

**UNIVERSIDAD DE LA REPÚBLICA
FACULTAD DE AGRONOMÍA**

**ENDOCRINOLOGÍA MOLECULAR, METABÓLICA Y REPRODUCTIVA
EN VACAS DE LECHE CON DIFERENTES ESTRATEGIAS DE
ALIMENTACIÓN EN LACTANCIA TEMPRANA**

por

Ana Laura ASTESSIANO DICKSON

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RESUMEN

El objetivo fue estudiar los mecanismos biológicos que regulan el metabolismo de nutrientes y como impactan en las respuestas productivas y reproductivas de vacas lecheras alimentadas con diferentes estrategias durante lactación temprana. Se realizaron tres experimentos (Udelar). Exp1: vacas en pastoreo con diferentes tiempos y sesiones de acceso a la pastura + DTM vs. DTM *ad libitum*. Exp2: vacas alimentadas con DTM *ad libitum* durante lactación temprana, cambian, sin período de adaptación a un sistema mixto de alimentación (pastoreo + DTM). Exp3: vacas en pastoreo con cantidades crecientes de forraje + DTM vs. DTM *ad libitum*. Durante la transición, las vacas perdieron CC, aumentaron las concentraciones de AGNE, BHB y la expresión de genes relacionados a la oxidación de ácidos grasos y cetogénesis, disminuyeron las concentraciones de glucosa, insulina e IGF-I y también la sensibilidad de la insulina. La disminución de IGF-I acompañó los cambios a nivel transcripcional característicos del desacople del eje somatotrófico (disminución de ARNm *GHRIA*, *IGF1*, *IGFBP3* y aumento de *IGFBP1*, *IGFBP2*, *IGFBP4*, *IGFBP5*). En el posparto temprano, las vacas consumiendo DTM *ad libitum* presentaron mayores concentraciones de insulina e IGF-I, menores de AGNE, y BHB y de ARNm de *ACADVL* y *HMGCOA* que vacas en sistemas mixtos. Las vacas con dos sesiones de pastoreo (9 h) tuvieron menores concentraciones de AGNE, mayores de BHB, leptina y adiponectina comparadas con vacas con una única sesión de pastoreo (6 h). Las vacas que pastorearon en dos sesiones fueron las que tuvieron más comprometida la performance reproductiva, en términos de reinicio de la ciclicidad ovárica, asociada a una mayor expresión hepática de ARNm *IGFBP6*. Del mismo modo, las vacas que pastorearon asignaciones medias de forraje y con menor balance energético, tuvieron menor expresión endometrial de ARNm *ADIPOR1* y *ADIPOR2*. La inclusión del pastoreo en el Exp2, disminuyó el PV, la CC y la producción de leche, tendió a aumentar IGF-1 y aumentó la expresión hepática de ARNm *IGF1*, *IGFBP3* e *IGFBP5*. Vacas alimentadas con DTM *ad libitum* y pastoreando alta asignación tuvieron mayor expresión de ARNm *IGF1*, *IGFBP3*, *IGFBP4*, *IGFBP5*. Utilizando altas asignaciones de forraje y de buena calidad al inicio de la lactancia -teniendo en cuenta el gasto energético por actividad de caminatas y

pastoreo- se lograrían resultados productivos y reproductivos similares y comparables con sistemas de alimentación DTM *ad libitum*. Palabras claves: pastoreo, expresión hepática, expresión endometrial

MOLECULAR ENDOCRINOLOGY, METABOLIC AND REPRODUCTIVE IN DAIRY COWS WITH DIFFERENT FEEDING STRATEGIES IN EARLY LACTATION

SUMMARY

The aim was to study the biological mechanisms that regulate nutrient metabolism and their impact on productive and reproductive responses in dairy cows fed with different strategies during early lactation. Three experiments were carried out at the Experimental Station of the Faculty of Agronomy (UdelaR) using Holstein cows. Exp1: grazing cows with different times and sessions of access to pasture + TMR vs. TMR *ad libitum*. Exp2: cows fed TMR *ad libitum* during early lactation assigned without an adaptation period to a mixed feeding system (grazing + TMR). Exp3: grazing cows under different herbage allowances (increasing amounts) + TMR vs. TMR *ad libitum*. During the transition period, cows lost BCS, had increased concentrations of NEFA, BHB and expression of genes related to fatty acid oxidation and ketogenesis and decreased glucose, insulin, and IGF-I concentrations as well as insulin sensitivity. The decrease in IGF-I was accompanied by transcriptional changes characteristic of the uncoupling of the somatotrophic axis (decreased *GHRIA*, *IGF1*, *IGFBP3* mRNA and increase of *IGFBP1*, *IGFBP2*, *IGFBP4*, *IGFBP5* mRNA). During early postpartum, cows fed TMR *ad libitum* had higher concentrations of insulin and IGF-I, lower concentrations of NEFA and BHB and reduced abundance of *ACADVL* and *HMGCOA* mRNA than cows in mixed systems. Cows with two grazing sessions (9 h) had reduced concentrations of NEFA but greater of BHB, leptin and adiponectin compared to cows with only one grazing session (6 h). Cows that had two sessions of grazing were the ones that had more compromised their reproductive performance, in terms of reinitiation of ovarian cyclicity, which was associated with a greater hepatic *IGFBP6* mRNA. Similarly, cows that grazed medium herbage allowances that had a reduced energy balance, had a lower endometrial expression of *ADIPOR1* and *ADIPOR2* mRNA. The inclusion of grazing, without an adaptation period, in the diet of dairy cows fed TMR *ad libitum* during early lactation, decreased BW, BCS and milk production, increased IGF-I concentrations and hepatic expression of mRNA

IGF1, *IGFBP3* and *IGFBP5* mRNA. Cows fed TMR *ad libitum* and grazed a high herbage allowance had greater endometrial expression of *IGF1*, *IGFBP3*, *IGFBP4*, *IGFBP5* mRNA. The use high herbage allowances the good quality in early lactation - taking into account animal energy expenditure due to the activity of walking and grazing - had similar productive and reproductive performance that could be comparable to TMR *ad libitum* systems.

Key word: grazing, hepatic expression, endometrial expression

1. INTRODUCCIÓN

La producción lechera en Uruguay ha mostrado un proceso constante de crecimiento llegando en el 2015 a algo más de 2.182 millones de litros, lo que implica un aumento del 24% respecto a los valores alcanzados en el 2010. Asimismo, la producción media por vaca ha crecido, y si bien hay predios que superan los 6000 litros anuales/vaca masa, el promedio estimado es de 4700 litros anuales/vaca masa (DIEA, 2016), evolución que ha sido acompañada por el aumento de tecnologías y el perfeccionamiento en el uso de los recursos (manejo animal, nutrición). La productividad por vaca es el factor que en forma individual explica una mayor proporción del crecimiento ($> 60\%$), mientras que el aumento de carga explica un 25-30 % del incremento en productividad del sector. Esta estrategia de intensificación de la producción de leche en Uruguay se ha basado en un incremento significativo en el uso de concentrados y reservas de forraje (DIEA, 2016), y más recientemente en el uso de dietas totalmente mezcladas (DTM), particularmente cuando se produce un desbalance entre la producción de forraje y los requerimientos de los animales en determinadas épocas del año (Chilibroste et al., 2007; Wales et al., 2013).

Los sistemas pastoriles lecheros presentan ventajas frente a otros sistemas más intensivos. En primer lugar, el menor costo relativo que se puede lograr con un adecuado manejo de las pasturas que van a ser cosechadas directamente por el animal, comparado con el costo de los concentrados y/o las reservas forrajeras (Dillon, 2006). A su vez, la leche producida por vacas alimentadas con pasturas presenta altas concentraciones de compuestos nutraceuticos beneficiosos para la salud humana (Elgersma et al., 2006), y finalmente se considera que la alimentación en base a pasturas promovería un mayor bienestar de los animales, respecto a sistemas de confinamiento (Rushen et al., 2008). En contrapartida, en estos sistemas aumentan los requerimientos energéticos de mantenimiento de la vaca como resultado de caminatas más intensas necesarias para la cosecha del alimento (Bargo et al., 2002), lo que también dependerá de la capacidad de consumo y composición de la dieta, así como de la calidad, cantidad y manejo de las pasturas ofrecidas (Wales et al., 2013).

Por otra parte, está demostrado que las vacas lecheras consumiendo solo pasto no pueden alcanzar su máximo potencial genético de producción (Kolver y Muller, 1998; Chilibraste et al., 2012) y presentan dificultades para mantener una condición corporal (CC) adecuada (Dillon et al., 2003). Es así que, vacas de altas producción en pastoreo requieren del suministro diario de concentrados para cubrir la demanda nutricional y aumentar y estabilizar la producción de leche a lo largo del año, configurándose así, los sistemas mixtos de producción lechera. En este escenario, es clave contar con estrategias que aumenten el consumo de MS total así como el consumo y uso del forraje disponible para potenciar estos sistemas de alimentación. Maximizar el consumo de MS y de forraje al inicio de la lactancia permitirá reducir el balance energético negativo (BEN), característico de esta etapa, permitiendo el establecimiento exitoso de la lactancia y disminuyendo los efectos negativos de la movilización de reservas corporales sobre la reproducción (Bauman 2000; Butler 2000) y otros procesos fisiológicos. El establecimiento exitoso de la lactancia requiere cambios importantes en las concentraciones de hormonas y metabolitos en sangre, así como en el metabolismo hepático; cambios que acompañan una partición de los nutrientes hacia la glándula mamaria y un re-direccionamiento del tipo de combustible utilizado por los tejidos periféricos (Bauman 2000; Lucy et al., 2009; McCarthy et al., 2010).

Investigaciones internacionales en lactancia media (Bargo et al., 2002; Vibart, et al., 2008) y lactancia completa (Fontaneli et al., 2005) han comparado sistemas mixtos con sistemas estabulados (100% DTM) y han demostrado que la inclusión de pasturas en la dieta de vacas de alta producción disminuye el consumo de MS total, determinando menores ganancias de peso vivo (PV), CC y menor producción de leche (10-20 %). Estos resultados se asociaron a menores concentraciones de ácidos grasos no esterificados (AGNE) en los animales consumiendo pastura dado la menor producción de leche y por lo tanto, menor movilización de reservas corporales (Vibart et al., 2008). A su vez, trabajos realizados en Uruguay con diferentes tiempos de acceso a la pastura, demostraron que aumentar el tiempo de pastoreo aumenta el consumo de

MS y la producción de leche (Mattiauda et al., 2013). Asimismo, se ha demostrado que asignaciones de forraje altas y medias al inicio de la lactancia logran resultados productivos similares y comparables con DTM (<10 %) (Chilibroste et al., 2012; Meikle et al., 2013). Sin embargo, la eficiencia reproductiva –en términos de reinicio a la ciclicidad ovárica- de vacas pastoreando asignaciones medias de forraje, se vio comprometida debido a la partición diferencial de nutrientes para sostener la producción de leche en detrimento de las reservas corporales (Meikle et al., 2013). De aquí surge la importancia de investigar estrategias de pastoreo que permitan respuestas productivas y reproductivas comparables a las logradas con DTM.

En este marco, son pocos los trabajos que hemos encontrado que estudien los mecanismos biológicos que están detrás de estas respuestas productivas, y menos aún que relacionen estos mecanismos biológicos con la partición de nutrientes y su impacto en la reproducción. Se ha demostrado que la competencia por nutrientes y la señalización metabólica impactan sobre todas las funciones del animal (Lucy et al., 2009), por lo tanto, generar información que integre respuestas productivas, balances energéticos, señales endocrino/metabólicas, funcionalidad hepática y reproductiva, es un paso esencial para visualizar el impacto integrado de estrategias nutricionales, como lo son el pastoreo y su combinación con DTM. La hipótesis que nos planteamos es que vacas alimentadas con dietas que permitan la maximización el consumo de nutrientes y energía durante la lactancia temprana, reduciendo el BEN durante este período, presentarán mayor producción de leche, reinicio de ciclicidad ovárica más temprano y un perfil de expresión génica uterina favorable para el crecimiento e implantación embrionaria al momento del servicio. La información generada en la comprensión de estos mecanismos, permitirá proveer de herramientas para la manipulación de procesos complejos que puedan tener un impacto económico en el mediano y largo plazo.

1.1.OBJETIVO GENERAL

Contribuir al conocimiento sobre los mecanismos biológicos que regulan el metabolismo de nutrientes y como esos mecanismos impactan en las respuestas productivas y reproductivas, combinando diferentes estrategias de alimentación durante lactación temprana.

1.2. OBJETIVOS ESPECÍFICOS

1. Estudiar el efecto de diferentes estrategias nutricionales basadas en pastoreo con diferentes tiempos y sesiones de acceso a la pastura + DTM vs. DTM *ad libitum* durante la lactancia temprana, sobre los perfiles metabólicos, endocrinos y la expresión hepática de genes relacionados con la partición de nutrientes (sistema GH-IGF-I y adipokinas) y su asociación con las respuestas productivas y reproductivas.
2. Estudiar alteraciones en los perfiles metabólicos, endocrinos y en la expresión hepática genes relacionados con la partición de nutrientes y el metabolismo energético (gluconeogénesis y oxidación de ácidos grasos) cuando vacas lecheras alimentadas con DTM *ad libitum* durante lactación temprana, cambian, sin período de adaptación a un sistema mixto de alimentación (pastoreo + DTM).
3. Estudiar el efecto de diferentes estrategias nutricionales basadas en pastoreo con cantidades crecientes de forraje + DTM vs. DTM *ad libitum* durante la lactancia temprana, sobre la expresión endometrial de genes relacionados con el sistema IGF-I, adipokinas, los receptores de progesterona (PGR) y estrógenos (ER α) al final del período de espera voluntario.

1.3. PROPUESTA DE LA TESIS

Esta propuesta de tesis surge por el interés, tanto a nivel nacional como internacional en sistemas mixtos de producción lechera (inclusión de pasturas en la dieta) debido a sus ventajas económicas, ambientales y de bienestar animal (Dillon et al., 2003), por

la importancia en la maximización del consumo de MS al inicio de la lactancia para el establecimiento exitoso de la misma y por el interés de entender el proceso de adaptación metabólica que impacta tanto a nivel productivo como reproductivo.

Es así que, la estructura central consiste en tres artículos científicos que estudian el efecto de diferentes estrategias nutricionales (pastoreo con diferentes asignaciones y sesiones + DTM vs. DTM *ad libitum*) durante el posparto en lactación temprana. Los dos primeros lo hacen en respuesta a variables metabólicas, endocrinas y de expresión génica hepática a inicio de lactancia (capítulo 3.1) o frente a cambios de dieta (capítulo 3.2) y el último estudia el efecto de la nutrición sobre la expresión génica endometrial al día 7 del ciclo estral en fase luteal temprana luego del período de espera voluntario siguiendo un enfoque de genes candidatos (capítulo 4.1).

2. ANTECEDENTES

1.1.1. Bases metabólicas de la adaptación a cambios en el balance energético durante gestación y lactación en vacas de leche

En el último tercio de gestación y/o durante las primeras semanas posparto, de manera similar a lo que ocurre en muchas especies de mamíferos -agravado por la selección genética para producir leche- se establece un BEN en las vacas lecheras porque el aumento de los requerimientos nutricionales no está acompañado de un aumento inmediato o suficiente en el consumo de alimentos. El incremento en el consumo de materia seca (MS) posterior al parto ocurre a una tasa menor que el aumento en la producción de leche estableciéndose un BEN (Bell, 1995; Bell y Bauman, 1997). La capacidad del animal para adaptarse y sobrellevar este BEN -a través de los mecanismos endócrinos y metabólicos para mantener la homeostasis- no sólo afecta la capacidad productiva de la vaca, sino también compromete la respuesta reproductiva durante el siguiente ciclo productivo (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 fisiológicos, bioquímicos y génicos (McCarthy et al., 2010). 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 la glándula mamaria y una creciente utilización de AGNE por los tejidos maternos (Bell, 1995). El inicio de la lactancia 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, provoca la movilización de los depósitos de grasa y 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).

1.1.1.1. Eje Somatotrófico

La principal hormona responsable de la partición de nutrientes es la hormona del crecimiento (GH; Etherton y Bauman, 1998). El eje somatotrófico que consiste en la

GH, los factores de crecimiento similar a la insulina tipo I y II (IGF-I e IGF-II), sus proteínas de unión (IGFBP 1 a 6) y sus receptores (IGFR), juega un rol crítico en la regulación del metabolismo y de varios procesos fisiológicos (Renaville et al., 2002). En contraste con la típica asociación directa y positiva entre GH e IGF-I, mientras la GH en sangre comienza a aumentar previo al parto, el IGF-I disminuye, disociación que coincide con el establecimiento del BEN de inicio de lactación y con la disminución de la insulina en sangre (Rhoads et al., 2004; Lucy 2008). El incremento de GH postparto estimula la gluconeogénesis en el hígado y antagoniza con la acción de la insulina en los tejidos periféricos. En particular, en el tejido adiposo, las concentraciones altas de GH y bajas de insulina estimulan la lipólisis y disminuyen la utilización de la glucosa, reservando así la misma para la síntesis de leche (Lucy et al., 2009).

A pesar de los considerables esfuerzos de la investigación internacional, el mecanismo molecular responsable del desacople del eje GH-IGF-I no está completamente claro. La disminución de los receptores de GH hepáticos son la principal causa de resistencia a GH en el posparto temprano (Lucy et al., 2009). La expresión hepática del transcripto de GHR, especialmente de la isoforma 1A (GHR- 1A), juega un papel importante en la regulación del desacople del eje, dado que disminuye dramáticamente en el postparto temprano conjuntamente con la disminución del ARNm y las concentraciones sanguíneas de IGF-I (Kobayashi et al., 1999). Las bajas concentraciones de insulina, características del posparto temprano, jugarían un rol clave en la disminución de la abundancia de GHR y por lo tanto en el desacople hormonal (Mashek et al., 2001; Butler et al., 2003; Rhoads et al., 2004). Asimismo, Looor et al. (2005) y Carriquiry et al. (2009) trabajando con vacas de leche, sugirieron que la expresión hepática de ARNm de *IGFBP3* también sería clave en este mecanismo.

Comparando animales con diferente potencial genético (NA90 vs. NZ90) los animales más productivos, pierden más CC, presentan mayores concentraciones de GH y menores de IGF-I, indicando un desacople del eje somatotrófico más severo (Lucy et al., 2009). A su vez, las vacas de menor producción tienen menores concentraciones de GH y mayores de insulina e IGF-I (Gong et al., 2002). Este perfil hormonal

determina que la capacidad de movilizar AGNE es menor y que una mayor cantidad de glucosa es destinada a tejidos extra-mamarios, características que llevan a la menor producción láctea (Bauman, 2000). Por otra parte, la suplementación en vacas lecheras cumple un rol clave, Grala et al. (2011) demostraron que la suplementación de vacas en pastoreo además de aumentar la producción de leche, presentó un menor desacople del eje somatotrófico a nivel de los metabolitos sanguíneos y la expresión génica hepática.

Resultados recientes muestran que la expresión de genes que codifican para enzimas de la oxidación de ácidos grasos, cambia durante el período de transición y lactación temprana y están moduladas por las reservas corporales en vacas de leche en pastoreo (Carriquiry et al., 2010). Por otro parte, a pesar que las concentraciones de insulina e IGF-I disminuyen luego del parto (Meikle et al., 2004; Pereira et al., 2010; Adrien et al., 2011), los cambios en la expresión hepática de ARNm de *GHR*, *GHR1A* e *IGF1* durante el pre y posparto inmediato en vacas lecheras en pastoreo, no son tan marcados como en sistemas de confinamiento con alimentación en DTM (Carriquiry et al., 2010; Grala et al., 2011).

1.1.1.2. Rol de las Adipokinas

El tejido adiposo cumple un rol no sólo como reserva de energía sino endócrino regulando el metabolismo. La detección por primera vez en rumiantes de la leptina, hormona proteica secretada principalmente por el tejido adiposo (Delavaud et al., 2000), puso de manifiesto el importante papel endócrino de este tejido y en los últimos años se han descubierto varias proteínas secretadas por este tejido llamadas adipokinas (ej. leptina, adiponectina, resistina, visfastina) (Lemor et al., 2009). Estas adipokinas, se han asociado a la homeostasis metabólica, regulando el metabolismo de lípidos y carbohidratos (Kubota et al., 2007; Lemor et al., 2009). La investigación sobre los mecanismos de regulación del metabolismo energético y en particular el rol de las adipokinas en el metabolismo energético es escasa en el modelo rumiante. En este sentido, se ha demostrado que durante el BEN en la transición de la vaca lechera, las concentraciones de leptina en sangre disminuyeron mientras que la expresión de

ARNm del receptor de leptina (*LEPR*) aumentaron tanto a nivel hepático (Thorn et al., 2008) como en tejido adiposo (Lemor et al., 2009) y que este aumento estaba parcialmente regulado por las concentraciones disminuidas de insulina (Thorn et al., 2008). Estos autores sugieren que el incremento en ARNm de *LEPR* durante períodos de BEN puede ser un mecanismo para atenuar la acción de la leptina central.

La adiponectina es producida principalmente por los adipocitos y circula en sangre en altas concentraciones. Esta hormona ha sido correlacionada negativamente con la disminución de la adiposidad ya que incrementa luego de la reducción de peso y promueve la secreción y sensibilidad a la insulina incrementando el consumo de alimento (Kubota et al., 2007). Se ha demostrado que las concentraciones de adiponectina se incrementan en el posparto de la vaca lechera (Raddatz et al., 2008; Singh et al., 2014). Sin embargo, Lemor et al. (2009) no encontraron un aumento de la expresión de ARNm de adiponectina (*ADIPO*) en tejido adiposo en el posparto de vacas lecheras. Con respecto a los receptores de adiponectina, la expresión de ARNm de *ADIPOR2* se incrementó en el hígado luego de la transición (Saremi et al., 2012), y durante el posparto en vacas lecheras con alimentación restringida en el preparto pero no en vacas alimentadas *ad libitum* (Loor et al., 2010). Estos resultados sugieren una regulación diferencial de estas hormonas y sus receptores en el tejido adiposo e hígado como reguladores de la homeostasis energética.

