results, the IGF-I /mTOR pathway inhibition, due to the nutritional stress during winter-gestation in rangeland beef cows, would determine increased hyperplasia but decreased hypertrophy of the liver. Hence, this coordinated expression pattern of down-regulation is likely to have a negative impact on tissue growth and repair mechanisms during pregnancy period. McCarthy *et al.* (2010) also reported down-regulation of genes that provide evidence that cell growth and proliferation are compromised in severe NEB dairy cows, and it has been reported that liver growth is reduced during pregnancy in nutrient-restricted when compared with adequate-fed beef cows (Meyer *et al.*, 2010). Interestingly, during the lactation period (+15 to +60 days), cell proliferation, through down-regulation of p-53 stabilization, cyclin E G1/S transition of the cell cycle, M/G1 transition, p-53 independent DNA damage response pathways, were also increased. However, during this period the IGF-I/mTOR pathway was not down-regulated, suggesting an

increased liver mass during lactation (reported previously in dairy cows; Reynolds *et al.*, 2004), when dry-matter intake increased as herbage mass of native pastures increased in spring and summer (Berreta *et al.*, 2000; Chapman *et al.*, 2007) in response to hepatocyte hypertrophy.

In addition, in the present study, the transforming growth factor- β (TGF-β) signaling pathway was also down-regulated during winter-gestation but not during the lactation period. The TGF-β that involves in both the negative and positive regulations of cell proliferation, differentiation, immune responses, and apoptotic processes (Chen *et al.*, 2003). Particularly, we found down-regulation of the ligands *inhibins* (*INHBA* and *INHBE*, 3-fold down-regulated), and *bone morphogenetic proteins* (BMPs, here *BMP7*) as well as of the *SMAD proteins* (family member 1, 4 and 9) that mediate TGF-β signaling pathway.

3.5.4 Effects of forage allowance of native pastures and genetic group on hepatic gene expression

Differences in forage allowances of native pastures determine changes in herbage mass, height, and quality as well as in plant growth rate that can affect cow energy intake (Chapman *et al.*, 2007), maintenance energy requirements (Brosh, 2007) and grazing behavior (Provenza, 2003). We found *ABCA4* mRNA expression down-regulated in HI, compared to LO cows. The ABCA4 is a member of the ATP-binding cassette transporter gene sub-family A (ABC1) found exclusively in multicellular eukaryotes, which function is the facilitated transport of retinoid compounds and although highly expressed in retina (Tsybovsky *et al.*, 2010). This transcript has been identified in liver cells (Ye *et al.*, 2008) and it has been reported that its expression is up-regulated in the liver in *ABCC6-/-* knockout mice which phenotype is the metabolic syndrome PXE (Li and Uitto, 2011). Although retinoids are involved in lipid metabolism (Bonet *et al.*, 2011), we could not find an explanation between the differential expression of this transcript between cows grazing different forage allowances. In addition, cluster and GSEA analyses, identified *ALDOC* and *ENOPH1* mRNA (2-fold increase) and intermediate

metabolism pathways related with gluconeogenesis, glucose and pyruvate metabolism up-regulated in HI than LO cows, probably associated with greater dry-matter intake and therefore availability of precursors as well as metabolic activity in these cows. In addition, there was more than 2.5-fold increase in *FASN* and *DAGLB* mRNA, which encode for enzymes of *de novo* fatty acid synthesis (*FASN*) and hydrolysis of diacylglycerol (*DAGLB*). However, the biological meaning of this up-regulation is not clear. Moreover, these four genes (*ALDOC*, *ENOPHI*, *FASN*, *DAGLB*) were among the 12 genes identified with the Gene Ontology analysis of the differentially expressed genes of the interaction between forage allowance of native pasture and cow genetic group, being more than 2-fold up-regulated in HI-CR than HI-PU cows.