1.1.2. Estrategias nutricionales en base pastoril al inicio de la lactancia

A modo general, se puede caracterizar el sistema de producción lechero uruguayo por explotar rodeos en pastoreo directo y caminatas diarias, conformando dietas que provienen en un 70% de la cosecha de forraje (pastoreo + reservas; Chilibróste et al., 2015). En sistemas lecheros de base pastoril, la cantidad y calidad de las pasturas ofrecidas son variables a lo largo del año y se produce un desequilibrio entre la oferta y la demanda de nutrientes en diversas etapas del ciclo de producción (Chilibróste et al., 2012), determinando que el consumo de pasturas no sea suficiente para alcanzar el máximo potencial genético de producción (Kolver y Muller, 1998) y que las vacas de alta producción presenten dificultad para mantener una CC adecuada (Dillon et al.,

2003). En este sentido, la inclusión de concentrados en la dieta ha aumentado para satisfacer los requerimientos de nutrientes de los animales que no son aportados por la pastura. Más aún, el uso de DTM en la lactancia temprana permite que las vacas lecheras alcancen el máximo rendimiento en momentos que la producción de MS del pasto es reducida (Wales et al., 2013)

1.1.2.1. Dietas totalmente mezcladas vs. dietas totalmente mezcladas más pastoreo

A nivel internacional hay varios trabajos en lactancia media (Soriano et al., 2001; Bargo et al., 2002; Vibart et al., 2008; Kennedy et al., 2015) y completa (White et al., 2002; Fontaneli et al., 2005) que comparan niveles de consumo de MS y de producción de leche cuando se usa DTM vs. pastura o pastura más DTM o más concentrado, encontrando resultados variables dependiendo de la dieta, del nivel de inclusión de pastura en la dieta y del tiempo de acceso. Bargo et al. (2002) combinando 50% pastura más 50% DTM observaron reducciones de 6% en el consumo de MS y 16% en producción de leche, mientras que Vibart et al. (2008) con diferentes niveles de inclusión de pastura (15 vs. 30 vs. 45%) observaron reducciones entre 18-21% en CMS y 10-12% en producción. Por otro lado, Kennedy et al. (2015) con pastoreo más suplementación con concentrado (8 kg MS/v/d) observaron reducciones del 8%, tanto en CMS como en producción, comparado con el sistema 100% DTM. Estos trabajos han demostrado que la inclusión de pasturas en la dieta de vacas de alta producción disminuye el CMS total y la producción de leche, sin modificar mayormente su composición, determinando menores ganancias de PV y CC.

En este contexto y dado el creciente interés por aumentar el uso combinado de DTM y pastura en la alimentación de la vaca lechera -aumentando el consumo de MS total y uso del forraje disponible para potenciar sistemas de alimentación mixtos- es que se han realizado diversos estudios con énfasis en la intervención de lactación temprana (primeros 60 días de lactancia). Estos trabajos incluyen el uso estratégico de DTM y evalúan la utilización de pasturas y suplementos en diferentes combinaciones, tanto en cantidades como en rutinas de alimentación (Chilibroste et al., 2012; Meikle et al., 2013; Fajardo et al., 2015; Mendoza et al., 2016), demostrando el impacto positivo de

la inclusión de DTM sobre la producción de leche, grasa y proteína al inicio de la lactancia. Sin embargo, se ha demostrado que pastoreo de asignaciones de forraje altas y medias suplementados con DTM al inicio de la lactancia (Chilibroste et al., 2012; Meikle et al., 2013) así como 4 horas de acceso a una pastura cortada de alta calidad (90% DTM + 10% pastura) Mendoza et al., 2016) logran resultados productivos similares y comparables con 100% DTM. Sin embargo, hay trabajos en lactancia temprana y media (primeros 90 o 130 días; Morales-Almarás et al., 2010; Dall-Orsolleta et al., 2016) que comparan DTM vs. DTM más diferentes tiempos de acceso a la pastura (6 y 12 h) y no encuentran diferencias mayores ni en CMS ni en producción de leche.

Asimismo, son pocos los trabajos que asocian estos resultados a los cambios metabólicos que ocurren en el animal. Vibart et al. (2008) reportaron menores concentraciones de glucosa y AGNE en los animales consumiendo pastura dado la menor producción de leche y, por lo tanto, menor movilización de reservas corporales de esos animales. En contraposición, Kennedy et al. (2008) reportaron mayores concentraciones de AGNE en animales con menor asignación de forraje dado la menor producción de leche y, por lo tanto, mayor grado de movilización de reservas corporales. En este mismo sentido, a nivel nacional, trabajando con asignaciones crecientes de forraje al inicio de la lactancia se reportaron aumentos en las concentraciones de AGNE y BHB al día 15 en vacas consumiendo asignaciones medias de forraje en comparación con los que consumieron 100% DTM, reflejando un balance energético negativo más severo en estos animales que impactó negativamente en la reproducción – en términos de reinicio de la ciclicidad ovárica (Meikle et al., 2013). También se ha reportado que vacas primiparas con un adecuado manejo de las estrategias de pastoreo y de las suplementaciones –maximizando el consumo de MS y de forraje al inicio de la lactancia (ej. pastoreo a altas asignaciones de forraje) lograron mejorar las respuestas productivas y reproductivas de los animales (Meikle et al., 2013), que podrían ser clave para mejorar la rentabilidad en sistemas lecheros de base pastoril.

Si bien es claro que el consumo y la producción de la vaca lechera son menores cuando se alimenta con alguna combinación nutricional respecto a una dieta DTM, también es claro que los resultados varían dependiendo de la asignación de forraje y la calidad del mismo. Por lo tanto, en sistemas de producción lecheros como el nuestro, combinar el uso de estrategias nutricionales en momentos claves del ciclo de producción, es fundamental para alcanzar el potencial productivo de los animales sin aumentar demasiado los costos de producción. Hasta el momento, no hemos encontrado reportes que evalúen el efecto de diferentes estrategias nutricionales -basadas en pastoreo con diferentes manejos de la pastura + DTM vs. DTM *ad libitum*-, sobre los mecanismos metabólicos y endocrinos que explican la partición de nutrientes (sistema GH-IGF-I y adipokinas) en vacas de leche.

1.1.2.2. Inclusión de pastoreo en dietas totalmente mezcladas

Está demostrado que la transición de una DTM a un sistema mixto (DTM + pastura) implicó cambios significativos en el consumo de MS (Schären et al., 2016a), en el comportamiento y actividad de la vaca (Peyraud et al., 1996; Chilibraste et al., 2012) y en la microflora ruminal (Schären et al., 2016b), causando cambios en la producción y composición de leche.

Poco se sabe sobre las adaptaciones metabólicas de las vacas lecheras a cambios en el régimen de alimentación al pasar de una DTM en lactancia temprana (0-60 días en leche) a un sistema de alimentación mixto en una etapa posterior de la lactancia. Se han reportado adaptaciones metabólicas durante la transición de una DTM a una dieta en base pastoril (+1.75 o 3.6 kg de MS de concentrado) en lactancia media (160 a 200 DIM; Schären et al., 2016a). Estos autores demostraron que las concentraciones de insulina disminuyeron, mientras que los AGNE y BHB aumentaron, sugiriendo una mayor lipomobilización durante los primeros días del cambio de dieta, asociados a la disminución en el consumo de MS, en la producción de leche y CC (Agenäs et al., 2002; Schären et al., 2016a). Sin embargo, los cambios a nivel hepático del eje somatotrófico y del metabolismo energético cuando a las vacas lecheras alimentadas con DTM se las cambia a un régimen de alimentación que incluya pastoreo y una

suplementación apropiada están poco estudiados.

1.1.3. Efecto de la nutrición sobre la reproducción

En los mamíferos, el nacimiento de una progenie viable y saludable implica una secuencia compleja de procesos biológicos, donde la hembra es la encargada de producir los gametos, recibir el embrión, promover el desarrollo y crecimiento del feto y mantener la gestación hasta el momento del parto. Factores como la nutrición y alteraciones endocrinas, afectan tanto la calidad de los gametos y la fertilización, como la vida libre del embrión a través del oviducto y la interacción entre el ambiente uterino y el embrión (Leroy et al., 2008; Spencer et al., 2015).

Las principales limitantes para el éxito reproductivo son el reinicio de la ciclicidad ovárica luego del parto y la mortalidad embrionaria temprana (Santos et al., 2004). Un estudio realizado a nivel nacional (n=899 vacas lecheras de uno, dos o tres lactancias pertenecientes a tambos comerciales), indicó que con un buen manejo nutricional las vacas lecheras reinician su ciclicidad ovárica dentro de los primeros 30 a 40 días posparto (Carriquiry et al., 2012). No hemos encontrado reportes sobre mortalidad embrionaria a nivel nacional, sin embargo, a nivel internacional, Santos et al. (2004) en vacas lecheras, reportan que cerca del 40% de las preñeces fallan alrededor del momento del reconocimiento materno de la gestación durante la lactancia, siendo menor esta pérdida en vacas secas.

Resultados de nuestro equipo de investigación han descrito el efecto del número de lactancias (primíparas vs. multíparas) y de la CC al parto (natural o provocado) sobre el reinicio de la ciclicidad ovárica en vacas Holando (Meikle et al., 2004; Adrien et al., 2012) encontrando que estas diferencias se reflejan en algunas variables endocrinas y metabólicas. Es así que, se ha reportado un BEN más severo en vacas lecheras primíparas vs. multíparas en condiciones pastoriles o de baja vs. alta CC, asociado a menores concentraciones de insulina y de IGF-I y anestros más largos (Meikle et al., 2004; Adrien et al., 2012). Meikle et al. (2004) trabajando en condiciones pastoriles, reportaron que las vacas lecheras que ovularon en lactación temprana presentaron mayores concentraciones de IGF-I que aquellas en las cuales la ovulación falló.

Por otra parte, se ha reportado que la mayor causa de mortalidad embrionaria en la etapa de pre implantación se debe a problemas en la señalización entre el embrión y la madre, lo que conduce a un desarrollo asincrónico, con retraso en el crecimiento del embrión (Thatcher et al., 2003). Hasta la implantación, el embrión se desarrolla libremente en el oviducto y en el útero y depende de sus secreciones (Ashworth, 1995; Spencer et al., 2015). Una sincronía estricta entre el ambiente materno y el embrión es esencial para asegurar la supervivencia embrionaria: tanto el endometrio como el embrión, sintetizan y secretan a la interfase embrio-maternal una multitud de factores de crecimiento, proteínas, citoquinas, hormonas y otras sustancias que afectan a ambas partes, ya sea en forma autócrina o parácrina, y que determinan dicha sincronía (Martal et al., 1997).

Entre los factores de crecimientos promotores del desarrollo embrionario, el sistema IGF tiene un rol primordial. El IGF-I actúa como factor mitogénico endócrino y es el mediador de los efectos de GH (Schoenle et al., 1982). Se ha comprobado la producción local de IGF-I en diferentes tejidos, por lo tanto, IGF-I tendría además acciones independientes de la GH (Han et al., 1987). Meikle et al. (2001) demostraron que la expresión endometrial de ARNm de *IGF1* fue más alta al estro y fase luteal muy temprana (día 0 y 5, respectivamente), que durante la fase luteal media o tardía (día 12 y 19, respectivamente). Estos cambios temporales estuvieron asociados con la expresión de ARNm del receptor de estrógenos alfa (*ER α*) y de progesterona (*PGR*) y con las concentraciones circulantes de P4 y 17 β estradiol. Estos hallazgos en la expresión endometrial de ARNm de *IGF1* fueron confirmados por otras metodologías posteriormente (Sosa et al., 2010), sugiriendo la relevancia de IGF-I durante la fase luteal temprana. Sin embargo, la expresión de ARNm de *IGF2* e *IGFR* presentaron sus máximos niveles durante la fase luteal media, sugiriendo un rol diferencial de IGF-I e IGF-II en el crecimiento del embrión y/o regulación de la función uterina. Asimismo, Sosa et al. (2009) observaron un aumento la expresión endometrial de ARNm de *IGF1* e *IGF2* al día 14 en ovejas preñadas comparadas con ovejas ciclando. Además, la subnutrición provocó una alteración en la expresión endometrial de ARNm de *IGF2* en ovejas cíclicas, duplicando la expresión de ARNm de *IGF2* comparado con el grupo

control.

Rhoads et al. (2008) encontraron que la expresión endometrial de ARNm de *GHR*, *IGF1*, *IGFBP2* no fue modificada durante los primeros 160 días de lactancia ni tampoco difirió en vacas preñadas vs. no preñadas. Por otro lado, Wathes et al., (2011) estudiando la expresión endometrial del sistema IGF en la segunda semana luego del parto en vacas con BEN moderado y severo, encontraron mayor expresión de ARNm de *IGFBP1* e *IGFBP4* y menor expresión de ARNm de *IGFBP6* en animales con BEN severo, sugiriendo efectos directos del BEN sobre la funcionalidad uterina. No hemos encontrado reportes de los efectos de diferentes planos nutricionales sobre la expresión de estos genes en el útero de vacas lecheras en el período clave donde debe estar apto para promover el desarrollo embrionario y mantener la preñez, al finalizar el período de espera voluntario.

Si bien son escasos los estudios de expresión génica endometrial en vacas lecheras durante la lactancia, hay evidencia sólida de que un adecuado aumento en los niveles de P4 luego de la ovulación, inducen cambios en el transcriptoma endometrial necesarios para establecer la receptividad uterina y promover el crecimiento del blastocisto (Lonergan y Forde, 2014). Estos cambios ocurren entre los días 7 y 13 en respuesta a la P4 ovárica, independientemente de si existe o no un embrión adecuadamente desarrollado (Forde et al., 2009; Simmons et al., 2009; Forde y Lonergan, 2012).

Respecto al rol de las adipokinas, secretadas por el tejido adiposo (ej. leptina), en la fertilidad bovina hemos encontrado poca información endometrial disponible respecto a los receptores de las mismas. Se ha descrito la expresión de ARNm de *LEPR* durante el ciclo estral, reportándose niveles más bajos al día 5 (Sosa et al., 2010). Sin embargo, hay evidencia en humanos del rol que tienen las adipokinas en la fertilidad (Mitchel et al., 2005). En este sentido, Takemura et al. (2006) describen un aumento de ARNm de *ADIPOR1* y *ADIPOR2* en el endometrio durante fase luteal media, sugiriendo un rol en la implantación del embrión. A su vez, Dos Santos et al. (2012) reportaron en mujeres con fallas recurrentes en la implantación del embrión vs.

mujeres fértiles, que la expresión endometrial de ARNm y proteína de leptina fue menor, de *LEPRB* fue mayor, de *ADIPOR1* y *ADIPOR2* fue menor, y no hubo diferencias en la expresión de adiponectina, sugiriendo un claro rol de las adipokinas y/o sus receptores en la receptividad del útero humano a la implantación. El impacto de las adipokinas en el útero de especies productivas es casi desconocido. Maleszka et al. (2014) y Smolinska et al. (2014) sugieren que cambios en la expresión de adiponectina en el útero porcino y variaciones en los niveles plasmáticos en cerdas cíclicas, son reguladas por los esteroides gonadales u otras hormonas que controlan el ciclo estral.

1.1.4. Enfoque Nutrigenómico: impacto de las estrategias de alimentación en lactación temprana sobre la expresión génica uterina en vacas lecheras

La nutrigenómica es la ciencia que estudia el rol de los nutrientes y componentes bioactivos de los alimentos sobre la expresión génica, incluyendo el efecto sobre la estabilidad del genoma, alteraciones epigenómicas, y en la expresión de ARN (transcriptómica), proteínas (proteómica) y metabolitos (metabolómica) (Fenech et al., 2011). Los nutrientes y componentes de la dieta pueden afectar el estado corporal de los individuos influyendo sobre los diferentes niveles que son objeto de estudio de las diferentes ciencias ómicas (Kato, 2008). Una de estas ciencias es la transcriptómica que estudia el transcriptoma, definiéndose al mismo como el conjunto de moléculas de ARN que se expresan en una célula, tejido u organismo (Blencowe et al., 2009), incluyendo la clasificación de los diferentes transcritos, la determinación de la estructura transcripcional de los genes y la cuantificación de los cambios en la expresión génica durante el desarrollo o bajo determinadas condiciones (Wang et al., 2009). Si bien el transcriptoma representa uno de los múltiples aspectos de los fenómenos biológicos que se modifican en respuesta a los nutrientes, Kato (2008) sostiene que el análisis del mismo puede emplearse como marcador global de estado y respuesta de células, órganos y estado corporal resultante de cambios en el proteoma y metaboloma. Es así que el análisis del transcriptoma es esencial para la interpretación de los elementos funcionales del genoma de la célula y los tejidos, y así comprender la complejidad de los fenotipos de los animales. La técnica llamada ARN-Seq (*high*

throughput next generation sequencing technologies) proporciona una herramienta para cuantificar y anotar el transcriptoma completo (Marioni et al., 2008, Mortazavi et al., 2008, Wang et al., 2009).

Bauersachs et al. (2005), estudiaron el transcriptoma del endometrio bovino durante el estro (día 0) y diestro (día 12) e identificaron más de 130 genes diferencialmente expresados. Durante el estro encontraron mayor expresión de ARNm *UTMP* (proteína uterina de la leche) y de genes relacionados con la adhesión celular, motilidad celular y la matriz extracelular y durante el diestro mayor expresión de genes que codifican para varias enzimas y proteínas de transporte, todos ellos potencialmente implicados en la regulación de los cambios del endometrio durante el ciclo estral.

Los mayores cambios transcripcionales del endometrio en vacas que quedaron o no quedaron preñadas, se dan en la fase luteal temprana del ciclo estral (Salilew-Wondim et al., 2010; Forde et al., 2011a), y son aún más marcados cuando se afectan las concentraciones circulantes de P4, lo que conduce a un ambiente uterino alterado. Forde et al. (2011a) han demostrado que bajas concentraciones circulantes de P4 en los primeros días luego del estro generan un ambiente uterino subóptimo con capacidad reducida para soportar la elongación del concepto. Asimismo, se ha reportado que el transcriptoma endometrial no cambia debido a la presencia de un embrión hasta aproximadamente el día 16 del ciclo estral, día del reconocimiento materno de la preñez (Forde et al., 2011b; Forde y Lonergan 2012). Por lo tanto, alteraciones en el endometrio cíclico se pueden utilizar para entender los cambios asociados con el establecimiento exitoso de la preñez.

Sin embargo, recientemente Binelli et al. (2015) estudiaron el transcriptoma uterino al día 6 post inseminación artificial de vacas gestantes vs. no gestantes, encontrando vías relacionadas a la biosíntesis de los esteroides y a la proliferación celular up-reguladas en vacas gestantes, indicando un estado más receptivo del endometrio en esos animales. Consistente con hallazgos anteriores del mismo grupo (Binelli et al., 2014), quienes sugieren que el endometrio receptivo debe tener características de un tejido proliferativo, con el fin de proporcionar un micro-ambiente más maduro y diferenciado

para el embrión.

Si bien no hemos encontrado trabajos que estudien el efecto de la nutrición durante el posparto temprano sobre el transcriptoma del útero de vacas lecheras ciclando (aptas para ser preñadas), varios trabajos han demostrado el efecto la nutrición sobre la expresión de genes candidatos en el útero (ovejas subnutridas Sosa et al. 2009; vacas en el posparto temprano Wathes et al., 2011). Por lo tanto, identificar factores que determinen la receptividad del endometrio en la fase luteal temprana del ciclo estral es fundamental para maximizar el rendimiento reproductivo de las vacas y así, mejorar la supervivencia embrionaria. En este marco, y considerando que una nutrición apropiada y la comprensión de la funcionalidad uterina durante el postparto temprano son cruciales para maximizar la productividad y la fertilidad en las vacas lecheras, es que este tipo de estudios son necesarios.

3. RESPUESTAS METABÓLICA, ENDÓCRINA Y EXPRESIÓN GÉNICA HEPÁTICA DURANTE EL POSPARTO

3.1. METABOLIC AND ENDOCRINE PROFILES AND HEPATIC GENE EXPRESSION OF HOLSTEIN COWS FED TOTAL MIXED RATION OR PASTURE WITH DIFFERENT GRAZING STRATEGIES DURING EARLY LACTATION. 2015. Astessiano AL, Meikle A, Fajardo M, Gil J, Mattiauda DA, Chilibraste P, Carriquiry M. Este artículo fue publicado en la revista Acta Veterinaria Scandinavica.

El objetivo fue evaluar los perfiles metabólicos, endocrinos y la expresión hepática de genes relacionados con la partición de nutrientes (ARNm de sistema *IGFs*, *INSR*) y las adipokinas (*LEPRb*, *ADIPOR1*, *ADIPOR2*) de vacas Holstein en lactancia temprana. Las vacas fueron alimentadas con DTM (100% *ad libitum* sin pastoreo) o dietas combinando DTM (50% *ad libitum*) y pastura con diferentes estrategias de pastoreo (6 h en una sesión de pastoreo o 9 h en dos sesiones de pastoreo).

La hipótesis que nos planteamos fue que vacas lecheras alimentadas con DTM *ad libitum* tienen una mejor performance productiva y reproductiva que vacas en pastoreo y que el aumento del tiempo de acceso a la pastura junto con el número de sesiones de pastoreo durante los primeros 60 días de lactancia, maximizará el consumo de MS de la pastura y se reflejará en un BEN más atenuado de las vacas lecheras en lactancia temprana.

Los resultados sugieren que los perfiles endocrino-metabólicos de estas vacas reflejaron no sólo cambios en la producción de energía en leche y el balance energético, sino también en la actividad (caminata y pastoreo). Las menores demandas energéticas, reflejaron el mejor balance energético que tuvieron las vacas alimentadas 100% DTM (mayor insulina e IGF-I/menor NEFA y BHB) comparado con los grupos de dietas combinadas. A pesar de ello, las vacas alimentadas con una sesión de pastoreo, reiniciaron la actividad ovárica al mismo tiempo que las vacas DTM. A su vez las mayores demandas energéticas se dieron en las vacas con dos sesiones de pastoreo, sugiriendo un estrés metabólico evidenciado en menores concentraciones de

insulina e IGF-1, que se asociaron negativamente en la función reproductiva (retraso en la reiniciación de la actividad ovárica y menor probabilidad de vacas ciclando).