Little changes were observed in hepatic gene expression between the cow genetic groups, suggesting that phenotypic differences during the gestation-lacation cycle of beef cows (greater milk production and shorter postpartum anestrus, Laporta *et al.*, 2011) are not largely associated to transcription differences. Only two genes were differentially expressed among CR and PU cows. *Transmembrane protein 149* (*TMEM149*) and *fat mass and obesity associated* (*FTO*). Poritsanos *et al.* (2010) reported that fasting increased *FTO* mRNA in mice liver and suggested that this transcript may participate in the feedback regulation of glucose metabolism via gluconeogenesis. The greater mRNA expression of *FTO* in CR than PU cows could be associated or explain metabolic differences and mechanisms of adaptation to periods of NEB between genotypes, since greater mRNA expression of gluconeogenic enzymes (PC and PCK1) between CR and PU cows reported by Laporta *et al.* (2011). Nevertheless, the expression and function of these genes in beef cattle liver requires further analysis

3.6 CONCLUSION

In conclusion, beef cows in rangeland conditions experience a NEB status, particularly during winter-gestation. The application of the present array system to beef cow liver has revealed that large changes in the expression patterns of genes

were taking place particularly during this period. We found increased expression of genes associated with lipid catabolism and glucose synthesis, and to a negative or inhibitory impact on cell growth and repair. Also, we demonstrate that higher forage allowances of native pastures enhance hepatic expression, particularly of genes associated with energy and glucose metabolism. This information will contribute to our knowledge of the biology of energy balance in beef cattle, and might even highlight genes and entire metabolic pathways that can be targeted for future studies.

3.7 ACKNOWLEDGMENTS

The authors thank Lic. P. Zorrilla and G. Greif from Institut Pasteur-Montevideo for their assistance in the microarray assay. This study was supported by research project INIA-FPTA # 262 awarded to M. Carriquiry.

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4. DISCUSIÓN Y CONCLUSIONES

4.1 DISCUSIÓN GENERAL

Considerando ambos abordajes de esta tesis, en vacas de carne en sistemas de producción basados en el pastoreo de campo nativo, los cambios en los perfiles metabólico-endócrino-moleculares estuvieron fuertemente modulados por la oferta de forraje y en su mayoría por el estado fisiológico (gestante/lactante) de las vacas a lo largo del año.

4.1.1 **Efecto del tiempo**-Balance energético negativo durante la gestación invernal

En el primer abordaje, los mayores cambios en la dinámica de la CC y los perfiles endocrino-metabólicos, se dieron durante la última mitad de la gestación durante el invierno (-165 a -45 días, correspondiendo a los meses de Mayo a Setiembre). Todos los animales, independientemente del tratamiento, perdieron CC y movilizaron reservas corporales, reflejado en el aumento de las concentraciones sanguíneas de AGNE, indicando que las condiciones climáticas de los meses de invierno no permitieron una producción de forraje suficiente en cantidad y/o calidad (Berretta *et al.,* 2000), para evitar esa caída de CC, aún en las vacas que pastorearon alta oferta de forraje. El aumento de AGNE se acompañó de una disminución marcada de las concentraciones plasmáticas de glucosa, y séricas de insulina (Rhoads *et al.,* 2004**;** Rukkwamushk *et al.*, 2006). Esto podría explicarse por una disminución del consumo de materia seca debido a la reducida disponibilidad y altura de forraje de las pasturas nativas durante el invierno (Chapman *et al.,* 2007), al aumento de los requerimientos de energía de pastoreo (Brosh, 2007), sumado a los altos requerimientos (fetal y mantenimiento de tejidos no uterinos) que establece el último tercio de la gestación (Bell, 1995**;** Houghton *et al.,* 1990). Estos patrones metabólicos y hormonales reflejan un claro período de BEN el cual, a diferencia del BEN de la vaca de leche durante el periparto y lactación temprana que se caracteriza por ser severo, pronunciado y concentrado en el tiempo, en la vaca de carne en condiciones de pastoreo, se establece durante los

últimos meses de gestación invernal siendo mas moderado pero prolongado y sostenido en el tiempo.

Las bajas concentraciones de insulina durante el invierno favorecieron la gluconeogénesis hepática (Drackley *et al.,* 2001) y la movilización de reservas del tejido adiposo y su posterior oxidación a nivel hepático (Bauman, 2000), lo que se vió reflejado en el aumento de la expresión hepática de transcriptos que codifican enzimas claves en la oxidación de acidos grasos (*CPT-1, ACOX, y ACADVL*) y gluconeogénesis (*PC* y *PCK1*) ampliamente reportado por varios autores en vacas de leche en el periparto y/o lactancia temprana (Loor *et al.,* 2005 y 2006; van Dorland *et al.,* 2009 y 2011, McCarty *et al.,* 2010). Además, la correlación positiva entre la expresión de estos transcriptos refleja la regulación coordinada de estas dos vías metabólicas (Gonzalez-Manchon *et al.,* 1989) durante el período de subnutrición-invernal en vacas de carne en condiciones pastoriles. En este estudio, demostramos que en vacas de carne el desacople del eje somatotrófico (GH-IGF), típicamente descrito en vacas de leche en el periparto (Meikle *et al.,* 2004; Rhoads *et al.*, 2004), solo se observó en vacas que pastoreaban alta oferta de forraje (ver discusión abajo) y los cambios más notorios en sus componentes, medidos en el primer abordaje, tanto a nivel hormonal (niveles séricos de IGF-I) y transcripcional (ARNm de GHR, GHR1A, IGF-I, IGFBP-2 y -3) se observaron también durante la gestación invernal.

De manera consitente con los resultados obtenidos en el primer abordaje, en el segundo abordaje de genómica funcional, el efecto que más influyó sobre la expresión génica hepática fue el tiempo, reflejándse en más de 4000 transcriptos diferencialmente expresados y más de 60 vías metabolicas, que se encienderon y/o se apagaron, como consecuencia del estado fisiológico de las vacas/cambios disponibilidad y altura de forraje a lo largo del ciclo gestación-lactación. Este abordaje no solo confirma la importancia de la gluconeogénesis y el metabolismo de los ácidos grasos (exploradas en el primer abordaje) durante períodos de BEN en ganado de carne, sino que también revela numerosos y novedosos genes y nuevas vías metabólicas diferencialmente expresadas a lo largo del tiempo que aún no han sido exploradas en ganado de carne, y que podrían ser en parte responsables de las

diferentes respuestas fenotípicas de los animales en nuestras condiciones de pastoreo. Los genes candidatos evaluados en el primer abordaje (con excepción de GHR y PPAR-α) fueron hallados diferencialmente expresados a lo largo del tiempo, confirmando la validéz de los resultados obtenidos. Encontramos además numerosos genes que participan en la regulación de mecanismos moleculares que regulan la gluconeogénesis hepática (Ej. *PDK4* y *G6PC;* Bradford y Allen, 2005), genes con comportamiento muy diferente al reportado en ganado de leche en BEN durante el periparto y lactancia temprana, y en otros otros modelos de animales experiementales que podrían explicar las adaptaciones a las restricciones alimenticias durante el invierno (Ej. *FGF-21***,** Estall *et al.,* 2009). Encontramos receptores nucleares y coactivadores (*PGC-1A*, Rhee *et al.*, 2003) con numerosas funciones en el metabolismo y la regulación homeorhetica del hígado, numerosos transportadores (*SLCs*) de glucosa, ácidos grasos y amino ácidos, y genes que participan en la oxidación mitocondrial, peroxisomal y microsomal (*ACADM, CYP4A11*, *EHHADH* Kohijima *et al.,* 2007), asi como en la síntesis de cuerpos cetónicos (*HMGCOAS2;* Voet y Voet, 2004).