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Metabolic and endocrine profiles and hepatic gene expression of Holstein cows fed total mixed ration or pasture with different grazing strategies during early lactation

Ana Laura Astessiano^{1*}, Ana Meikle², Maite Fajardo¹, Jorge Gil³, Diego Antonio Mattiauda¹, Pablo Chillibroste¹ and Mariana Carriquiry¹

Abstract

Background: In dairy mixed production systems, maximizing pasture intake and total mixed ration (TMR) supplementation are management tools used to increase dry matter and energy intake in early lactation. The objective was to evaluate metabolic and endocrine profiles and hepatic gene expression of Holstein cows fed either TMR ad libitum (without grazing) or diets combining TMR (50 % ad libitum DM intake) and pasture with different grazing strategies (6 h in one grazing session or 9 h in two grazing sessions) in early lactation. Pluriparous cows were grouped by calving date, blocked within group by body weight and body condition score (BCS) and randomly assigned to one of three feeding strategies from calving (day 0) to 60 days postpartum: control cows fed TMR ad libitum (G0; confined cows fed 100 % TMR without access to pasture), pasture grazing with 6 h of access in one session supplemented with 50 % TMR (G1), and 9 h of access in two sessions supplemented with 50 % TMR (G2).

Results: Net energy (NE), but not metabolizable protein (MP), demands for maintenance and/or milk increased in G2 when compared with G1 and G0 cows, respectively. However, NE and MP balances were lower in G1 and G2 than G0 cows. Cow BCS at +55 days was greater in G0 than G2 cows and probability of cows cycling during the first month was greater in G0 and G1 than G2 cows. During the postpartum period, non-esterified fatty acids were greater in G1 than G2 and G0 and β -hydroxybutyrate was greater in G1 and G2 than G0 cows. Plasma insulin was greater and insulin-like growth factor (IGF)-I tended to be greater in G0 than G2 cows, leptin was greater in G2 and G0 and adiponectin were greater in G2 cows. Hepatic expression of growth hormone receptor-1A and *IGF1* mRNA decreased while *IGF binding proteins 1,2,4,5 and 6 (IGFBP)* mRNA as well as mRNA expression of insulin, leptin (*LEPRb*) and adiponectin-2 receptors increased from pre to postpartum in all cows. However, only hepatic *IGFBP6* and *LEPRb* mRNA were greater in G2 than G0 and G1 cows, respectively.

Conclusion: Metabolic-endocrine profiles of cows with different feeding strategies in early lactation reflected not only changes in milk energy output and energy balance but also in walking and grazing activity. Concentrations of insulin and IGF-I were increased in G0 cows whereas plasma adiponectin and both, insulin and leptin sensitivity were improved G2 cows. Increased NE demands in G2 cows when compared to G1 and G0 cows, implied a metabolic stress that impacted negatively on reproductive function.

*Correspondence: lauaste@gmail.com

¹ Department of Animal Production and Pastures, School of Agronomy, Universidad de la República (UdelaR), Av. E. Garzón 780, C.P. 12900 Montevideo, Uruguay

Full list of author information is available at the end of the article



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Background

Pasture-based dairy production systems have gained interest during the last decade due to their economic, environmental and animal-welfare advantages [1]. However, these production systems do not allow cows to approach their genetic potential for milk production and dry matter intake (DMI), especially in early lactation [2]. Maximizing DMI is crucial for a successful establishment of high lactation as the onset of lactation is accompanied by a period of negative energy balance during which, the high-producing dairy cows mobilize body energy reserves and lose body condition score (BCS) to support the copious amount of milk produced [3, 4].

The transition between late pregnancy and early lactation involves coordination among organs and tissues, including the mammary gland and metabolically active tissues like liver and adipose tissue. This is achievable, among other mechanisms, because the liver becomes refractory to growth hormone (GH) uncoupling the somatotrophic axis. The uncoupling of the GH-IGF axis is associated to a catabolic endocrine state of increased GH and decreased IGF-I concentrations that, together with low concentrations of insulin, support tissue mobilization, increased liver gluconeogenesis, and high peak milk production [3, 5]. In addition, adipose tissue plays its role not only in the storage and mobilization of lipids but also as an active endocrine tissue sensing metabolic signals and secreting hormones (i.e. leptin and adiponectin) that affect whole-body energy homeostasis through modulation of glucose and fatty acids metabolism in peripheral tissues, and central regulation of feed intake and energy expenditure [6–8]. Improving nutrition of the postpartum dairy cow will not only impact on milk production but also will minimize the negative effects of the catabolic situation (i.e. IGF-I and insulin) on reproductive performance [9].

In grazing dairy cows, it has been reported that energy intake is the primary determinant of milk production. Thus, most grazing systems incorporate supplementary feeds in the form of forage and concentrates and more recently as mixed rations [10]. Supplementing grazing dairy cows with mixed rations has the potential to capitalize on the benefits of formulated total mixed rations (TMR) while maintaining a relatively low-cost feeding system based on grazed pasture. Previous research has demonstrated that high levels of supplement, as TMR, increased DMI and milk production of grazing dairy cows [10].

Herbage intake in grazing dairy cows is not only limited by physiological and behavioral constraints but also by sward characteristics and grazing management constraints [10, 11]. Previous reports indicated that daily herbage allowance had a major role in milk production

and/or days to first ovulation in supplemented primiparous cows in early lactation, although the very low proportion of the allowed grazing time is spent grazing [9, 12]. Thus, daily access time to pasture and number of grazing sessions could be also used as grazing management tool to improve pasture DMI and milk production, as well as to increase grazing efficiency in dairy cows [11].

The hypothesis of this work was that TMR feeding will result in greater milk production and better reproductive performance than grazing dairy cows, and that the increase of the daily access time at pasture together with the number of grazing sessions during the first 60 days of lactation will maximize pasture DMI and improve energy balance of dairy cows in early lactation. The aim of the present study was to evaluate metabolic and endocrine profiles and hepatic gene expression of Holstein cows fed either TMR *ad libitum* (without grazing) or diets combining TMR (50 % *ad libitum* DM intake) and pasture with different grazing strategies (6 h in one grazing session or 9 h in two grazing sessions) in early lactation.

Methods

The experiment was carried out at the Experimental Station “Dr. Mario A. Cassinoni” of the School of Agronomy (EEMAC, Paysandú, Uruguay) from March to June 2011. Animal procedures were approved by the Animal Experimentation Committee of the Universidad de la República.

Animals and experimental design

Pluriparous dairy cows ($n = 27$, third-lactation cows, body weight (BW) = 709 ± 52.5 kg, BCS = 3.25 ± 0.25) were used in a complete randomized block design. Cows were grouped according with their expected calving date ($3/22/11 \pm 2.3$ days $n = 15$), and $4/11/11 \pm 4.8$ days, $n = 12$) blocked within groups according with BW and BCS and randomly assigned within block to one of three feeding strategies from calving (day 0) to 60 days postpartum (days): control cows fed TMR *ad libitum* (G0), pasture grazing with 6 h of access to paddock in one grazing session (8:00–14:00 h) and supplemented with TMR (G1), and pasture grazing with 9 h of access to paddock in two grazing sessions (8:00–14:00 and 17:00–20:00 h) and supplemented with TMR (G2). Two cows of G2 were removed from the study due to lameness thus the final treatment groups were TMR ($n = 9$), G1 ($n = 9$) and G2 ($n = 7$).

During the prepartum period (from -40 ± 6 days to calving) the experimental herd was managed to achieve a BCS at calving between 3.0 and 3.5 (1–5 scale; [13]). During this period, a TMR of corn silage and concentrate was fed to the cows to prevent BCS loss. During the postpartum period, all cows were assigned to experimental diets and were offered the same amount of DM (*ad libitum*,

approximately 30.7 kg DM/cow/d) only as TMR (for G0 cows) or 50 % DM as pasture and 50 % as TMR (for G1 and G2 cows). Cows (G1 and G2) grazed a second year perennial pasture of *Festuca arundinacea*, *Trifolium repens* and *Lotus corniculatus* (located 1.7 km from the milking parlour) in a 7-d rotational system with a mean herbage allowance of 15 kg DM/cow/d (4 cm above ground level) with 325 ± 1.8 g/kg of DM, 165 ± 1 g/kg DM of crude protein (CP), 479 ± 3.1 g/kg DM of neutral detergent fiber (NDF), 236 ± 0.5 g/kg DM of acid detergent fiber (ADF), and 1.6 Mcal/kg of net energy of lactation (NEL). The TMR, had a forage/concentrate ratio of 45/55 (DM basis) and was composed by corn or sorghum silage and a concentrate that included dry ground corn (19 %), wheat grain (12 %), soybean expeller (9 %), sunflower expeller (11 %), urea (3 %), minerals and vitamins (9 %) with a chemical composition of 544 ± 36 g/kg of DM, 158 ± 20 g/kg DM of CP, 309 ± 31 g/kg DM of NDF, 165 ± 16 g/kg DM of ADF, and 1.6 Mcal/kg of NEL. This TMR was formulated according to NRC [14] for a milk production target of 40 kg/d and a 15 % feed refusal. The TMR was offered once a day in the afternoon to G1 and G2 cows and twice a day (40 % in the morning and 60 % in the afternoon) to the G0 cows.

The proportion of TMR and pasture in the diet (DM basis) calculated for each treatment after the DM intake of TMR (based on difference between feed offered and refused) and pasture (based on alkane-dosing) was determined, indicated that diet was composed of 27 % pasture and 73 % TMR for G1 cows and 34 % pasture and 66 % TMR for G2 cows [15]. Nutrient composition of estimated diets are presented in Table 1.

Cows were milked twice a day (5:00 and 15:00 h), milk production was determined daily and milk samples

were obtained weekly to determine fat, protein and lactose composition. Cow BCS and BW were measured weekly and blood samples were obtained weekly from -40 to +60 days immediately after the morning milking by venipuncture of the coccygeal vein in heparinized tubes. Samples were centrifuged ($2000 \times g$ for 15 min at 4 °C) within 2 h after collection and plasma was stored at -20 °C until assayed. Liver biopsies were obtained from cows at -40 $\pm$ 6, -20 $\pm$ 3, +10 $\pm$ 4 and +55 $\pm$ 4 days using a 14-gauge biopsy needle (Tru-Core®-II Automatic Biopsy Instrument; Angiotech, Lausanne, Switzerland) as described by Carriquiry et al. [16]. Liver samples were immediately frozen in liquid nitrogen and stored at -80 °C until total RNA was isolated.

Metabolite and hormone analyses

The metabolic profiles (non-esterified fatty acids (NEFA), β -hydroxybutyrate (BHB), glucose and urea) were determined by colorimetric assays on Vitalab Selectra II auto-analyzer (Vital Scientific, Dieren, The Netherlands) using commercial kits (Wako NEFA-HR(2), Wako Pure Chemical Industries Ltd., Osaka, Japan for NEFA; Randox Laboratories Limited, 55 Diamond Road, Crumlin, Country Antrim, BT29 4QY, United Kingdom for BHB; Wiener Laboratories S.A.I.C. Riobamba, Rosario, Argentina for glucose and urea). All samples were determined in the same assay for each metabolite, the intra-assay CV for all determinations was less or equal than 10 %.

Concentrations of insulin and IGF-I were measured using immunoradiometric assays (IRMA) with commercial kits (INS-IRMA; DIA Source Immune Assays S.A., Belgium and IGF-I-RIACT Cis Bio International, GIF-SUR-YVETTE CEDEX, France, respectively) previously used in bovine [17]. All samples were determined in a single assay for each hormone. For insulin, the assay detection limit was 0.7 μ U/ml, and intra-assay CV for control 1 (22.3 μ U/ml) and 2 (55.5 μ U/ml) were 8.2 and 8.3 %, respectively. For IGF-I, the assay detection limit was 0.3 ng/ml, and intra-assay CV for control 1 (41.1 ng/ml) and control 2 (521.5 ng/ml) were 7.8 and 7.9 %, respectively.

Leptin concentrations were determined by a liquid-phase radioimmunoassay (RIA) using a commercial Multi-Species Leptin kit (RIA kit, Millipore, USA) previously reported in bovines [17, 18]. The RIA had a sensitivity of 2.9 ng/ml. All samples were determined in the same assay and the intra-assay CV for control 1 (4.2 ng/ml) and control 2 (18.8 ng/ml) were 8.2 and 7.4 %, respectively. In the absence of purified bovine adiponectin, concentrations of adiponectin were measured with a human RIA kit (HADP-61 HK, Millipore, USA) using undiluted plasma samples [18, 19]. The sensitivity of the assay was 1.54 ng/ml. All samples were determined in the same

Table 1 Estimated nutrient composition of diets according to feeding strategy in early lactation

Component ^a	Treatments ^b		
	G0	G1	G2
DM, g/kg	613	532	512
CP, g/kg	156	156	159
Digestible RUP, % of DM	79.5	78.0	77.7
NDF, g/kg	336	308	301
ADF, g/kg	204	216	219
Ash, g/kg	45	62	66
NEL, Mcal/d	1.55	1.58	1.60

^a Nutrient composition calculated from TMR and pasture DMI estimated by Fajardo et al. [15] and feed sample chemical analyses

^b Feeding strategies from calving (day 0) to 60 days postpartum: G0 DM offered as 100 % total mixed ration (TMR; n = 9), G1 DM offered as 50 % pasture in one (am) grazing session (6 h) plus 50 % TMR (n = 9), G2 DM offered as 50 % pasture in two (am/pm) grazing sessions (9 h) + 50 % TMR (n = 7)

assay and the intra-assay CV for control 1 (12.2 ng/ml) and control 2 (95.4 ng/ml) were 6 and 12 %, respectively.

Days to first ovulation were determined by progesterone milk concentrations twice a week. Milk was skimmed at 3000 rpm at 4 °C for 15 min. Progesterone concentrations in skim milk were measured by a solid-phase RIA using a commercial kit (Coat and Count; Diagnostic Products, Los Angeles, CA, USA). All samples were analyzed in a single assay; the sensitivity was 0.01 ng/ml, the intra-assay CV was not greater than 10.6 %. Days to first ovulation was defined as the day in which progesterone concentration in milk had two consecutive samples greater than 1 nmol/l.

Isolation and purification of RNA

Isolation of total RNA from hepatic tissue and synthesis of cDNA by reverse transcription was performed according with Carriquiry et al. [16] (see Additional file 1). Primers (Additional file 1) to specifically amplify cDNA of target genes: *GHR*, *GHR1A*, *IGF1*, *IGF2*, *IGF binding proteins-1 to 6* (*IGFBP1*, *IGFBP2*, *IGFBP3*, *IGFBP4*, *IGFBP5*, *IGFBP6*), insulin receptor (*INSR*), long form of the leptin receptor (*LEPRb*), adiponectin receptor 1 and 2 (*ADIPOR1*, *ADIPOR2*), and from endogenous controls: β -actin (*ACTB*), hypoxanthine phosphoribosyltransferase (*HPRT*), and ribosomal protein S9 (*RPS9*), were obtained from literature or specifically designed using the Primer3 website (<http://frodo.wi.mit.edu/primer3/>) based on bovine nucleotide sequences available from NCBI (<http://www.ncbi.nlm.nih.gov/>). Before use, primer product sizes (1 % agarose gel separation) and sequences (Macrogen Inc., Seoul, Korea) were determined to ensure that primers produced the desired amplicons.

Real time PCR reactions were performed in a total volume of 15 μ l using KAPA SYBR® FAST Universal 2X qPCR Master Mix (Kapa Biosystems, inc. Woburn, MA, USA) according with Astessiano et al. [20], using the following standard amplification conditions: 10 min at 95 °C and 40 cycles of 15 s at 95 °C, 45 s at 60 °C, and 20 s at 72 °C. Dissociation curves were run on all samples to detect primer dimers, contamination, or presence of other amplicons. Each disk included a pool of total RNA from bovine liver samples analyzed in triplicate to be used as the basis for the comparative expression results (exogenous control) and duplicate tubes of water (non-template control). Gene expression was measured by relative quantification [21] to the exogenous control and normalized to the geometric mean expression of the endogenous control genes (*HPRT*, *ACTB* and *RPS9*). Expression stability of 3 selected housekeeping genes was evaluated using MS-Excel add-in Normfinder (MDL, Aarhus, Denmark). The stability values obtained with Normfinder they were 0.144, 0.121, and 0.178 for

HPRT, *ACTB*, and *RPS9*, respectively. Amplification efficiencies or target and endogenous control genes were estimated by linear regression of a dilution cDNA curve ($n = 5$ dilutions, from 100 to 6.25 ng/tube; Additional file 1). Intra and inter-assay CV values were 1.9 and 4.2 %, respectively.

Calculations and statistical analyses

Net energy (NE) and metabolizable protein (MP) calculations were based on NRC [14]. Maintenance NE requirements were calculated as $NEM = 0.08 \times BW^{0.75} + NEmact$, where $NEmact = (((Distance/1000) \times Trips) \times (0.00045 \times BW)) + (0.0012 \times (BW))$. Lactation NE requirements were calculated as $NEL = milk\ yield \times [(0.0929 \times fat\ \%) + (0.0563 \times true\ protein\ \%) + (0.0395 \times lactose\ \%)]$, using composition data derived from analysis of samples collected weekly. Metabolizable protein required for maintenance was calculated as $MPM = 4.1 \times (BW^{0.50}) + 0.30 \times (BW^{0.60}) + ((DMI \times 30) - [0.50 \times (bacterial\ MP/0.8)]) - bacterial\ MP + endogenous\ MP/0.67$. Metabolizable protein required for lactation (MPL) was calculated as $MPL = (milk\ yield \times true\ protein\ \%)/0.67$.

Estimation of the revised quantitative insulin sensitivity check index (RQUICKI) was done according to Perseghin et al. [22], i.e. $RQUICKI = 1/[log(Glucose, mg/dl) + log(Insulin, \mu U/ml) + log(NEFA, mmol/l)]$, in which a low RQUICKI index indicates decreased insulin sensitivity.

Data were analyzed in a randomized block design using the SAS System program (SAS Institute Inc., Cary, NC, USA). Univariate analyses were performed on all variables to identify outliers and inconsistencies and to verify normality of residuals. Data of BCS, energy and MP balances and their components, plasma metabolite and hormone concentrations and hepatic mRNA expression were analyzed by repeated measures using the MIXED procedure with days postpartum as the repeated effect, and the appropriate covariance structure [first-order autoregressive (AR(1)) for evenly spaced data or spatial power (SP(POW)) for unevenly spaced data]. The Kenward-Rogers procedure was used to adjust the denominator degree of freedom. Data were analyzed with a model that included period (pre or postpartum), nutritional treatment within period (no treatment for period prepartum and G1, G2 and G0 for period post partum), days postpartum within period, and the interaction between treatment and days within period as fixed effects and replication and block within replication as random effects. The interaction between treatment and replication was included in the model as a random effect but as covariance parameter estimates were zero or close to zero it was removed from the model.

Days to first ovulation was defined as the number of days from calving to reinitiation of ovarian cyclicity. The probability for first ovulation was estimated by the proportion of cows with first ovulation confirmed every 5 days from day 15 to 60 postpartum. Days to first ovulation and probability of cows cycling during the first month were analyzed with a generalized lineal model using the GENMOD procedure with a model that included the fixed effect of nutritional treatment and with the Poisson and a log link or binomial distribution and a logit link specified, respectively. Tukey–Kramer tests were conducted to analyze differences between groups ($\alpha = 0.05$). For all results, means were considered to differ when $P \leq 0.05$, and trends were identified when $0.05 < P \leq 0.10$. Data are presented as least square means $\pm$ pooled standard errors.

Results

Cow productive and reproductive performance

Milk energy output tended ($P = 0.09$) to be greater for G0 than G1 cows but not for G2 cows (Table 2; Fig. 1a), whereas, estimated NEM during the first 60 days was the greatest ($P < 0.05$) for G2 and the lowest for G0 cows due to differences in NE requirement for waking and grazing activity. In contrast, milk MP requirement did not differ among treatments (Table 2). However, both estimated NEL and MP balances were greater ($P = 0.01$) for G0 than G1 and G2 cows.

Cow BCS decreased ($P < 0.05$) from -20 to $+55$ days and at $+55$ days was greater ($P < 0.05$) in G0 than G2 cows (Table 2; Fig. 1b). Days to first ovulation were greater ($P = 0.02$) for G2 than G1 cows and intermediate for G0 ($21, 40$ and 28 ± 5 days for G1, G2 and G0, respectively) while probability of cows cycling during the first month of lactation was greater ($P < 0.01$) for G0 and G1 than G2 cows ($89, 43$ and 71% for G1, G2 and G0, respectively) (Fig. 1c).

Plasma metabolites and hormones

Concentrations of NEFA peaked between $+5$ and $+12$ days and during the postpartum were greater ($P = 0.02$) in G1 than G2 and G0 cows, as NEFA concentrations remained elevated at $+30$ and $+40$ days only in former ones (Fig. 2a). Concentrations of BHB peaked between $+15$ and $+30$ days and during the postpartum were greater ($P < 0.05$) in G1 and G2 than G0 cows (Fig. 2b). Plasma glucose and urea were not affected by treatment, but varied during the transition and early lactation periods. Plasma glucose concentrations increased ($P < 0.01$) from calving to $+55$ days, while plasma urea concentrations decreased ($P < 0.01$) from -40 to calving and then increased ($P < 0.01$) until $+55$ days (data not shown).