Asimismo, encontramos numerosas vías metabólicas relacionados con el ciclo celular (proliferación, crecimiento y apoptosis) diferencialmente expresadas durante la gestación-invernal. En particular, una vía metabólica muy interesante que se apaga duranate la gestación invernal fue la *IGF/mTOR*, la cual responde a varias señales ambientales, incluyendo los nutrientes, factores de crecimiento y hormonas (Feng, 2010) y provee una señal positiva para el crecimiento y la división celular y la síntesis de proteínas. La inhibición de la vía de IGF-I /mTOR, debido al estrés nutricional durante el invierno, tuvo un impacto negativo en el crecimiento celular y los mecanismos de reparación durante el período de gestación, al igual que lo reportado por McCarthy *et al.* (2010) en ganado de leche en BEN severo. Por otra parte, la proteína p53 que inhibe esta vía debido al estrés, y regula negativamente el crecimiento y la división celular se encontraba desactivado (debido a la disminución de su estabilización) lo que explica la desregulación de vías relacionadas con la detención del ciclo celular (G1 ciclina E/ S de

transición del ciclo celular, M/G1 transición) y la apoptosis durante períodos de BEN.

4.1.2 Efecto del tiempo-periparto y lactancia temprana

En términos generales, no se observaron grandes cambios en el perfil metabólico, endócrino y de expresión génica hepática, durante el período de periparto y lactancia temprana (-15 *vs.* 15 y 15 *vs*. 60), en comparación con los drásticos cambios que ocurrieron durante la gestación-invernal. De todas maneras, la disminución de las concentraciones circulante de AGNE y aumento de glucosa, estarían indicando una disminución de la tasa de lipolisis y recuperación del balance energético de las vacas al comenzar la primavera. Esta recuperación podría explicarse por el incremento en la disponibilidad, altura y calidad de forraje, asociado al aumento de la temperatura en primavera-verano (Berretta *et al,* 2000) y por ende a un aumento del consumo de materia seca (Chapman *et al.,* 2007). Sin embargo, tanto la CC como los niveles de insulina e IGF-I se mantuvieron bajos, indicando una partición de nutrientes posiblemente hacia la glándula mamaria (Bauman, 2000). No obstante, a pesar que los niveles de IGF-I disminuyeron en el periparto y lactación temprana, a nivel molecular, el transcripto de IGF-I incrementó su expresión pero este aumento no se acompanó de un aumento de los transcriptos de GHR y su isoforma 1A que continuaron bajos, y la expresión de ARNm de IGFBP2 (asociado a restricciones nutricionales; Straus, 1994) si bien disminuyó su expresión a los 15 días aumentó nuevamente cercano al pico de producción de leche (60 días). En el mismo sentido, la maquinaria de la oxidación de ácidos graso se activó nuevamente a los 60 días. Esto podría indicar que si bien hay una mayor disponibilidad de forraje en primavera-verano (tanto en alta como en baja oferta) los nutrientes disponibles se dirijen hacia la producción de leche y mantenimiento de otras funciones (como por ejemplo la reproducción, Short, 1990). En otras palabras, los requerimientos de lactación provocan que las vacas gasten lo que consumen para mantenimiento y producción de leche sin reponer las reservas corporales durante este período.

En lo que respecta al desempeño reproductivo de los animales en el posparto, la duración del anestro posparto se correlacionó negativamente con la CC (Short, 1990), con las concentraciones de IGF-I e insulina (Meikle *et al.,* 2004) y positivamente con la expresión hepática de *IGFBP2* (Roberts *et al.,* 1997).

4.1.3 Efecto de las distintas ofertas de forraje de campo natural

La mayor oferta, disponibilidad, asignación y altura de forraje de las pasturas podría afectar el consumo de energía (Chapman *et al.,* 2007), los requerimientos de mantenimiento (Brosh, 2007) y/o comportamiento de pastoreo (Provenza *et al.,* 2003). En este trabajo, la mayor CC junto a los mayores niveles circulantes de IGF-I e insulina y la mayor abundancia de ARNm de *IGFBP2* en las vacas que pastorearon alta oferta de forraje a lo largo del año se reflejó en un mejor status nutricional de los animales a lo largo de todo el período evaluado, lo que se tradujo en una mayor producción de leche y un mejor desempeño reproductivo en el posparto, de acuerdo a lo previamente reportado por Soca *et al.* (2008). El desacomple del eje somatotrófico en vacas de carne en pastoreo, que interviene en la regulación homeorhetica de la partición de los nutrientes en períodos de BEN, solo se observó en las vacas que pastorearon alta oferta de forraje. En cambio, en las vacas que pastorearon baja oferta de forraje no se dieron grandes variaciones a nivel tanto hormonal como de los transcriptos relacionados a este eje. Esto podría estar reflejando mecanismos de adaptación y partición de los nutrientes diferenciales dependiendo del aporte nutriconal.

En el segundo abordaje, esto se reflejó, en 225 genes diferencialmente expresados entre alta y baja oferta de forraje. El análisis de genes diferencialmente expresados, clusters y vías metabólicas (GSEA), identificaron transcriptos del metabolismo de glucosa y gluconeogénesis como *ALDOC* y *ENOPH1* (mas de 2 veces) y vías del metabolismo intermediario relacionados con la gluconeogénesis y del metabolismo del piruvato encendidos en la oferta alta de forraje. Además el incremento (más de 2.5 veces) en las vacas que pastorearon alta oferta de *FASN* y *DAGLB*, cuyas enzimas participan en la síntesis de ácidos grasos *de novo* y la hidrólisis de diacilglicerol, respectivamente. Estos resultados podrían asociarse con,

y/o explicarse por, el mayor consumo de materia seca y mayores niveles de precursores e intermediarios metabólicos en estas vacas que permitirían un metabolismo y maquinaria más activa en estos animales.

4.1.4 Efecto del grupo genético

Cuando se practican cruzamientos entre razas diferentes se potencian los efectos genéticos no aditivos en características de baja heredabilidad (Morris *et al.,* 1987). En este sentido, el grupo genético como efecto principal fue notorio en los parámetros productivos y reproductivos, particularmente, las vacas cruza fueron más pesadas y tuvieron mayor CC durante todo el período evaluado, produjeron más leche y tuvieron un mejor desempeño reproductivo en el posparto. Esta superioridad de las vacas cruza en variables reproductivas y productivas ha sido ampliamente documentada tanto a nivel nacional (Espasandin *et al.,* 2006) como internacional (Morris *et al.,* 1987). Sin embargo, en ambos abordajes se observaron pocos cambios respecto al grupo genético en los parámetros metabólicos, endócrinos y de expresión génica.

En primer abordaje, se observaron interacciones particulares del grupo genético con los días relativos al parto en los niveles de glucosa e insulina y en la abundancia de ARNm de IGFBP3 y PC. En el segundo abordaje, se encontraron dos transcriptos (gen asociado a la obesidad, *FTO* y una proteína transmenbrana, *TEM149*) más expresados en las cruzas que en las puras que podrían asociarse o explicar mecanismos de adaptación diferenciales a períodos de BEN entre los genotipos. Sin embargo, en su interacción con el ambiente (oferta de forraje), 148 transcriptos estuvieron diferencialmente expresados, donde 10 de los 12 genes encontrados en el análisis de ontología estuvieron más expresados en las vacas cruza en alta oferta de forraje. Los transcriptos *ALDOC*, *ENOPHI*, *FASN*, *DAGLB* estuvieron más expresados en las vacas cruza en alta oferta de forraje, por lo que parecería ser que el efecto de la oferta de forraje podría estar parcialmente explicado por el comportamiento de las cruzas en alta oferta de forraje.

El anestro posparto más corto reportado en las vacas cruzas en comparación con las puras se asoció a un mejor estado metabólico (mayor CC, mayores niveles de insulina y abundancia de ARNm hepático de GHR en particular al final del

otoño (-165 días) y menor abundancia de ARNm de IGFBP3 al final del invierno (-75 y -45 días) y lactancia temprana (+15 días). Estos resultados apoyarían el concepto de la existencia de una "memoria metabólica" en estos animales (Chilliard *et al.*, 2005).

4.2 CONCLUSIONES GLOBALES

Este es el primer trabajo a nivel nacional e internacional que aplica la tecnología de microarreglos con el objetivo de estudiar el control molecular de la adaptación homeorhetica del hígado a cambios en el balance energético durante el ciclo de gestación-lactación en ganado de carne en condiciones pastoriles desde una visión de la genómica funcional, relacionándolo con cambios en el perfil metabólico/endocrino y con las respuestas productivas y reproductivas.