Table 2 Effect of early feeding strategy on cow BW, BCS, and estimated net energy of lactation (NEL) and metabolizable protein (MP) balances

Variable	Treatments ¹			SE	P value
	G0	G1	G2		
Estimated NEL balance ²					
Maintenance, Mcal of/d	10.3 ^c	12.8 ^b	13.9 ^a	0.03	0.01
Milk, Mcal/d	27.8 ^x	24.0 ^y	26.5 ^{xy}	1.25	0.09
Balance, Mcal/d	2.7 ^a	−5.2 ^b	−5.6 ^b	1.30	0.01
Estimated MP balance ²					
Milk, g of MP/d	1780	1620	1660	70.24	0.30
Balance, g of MP/d	0.08 ^a	−0.26 ^b	−0.34 ^b	0.08	0.01
BW, kg	646	655	618	15	0.23
BCS, units	2.9	2.8	2.8	0.07	0.53

¹ Feeding strategies from calving (day 0) to 60 days postpartum: DM offered as 100 % total mixed ration (TMR; n = 9), G1 DM offered as 50 % pasture in one (am) grazing session (6 h) plus 50 % TMR (n = 9), G2 DM offered as 50 % pasture in two (am/pm) grazing sessions (9 h) + 50 % TMR (n = 7)

² Required NEL and MP for maintenance (including NEL for activity) and milk, values were calculated according to NRC [14]

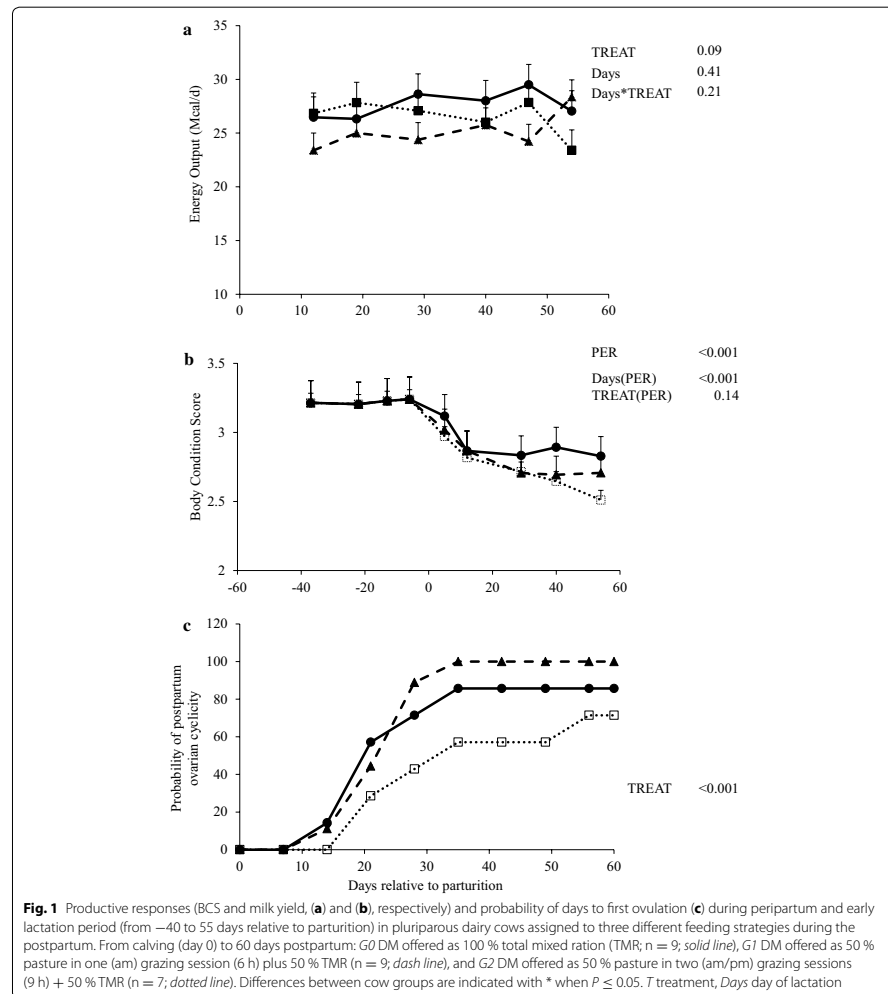
^{a,b,c} Letters denote least squares means differ ($P \leq 0.05$)

^{xy} Indicate a tendency $0.05 < P > 0.10$, for the interaction between cow groups and treatments

Plasma insulin concentrations decreased ($P = 0.02$) around calving and during the postpartum period were greater ($P = 0.02$) in G0 than G2 cows (Fig. 2c). Plasma IGF-1 concentrations decreased ($P < 0.01$) from -40 to $+5$ days and increased thereafter until $+55$ days, but prepartum concentrations were not recovered at the end of the experimental period, and during the postpartum period tended to be greater ($P = 0.06$) in G0 and G1 than G2 cows (Fig. 2d). Leptin concentrations decreased ($P < 0.01$) from -40 days to calving, increasing thereafter and during the postpartum were greater ($P < 0.01$) in G2 and G0 than G1 cows (Fig. 2e). Adiponectin concentrations increased ($P < 0.01$) from pre to postpartum and during the postpartum were greater ($P = 0.01$) in G2 than G1 and G0 cows as plasma adiponectin were elevated at $+15$ and $+30$ days only in the former ones (Fig. 2f). However, the adiponectin to leptin ratio was not affected by treatment or days of lactation (averaged $95.6, 83.3, 70.0 \pm 24.4$ for G1, G2 and G0 cows, respectively). The RQUICKI index decreased ($P < 0.01$) from -40 days to calving, and during the postpartum was greater ($P = 0.02$) in G2 than G1 and intermediate in G0 cows.

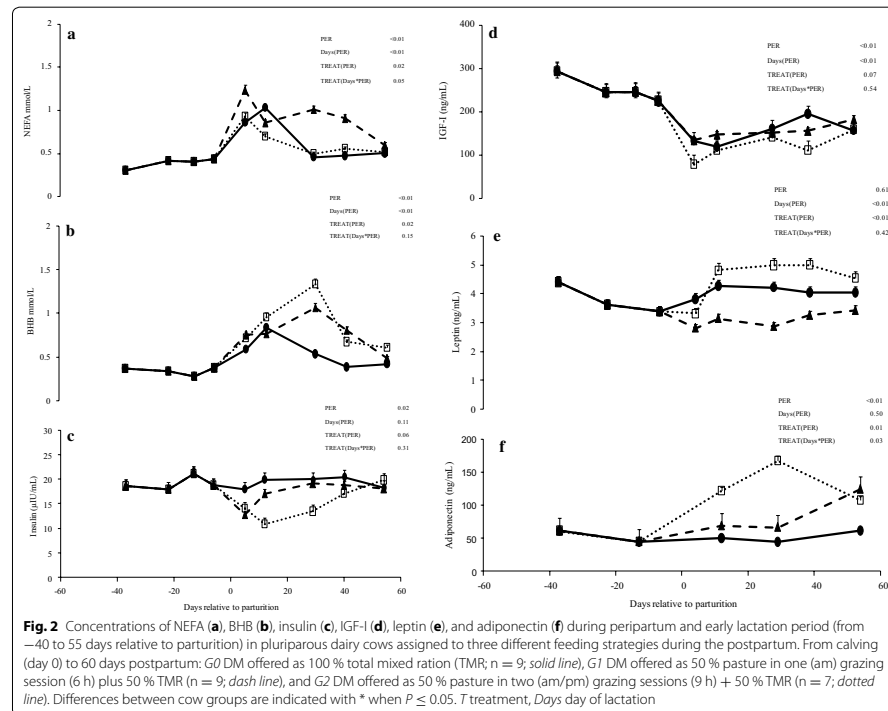
Hepatic gene expression

Hepatic *GHR* mRNA was not affected by period, but tended to be affected by treatment ($P = 0.09$) being 1.8-fold greater in G1 than G2 cows during the postpartum (Table 3). However, expression of *GHR1A*



decreased ($P < 0.01$) and *IGF1* mRNA tended to decrease ($P = 0.08$) from pre to postpartum while *IGF2* mRNA increased ($P = 0.002$) at +55 days for all cows (Table 3). Hepatic *IGFBP1*, *IGFBP4* and *IGFBP5* mRNA increased ($P \leq 0.04$) from pre to postpartum while *IGFBP2* mRNA increased ($P < 0.01$) from -20 vs. +10 days and tended

to decrease again ($P = 0.06$) at +55 days (Table 3). In contrast, *IGFBP3* mRNA tended ($P = 0.08$) to increase from -40 to -20 days and decreased ($P < 0.01$) from -20 to +10 days (Table 3). Expression of *IGFBP6* mRNA tended ($P = 0.07$) to increase from pre to postpartum and to be affected by treatment, being greater in G2 than



G0 and intermediate in G1 cows during the postpartum (Table 3).

Expression of *INSR* mRNA in the liver increased ($P < 0.03$) at + 55 days for all cows (Table 4). Hepatic *LEPRb* mRNA increased ($P < 0.01$) from pre to postpartum and was greater ($P < 0.05$) in G2 than G1 cows during early lactation (Table 4). Although *ADIPOR1* mRNA did not vary due to feeding strategies or days of lactation, *ADIPOR2* mRNA increased ($P < 0.01$) from pre to postpartum in all cows (Table 4).

Discussion

Days of lactation on metabolic and endocrine profiles and liver gene expression

During the peripartum and early lactation, all cows entered a period of negative energy balance, as the rapid increase in milk yield was not met by DMI, determining various metabolic and endocrine adaptations to provide

energy and nutrients to the early lactating mammary gland [3]. Cows mobilized body reserves, reflected by the loss of BCS and the elevated plasma concentrations of NEFA and BHB in blood that indicated increased lipolysis and ketogenesis [4, 9]. In addition, plasma urea decreased around calving and increased during early lactation probably, reflecting enhanced tissue protein breakdown to provide gluconeogenesis precursors as well as the increase feed intake [23]. Insulin and IGF-I concentrations decreased around calving which was consistent with the reduction in DMI, characteristic of this period and with an uncoupled somatotrophic axis, which mediates nutrient partitioning towards milk production [3, 5]. Indeed, the IGF-I decrease at calving was associated with *GHR1A* and *IGF1* mRNA decreases during the early postpartum while no changes were detected in the abundance of hepatic *GHR* mRNA as reported before [24]. Decreased insulin concentrations are hypothesized to

Table 3 Hepatic expression of genes related to the GH-IGF axis during the peripartum and early lactation periods (from –40 to 55 days relative to parturition) of dairy cows assigned to three different feeding strategies during the first 60 days of lactation

Gene ³	Days	All ⁴	Treatments ¹			SE	P value ²		
			G0	G1	G2		PER	Days (PER)	TREAT (PER)
<i>GHR</i>	–40	1.00	–	–	–	–	0.39	0.27	0.09
	–20	0.88	–	–	–	–			
	10	0.75	0.78 ^{ab}	0.91 ^{ab}	0.56 ^b	0.57			
	55	1.01	0.88 ^{ab}	1.47 ^a	0.69 ^b	0.56			
<i>GHR1A</i>	–40	1.00 ^a	–	–	–	–	<0.01	0.10	0.97
	–20	0.91 ^a	–	–	–	–			
	10	0.56 ^b	0.58	0.58	0.51	0.19			
	55	0.41 ^c	0.42	0.40	0.41	0.18			
<i>IGF1</i>	–40	1.02 ^{xy}	–	–	–	–	0.08	0.70	0.20
	–20	1.12 ^x	–	–	–	–			
	10	0.84 ^y	0.58	0.79	1.17	0.32			
	55	0.92 ^{xy}	0.74	1.11	0.91	0.31			
<i>IGF2</i>	–40	1.00 ^b	–	–	–	–	<0.01	0.05	0.91
	–20	1.20 ^b	–	–	–	–			
	10	1.29 ^b	1.20	1.27	1.39	0.43			
	55	1.76 ^a	1.80	1.98	1.51	0.48			
<i>IGFBP1</i>	–40	1.00 ^b	–	–	–	–	<0.01	0.42	0.25
	–20	1.15 ^b	–	–	–	–			
	10	1.96 ^a	2.09	1.56	2.25	0.93			
	55	2.44 ^a	3.25	1.85	2.20	0.97			
<i>IGFBP2</i>	–40	1.00 ^b	–	–	–	–	<0.01	0.10	0.94
	–20	1.24 ^b	–	–	–	–			
	10	3.35 ^a	3.46	3.20	3.38	0.79			
	55	2.43 ^a	2.25	2.78	2.26	0.81			
<i>IGFBP3</i>	–40	1.00 ^{ab}	–	–	–	–	0.09	0.10	0.54
	–20	1.25 ^a	–	–	–	–			
	10	0.91 ^b	1.10	0.74	0.90	0.24			
	55	1.05 ^{ab}	1.00	1.04	1.10	0.24			
<i>IGFBP4</i>	–40	1.00 ^{ab}	–	–	–	–	0.04	0.54	0.73
	–20	0.97 ^b	–	–	–	–			
	10	1.14 ^{ab}	1.05	1.04	1.32	0.41			
	55	1.35 ^a	1.25	1.34	1.45	0.40			
<i>IGFBP5</i>	–40	1.00 ^b	–	–	–	–	<0.01	0.62	0.74
	–20	1.10 ^b	–	–	–	–			
	10	2.03 ^a	2.09	1.60	2.41	0.84			
	55	1.75 ^a	1.36	2.03	1.85	0.94			

Table 3 continued

Gene ³	Days	All ⁴	Treatments ¹			SE	P value ²		
			G0	G1	G2		PER	Days (PER)	TREAT (PER)
IGFBP6							0.08	0.71	0.06
	—40	1.00 ^y	—	—	—	—			
	—20	0.97 ^y	—	—	—	—			
	10	1.13 ^{xy}	0.93 ^b	1.01 ^{ab}	1.46 ^{ab}	0.34			
	55	1.29 ^x	0.86 ^b	1.33 ^{ab}	1.68 ^a	0.34			

¹ Feeding strategies from calving (day 0) to 60 days postpartum: G0 DM offered as 100 % total mixed ration (TMR; n = 9), G1 DM offered as 50 % pasture in one (am) grazing session (6 h) plus 50 % TMR (n = 9), G2 DM offered as 50 % pasture in two (am/pm) grazing sessions (9 h) + 50 % TMR (n = 7)

² PER period (pre vs. postpartum), TREAT treatment. The interaction TREAT (PER) × Days was not significant (P > 0.25) for any of the genes

³ GHR = growth hormone receptor, GHR1A = growth hormone receptor 1A, IGF1 = insulin-like growth factor-1, IGF2 = insulin-like growth factor-2, IGFBP = IGF-binding protein 1 to 6. Relative units, data are expressed as fold difference relative to first sample (—40 days)

⁴ Lsmeans for Days(PER) effect, including all treatments

^{a,b,c} Letters denote least squares means differ (P ≤ 0.05)

^{xy} Indicate a tendency 0.05 < P > 0.10, according to Tukey–Kramer test for the interaction between PER, Days and TREAT

Table 4 Hepatic expression of genes related to the energy metabolism during the peripartum and early lactation periods (from —40 to 55 days relative to parturition) in multiparous dairy cows assigned to three different feeding strategies during the first 60 days of lactation

Gene ³	Days	All ⁴	Treatments ¹			SE	P value ²		
			G0	G1	G2		PER	Days (PER)	TREAT (PER)
INSR							<0.01	0.04	0.92
	—40	1.00 ^b	—	—	—	—			
	—20	0.91 ^b	—	—	—	—			
	10	1.12 ^{ab}	1.21	1.00	1.14	0.25			
	55	1.55 ^a	1.42	1.84	1.39	0.24			
LEPRB							<0.01	0.15	0.02
	—40	1.00 ^b	—	—	—	—			
	—20	1.41 ^{ab}	—	—	—	—			
	10	1.49 ^a	1.61 ^{abc}	1.05 ^c	1.80 ^{ab}	0.47			
	55	1.58 ^a	1.55 ^{abc}	1.17 ^{bc}	2.03 ^a	0.48			
ADIPO1							0.33	0.40	0.99
	—40	1.00	—	—	—	—			
	—20	0.95	—	—	—	—			
	10	0.98	0.98	0.96	1.00	0.67			
	55	1.16	1.15	1.18	1.14	0.72			
ADIPO2							<0.01	0.59	0.91
	—40	1.02 ^b	—	—	—	—			
	—20	0.92 ^b	—	—	—	—			
	10	2.27 ^a	2.38	2.31	2.10	0.30			
	55	2.06 ^a	1.83	2.26	2.09	0.30			

¹ Feeding strategies from calving (day 0) to 60 days postpartum: G0 DM offered as 100 % total mixed ration (TMR; n = 9), G1 DM offered as 50 % pasture in one (am) grazing session (6 h) plus 50 % TMR (n = 9), G2 DM offered as 50 % pasture in two (am/pm) grazing sessions (9 h) + 50 % TMR (n = 7)

² PER period (pre vs. postpartum), TREAT treatment. The interaction TREAT(PER) × Days was not significant (P > 0.25) for any of the genes

³ INSR = insulin receptor, LEPRB = full-length leptin receptor, ADIPO1 = adiponectin receptor 1, ADIPO2 = adiponectin receptor 2. Relative units, data are expressed as fold difference relative to first sample (—40 days)

⁴ Lsmeans for Days (PER) effect, including all treatments

^{a,b,c} Letters denote least squares means differ (P ≤ 0.05)

be a key signal regulating this mechanism, as stimulates hepatic IGF-I secretion by modifying hepatic GHR and IGFBP secretion [25].

Most IGF-I is bound to IGFBP3 in a ternary complex, the negative energy balance would induce a shift of this complex to binary complexes in which IGF-I is bound to IGFBP1 and particularly to IGFBP2, which would in turn reduce half-life of IGF-I in blood [26]. The increased hepatic *IGFBP1* and *IGFBP2* mRNA expression and the simultaneous decrease of *IGFBP3* mRNA in all cows have been shown before [20, 27] and support this hypothesis. Indeed, Kessler et al. [28], demonstrated that as plasma IGF-I concentrations dropped during the lactation negative energy balance or during feed restriction, binding affinity of IGF-I to IGFBP1 and IGFBP2 in plasma increased while its binding affinity to IGFBP3 decreased. Moreover, *IGFBP4* and *IGFBP5* mRNA also increased during the postpartum (at +55 and +10 DPP, respectively) and it has been reported that these proteins may have also inhibitory effects on IGF-I activity [29, 30]. On the other hand, in the present study, IGF-I concentrations started to increase after the first month postpartum while *GHR1A* mRNA expression remained low and hepatic mRNA of low molecular weight *IGFBP* were still elevated, thus, other factors would be responsible for the increasing IGF-I concentrations during the second month after calving.

Interestingly, hepatic *IGF2* and *IGFBP6* mRNAs, a binding protein with high affinity to IGF-II, were low during peripartum and increased at +55 days for all cows. Relatively few studies have examined IGF-II during the transition period of the dairy cow, although its transcript is expressed at comparatively high levels in the liver [31]. The greater expression of *IGF2*, *IGFBP6* and also *IGFBP4* mRNA together with the greater probability of cycling after the first month postpartum is consistent a role of these genes in reproduction [32–34].

The reduced insulin and elevated NEFA concentrations during the early postpartum, determined that the in the present study RQUICKI decreased during this period, which is indicative of increased reduced insulin resistance [35]. Indeed, the adipokines adiponectin and leptin, are considered positive regulators of insulin sensitivity in various species [6, 7]. Reduced concentrations of these hormones around calving, as reported here, may increase insulin resistance of peripheral tissues, which would decrease glucose uptake by skeletal muscles and adipose tissues and increase hepatic gluconeogenesis [7], improving glucose supply to the mammary gland for enhanced milk production. Although adiponectin and leptin profiles during early postpartum period depended of the treatment (see “Discussion” below), the concentrations increased rapidly during the early postpartum.

Similar findings were reported for adiponectin [36], but not for leptin [6]. Differences in leptin profiles during the postpartum between this and other reports could be the result of the restricted feeding dry period nutrition (vs. ad libitum) in the present study. In addition, Weber et al. [37] reported elevated leptin concentrations in dairy cows with moderate hepatic lipid fat associated with reduced abdominal fat mobilization, and in agreement with our results, with reduced insulin and NEFA concentrations and greater insulin sensitivity based on RQUICKY.

Insulin and adipokines allow for the communication of pancreas and adipose tissue with other body organs such as liver, to regulate, among others, energy metabolism and immune functions through not only alteration in blood concentrations, but also differential tissue regulation of receptor expression during the lactation cycle. Effects of leptin are mainly mediated by *LEPRb* [8] and of adiponectin via *ADIPOR1* and *ADIPOR2*, with *ADIPOR1* predominantly expressed in skeletal muscle and *ADIPOR2* in liver [7]. Consistent with the literature [8, 38], in the present study, hepatic *ADIPOR1* mRNA abundance was stable whereas *ADIPOR2* and *LEPRb* mRNA increased in early lactation, counteracting reduced adiponectin and leptin concentrations around calving. This increase in *ADIPOR2* and *LEPRb* mRNA would favor a role of these hormones on insulin sensitivity in the liver, inducing fatty acid oxidation and preventing lipid accumulation [7, 38]. Hepatic *INSR* mRNA increased during postpartum and was greater at +55 days in all cows. Our data are consistent with reports that found greater liver *INSR* mRNA in control cows than cows with fatty liver disease or ketotic dairy cows [39], which suggested that reduced expression of *INSR* mRNA in the liver during early postpartum indicates that responses to insulin are markedly decreased, which might be due to insulin resistance.

Effect of treatment on metabolic and endocrine profile and hepatic gene expression

In the present study, although there were differences in milk NE and MP output as well as NE maintenance requirements between cow groups, the greater estimated DMI [14], thus, the greater estimated NEL and MP intakes, would explain differences in NE and MP balances between G0 and grazing cows (G1 and G2). The NE and MP balances indicated that grazing cows (G1 and G2 cows) mobilized greater amounts of tissue which was partially reflected in different BCS at +55 days between G2 and G0 cows.

Nevertheless, plasma NEFA concentrations in early lactation did not differ between G0 and G2 cows and were less than in G1 cows. The reduced NEFA and BHB concentrations in G0 cows would indicate a low rate of

lipolysis and hepatic ketogenesis in agreement with their better energy balance [9]. In contrast, the reduced NEFA concentrations in G2 cows (when compared to G1 cows) that were accompanied with elevated BHB concentrations (not different than the observed in G1 cows), could probably be related to a differential tissue utilization of NEFA due to the increased walking and grazing activities in these cows. Plasma NEFA fuel, through mitochondrial fatty acid oxidation and Krebs cycle, energy production in muscle for muscle contraction or exercise, [3, 4]. Indeed, it has been suggested that increased walking activity could reduce plasma NEFA in dairy cows [40]. In addition, the greater plasma BHB in grazing cows (G1 and G2) could not only be related to an increased hepatic ketogenesis but also could reflect an increased synthesis of butyric acid in the rumen as fresh pastures were included in the diet [41]. The greater plasma insulin observed in G0 than G2 cows and IGF-I concentrations in G0 and G1 than G2 cows during the postpartum, was consistent with the greater non-fiber carbohydrate concentration, the reduced energy requirements for activity and the greater estimated energy balance in the former ones [10, 42].

In the present study, the lowest concentrations of insulin and NEFA in G2 cows determined an increased RQUICKI in this group indicative of greater insulin sensitivity [35]. Adiponectin and leptin, which have insulin sensitizing actions, were the greatest in G2 cows during early lactation. Circulating adiponectin concentrations, insulin sensitivity, and basal fat oxidation rate have been showed to increase with exercise in humans [43]. Therefore, greater walking and grazing activity because of the increased number of sessions (two grazing sessions) and access time to pasture (9 h) in G2 cows may explain these results.

We found and elevated expression of hepatic *IGFBP6* mRNA in G2 cows consistent with Fenwick et al. [27] that highlight a role for this binding protein in undernutrition or energy deficiency. Moreover, Cummins et al. [44] reported that cows with lower fertility had greater abundance of *IGFBP6* mRNA in the liver during the transition period in comparison with higher fertility cows. In agreement with the latter result, days to first ovulation were the longest and probability of cycling cows was the lowest in G2 cows. Indeed, Meikle et al. [9] reported that although there were no differences in milk production, dairy cows grazing medium sward herbage allowance showed a delayed reinitiation of ovarian cyclicity and lower BCS postpartum when compared to TMR and high herbage allowance grazing cows.

Conclusion

In this work, NE, but not MP, demands for maintenance and/or milk increased in G2 cows when compared with G1 and G0 cows, respectively. However, both NE and MP

balances were less in grazing cows (G1 and G2) than in G0 cows. Metabolic-endocrine profiles of cows with different feeding strategies in early lactation reflected not only changes in milk energy output and energy balance but also in walking and grazing activity. Concentrations of insulin and IGF-I were increased in G0 cows whereas plasma adiponectin and both, insulin and leptin sensitivity were improved G2 cows. The greater energy demands and/or reduced energy balance in G2 cows when compared to both G1 and G0 cows implied a metabolic stress that impacted negatively on reproductive function (delayed reinitiation of ovarian cyclicity and reduced probability of cycling cows).

Additional file

Additional file 1. Primers used for the quantification of target and endogenous control gene cDNA.

Authors' contributions

AA participated in the design of the study and field work, performed all laboratory analyses, carried out the statistical analysis and drafted the manuscript. MC participated in the study design, supervised and advised AA in all the stages of the work (field work, laboratory and statistical analyses), and helped to draft the manuscript and corrected it. PC conceived the study, participated in its design and revised the manuscript. AM participated in the study design, supervised and advised in metabolite and hormone analyses, and help to draft the discussion of the manuscript and corrected the drafted manuscript. DM, MF and JG participated in the coordination of the field work and in the acquisition of data and samples. All authors read and approved the final manuscript.

Author details

¹ Department of Animal Production and Pastures, School of Agronomy, Universidad de la República (UdelaR), Av. E. Garzón 780, C.P. 12900 Montevideo, Uruguay. ² Laboratory of Nuclear Techniques, School of Veterinary Medicine, Universidad de la República (UdelaR), C/Lasplacas 1550, C.P. 11600 Montevideo, Uruguay. ³ Department of Health in Livestock Systems, School of Veterinary Medicine, Universidad de la República (UdelaR), EEMAC, Paysandú C.P. 6000, Uruguay.

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Competing interests

The authors declare that they have no competing interests.

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3.2. METABOLIC ADAPTATIONS DUE TO THE INCLUSION OF PASTURE IN THE DIET OF DAIRY COWS FED TOTAL MIXED RATION DURING EARLY LACTATION. 2017. Astessiano A.L, Meikle A, Chilbroste P, Mattiauda D. A, Fajardo M, Carriquiry M. Este artículo fue publicado en *Open Journal of Animal Science*.

El objetivo fue estudiar las alteraciones en los perfiles metabólicos y endocrinos, así como la expresión hepática de los genes relacionados con la partición de nutrientes (ARNm de sistema *IGFs*, *INSR*, *LEPRB*) y el metabolismo energético (*G6PC*, *PC*, *PDH1A*, *ACOX*, *CPT1A*, *ACADVL*, *HMGC_oA*, *CS*) en vacas lecheras que cambiaron de dieta sin un período de adaptación. Las vacas fueron alimentadas 100% DTM durante los primeros 60 días, pasaron a un régimen de alimentación que incluyó una sesión de pastoreo (6 hrs) y con una reducción del 50% de la DTM.

La hipótesis que nos planteamos fue que el metabolismo de vacas lecheras alimentadas 100% DTM durante la lactancia temprana, se adaptaría rápidamente a cambios en el régimen de alimentación para hacer frente a cambios en la cantidad y tipo de nutrientes absorbidos.

Los resultados confirmaron que la regulación metabólica en vacas lecheras es un sistema dinámico, y los cambios en la estrategia de alimentación sin un período de adaptación modificaron el metabolismo animal (tendió a aumentar las concentraciones plasmáticas de IGF-I, aumentó la expresión hepática de ARNm *IGFBP5* e *IGFBP6*, mientras que ARNm *IGF1* e *IGFBP3* tendieron a ser mayores comparado con las vacas que no modificaron su alimentación). La inclusión del pastoreo en vacas que fueron alimentadas con DTM durante la lactancia temprana, indicó una redistribución de nutrientes y energía hacia requerimientos de mantenimiento (aumento caminata y pastoreo) en detrimento de la producción de leche.

Metabolic Adaptations Due to the Inclusion of Pasture in the Diet of Dairy Cows Fed Total Mixed Ration during Early Lactation

Ana Laura Astessiano^{1*}, Ana Meikle², Pablo Chilbroste¹, Diego Antonio Mattiauda¹, M. Fajardo¹, Mariana Carriquiry¹

¹Facultad de Agronomía, Universidad de la República, Montevideo, Uruguay

²Facultad de Veterinaria, Universidad de la República, Montevideo, Uruguay

Email: *lauaste@gmail.com

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Abstract

The aim of this study was to evaluate the metabolic adaptations due to the inclusion of pasture in the diet of dairy cows fed on a total mixed ration (TMR) *ad libitum* during early lactation. Multiparous cows (n = 18) were used in a randomized complete block design and were randomly assigned according to parity, BW and BCS to one of two feeding strategies from calving to 60 DIM: 1) cows fed TMR *ad libitum* (without access to pasture; 100% TMR) and 2) cows fed on a mixed system with pasture grazing (6 h of access to paddock in one grazing session, 8:00 to 14:00 h) and supplemented with 50% of *ad libitum* TMR (Pasture Group, PG). At 61DIM, TMR fed cows were assigned without an adjustment period to a similar feeding and management routine than PG group (Post-TMR), while PG cows remained in their original routine throughout the experiment. Thus, at 61DIM and thereafter, both, PG and Post-TMR cows grazed a second-year pasture and were supplemented with 50% TMR (DM basis). Milk production was determined daily until 80 DIM, and cow BCS and BW were registered and blood samples and liver biopsies were obtained one week before and one week after dietary change (−1 to +1 wk; +55 and +69 DIM). Milk yield, BW and BCS did not differ between treatments but decreased or tended to decrease from −1 to +1 wk only in Post-TMR cows. Serum IGF-1 tended to increase in Post-TMR cows. Hepatic expression of *IGFBP5* and *IGFBP6* mRNA, were greater while *IGF1* and *IGFBP3* mRNA tended to be greater for Post-TMR than PG cows. Hepatic expression of *IGF1*, *IGFBP5* and *IGFBP6* mRNA increased from −1 to +1 wk only in Post-TMR cows. Expression of *ACADVL* and *PDHFA* mRNA had a 2-fold increase in both groups from wks −1 to +1. The results confirm that changes in feeding strategy without an adaptation period modified animal metabolism. The inclusion of grazing to cows that were fed TMR during early

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lactation, increased IGF-1 concentrations and modified hepatic expression of genes related with IGF system and fatty acid metabolism indicating redistribution of nutrients and energy towards maintenance requirements (increased due to walking and grazing activity) in detriment of milk production.

Keywords

Turnout to Pasture, Hepatic Expression, Dairy Cattle

1. Introduction

Pasture-based systems allow low-cost and high-nutritional value feeding in dairy cows, and offer benefits for animal welfare and environmental care [1]. However, the quantity and quality of the pasture offered is variable throughout the year, determining an imbalance between nutrient supply and demand at various stages of the production cycle of the modern dairy cow [2]. Thus, the inclusion of concentrates and silages in the dairy cow diet has increased to meet the requirement of the milking herd [3] [4]. In this regard, the use of a total mixed ration (TMR) in early lactation allows dairy cows achieve maximum performance in times of reduced pasture dry matter (DM) production, determining an increase in milk yield, cow body condition score (BCS), metabolic status and reproductive performance [5] [6] [7].

The transition from a TMR to a pasture based systems involved significant changes in feed intake [8], cow behavior and activity [3], and rumen microbiota [9] causing changes in milk production and composition [10] [11]. However, little is known about dairy cow metabolic adaptations to changes in feeding regimes, from a TMR diet in early lactation (0 - 60 days in milk; DIM) to a pasture-based diet in a later stage of lactation. Metabolic adaptations during the transition from a TMR to a pasture-based diet (+1.75 or 3.6 kg DM of concentrate) in mid-lactation cows (160 to 200 DIM) included decreased insulin and increased non-esterified fatty acids (NEFA) and beta-hydroxybutyrate (BHB), indicating an increased lipomobilization during the first days or weeks on pasture, associated to decreased dry matter intake (DMI), milk yield and BCS [11] [8]. Nevertheless, liver changes on the somatotrophic axis and energy - glucose and fatty acid - metabolism in response to this dietary change has been scarcely reported and understood. Better understanding of the effects of different feeding strategies on animal metabolism and performance will promote improvements in actual feeding practices. To our knowledge, there are no studies that evaluated energy metabolism when TMR-fed dairy cows are changed to a feeding regime that included pasture grazing and an appropriate supplementation.

We hypothesized that when cows in early lactation change from a 100% TMR to a pasture + 50% TMR feeding regime, metabolism would rapidly adapt in order to cope with changes in amount and type of nutrients absorbed as well as in energy balance. Therefore, the aim of this study was to examine alterations in

metabolic and endocrine profiles as well as hepatic expression of genes related with nutrient partitioning and energy metabolism when dairy cows fed TMR *ad libitum* during the first 60 DIM (100% TMR) changed to a feeding regime that included pasture grazing (6 h/d) and supplementation with a reduced allowance of TMR (50%) without an adaptation period.

2. Materials and Methods

The experiment was performed according to the protocol approved by the Animal Experimentation Committee (CHEA) of the Universidad de la República (UdelaR, Uruguay). It was carried out the Experimental Station “Dr. Mario A. Cassinoni” of the School of Agronomy (Paysandú, Uruguay).

2.1. Animals and Experimental Design

Eighteen multiparous Holstein cows (fall calving; body weight (BW) = 709 ± 52.5 kg and BCS = 3.25 ± 0.25 units and parity = 4.5 ± 1.7) were used in a randomized block design with two replications (groups). Cows were grouped according to their due calving date (beginning and end of fall) and were randomly assigned within group according to their parity, BW and BCS to one of two feeding strategies from calving (day 0) to 60 DIM: 1) cows fed a TMR *ad libitum* (without access to pasture) and 2) cows fed on a mixed system with grazing (6 h of access to paddock in one grazing session, 8:00 to 14:00 h) and supplemented with 50% of *ad libitum* TMR (PG). At 61 ± 2 DIM, TMR fed cows were assigned without an adjustment period to a similar feeding and management routine than PG group (Post-TMR), while PG cows remained in their original routine throughout the experiment. Therefore, in the present study we evaluated two treatments: Post-TMR (0 to 60 DIM fed TMR and 61 to 80 DIM fed pasture grazing + 50% TMR) and PG as a control group (0 to 80 DIM pasture grazing + 50% TMR). Details on cow feeding and management during the prepartum and early postpartum as well as productive, metabolic, endocrine and reproductive responses during the first 60 DIM have been previously reported [6] [7].

At 61 DIM, all cows (PG and Post-TMR) grazed a second-year pasture of *Festuca arundinacea*, *Trifolium repens* and *Lotus corniculatus* (located 1.7 km from the milking parlour) in a 7-d rotation system with a mean herbage allowance at 4 cm above the ground level of 15 kg DM/cow/d (279 ± 14 DM g/kg, and 184 ± 14 g/kg DM of crude protein (CP), and 201 ± 2.5 g/kg DM acid detergent fiber (FDA)). The TMR was offered once a day in the afternoon (after pm milking), had a forage/concentrate ratio of 45/55 (dry basis) and was composed of sorghum silage (0.45) and a concentrate that included ground corn (0.19), wheat (0.12), soybean expeller (0.09), sunflower expeller (0.11), urea (0.003) and minerals and vitamins (0.009) (597 ± 43 DM g/kg, 167 ± 18 g/kg DM PC, and 141 ± 16.0 g/kg DM FDA).

Cows were milked twice a day (5:00 and 15:00 h) and milk production was determined daily from 61 until 80 DIM of lactation. Milk samples to determine fat, protein and lactose composition and BCS (score 1 a 5) [12], BW, blood sam-

ples and liver biopsies were obtained one week before and one week after dietary change (−1 and 1 wk relative to dietary change or +55 and +69 DIM). Blood samples were obtained in heparinized tubes by venipuncture of the coccygeal vein and were centrifuged (2000 x g for 15 min at 4°C) within 2 h after collection and serum was stored at −20°C until assayed. After blood collection, liver biopsies (500 mg) were obtained using a 14-gauge biopsy needle (Tru-Core®-II Automatic Biopsy Instrument; Angiotech, Lausanne, Switzerland) as described by Carriquiry *et al.* [13]. Liver samples were immediately frozen in liquid nitrogen and stored at −80°C until total RNA was isolated.

2.2. Metabolite and Hormone Analyses

Concentrations of serum NEFA, BHB, glucose and urea were determined by colorimetric assays using commercial kits according to [7]. Concentrations of insulin, IGF-1 and leptin were measured using commercial kits by immunoradiometric assays (IRMA, for insulin and IGF-1) or by a liquid-phase radioimmunoassay (RIA, for leptin). Assay details and detection limits as well as intra-assay CV were described in Astessiano *et al.* [7].

2.3. Isolation and Purification of RNA

Isolation of total RNA from hepatic tissue and synthesis of cDNA by reverse transcription was performed according with Astessiano *et al.* [7]. Primers (**Supplementary Table S1**) to specifically amplify cDNA of target genes: *IGF1*, *IGF2*, *IGF binding proteins-1 to 6* (*IGFBP1*, *IGFBP2*, *IGFBP3*, *IGFBP4*, *IGFBP5*, *IGFBP6*), insulin receptor (*INSR*), and long isoform of leptin receptor (*LEPRB*), glucose 6-phosphatase (*G6PC*), pyruvate carboxylase (*PC*), pyruvate dehydrogenase (*PDHA*), acyl-CoA oxidase (*ACOX*), carnitine palmitoyltransferase (*CPT1A*), acyl-CoA dehydrogenase very long chain (*ACADVL*), hydroxymethylglutaryl-CoA synthase (*HMGCoA*), citrate cintase (*CS*), and from endogenous controls: β -actin (*ACTB*), hypoxanthine phosphoribosyl transferase (*HPRT*), and ribosomal protein S9 (*RPS9*) were used [7].

Real time PCR reactions were performed in a total volume of 15 μ L using KAPA SYBR® FAST Universal 2X qPCR Master Mix (KapaBiosystems, inc. Woburn, MA, USA) according with [14]. Gene expression one week after dietary change (+1 wk) was measured by relative quantification [15] to the expression of one week before dietary change (−1 wk) and normalized to the geometric mean expression of the endogenous control genes (*HPRT*, *ACTB* and *RPS9*). Expression stability of 3 selected housekeeping genes was evaluated using MS-Excel add-in Normfinder (MDL, Aarhus, Denmark). The stability values obtained with Normfinder they were 0.144, 0.121, and 0.178 for *HPRT*, *ACTB*, and *RPS9*, respectively. Amplification efficiencies of target and endogenous control genes were estimated by linear regression of a dilution cDNA curve (n = 5 dilutions, from 100 to 6.25 ng/tube). Intra and inter-assay CV values were 1.9% and 4.2%, respectively.

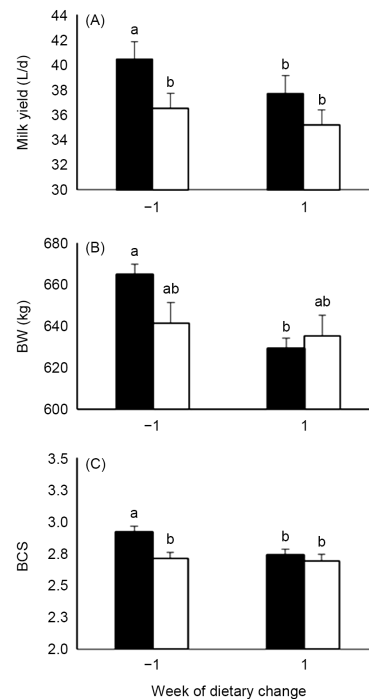


Figure 1. Changes in milk yield, BW and BCS one week before and one week after dietary change (+55 and +69 DIM, respectively) in multiparous dairy cows (Post-TMR; ■ and PG; □). Within treatments: a vs b indicates significant differences ($p < 0.05$).

Table 1. Effect of dietary change on serum metabolite and hormone concentrations in multiparous dairy cows.

	Treatments ¹			P value		
	Post-TMR	PG	SE	T	WK	T x WK
NEFA (mmol/L)	0.6	0.5	0.06	0.66	0.56	0.20
BHB (mmol/L)	0.4	0.5	0.04	0.65	0.14	0.25
Urea (mmol/L)	5.3	5.3	0.50	0.96	0.88	0.56
Glucosa (mmol/L)	4.2	4.0	0.04	0.23	0.57	0.58
IGF-I (ng/mL)	148.5	143.5	13.40	0.83	0.91	0.06
Insulina (μIU/mL)	17.5	18.4	1.50	0.69	0.47	0.62
Leptina (ng/mL)	4.1	3.6	0.50	0.53	0.45	0.47

¹**Post-TMR:** Cows changed at 61 DIM to a mixed feeding regime that included pasture grazing (one am grazing session of 6 h) + 50% TMR (DM basis) after being fed 100% TMR (*ad libitum*) during the first 60 DIM. **PG:** control group, cows fed a mixed feeding regime of pasture grazing (one am grazing session of 6 h) + 50% TMR (DM basis) from calving. T: treatment; WK: week relative to dietary change.

2.4. Calculation and Statistical Analyses

Net energy (NE) calculations was based on [16]. Maintenance NE requirements were calculated as $NEM = 0.08 \times BW^{0.75} + NEmact$, where $NEmact = (((Distance/1000) \times Trips) \times (0.00045 \times BW)) + (0.0012 \times (BW))$. Lactation NE requirements were calculated as $NEL = milk\ yield \times [(0.0929 \times fat\ %) + (0.0563 \times true\ protein\ %) + (0.0395 \times lactose\ %)]$, using composition data derived from analysis of samples collected weekly.

Data were analyzed in a randomized block design using the SAS System program (SAS Institute Inc., Cary, NC, USA). Univariate analyses were performed on all variables to identify outliers and inconsistencies and to verify normality of residuals. Data of milk production, BW, BCS, and serum metabolite and hormone concentrations were analyzed by repeated measures using the MIXED procedure with DIM as the repeated effect, and the unrestricted (UN) covariance structure. The model included the treatment (PG vs. Post-TMR), week (before and after dietary change), and the interaction between treatment and week as fixed effects, replication as a random effect and calving date as a covariate. The Kenward-Rogers procedure was used to adjust the denominator degree of freedom. Tukey-Kramer tests were conducted to analyze differences between treatments and weeks ($\alpha = 0.05$). Data of hepatic gene expression were analyzed using the MIXED procedure with a model that included treatment as fixed effect, replication as a random effect and calving date as a covariate. One-way t-test were conducted to assess week effect within treatment. Correlation coefficients were estimated using the CORR procedure. Data are presented as least square means $\pm$ pooled standard errors.

3. Results

Milk yield did not differ between treatments ($P = 0.16$) but decreased ($P < 0.05$) from -1 to $+1$ wk only in Post-TMR (**Figure 1(A)**) whereas milk NEL output and milk composition was not affected ($P > 0.10$) by treatment or week. Cow BW and BCS tended to be affected by treatment and week ($P = 0.12$) as they decreased ($P = 0.03$) from -1 to $+1$ wk only in Post-TMR cows (**Figure 1(B)** and **Figure 1(C)**). Maintenance NEL requirement was affected by the interaction between treatment and week ($P = 0.01$) as while it did not change from -1 to $+1$ wk in PG cows (12.81 to 12.66 ± 0.3 Mcal/d), an increase ($P < 0.05$) was observed in Post-TMR cows (10.73 to 11.91 ± 0.3 Mcal/d).

Serum concentrations of NEFA, BHB, urea and glucose were not affected by treatment or week (**Table 1**). Serum IGF-1 concentrations were not affected by treatment or week but tended to be affected by the interaction between treatment and week ($P = 0.06$) as they tended to increase in Post-TMR cows with the change of diet (129.5 vs. 167.5 ± 13.5 ng/mL from -1 to $+1$ wk, respectively). Concentrations of insulin and leptin were not affected by treatment, week or their interaction (**Table 1**).

Hepatic expression of *IGFBP5* and *IGFBP6* mRNA ($P < 0.05$), were greater while IGF1 and *IGFBP3* mRNA tended to be greater ($P = 0.14$) for Post-TMR

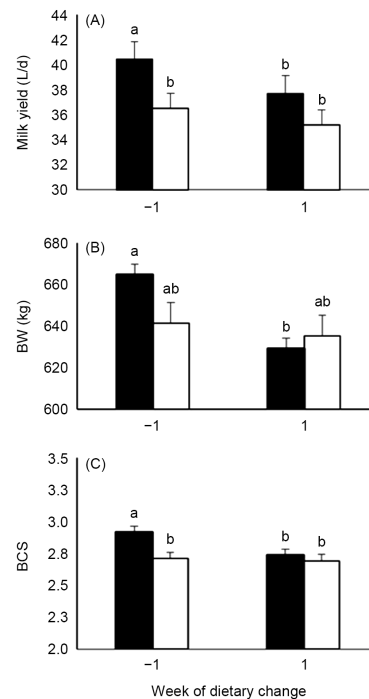


Figure 1. Changes in milk yield, BW and BCS one week before and one week after dietary change (+55 and +69 DIM, respectively) in multiparous dairy cows (Post-TMR; ■ and PG; □). Within treatments: a vs b indicates significant differences ($p < 0.05$).