Caracterizamos los cambios a niveles metabólicos, hormonales y de expresión hepática de genes a lo largo del año asociados al establecimiento de un BEN durante los últimos meses de gestación invernal en las vaca de carne en nuestras condiciones de pastoreo de campo nativo. El inicio de la lactación, y el momento cercano al pico de producción de leche (60 dias), implican cambios leves a moderados, en relación al período de gestación, para la vaca de carne.

Claramente las diferencias en términos productivos y reproductivos se reflejaron en perfiles metabólicos, hormonales y moleculares diferentes asociados a mecanismos de adaptación o partición de nutrientes diferenciales que favorecieron las respuestas observadas en las en las vacas que pastorearon alta oferta de forraje. La oferta parece ser una señal muy fuerte para los animales, los cuales reponden los cambios estacionales en la disponibilidad de forraje. Sin embargo, el genotipo animal, quizás por la cercanía genética de las razas usadas en este experimento, no paracería ser así, ya que las notorias diferencias fenotípicas en las variables productivas y reproductivas no se reflejan en los perfiles metabólicos, endócrinos ni moleculares, sugiriendo que quizás podrían existir otros mecanismos o interacciones moleculares no explicadas o abordadas por la transcriptómica (ej. epigenética y/o modificaciones pos-transcripcionales) que estarían explicando la heterosis. Aún más, la interaccion genotipo-ambiente encontrada a nivel del

genoma en las condiciones de este trabajo, refleja que el genotipo cruza en un mejor ambiente nutricional (alta oferta de forraje) se adapta mejor a las condiciones cambiantes sin alterar su condición ni su respuesta productiva y reproductiva. La aplicación de esta nueva herramienta genómica a esta temática nos permitó identificar novedosos genes (que no son claves en las vías metabólicas en las que participan, descritos en el segundo artículo) con grandes o pequeños cambios en sus niveles de expresión que podrían impactar fuertemente en las respuestas observadas a nivel fenotípico en nuestras condiciones ambientales.Los genes involucrados podrian ser los responsables de esta mejor adaptación, aunque esto requiere una de validación correspondiente. Si bien destacamos la participación de vías metabólicas asociadas al metabolismo de la glucosa y los ácidos grasos, destacamos las novedosas vías metabólicas relacionadas con el control del ciclo celular, todas ellas más inactivas durante el período de BEN, resaltando la importancia a nivel celular (turn-over) en este escenario. Deberíamos continuar y ahondar en esta nueva línea de trabajo. Además surgen numerosos posibles genes candidatos (FTO, PDK4, PGC-1α, entre otros) para la búsqueda de marcadores moleculares.

De esta manera este abordaje de estudio exploratorio con el objetivo primero de intentar comprender mejor la biología detrás de las respuestas que observamos, nos abre un nuevo abanico de posibilidades y nuevas líneas de investigación futura en otras áreas como la proteómica y la ingeniería genética. Conociendo mejor mecanismos internos moleculares intrínsecos responsables de ligar el estatus endocrino y metabólico con la respuesta productiva y/o reproductiva en vacas de carne en condiciones de pastoreo de campo nativo, podremos intervenir más eficientemente en el mediano y largo plazo desde un abordaje funcional e integrativo para mejor la eficiencia global del sistema criador en nuestro país.