Table 1. Effect of dietary change on serum metabolite and hormone concentrations in multiparous dairy cows.

	Treatments ¹			P value		
	Post-TMR	PG	SE	T	WK	T x WK
NEFA (mmol/L)	0.6	0.5	0.06	0.66	0.56	0.20
BHB (mmol/L)	0.4	0.5	0.04	0.65	0.14	0.25
Urea (mmol/L)	5.3	5.3	0.50	0.96	0.88	0.56
Glucosa (mmol/L)	4.2	4.0	0.04	0.23	0.57	0.58
IGF-I (ng/mL)	148.5	143.5	13.40	0.83	0.91	0.06
Insulina (μIU/mL)	17.5	18.4	1.50	0.69	0.47	0.62
Leptina (ng/mL)	4.1	3.6	0.50	0.53	0.45	0.47

¹**Post-TMR:** Cows changed at 61 DIM to a mixed feeding regime that included pasture grazing (one am grazing session of 6 h) + 50% TMR (DM basis) after being fed 100% TMR (*ad libitum*) during the first 60 DIM. **PG:** control group, cows fed a mixed feeding regime of pasture grazing (one am grazing session of 6 h) + 50% TMR (DM basis) from calving. T: treatment; WK: week relative to dietary change.

than PG cows (**Table 2**). In addition, expression of *IGF1*, *IGFBP5* and *IGFBP6* mRNA had a 2.3 to 3.4-fold increase ($P < 0.025$) from -1 to +1 wk only in Post-TMR cows. Expression of *IGF2*, *IGFBP1*, *IGFBP2*, *IGFBP4*, *INSR*, and *LEPRB* mRNA were not affected by treatments ($P > 0.22$; **Table 2**). Expression of the genes related to glucose and fatty acid metabolism in the liver did not differ ($P > 0.50$) between treatments (**Table 2**), but *ACADVL* and *PDH1A* mRNA had a 2-fold increase ($P < 0.01$) in both groups from -1 to +1 wk. In addition, the ratio *PDH1A* to *PC* mRNA tended ($P = 0.07$) to have a 1.5-fold increase from -1 to +1 wk only in Post-TMR cows. Independently of treatment, hepatic *ACADVL* mRNA was positively linearly correlated with serum NEFA concentrations ($r = 0.71$, $P = 0.07$).

Table 2. Effect of dietary change on relative hepatic mRNA expression of genes related to IGF system, glucose and fatty acid metabolism in multiparous dairy cows.

	Treatments			P value
	Post-TMR	PG	SE	
<i>IGF1</i>	2.41	1.00	0.25	0.14
<i>IGF2</i>	1.00	0.97	0.20	0.96
<i>IGFBP1</i>	0.60	1.22	0.13	0.22
<i>IGFBP2</i>	0.95	1.00	0.12	0.93
<i>IGFBP3</i>	1.33	0.76	0.20	0.14
<i>IGFBP4</i>	0.84	1.05	0.16	0.66
<i>IGFBP5</i>	3.43	0.75	0.35	0.01
<i>IGFBP6</i>	2.32	0.66	0.12	0.04
<i>INSR</i>	1.03	0.83	0.11	0.65
<i>LEPRB</i>	1.46	1.46	0.12	0.99
<i>ACADVL</i>	2.01	1.85	0.23	0.80
<i>ACOX</i>	1.20	1.47	0.12	0.46
<i>CS</i>	1.18	1.49	0.24	0.65
<i>PC</i>	1.32	1.47	0.24	0.80
<i>G6PC</i>	1.16	1.48	0.11	0.63
<i>HMGCoA</i>	1.92	1.56	0.12	0.79
<i>PDH1A</i>	2.09	1.78	0.14	0.62
<i>CPT1A</i>	1.05	1.05	0.12	0.99

Post-TMR: Cows changed at 61 DIM to a mixed feeding regime that included pasture grazing (one am grazing session of 6 h) + 50% TMR (DM basis) after being fed 100% TMR (*ad libitum*) during the first 60 DIM. **PG:** control group, cows fed a mixed feeding regime of pasture grazing (one am grazing session of 6 h) + 50% TMR (DM basis) from calving. ²Genes: *IGF1*, *IGF2* = insulin like growth factor 1 and 2; *IGFBP1*, *IGFBP2*, *IGFBP3*, *IGFBP4*, *IGFBP5*, *IGFBP6* = IGF binding proteins-1, 2, 3, 4, 5 and 6; *INSR* = insulin receptor; *LEPRB* = full-length leptin receptor; *ACADVL* = Acyl-CoA dehydrogenase very long chain; *ACOX* = Acyl-CoA oxidase; *CS* = citrate cintase; *PC* = pyruvate carboxylase; *G6PC* = glucose 6-phosphatase; *HMGCoA* = hydroxymethylglutaryl-CoA synthase; *PDH1A* = pyruvate dehydrogenase; *CPT1A* = carnitinepalmitoyltransferase.

4. Discussion

The inclusion of grazing, without an adaptation period, in the diet of dairy cows that were fed only TMR during early lactation, modified hepatic IGF system associated with changes in productive performance. Milk production decreased only in Post-TMR group after dietary change. Although this decrease could accompany the normal decline in the lactation curve around the peak of milk production [17], as it was only observed in Post-TMR cows, it was probably associated to a restriction in DMI and/or increased in energy requirements after the change of the diet [6]. It has been reported that not only characteristics of the pasture, but also its management can limit DMI of grazing animals [4] [18]. Bargo *et al.* [19] reported decreased milk production and DMI for cows grazing pasture and supplemented with TMR (as 17 kg DM/d per cow) when compared to cows fed only TMR. In addition, grazing as such produces an increase in energy expenditure (for walking and grazing) compared to animals with food easily available as a TMR [20]. This idea is supported by the fact that cows in the Post-TMR group increased estimated energy requirements maintenance [16] by 10% from -1 to +1 wk (+55 to +69 DIM).

The observed decreases in milk yield, BW and BCS from -1 to +1 wk only in Post-TMR cows suggested that the reduction in TMR intake and the greater energy maintenance demands were not compensated by an adequate intake from pasture in these cows. Indeed, DMI and grazing behavior of the same experimental treatments were reported by [6] that despite not finding differences in average DMI and grazing behavior between Post-TMR and PG cows after the change of diet, they reported lower grazing activity on Post-TMR during the last hours in the pasture. In addition, it is also known the transition from one diet type to another causes changes in the rumen microbiota and rumen stratification [21] [22]. These changes could lead to alterations in fermentation patterns [23] and physiological and structural adaptations of the rumen epithelium [24] that could affect nutrient utilization in the gastrointestinal tract and also explain the decline in performance (milk yield, BW and BCS) in Post-TMR cows.

Dietary change in this study was done after peak milk yield and nadir of BCS [7], thus, no changes in BCS would have been expected according to the lactation stage. Nevertheless, as reported before, both, BW and BCS decreased for Post-TMR cows after the change of diet, indicating mobilization of body reserves, probably to cope with the increased maintenance requirements. Indeed, milk fatty acid profile of these cows showed an increase of preformed fatty acids, particularly of stearic acid (Barca *et al. unpublished results*), which may reflect lipomobilization [25]. However, NEFA concentrations did not increase in Post-TMR cows, which suggested that lipid mobilization was not extensive and/or there was a differential tissue NEFA utilization due to the increased walking and grazing activities in these cows [7]. The oxidation of mitochondrial fatty acids produces energy in muscle for muscle contraction or exercise [26].

In agreement with the inverse association between serum IGF-1 and milk production in dairy cows [27], we found that the concentrations of IGF-1 tended

to increase after the dietary change in Post-TMR cows. The role of IGF-1 around calving, when its concentrations decreased consistent with an uncoupled somatotrophic axis, mediating nutrient partitioning towards milk production has been extensively reviewed [27] [28]. Therefore, considering that in the present study the change of diet occurred after the peak of milk production, results suggested that the increase in circulating IGF-1 would modify nutrient redistribution in detriment of milk production. The increased serum IGF-1 concentrations were also consistent with an increased hepatic mRNA expression of *IGF1* and three of its binding proteins, *IGFBP3*, *IGFBP5* and *IGFBP6*, after the change of diet only in Post-TMR cows. The association between hepatic *IGF1* mRNA and circulating concentrations of IGF-1 has been previously reported [29] [30]. It is known that, once released from the cell, the majority of IGF-1 found in circulation is bound with high affinity to one or more of the 6 known IGF binding proteins. However, binary complexes that IGF-1 form with IGFBP-3 or IGFBP-5 (including the binding with the acid-labile) extend IGF-1 half-life from 10 min to 16 hours or longer increasing availability of IGF to cellular functions in the body [31] [32] [33]. Thus, increased serum IGF-1 could have been responded not only to a greater hepatic IGF-1 secretion, but also to an increased circulation of this hormone in blood as a ternary complex due to greater availability of IGFBP3 and IGFBP5. Consistent with our results, Fenwick *et al.* [30] reported increased *IGFBP3*, *IGFBP5* and *IGFBP6* mRNA expression in the liver of early lactation dairy cows with reduced milk production and moderate negative energy balance when compared with cows with greater milk production and severe negative energy balance. In addition, Coyne *et al.* [34] reported increased hepatic *IGFBP5* mRNA in heifers fed a high n-3 polyunsaturated diet. Indeed, in the present study n-3 PUFA intake increased after the change of diet (by the inclusion of pasture) for Post-TMR cows (Barca *et al.* unpublished results).

The increase in *ACADVL* mRNA in the liver at +1 wk in both treatments indicated a metabolic adaptation to promote β -oxidation of fatty acids [35]. Indeed, we found a positive correlation between serum NEFA concentrations and hepatic *ACADVL* mRNA. The increase in liver *ACADVL* mRNA as lactation progresses could reflect a better liver capacity to use NEFA as fuel as has been shown previously [35]. Interestingly, although no changes in hepatic *PC* mRNA were observed, *PDH1A* mRNA increased its expression in both treatments suggesting that the shift in the metabolism later in lactation was not directed to improve gluconeogenesis but to the complete oxidation of the carbons (Krebs Cycle). Moreover, the *PDH1A* to *PC* mRNA ratio tended to increase only in Post-TMR cows after the change of diet suggesting a greater flux of pyruvate to acetyl-coA than to oxaloacetate, probably due to a reduced demand for the generation of oxaloacetate via *PC* [36] as milk production, thus lactose production and the need for glucose, was reduced in this group.

The results confirm that metabolic regulation in dairy cows is a dynamic system, and changes in feeding strategy without an adaptation period modified animal metabolism. In the present study, the inclusion of grazing to cows that

were fed TMR during early lactation, increased IGF-1 concentrations and modified hepatic expression of genes related with IGF system and fatty acid metabolism indicating redistribution of nutrients and energy towards maintenance requirements (increased due to walking and grazing activity) in detriment of milk production.

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4. EFECTO DE LA NUTRICIÓN SOBRE LA EXPRESIÓN GÉNICA ENDOMETRIAL AL DÍA 7 DEL CICLO ESTRAL

4.1. ENDOMETRIAL GENE EXPRESSION IN PRIMIPAROUS DAIRY COWS AT THE END OF THE VOLUNTARY WAITING PERIOD IS AFFECTED BY NUTRITION: TOTAL MIXED RATION VS INCREASING LEVELS OF HERBAGE ALLOWANCE. 2017. Astessiano AL, Carriquiry M, Mattiauda DA, Adrien ML, Chilibraste P, Meikle A. Este artículo fue publicado en *Reproduction in Domestic Animal*.

El objetivo fue evaluar cambios en la expresión génica endometrial de genes candidatos (ARNm de sistema *IGFs*, *INSR*, *LEPRB*, *ADIPOR1*, *ADIPOR2*, *ER*, *PGR*) al día 7 del ciclo estral inducido (fin del período de espera voluntario) en vacas lecheras primíparas con diferentes estrategias de alimentación. Las vacas fueron alimentadas con DTM (100% *ad libitum*) o por dietas combinado DTM y pastoreo con cantidades crecientes de forraje.

La hipótesis que nos planteamos fue que las estrategias de alimentación determinan balances energéticos diferentes, que impactan no solo en la producción de leche, sino también en la funcionalidad uterina al momento del servicio. Por lo tanto, vacas alimentadas con dietas que maximicen la ingesta de nutrientes y energía en lactación temprana, reducirían el BEN y mostrarían una expresión genética endometrial favorable para el crecimiento del embrión.

Este estudio proporcionó evidencia sólida de la importancia del manejo nutricional durante la lactancia temprana sobre el ambiente uterino al final del período voluntario. La mayor expresión de genes relacionados con el crecimiento embrionario y la función uterina (sistema IGF y receptores de progesterona y adiponectina) en vacas alimentadas con DTM y alta asignación de forraje sugiere un ambiente favorable para el crecimiento embrionario. Estos resultados, son consistentes con la mejor performance reproductiva y balance energético de estas vacas comparadas con las vacas

alimentadas con menores asignaciones de forraje (Meikle et al., 2013).

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ORIGINAL ARTICLE

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Endometrial gene expression in primiparous dairy cows at the end of the voluntary waiting period is affected by nutrition: Total mixed ration vs increasing levels of herbage allowance

AL Astessiano¹  | M Carriquiry¹ | DA Mattiauda¹ | ML Adrien² | P Chilibroste¹ | A Meikle²

¹School of Agronomy, Universidad de la República, Montevideo, Uruguay

²School of Veterinary Medicine, Universidad de la República, Montevideo, Uruguay

Correspondence

AL Astessiano, School of Agronomy, Universidad de la República, Montevideo, Uruguay.
Email: lauaste@gmail.com

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Contents

The study postulated that differential nutritional management during the early lactation period would be reflected in endometrial expression of genes related to embryo growth at the end of the voluntary waiting period. Thus, the effect of the combined use of total mixed ration (TMR) and grazing under different herbage allowances during the first 75 days post-partum (DPP) on endometrial gene expression was evaluated in primiparous dairy cows. Cows were blocked by body weight, age and body condition score and randomly assigned to three grazing treatments: high (HA, 30 kg DM per cow per day), medium (MA, 15 kg DM per cow per day) and low (LA, 7.5 kg DM per cow per day) herbage allowance (mixed pasture, 2,600 kg DM per ha) plus 8 kg DM of supplement or TMR (55% forage, 45% concentrate) fed *ad libitum* (TMR) from calving to 75 DPP. At 57 DPP, cows were synchronized for oestrus (day 0, 68 DPP) and at day 7, endometrial biopsies were obtained. The nutritional treatment did not affect insulin, IGF-1 and leptin concentrations on days 0, 4 or 7. Expression of *IGF1*, *IGFBP3*, *IGFBP4*, *ADIPOR1* and *ADIPOR2* mRNA was significantly affected by the nutritional treatment. Endometrial *IGF1* and *IGFBP4* mRNA were twofold greater in TMR and HA than MA and LA cows. Expression of *IGFBP3* and *ADIPOR1* mRNAs was greater in TMR and HA than MA cows, but did not differ from LA cows. All groups had greater expression of *ADIPOR2* mRNA than MA cows. This study provided solid evidence of the importance of nutritional management during early lactation on uterine environment at the end of the voluntary waiting period. The greater expression of genes related to embryo growth and uterine function (IGF system, progesterone and adiponectin receptors) in cows fed diets maximizing energy intake suggests a favourable environment for embryonic growth, which may explain the improved reproductive performance of cows in good energy balance.

1 | INTRODUCTION

Reproductive performance of high-yielding dairy cows underwent a major decline during the last 50 years of the 20th century (1950 to 2000; Butler, Cummins, & Moore, 2015); more recently selecting for improved fertility has halted this continuous decline and has even

started to improve (Norman, Wright, Hubbard, Miller, & Hutchison, 2009). The reproductive success after calving is limited by the resumption to ovarian cyclicity and the early embryo mortality which are both affected by the typical negative energy balance of early lactation (Santos, Thatcher, Chebel, Cerri, & Galvão, 2004). It is known that pregnancy establishment is vulnerable during the period of maternal

recognition of pregnancy and that 40% of pregnancy failures occurred during this period (Diskin, Murphy, & Sreenan, 2006), and the endometrium must produce an appropriate environment to stimulate embryo development and to allow implantation (Beltman et al., 2010).

The main regulators of uterine function are the oestrogens and progesterone that act via their specific nuclear receptors (ESR and PGR). In addition to the influence from the ovary, the post-partum uterus is exposed to the prevailing metabolic environment within the animal (Wathes et al., 2007). Several hormones such as growth hormone (GH)-IGF axis, leptin and adiponectin have been associated not only with post-partum ovarian cyclicity but also with uterine function (Meikle et al., 2004; Tabandeh, Hosseini, Saeb, Kafi, & Saeb, 2010). Reduced early post-partum insulin and IGF-1 concentrations due to feed restriction (20%) affected oviduct and endometrium expression of genes related with tissue remodelling processes in dairy cows at day 80 post-partum, which by impairing the normal functioning of the tract may explain the reduced reproductive performance of underfed cows (Valour et al., 2013). Indeed, recent data indicated that the mammalian endometrium appear to be a dynamic and reactive tissue which its compromised or suboptimal physiology can affect embryo development before implantation, with visible and sometimes severe consequences for the placentation process, foetal development and pregnancy outcome (Sandra et al., 2015). Many members of the GH-IGF axis are expressed locally within the endometrium, and their expression is altered by the stage of the oestrous cycle and pregnancy (Llewellyn, Fitzpatrick, Kenny, Patton, & Wathes, 2008; McCarthy, Roche, & Forde, 2011; Sosa et al., 2010). Moreover, in agreement with the known stimulatory effect of IGF-1 on both the embryo and the endometrium (Wathes, Reynolds, Robinson, & Stevenson, 1998), the endometrial expression of IGF1 mRNA was positively associated with cow pregnancy at day 17 (Kirby, Thatcher, Collier, Simmen, & Lucy, 1996). Leptin and adiponectin—two of the better-studied adipokines—have been described to affect early embryonic development (porcine; Chappaz et al., 2008) directly or indirectly through their hormone receptors in the uterus (mice; Mirkin et al., 2005; Kim et al., 2011). In the bovine uterus, leptin receptor (*LEPR*) has been described in heifers (Sosa et al., 2010), but we did not find any reports on adiponectin receptors (*ADIPOR*).

It has been reported that grazing dairy cows do not get sufficient dry matter intake (DMI) to sustain the high milk production that could be achieved with their actual genetic potential (Kolver & Muller, 1998). Moreover, in mixed dairy systems, grazing management is key to sustain high milk yield and reduce production costs. Meikle, Adrien, Mattiauda, and Chillbroste (2013) showed greater milk production (20% more) when daily herbage allowance was increased from low (7.5 kg DM per cow per day) to medium (15 kg DM per cow per day), but no response was obtained when daily herbage allowance was increased to 30 kg DM per cow per day. Indeed, cows grazing medium herbage allowance sustained their high milk production by mobilizing energy reserves, which had a negative impact on the post-partum reinitiation of ovarian cyclicity. This led us to further hypothesize that the differential negative energy balance observed according to the different nutritional treatments, and consequent milk production,

affected also uterine functionality at breeding time (end of the voluntary waiting period, at 68 DPP). Thus, this study aimed to analyse, in primiparous dairy cows at day 7 of oestrous cycle (critical period of early embryo loss; Diskin & Morris, 2008), changes on the endometrial gene expression induced by the combined use of total mixed ration and grazing under different sward herbage allowances. It was expected that cows fed diets that maximized nutrient and energy intake and reduced negative energy balance during early lactation would show an endometrial gene expression favourable to embryo growth.

2 | MATERIALS AND METHODS

The experiment was carried out at the EEMAC Research Station, School of Agronomy, Uruguay (30°S, 53°W). Animal procedures were approved by the Animal Experimentation Committee of University of Uruguay (UdelaR, Montevideo, Uruguay).

2.1 | Animals and experimental design

Primiparous Holstein dairy cows ($n = 44$, 595 ± 41 kg of BW, 3.7 ± 0.3 of BCS) calving within 20 days during fall (May–June) were used. Cows were blocked by BW, age and BCS and randomly assigned to three grazing treatments ($n = 11$ each): high (HA, 30 kg DM per cow per day), medium (MA, 15 kg DM per cow per day) and low (LA, 7.5 kg DM per cow per day) herbage allowance (mixed pasture, 2,600 kg DM per ha) plus 8 kg DM of TMR supplement or to TMR (55% forage, 45% concentrate) fed ad libitum (TMR) from calving to 75 days post-partum. The TMR supplement fed (i.e., 8 kg DM) once per day to the grazing treatments included, on a fresh weight basis, corn silage (10 kg) compound feed (4.8 kg) and grass hay (0.4 kg; roughage to concentrate ratio close to 55:45) and was formulated to meet maintenance metabolizable energy needs plus 8–10 L/d of milk (NRC 2001), leaving any difference in cow performance to the effect of grazing treatments.

Cows were milked twice a day (5:00 and 16:00), and milk yield was measured daily, and samples were obtained weekly for the analysis of protein, fat and lactose, and cow BCS was registered weekly (Edmonson, Lean, Weaver, Farver, & Webster, 1989). Data of BCS, milk yield, metabolite and hormone concentrations during the first 60 days of lactation and days to first ovulation have been published previously. At 60 days of lactation, milk yield and BCS were the greatest in TMR, the lowest in LA and intermediate in HA and MA cows which did not differ (29.5 , 24.7 , 23.2 , 19.6 ± 0.4 L/d milk yield and 3.2 , 2.8 , 2.7 , 2.4 ± 0.1 BCS in TMR, HA, MA and LA cows respectively; Meikle et al., 2013).

2.2 | Tissue collection and blood sample

At 57 days post-partum, cows were synchronized for oestrus. Oestrous synchronization was conducted in two groups of animals to homogenize days post-partum at the time of initiation of treatment. Hormonal treatment involved placing at day 9 (day 0 = oestrus), a natural

progesterone intravaginal device (Terapress[®]) with an intramuscular injection of 2.5 ml of Fertagyl[®] (0.25 mg of synthetic gonadotrophin hormone releasing hormone/cow). The implant was removed at day 2, and 2 ml of Enzaprost DC[®] (D-Cloprostenol/cow 0.15 mg) was injected intramuscularly, and patches were placed in the sacro-coccygeal region to aid in oestrous detection. Oestrous detection was conducted for 5 days after implant removal, by observing the animals twice daily (after each milking) for 40 min. At day 7 of the oestrous cycle (day 0 = oestrus) in a subsample of nine cows of each group endometrial biopsies ipsilateral to the corpus luteum were obtained as described by (Meikle et al., 2001). Endometrial samples were immediately frozen in liquid nitrogen and stored at -80°C until total RNA was isolated.