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6. ANEXOS

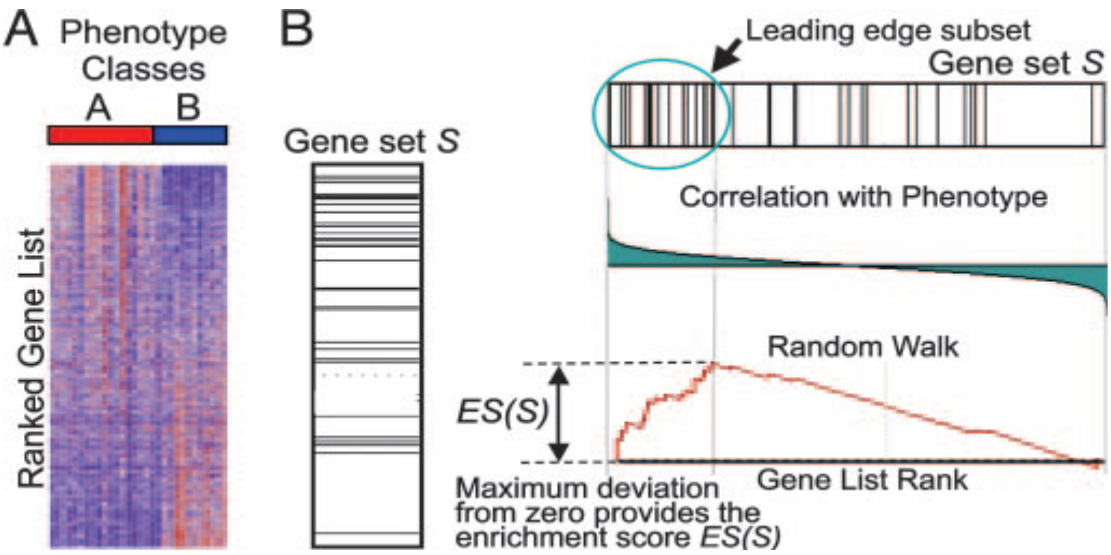
6.1 Tabla ANOVA con p-valores de los efectos principales: grupo genético (GG), oferta de forraje (OF), días, y sus interacciones. Se muestran las variables productivas y reproductivas, metabólicas y homonales y de expresión génica analizadas en el primer artículo de la tesis.

	p- valores de los efectos fijos y sus interacciones						
Variables	OF	GG	Dias	OF*GG	OF*Dias	GG*Dias	OF*GG*Dias
Productivas y reproductivas							
Peso Vivo (kg)	0.018	0.059	< .0001	0.475	0.051	0.778	0.352
Peso nacer del ternero (kg)	0.419	0.345		0.125			
Condición Corporal (unidades)	< .0001	0.048	< .0001	0.245	0.852	0.655	0.887
Producción de leche (lts)	0.031	0.011	-	0.973	-	-	-
Reinicio de la ciclicidad ovárica	< 0.001	0.022	-	0.428	-	-	-
Metabólicas y hormonales							
AGNE (nmol/L) [1]	0.786	0.542	< .0001	0.279	0.15	0.447	0.463
Glucosa (mmol/L)	0.391	0.726	0.008	0.633	0.836	0.037	0.128
Insulina (uUI/mL)	0.027	0.263	< .0001	0.103	0.204	0.033	0.671
IGF-I (ng/mL)	0.005	0.294	< .0001	0.237	0.068	0.629	0.675
Expresón génica [2]							
> Eje somatotrófico							
GHR total	0.373	0.192	0.588	0.942	0.082	0.841	0.021
GHR-1A	0.568	0.283	0.003	0.924	0.384	0.195	0.166
IGF-I	0.864	0.934	0.158	0.854	0.049	0.923	0.418
IGFBP3	0.893	0.374	0.007	0.851	0.656	0.021	0.665
IGFBP2	0.084	0.796	< 0.001	0.900	0.819	0.914	0.787
> Oxidación de ácidos grasos							
ACOX	0.523	0.423	< .0001	0.827	0.502	0.019	0.571
ACADVL	0.562	0.835	0.011	0.253	0.456	0.612	0.615
CPT-1	0.685	0.770	0.027	0.592	0.317	0.855	0.524
PPARA-α	0.600	0.467	0.056	0.759	0.222	0.851	0.549
> Gluconeogénesis							
PC	0.458	0.809	0.011	0.388	0.015	< .0001	< .0001
PCK1	0.948	0.198	< 0.001	0.877	0.598	0.730	0.056
> Controles endógenos							
HPRT	0.631	0.823	0.391	0.336	0.356	0.120	0.878
ACTB	0.582	0.743	0.598	0.473	0.639	0.190	0.640