In the same cows in which endometrial biopsies were obtained, blood was collected at day 0 (oestrous or at 48 hr after to implant removed), 4 and 7 (day of biopsy) of the oestrous cycle by venipuncture of the coccygeal vein using Vacutest[®] tubes (8 ml, Vacutest Kima, Arzergrande, Italy) that contained clot activator gel. Samples were centrifuged (2,000× g for 15 min at 4°C) within 1 hr after collection, and serum was stored at -20°C until hormone analyses were performed.

2.3 | Isolation, purification of RNA, and real-time PCR

Although endometrial biopsies were collected from each animal, the amount of tissue was not enough for mRNA extraction for some of the samples, and therefore 7, 8, 8 and 6 samples were analysed for TMR, HA, MA and LA respectively.

Total RNA from uterine 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 the absorbance at 260 nm (NanoDrop ND-1000 Spectrophotometer; Nanodrop Technologies, Wilmington, DE, USA), and purity and integrity of RNA isolates were assessed from 260/280 and 260/230 absorbance ratios (greater than 1.8) and by electrophoresis in 1% agarose gel. Isolated RNA was stored at -80°C until analysed by quantitative real-time PCR.

The SuperScript[®] III First-Strand Synthesis System kit (Invitrogen) was used to conduct the reverse transcription using random hexamers and 1 µg of total RNA as a template. The cDNA was stored at -20°C until its use in the real-time PCR. Primers (Table S1) to specifically amplify cDNA of target genes: *GHR*, *IGF1*, *IGF2*, *IGF1R*, *IGF binding proteins 1 to 6* (*IGFBP1*, *IGFBP2*, *IGFBP3*, *IGFBP4*, *IGFBP5*, *IGFBP6*), insulin receptor (*INSR*), *LEPRB*, *ADIPOR1*, *ADIPOR2*, oestrogen and progesterone receptors (*ESR* and *PGR* respectively) and from endogenous controls: β -actin (*ACTB*), hypoxanthine phosphoribosyltransferase (*HPRT*) and ribosomal protein *S9* (*RPS9*) were obtained from literature or specifically designed using the Primer3 software (<http://bioinfo.ut.ee/primer3-0.4.0/primer3/>) based on bovine nucleotide sequences available from NCBI (<http://www.ncbi.nlm.nih.gov/>). Before use, primer product sizes (1% agarose gel separation) and sequence (Macrogen Inc., Seoul, Korea) were determined to ensure that primers produced the desired amplicons.

Real-time PCR reactions were performed in a total volume of 15 µl using KAPA SYBR[®] FAST Universal 2× qPCR Master Mix (Kapa Biosystems, inc. Woburn, MA, USA) according with Astessiano, Perez Clariget, Quintans, Soca, and Carriquiry (2011) using the following standard amplification conditions: 10 min at 95°C and 40 cycles of 15 s at 95°C, 45 s at 60°C and 20 s at 72°C. Dissociation curves were run on all samples to detect primer dimers, contamination or presence of other amplicons. Each disc included a pool of total cDNA from bovine uterine samples analysed in triplicate to be used as the basis for the comparative expression results (exogenous control) and duplicate tubes of water (non-template control). Gene expression was measured by relative quantification (Pfaffl, 2009) to the exogenous control and normalized to the geometric mean expression of the endogenous control genes (*HPRT*, *ACTB* and *RPS9*). Expression stability of three selected housekeeping genes was evaluated using MS-Excel add-in Normfinder (MDL, Aarhus, Denmark), and the stability values obtained were 0.435, 0.219 and 0.295 for *HPRT*, *ACTB* and *RPS9* respectively. In addition, these three genes have been used before as an endogenous control in tissues from ruminants (Casal et al., 2014; De Brun et al., 2013), and they proved to be good housekeeping genes as their expression did not varied among treatments in this study. Amplification efficiencies or target and endogenous control genes were estimated by linear regression of a dilution cDNA curve ($n = 5$ dilutions, from 100 to 6.25 ng/tube). Intra- and inter-assay CV values were 2.2% and 4.3% respectively.

2.4 | Hormone analyses

Progesterone (P4) was determined to confirm ovulation after synchronization and was measured by a solid-phase radioimmunoassay (RIA) using a commercial kit (Coat and Count; Diagnostic Products, Los Angeles, CA, USA). All samples were analysed in a single assay; the sensitivity was 0.01 ng/ml; the intra-assay coefficients of variation (CV) were 10.6%. Ovulation was defined as increased P4 concentrations > 1 ng/ml on day 7 of the oestrous cycle, only cows that ovulated were included in the statistical analysis (6, 6, 5 and 4 animals per treatment TMR, HA, MA and LA respectively).

Concentrations of insulin and IGF-1 were measured using immunoradiometric assays (IRMA) with commercial kits (INS-IRMA; DIA Source Immuno Assays S.A., Belgium and IGF-I-RIACT Cis Bio International, GIF-SUR-YVETTE CEDEX, France, respectively) previously used in bovines by Astessiano et al. (2015). All samples were determined in a single assay for each hormone. For insulin, the assay detection limit was 1.1 µIU/ml, and intra-assay CV for control 1 (22 µIU/ml) and 2 (55 µIU/ml) were 5.3% and 8.6% respectively. For IGF-1, the assay detection limit was 0.29 ng/ml, and intra-assay CV for control 1 (41 ng/ml) and control 2 (521 ng/ml) was 5.54% and 5.34% respectively. Leptin concentrations were determined by a liquid-phase radioimmunoassay (RIA) using a commercial Multi-Species Leptin kit (RIA kit, Millipore, USA) previously reported in bovines (Pinotti & Rosi, 2006). The RIA had a sensitivity of 1.4 ng/ml. All samples were determined in the same assay, and the intra-assay CV for control 1 (4.2 ng/ml) and control 2 (18.8 ng/ml) was 7% and 5.2% respectively.

	Treatments ¹				SE	p-value TREAT
	TMR	HA	MA	LA		
Progesterone ng/ml	2.60	2.16	1.85	1.47	1.23	.78
Insulin μ U/ml	10.75	11.41	13.25	10.58	1.10	.40
IGF-I ng/ml	78.52	87.82	80.61	90.52	8.70	.75
Leptin ng/ml	4.08	4.74	4.52	4.78	1.21	.79

¹Treatments = cows were assigned to three grazing treatments: high (HA, 30 kg DM per cow per day), medium (MA, 15 kg DM per cow per day) and low (LA, 7.5 kg DM per cow per day) herbage allowance (mixed pasture, 2,600 kg DM per ha) plus 8 kg DM of supplement or to a TMR (55% forage, 45% concentrate) fed *ad libitum* (TMR) from calving to 75 days post-partum. TREAT, Treatments.

TABLE 1 Hormone concentration in the beginning of the oestrous cycle in primiparous dairy cows in total mixed ration (TMR; $n = 7$), high (HA; $n = 8$), medium (MA; $n = 8$) and low (LA; $n = 6$) pasture allowances groups

2.5 | Statistical analyses

Data were analysed in a randomized block design using the SAS System programme (SAS Institute Inc., Cary, NC, USA). Univariate analyses were performed on all variables to identify outliers and inconsistencies and to verify normality of residuals. Data of hormone concentrations were analysed as repeated measures using the MIXED procedure with days as the repeated effect, the first-order autoregressive as the covariance structure and the Kenward–Rogers procedure to adjust the denominator degrees of freedom. The model included nutritional treatment, days, and their interaction, and block as random effect. As the interaction between treatment and days was not significant (except for progesterone concentrations), it was removed from the model. Uterine mRNA expressions were analysed using the same model without the repeated effect of day. Tukey–Kramer tests were conducted to analyse differences between groups ($\alpha = 0.05$). For all results, means were considered to differ when $p \leq .05$, and trends were identified when $.05 < p \leq .10$. Data are presented as least square means $\pm$ pooled standard errors.

3 | RESULTS

The nutritional treatment did not affect concentrations of P4, insulin, IGF-1 or leptin (Table 1).

The expression of *IGF1* mRNA was affected by the nutritional treatment ($p = .01$) and was twofold greater ($p < .05$) in TMR and HA cows than MA and LA cows (Figure 1a).

Although the effect of nutritional treatment on endometrial expression of *IGF1R* and *IGF2* mRNA did not reach significance (Table 2), when Tukey–Kramer tests were conducted, *IGF1R* mRNA was greater ($p < .05$) in HA than LA cows, while *IGF2* mRNA was greater ($p < .05$) in TMR than MA cows. Endometrial *IGFBP3* mRNA was greater ($p < .05$) in TMR and HA than MA cows (Figure 1b), but did not differ from LA cows, while *IGFBP4* mRNA was greater ($p < .05$) in TMR and HA than MA and LA cows (Figure 1c). Expression of *IGFBP5* mRNA was greater ($p < .05$) in TMR and HA than MA cows.

In addition, the expression of *ADIPOR1* and *ADIPOR2* mRNA was affected ($p \leq .04$) by the nutritional treatment. The *ADIPOR1* mRNA was greater ($p \leq .04$) in TMR and HA than MA cows, while LA cows

did not differ from any group (Figure 1d). In contrast, the expression of *ADIPOR2* mRNA was greater ($p < .01$) in TMR, HA and LA than MA cows (Figure 1e).

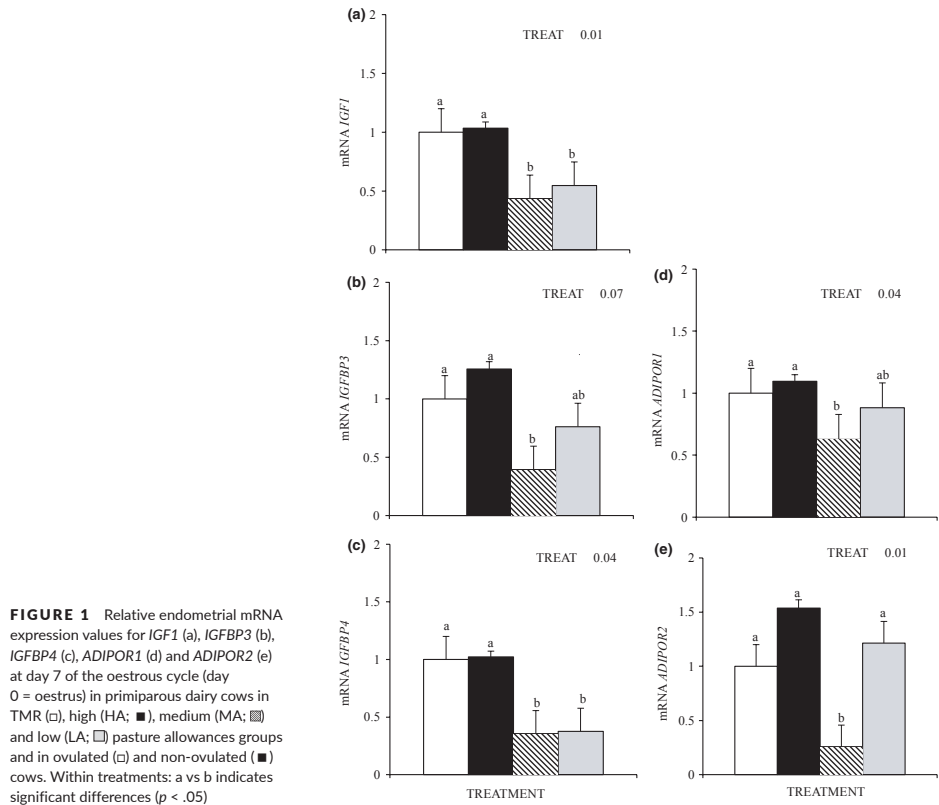
Although the effect of nutritional treatment on *PGR* mRNA expression did not reach significance (Table 2), when Tukey–Kramer test was conducted, expression tended to be twofold greater ($p = .06$) in TMR and HA than LA cows (Table 2). The expression of other genes did not differ among treatments (Table 2).

When cows that did not ovulate were also included in the analysis and ovulation was added as fixed effect, similar results were obtained (data not shown). Moreover, mRNA expression of *PGR*, *IGF2* and *IGFBP5* showed trends to differ among treatments when all cows were considered in the analysis ($p = .11$, 0.13 and 0.09 respectively) and Tukey–Kramer test was as shown in table 2.

4 | DISCUSSION

The present study provides novel information on changes induced by the combined use of TMR and grazing different sward herbage allowances on endometrial gene expression at day 7 of the oestrous cycle (critical period of early embryo loss; Diskin & Morris, 2008) of dairy primiparous Holstein cows at the end of the voluntary waiting period. The uterine endometrium plays a central role in early conceptus-maternal communication for establishment and maintenance of pregnancy which involves dynamic changes in the uterine cell types tightly regulated by changes in steroid hormones, cytokines and growth factors and their receptors. Hence, the endometrium has to be considered a critical epigenetic contributor to the embryo trajectory from the very earliest stages of pregnancy (Sandra et al., 2015).

Although hormone concentrations did not differ in the early stages of oestrous cycle, relevant differences were detected in endometrial mRNA expression among treatments. All hormone concentrations were within the normal range, consistent with previous reports in dairy cows (Rhoads, Meyer, Lamberson, Keisler, & Lucy, 2008) and heifers (Killeen et al., 2014) during the same days of the oestrous cycle. It should be considered that lack of differences due to treatments on hormone concentrations in the present study may be explained, at least partially, by the low differences on energy balance between groups at beginning of the synchronization period (Meikle et al., 2013)



and by blood sampling frequency which was not optimal, as only three samples were collected.

The physiological status of the endometrium just before the moment of blastocyst hatching (uterus at day 7) is key in the development and future elongation of the embryo. In beef heifers, Forde et al. (2011) proposed that P4 mediated its effects predominantly via the endometrium at a time the embryo is transferred (day 7) will affect the future process of elongation. In the current study, the greater expression of *IGF1* mRNA in TMR and HA than MA and LA cows—for example animals with better nutritional status during early lactation—suggests a favourable uterine environment for embryo development. Indeed, P4 increases embryo growth and induces the secretion of IFN τ by acting on the endometrium to stimulate the production of a variety of embryotrophic factors (Geisert et al., 1991). Indeed, it has been shown that *IGF1* mRNA expression is regulated, primarily, by the action of P4 on the uterine endometrium (Satterfield et al., 2010). The expression of *IGF1* mRNA in the uterus was consistent with *PGR* mRNA expression, suggesting a favourable uterine environment for embryonic

development in cows fed diets that maximized nutrient and energy intake during early lactation. The greater expression of these genes found in TMR and HA cows is consistent with the greater probability of post-partum ovarian cyclicity in these cows (Meikle et al., 2013).

Greater uterine *IGF1* mRNA (Meikle et al., 2001) and greater IGF-1 secretion into the uterine lumen (Wathes et al., 1998) around oestrus has been suggested to enhance the uterine environment on which the ruminant embryo is dependent during the pre-implantation period (Robinson, Fray, Wathes, Lamming, & Mann, 2006). Indeed, the endometrial expression of *IGF1* mRNA was positively associated with cow pregnancy at day 17 (Kirby et al., 1996). Further control of this growth factor is achieved via competitive binding to the IGFBPs, whose expression is regulated in a tissue-specific manner and influenced by animal metabolic status (Clemmons, 1998; Thissen, Ketelslegers, & Underwood, 1994). The lower endometrial expression of *IGF2* and *IGF1R* mRNA in MA and LA cows, respectively, is consistent with a poor endocrine and metabolic status previously reported in these animals (Meikle et al., 2013).

Gene	Treatments ¹				SE	p-value TREAT
	TMR	HA	MA	LA		
PGR	1.00 ^a	1.09 ^a	0.64 ^{ab}	0.48 ^b	0.12	.19
ESR	1.00	0.88	0.78	0.54	0.22	.76
GHR	1.00	0.89	0.97	0.47	0.18	.47
IGF1R	1.00 ^{ab}	1.47 ^a	0.82 ^{ab}	0.55 ^b	0.13	.14
IGF2	1.00 ^a	0.55 ^{ab}	0.45 ^b	0.51 ^{ab}	0.17	.18
LEPRB	1.00 ^a	0.72 ^a	0.81 ^a	0.30 ^b	0.12	.27
INSR	1.00 ^a	1.39 ^a	0.51 ^b	0.97 ^{ab}	0.23	.22
IGFBP1	1.00	1.79	2.35	2.32	0.02	.56
IGFBP2	1.00	1.39	1.44	1.25	0.42	.85
IGFBP5	1.00 ^a	1.03 ^a	0.47 ^b	0.57 ^{ab}	0.21	.17
IGFBP6	1.00	0.99	0.76	0.84	0.27	.50

Different letters between treatments: a vs b indicates significant differences ($p < .050$).

¹Treatments = cows were assigned to three grazing treatments: high (HA, 30 kg DM per cow per day), medium (MA, 15 kg DM per cow per day) and low (LA, 7.5 kg DM per cow per day) herbage allowance (mixed pasture, 2,600 kg DM per ha) plus 8 kg DM of supplement or to a TMR (55% forage, 45% concentrate) fed *ad libitum* (TMR) from calving to 75 days post-partum.

TREAT, treatments.

Genes: PGR, progesterone receptor; ESR, oestrogen receptors; GHR, growth hormone receptor; IGF1R, insulin-like growth factor 1 receptor; IGF2, insulin-like growth factor 2; LEPRB, leptin receptor; INSR, insulin receptor; IGFBP1, IGFBP2, IGFBP5, IGFBP6, IGF binding proteins 1, 2, 5 and 6.

In this study, endometrial expression of *IGFBP3*, *IGFBP4* and *IGFBP5* mRNA at day 7 of oestrous cycle was affected by nutritional treatments, being greater in TMR and HA than MA cows. The increased in *IGFBP3* mRNA supports a stimulatory role of this protein during early pregnancy as it has been reported that its expression responds to embryonic signals (Liu, Mele, Catz, Noyes, & Rosenwaks, 1995) and theorized that it may aid in the transportation of systemic IGF to its target site (Robinson et al., 2006). Several studies demonstrated that during development *IGF2* and *IGFBP4* mRNA are co-expressed with *IGF2* in many tissues indicating a potential interaction between *IGF2* and *IGFBP4* in growth and a possibly reservoir of IGF within tissues (Ning, Schuller, Conover, & Pintar, 2008). Contradictory with our results, Wathes, Cheng, Fenwick, Fitzpatrick, and Patton (2011) showed that *IGFBP4* mRNA endometrial expression was greater in cows in severe than mild negative energy balance; however, it should be noted that this study was conducted at 14 days post-partum when uterine involution was not complete, animals were not cycling, and difference between groups on energy balance was greater than in our study. The increased expression of *IGFBP5* mRNA found in TMR and HA than MA cows is consistent with its stimulatory effects on IGF-1 action (Robinson et al., 2006). Overall, data of the IGF system suggest that endometrial expression is modulated according to the energy status.

Female reproductive functions are closely associated with nutritional status, and adiponectin seems to be an important factor linking the energy homeostasis with reproductive function. The impact of adiponectin in the endometrium in productive species is almost unknown (Smolinska et al., 2014), and as far as we know this is the first description of adiponectin receptors in bovine uterus. In humans

TABLE 2 Relative endometrial mRNA expression values at day 7 of the oestrous cycle (day 0 = oestrus) in primiparous dairy cows in TMR ($n = 7$), high (HA; $n = 8$), medium (MA; $n = 8$) and low (LA; $n = 6$) pasture allowances groups cows

(Takemura et al., 2006) and rodent uterus (Mirkin et al., 2005), expression of adiponectin receptors (ADIPOR1/2) increased during the window of implantation suggesting a relevant role during this period. Data suggest that both ADIPOR1/ADIPOR2 are sensitive to energy status, as its expression was reduced in MA cows, reflecting the metabolic stress that these animals suffered during the transition period and are consistent with delayed reinitiation of ovarian cyclicity during the first month after calving (−30 to 30 DPP; Meikle et al., 2013).

Overall, the greater endometrial gene expression (IGF family and progesterone and adiponectin receptors) on day 7 of the oestrous cycle at the end of the voluntary waiting period found in TMR/LA when compared to MA/LA cows suggests a better physiological status of the endometrium in the former ones. This finding may improve embryo development and elongation, which in turn could impact on consequent delivery of IFNT and blockade of the luteolytic mechanism (e.g. embryo signalling, maternal recognition of pregnancy and lower embryo losses; Spencer, Forde, & Lonergan, 2015). Future research on the relevance of energy balance during the early post-partum period on uterine gene expression and function at the end of the voluntary waiting period may explain the lower fertility pregnancy rates found in lactating dairy cows.

5 | CONCLUSION

This study provides solid evidence of the importance of nutritional management during early lactation on uterine environment at the end of the voluntary waiting period. The greater expression of genes

related to embryo growth and uterine function (IGF system and progesterone and adiponectin receptors) in cows fed with diets allowing increased energy intake suggests a favourable environment for embryonic growth, which may explain the improved reproductive performance in cows in good energy balance.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHOR CONTRIBUTIONS

All the co-authors collaborated in the generation of the model, in the organization of the field work and in the interpretation of the results. In particular, Mariana Carriquiry and Pablo Chilbroste collaborated in the final discussion of the data, and Ana Meikle collaborated from the experimental design to the interpretation and discussion of the results and in the final writing of the article.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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5. DISCUSIÓN Y CONCLUSIONES

El estudio del efecto de diferentes estrategias nutricionales como lo son el pastoreo (diferentes tiempos de acceso u oferta de forraje) y su combinación con DTM durante lactación temprana, sobre los perfiles metabólicos/endocrinos, la expresión hepática de genes relacionados con el eje somatotrófico, la regulación del metabolismo energético, la gluconeogénesis y la expresión génica uterina durante la fase luteal temprana permitió comprender -en parte- la priorización de la partición de nutrientes hacia producción de leche en detrimento de la reproducción. Los resultados confirman que la regulación metabólica en vacas lecheras es un sistema dinámico, y que satisfacer los requerimientos del animal con una adecuada alimentación al inicio de la lactancia es fundamental para mejorar variables productivas, reproductivas y también favorecer el ambiente uterino al final del período de espera voluntario para un adecuado desarrollo y crecimiento embrionario.