[1]AGNE = ácidos grasos no esterificados. [2]GHR = receptor hormona de crecimiento, GHR1A= receptor hormona de crecimiento 1A, IGF-I = factor de crecimiento similar a la insulina-I, IGFBP-2 = IGF-binding protein-2, IGFBP-3 = IGF-binding protein-3, ACOX1 = acyl-CoA oxidase 1, palmitoyl, ACADVL = and acyl-CoA dehydrogenase, very long chain, CPT1 = carnitin palmitoil transferasa 1, PPAR-α =

peroxisome proliferator activator alpha, PC = piruvato arboxilasa, PCK1 = piruvato carboxilasa kinasa 1, ACTB= ß-actina y HPRT = ypoxanthin ephosphoribosyl transferase (controles endógenos).

6.2 ILUSTRACIÓN DEL MÉTODO GSEA (Gene Set Enrichment Analysis, fuente: Subramanian *et al.*, 2005).



Visión general ilustrativa del método GSEA. La expresión de conjunto de datos ordenados según la correlación con el fenotipo se muestra en el mapa de color (intensidad de expresión). Este método calcula una medida de distancia de expresión entre dos fenotipos A y B para todos los genes del array (intensidad del gen1 en muestra1 - intensidad del gen1 en muestra2 / desvio). El resultado se ordena de mayor a menor en una lista L. Una vez ordenada la lista, se "testea" la presencia/ausencia de cada gen, perteneciente al "gene set S" (vía metabólica) que se está evaluando, en la lista L, otorgadose un valor positivo en presencia o negativo en ausencia. El Enrichment Score (ES, valor de enriquesimiento) refleja el grado en que un gene set S esta sobrerepresentado en la parte superior o inferior de la lista L, y es la máxima desviación del cero (una vez que se suma/resta de acuerdo al perencia/ausencia de cada uno de los genes). En este caso, la mayoría de los genes

que están en la vía metabólica estudiada se expresan en la parte superior de la lista ordenada (ES positivo=vía metabólica más expresada en A). En nuestro trabajo, por ejemplo para la comparación entre Tiempo 1 (-165 días) *vs.* Tiempo2 (-15 días), un ES positivo para una vía metabólica *x* significa que los genes que la componen se encuentran más expresados en el Tiempo1 que en el 2. De lo contrario, si el ES es negativo, significa que están más expresados en el Tiempo 2.

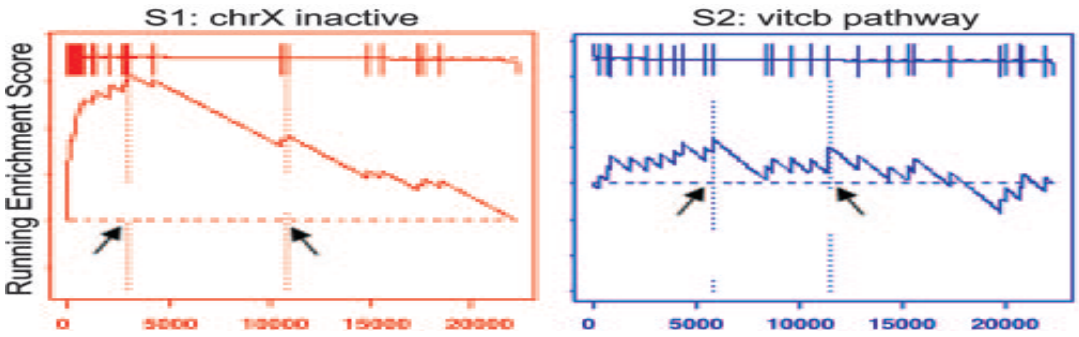


Fig. 2. Ejemplos de Valores de Enriquecimiento (ES). Se muestra la distribución de 2 vías metabólicas (gene sets) en células de la línea germinal de hembras vs. machos (fenotipos). En color rojo S1: genes de inactivación cromosómica, azul S2: importación de la vitamina C neuronal. S1=enriquecsido positiva y significativamente en hembras. S2=distribuido uniformemente, no enriquecsido en ningún género. Las flechas muestras los lugares donde se da el máximo ES.