Como se ha demostrado, durante la transición, las vacas entran en un período de BEN dado porque el rápido aumento en la producción de leche no es acompañado por un aumento suficiente en el consumo de alimento. Esto determina la necesidad de que ocurran varias adaptaciones metabólicas y endocrinas para proporcionar energía y nutrientes necesarios a la glándula mamaria (Bauman 2000; Kessel et al., 2008; van Dorland et al., 2009). En el presente trabajo, similar a lo reportado por otros autores (Loor et al., 2007; McCarthy et al., 2010; Graber et al., 2010) observamos que, para hacer frente al BEN, las vacas movilizaron reservas corporales que se reflejaron en pérdidas de CC, aumentos en las concentraciones de AGNE, BHB y de genes relacionados a la oxidación de ácidos grasos y cetogénesis (ARNm *ACOX*, *ACADVL*, *CPT1* y *HMGCOA*; Astessiano et al., 2014). Asimismo, la disminución de la glucosa e insulina encontradas alrededor del parto, son indicativos de la utilización prioritaria de la glucosa por la glándula mamaria debido alta demanda de lactosa para la producción de leche y a la disminución del suministro de propionato como resultado de la clásica disminución del consumo (Grummer, 1993, Graber et al., 2010) lo que conlleva a un aumento de la gluconeogénesis hepática (van Dorland et al., 2009). En

el presente trabajo, este aumento de glucosa se reflejó en el incremento simultáneo de la expresión de genes que codifican para enzimas claves en la gluconeogenesis (ARNm *PC*, *PCK* y *G6PC*; Astessiano et al., 2013). Por otra parte, a pesar de no haber encontrado diferencias en la expresión de ARNm *PDH* que cataliza la descarboxilación oxidativa de piruvato a acetil CoA, hubo una tendencia al aumento de ARNm *PDK* que cataliza la reacción de fosforilación de la PDH, contribuyendo a la regulación del metabolismo de la glucosa y aumentando así la capacidad gluconeogénica hepática (Loor et al., 2006; van Dorland et al., 2009).

El aumento de AGNE y la disminución en las concentraciones de insulina reportados durante el postparto temprano, indicaron un aumento de la resistencia a la insulina, consistente con otros autores (Holtenius y Holtenius 2007). Se ha reportado que las concentraciones reducidas de adiponectina y leptina alrededor del parto aumentan la resistencia a la insulina de los tejidos periféricos disminuyendo la captación de glucosa por el músculo esquelético y el tejido adiposo, favoreciendo la gluconeogénesis hepática (Block et al., 2001) y por lo tanto el suministro de glucosa a la glándula mamaria para la producción de leche. Los resultados del presente trabajo muestran que al inicio de la lactancia, no solo disminuye la concentración de insulina en sangre, sino que también disminuye la sensibilidad de la insulina para hacer frente a la alta demanda de la glándula mamaria.

La disminución de las concentraciones de insulina e IGF-I alrededor del parto, acompañan el característico desacople del eje somatotrófico para re-direccionar los nutrientes a la producción de leche (Bauman 2000; Lucy 2008). En este trabajo, a pesar de no detectar cambios en la expresión hepática del transcripto de *GHR* como se informó anteriormente (Kobayashi et al., 1999; Lucy 2008), hubo una disminución de la isoforma 1A del receptor (*GHR1A*), de *IGF1* y de *IGFBP3* con un aumento simultáneo en la expresión hepática de las proteínas de unión de bajo peso molecular (ARNm *IGFBP1*, *IGFBP2*, *IGFBP4*, *IGFBP5*). Estos cambios fueron descritos anteriormente por varios autores en el periodo de transición (Kobayashi et al., 1999; Loor et al., 2005; Fenwick et al., 2008; Carriquiry et al., 2009; Kessler et al., 2013) y son consistentes con el estado de resistencia a la GH en el hígado, la disminución de

IGF-I en sangre, el efecto estimulador que tiene la *IGFBP3* (Robinson et al., 2006) e inhibitorio de *IGFBP1*, *IGFBP2* e *IGFBP4* sobre la actividad de IGF-I (Rajaram et al., 1997; Beattie et al., 2006; Piechotta et al., 2013).

Durante el posparto temprano, las concentraciones plasmáticas de AGNE no difirieron entre vacas que consumieron DTM *ad libitum* vs. vacas en dos sesiones de pastoreo, pero fueron menores que en las vacas con una única sesión de pastoreo. Esta reducción de las concentraciones de AGNE y BHB en las vacas sin pastoreo podría estar indicando una baja tasa de lipólisis y cetogénesis hepática de acuerdo con su mejor balance energético (Kennedy et al., 2008). Consistente con la menor abundancia de ARNm de *ACADVL* y *HMGCOA* encontrada en las vacas consumiendo DTM *ad libitum* vs. vacas en sistemas mixtos (DTM + pastoreo) (Astessiano et al., 2014) y las mayores concentraciones de insulina e IGF-I plasmática que son indicativos de las menores necesidades energéticas y el mejor balance energético que tuvieron estas vacas (Loor et al., 2007; Wales et al., 2013). Por el contrario, las concentraciones reducidas de AGNE en las vacas con dos sesiones de pastoreo (comparadas con una sesión de pastoreo) acompañadas de concentraciones elevadas de BHB, podrían estar relacionadas con una utilización diferencial de los AGNE debido al aumento de las actividades de caminata y pastoreo en estas vacas. De hecho, han sugerido que el aumento de la actividad dado por la caminata podría reducir las concentraciones de AGNE en plasma (Adewuyi et al., 2005) y han reportado que durante el ejercicio los AGNE son utilizados para la producción de energía en el músculo y contracción muscular a través de la oxidación de ácidos grasos mitocondriales y del ciclo de Krebs (Drackley et al., 2001). En el mismo sentido, las mayores concentraciones de leptina y adiponectina encontradas en estas vacas, son similares a los resultados reportados en humanos por Simpson y Singh (2008) que encontraron aumentos en las concentraciones circulantes de adiponectina, la sensibilidad a la insulina y en la tasa de oxidación de la grasa basal con el ejercicio durante el posparto. Por lo tanto, la mayor actividad de estas vacas -dado por el mayor número de sesiones de pastoreo y el mayor tiempo de acceso a la pastura- podrían explicar estos resultados, demostrando la mayor exigencia metabólica que tienen las vacas lecheras en pastoreo comparada

con vacas en sistemas de confinamiento con alimentación en base a DTM. Resultados similares al comparar asignaciones crecientes de forraje fueron reportados por Meikle et al. (2013) que no encontraron diferencias entre vacas consumiendo DTM *ad libitum* vs. asignaciones altas de forraje, pero las concentraciones de AGNE y de BHB fueron mayores en vacas consumiendo asignaciones medias de forraje indicando el menor balance energético de esas vacas. Asimismo, reportaron mayores concentraciones de insulina e IGF-I en vacas consumiendo DTM *ad libitum* indicativo del mejor balance energético.

La inclusión del pastoreo, sin un período de adaptación, en la dieta de vacas lecheras alimentadas con DTM *ad libitum* durante la lactancia temprana, disminuyó el PV, la CC y la producción de leche, probablemente asociado a una restricción en el consumo de MS y al cambio de la dieta (Fajardo et al., 2015). En este sentido, varios autores han reportado disminución de la producción de leche y del consumo de MS en vacas alimentadas con dietas que combinan DTM e inclusión de pasturas vs. vacas alimentadas con DTM *ad libitum* (Bargo et al., 2002; Vibart et al., 2008; Kennedy et al., 2015). Asimismo, la inclusión del pastoreo, tendió a aumentar las concentraciones de IGF-1 en vacas que cambiaron de dieta, consistentes con una mayor expresión de ARNm hepático de *IGF1*, *IGFBP3* e *IGFBP5*. Considerando que el cambio de dieta ocurrió luego del pico de producción de leche, este aumento en el IGF-1 circulante podría sugerir una modificación en la redistribución de los nutrientes en detrimento de la producción de leche (Bauman 2000; Fenwick et al., 2008).

En el presente estudio, los resultados muestran que los cambios en la regulación endocrina y metabólica según la estrategia nutricional, afectaron no solo el reinicio a la ciclicidad ovárica sino también la funcionalidad uterina. Las vacas que pastorearon en dos sesiones fueron las que tuvieron más comprometida la performance reproductiva, -más días a primera ovulación y menor probabilidad de ciclar en el primer mes- comparado con los otros grupos y presentaron mayor expresión hepática de ARNm *IGFBP6*, consistente con Cummins et al. (2012) en vacas menos fértiles vs. vacas con mayor fertilidad. De manera similar a lo reportado por Meikle et al. (2013)

que vacas alimentadas con asignaciones medias de forraje presentan un reinicio a la ciclicidad ovárica más tardío comparado con vacas alimentadas con DTM *ad libitum* y asignaciones altas de forraje. Más aún en estas condiciones, a pesar de no encontrar diferencias en las concentraciones hormonales durante la primera etapa del ciclo estral (Rhoads et al., 2008), si se detectaron diferencias relevantes en la expresión génica endometrial entre los tratamientos, afectando la funcionalidad uterina. La mayor expresión de ARNm *IGF1*, *IGFBP3*, *IGFBP4*, *IGFBP5*, en las vacas alimentadas con dietas que maximizaron la ingesta de nutrientes y energía (DTM *ad libitum* y alta asignación) sugirió un entorno uterino favorable para el desarrollo del embrión. Estos resultados son consistentes con el rol estimulador que tiene ARNm *IGFBP3* durante la preñez temprana (Liu et al., 1995) y junto con *IGFBP5* sobre la acción de IGF-1 (Robinson et al., 2006). Asimismo, la menor expresión endometrial de ARNm *IGF2* e *IGFIR* en las vacas de media y baja asignación de forraje, es consistente con el peor estado endocrino y metabólico reportado previamente en estos animales (Meikle et al., 2013).

Se ha demostrado que las funciones reproductivas femeninas están estrechamente asociadas con el estado nutricional, y la adiponectina parece ser un factor importante que vincula la homeostasis energética con la función reproductiva. La menor expresión endometrial de ARNm *ADIPOR1* y *ADIPOR2*, encontrada en las vacas alimentadas con asignación media de forraje es consistente con lo reportado en útero por Takemura et al. (2006; en humanos) y por Mirkin et al. (2005; en roedores), que encontraron aumento de ambos receptores durante la ventana de implantación en condiciones de buen balance energético. Nuestros resultados reflejan el estrés metabólico que sufrieron estos animales durante el período de transición y son consistentes con el retraso a la ciclicidad ovárica durante el primer mes post-parto reportado anteriormente por Meikle et al. (2013). Resultados preliminares sobre el transcriptoma global del útero de vacas lecheras, comparando dos sistemas de alimentación (pastoreo + DTM vs. DTM *ad libitum*) al final del período de espera voluntario, muestran las vías metabólicas relacionadas a la remodelación tisular y regulación de la actividad enzimática up reguladas en vacas alimentadas en sistemas

mixtos, mientras que las vías relacionadas al sistema inmune están up reguladas en vacas alimentadas con DTM *ad libitum* (Astessiano et al., 2016). Estos resultados sugieren que la alimentación puede favorecer el ambiente uterino y consecuente desarrollo embrionario.

Los resultados de este estudio muestran que, utilizando altas asignaciones de forraje, de buena calidad y teniendo en cuenta el gasto energético de los animales por actividad (caminatas y pastoreo) al inicio de la lactancia, se logren resultados productivos y reproductivos similares y comparables con sistemas de alimentación DTM *ad libitum*. De esta manera es posible tener sistemas mixtos de producción lechera más competitivos, aprovechando las ventajas económicas, ambientales y de bienestar animal de dichos sistemas.

La mayor demanda energética en las vacas con doble acceso a la pastura al inicio de la lactancia (dado por la actividad de pastoreo) implicó un mayor estrés metabólico que impactó negativamente en la función reproductiva (retraso en el reinicio de la actividad ovárica y menor probabilidad de reinicio). Asimismo, cambios en la estrategia de alimentación sin un período de adaptación también modificaron el metabolismo animal, la inclusión del pastoreo en vacas que fueron alimentadas con DTM durante la lactancia temprana, indicó una redistribución de nutrientes y energía hacia requerimientos de mantenimiento (actividad de caminar y de pastoreo) en detrimento de la producción de leche. El estudio del ambiente uterino al final del período de espera voluntario, presento mayor expresión de genes relacionados con el crecimiento embrionario y la función uterina, en vacas alimentadas con dietas que permitieron maximizar el consumo de energía sugiriendo un ambiente favorable para el crecimiento embrionario.

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7. ANEXOS

7.1.

Cambios temporales durante el parto relacionados con la gluconeogenesis en vacas Holando con diferentes estrategias de alimentación en lactación temprana

A.L Astessiano^{1*}, P. Chilibroste¹, A Meikle², M. Fajardo¹, D.A Mattiauda¹, M Carriquiry¹

¹Facultad de Agronomía, UDELAR, Uruguay, ²Facultad de Veterinaria, UDELAR, Uruguay. *lauaste@gmail.com

Con el objetivo de estudiar los cambios temporales durante el parto y el efecto de diferentes estrategias nutricionales durante los primeros 60 días de lactancia sobre la expresión hepática de genes relacionados con la gluconeogenesis (*piruvato carboxilasa*, **PC**; *fosfoenolpiruvato carboxikinasa*, **PCK** y *glucosa-6-fosfatasa* **G6PC**), se utilizaron 25 vacas Holando multíparas en un diseño de bloques al azar. Los animales fueron asignados a tres tratamientos: **TMR**) ración totalmente mezclada (30 kgMS/d; 45% ensilaje, 55% concentrado); **P1**) 50% pastura en una sesión de pastoreo (am; 7 h; oferta de forraje = 15 kgMS/d) + 50% TMR (15 kgMS/d) y **P2**) 50% pastura en dos sesiones de pastoreo (am/pm; 11h; oferta de forraje = 15 kgMS/d) + 50% TMR (15 kgMS/d). Muestras de sangre e hígado fueron obtenidas a los -40, -20, 10, 30 y 55 días posparto. Los datos se analizaron en un modelo mixto y las medias se consideraron diferentes cuando $P \leq 0.05$. La producción de energía en leche no fue afectada por los tratamientos. La CC disminuyó del pre al posparto siendo a los 55 días mayor en TMR. Las concentraciones de glucosa disminuyeron a los 10 días, siendo menores ese día en P1 y P2 que en TMR. La insulina plasmática disminuyó del pre al postparto y tendió ($P=0.06$) a ser afectada por la interacción entre tratamiento y días, al ser menores en P2 que TMR y P1 a los 10 y 30 días. Estos resultados fueron consistentes con el consumo y/o requerimiento de energía. La abundancia de ARNm de *PC*, *PCK* y *G6PC* no difirió entre tratamientos y aumentó del pre al posparto, siendo máxima la expresión de ARNm de *PC* al día 10 y de *PCK* al día 55. El aumento de las necesidades de glucosa para producción de leche se reflejó en el incremento simultáneo de la expresión de genes que codifican para enzimas claves en la gluconeogenesis.

Palabras claves: pastoreo, expresión génica hepática

Cambios temporales durante el periparto relacionados con la gluconeogenesis en vacas Holando con diferentes estrategias de alimentación en lactación temprana



A.L Astessiano¹, P. Chilbroste¹, A Meikle², M. Fajardo¹, D.A Mattiauda¹, Carriquiry, M.¹

¹Departamento de Producción Animal y Pasturas Facultad de Agronomía, UdelaR, Montevideo Uruguay ²Departamento de Producción Animal y Pasturas EENAC Facultad de Agronomía, UdelaR, Paysandú Uruguay



INTRODUCCIÓN

Adaptaciones al balance energético negativo de inicio de lactancia, implican cambios internos con el fin de promover la disponibilidad de glucosa y aminoácidos para el metabolismo (Bell, 1995). Además de haber una utilización prioritaria de la glucosa por parte de la glándula mamaria, existe también proteólisis, con el fin de movilizar aminoácidos que contribuyan a la gluconeogenesis hepática (Bauman 2000, Lucy 2008). En vacas lecheras en pastoreo el consumo de MS no es suficiente para mantener la alta producción de leche que se podría lograr con su potencial genético real (Kolver y Muller 1998). Por lo tanto, el tiempo de acceso a las pasturas y el número de sesiones de pastoreo podrían ser utilizados como herramientas para mejorar la eficiencia de pastoreo y la producción de leche (Chilbroste et al 2007; Kennedy et al 2009; Perez-Ramirez et al. 2009)

OBJETIVOS

Estudiar los cambios temporales durante el periparto y el efecto de diferentes estrategias nutricionales durante los primeros 60 días de lactancia sobre la expresión hepática de genes relacionados con la gluconeogenesis

MATERIALES Y MÉTODOS

✓ El experimento se realizó en la Estación Experimental Mario A. Casinoni - Facultad de Agronomía (Paysandú, Uruguay).

✓ Se utilizaron 25 vacas Holando multiparas en un diseño de bloques al azar

✓ Los animales fueron asignados a tres (TRAT):

○ TMR) ración totalmente mezclada (30 kgMS/d; 45% pastura, 55% concentrado)

○ P1) 50% pastura en una sesión de pastoreo (am; 7 h; oferta de forraje = 15 kgMS/d) + 50% TMR (15 kgMS/d)

○ P2) 50% pastura en dos sesiones de pastoreo (am/pm; 11h; oferta de forraje = 15 kgMS/d) + 50% TMR (15 kgMS/d)

✓ Las muestras de sangre e hígado fueron obtenidas a los -40, -20, 10, 30 y 55 días posparto (DPP) para determinar:

• concentraciones de glucosa e insulina.
• abundancia mRNA *PC*, *PCK* y *G6PC* por PCR en tiempo real.

✓ Los datos se analizaron usando un modelo mixto anidado y las medias se consideraron diferentes cuando $P \leq 0.05$.

RESULTADOS

✓ La producción de energía en la leche no fue afectada por los tratamientos (27.4, 26.7 y 25.1 $\pm$ 1.4 Mcal/d en TMR, P1 y P2)

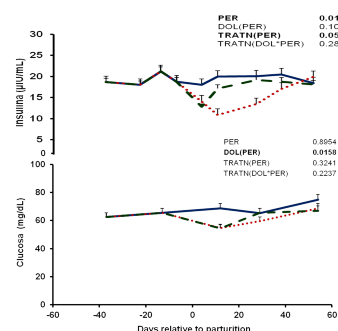
✓ La energía estimada para mantenimiento fue mayor en P2 que en P1 y que en TMR en P1

✓ La CC disminuyó del pre al posparto siendo a los 55 DPP mayor en TMR.

✓ Las concentraciones de **insulina** disminuyeron del pre al posparto y tendieron a ser afectadas por la interacción entre tratamiento y días, ya que fueron menores en P2 que TMR y P1 a los 10 y 30 DPP

✓ Las concentraciones de glucosa disminuyeron a los 10 días, siendo menores ese día en P1 y P2 que en TMR.

Gen	Días	Tratamientos			P-value			
		TMR	P2	P1	PER	DOL(PER)	TRATN(PER)	TRATN(DOL*PER)
PC	-40	1.00	c	1.00	c			
	-20	1.12	c	1.12	c			
	10	2.31	ab	2.39	a	2.26	ab	
PCK1	55	1.58	abc	1.40	bc	2.00	ab	
	-40	1.00	c	1.00	c			
	-20	0.78	c	0.78	c			
G6PC	10	1.53	b	1.74	bc	1.67	b	
	55	2.07	ab	2.83	a	2.03	ab	
	-40	1.00		1.00				
	-20	0.86		0.86				
	10	1.39		1.06				
	55	1.53		1.11				



✓ La abundancia de ARNm de *PC*, *PCK* y *G6PC* no difirió entre TRAT y aumentó del pre al posparto, siendo máxima la expresión de ARNm de *PC* al día 10 y de *PCK* al día 55.

El aumento de las necesidades de glucosa para producción de leche se reflejó en el incremento simultáneo de la expresión de genes que codifican para enzimas claves en la gluconeogenesis.

Hepatic expression of genes related with fatty acid metabolism in Holstein cows with different feeding strategies in early lactation

A.L. Astessiano¹, P. Chilibroste², M. Fajardo², D.A. Mattiauda¹, A. Meikle³, M. Carriquiry¹

¹School of Agronomy, UDELAR, Montevideo, Uruguay, ²School of Veterinary Medicine, UDELAR, Paysandú (EEMAC), Uruguay, ³School of Veterinary Medicine, UDELAR, Montevideo, Uruguay

Multiparous cows (n=25) were used in a randomized complete block design to study the effects of nutrition during the first 60 d postpartum (DPP) on hepatic expression of genes associated with fatty acid oxidation. Cows were assigned to 3 treatments (TREAT): TMR= total mixed ration (30 kg DM/d offered; 45% forage, 55% concentrate); G1= 50% pasture in one (am) grazing session (6 h; pasture allowance =15 kg DM/d) + 50% TMR (15 kg DM/d offered) and G2= 50% pasture in two (am/pm) grazing sessions (9 h; pasture allowance =15 kg DM/d) + 50% TMR (15 kg DM/d offered). Liver biopsies were collected at -40, -20, +10 and +55 DPP to measure the abundance of mRNA of pyruvate dehydrogenase (*PDH1A*) and pyruvate dehydrogenase kinase (*PDK*), acyl-CoA dehydrogenase, very long chain (*ACADVL*), acyl-CoA oxidase (*ACoAoX*), carnitine palmitoyltransferase-1A (*CPT1A*), β -hydroxy- β -methylglutaryl-coenzyme A (*HMGCOA*) and citrate synthase (*CS*) by SYBR-Green real time RT-PCR. Data were analyzed in a mixed model using a repeated measures analysis. Milk energy output did not differ among TREAT (27.8, 26.4 and 23.4 $\pm$ 1.4 Mcal/d for TMR, G1 and G2, respectively) while BCS decreased ($P<0.05$) during the postpartum and at +55 DPP was greater ($P=0.02$) in TMR than G1 and G2 cows. Hepatic expression of *ACADVL*, *ACOX*, and *CPT1A* and *HMGCOA* mRNA increased ($P<0.03$) from pre to postpartum. The *PDK* mRNA tended ($P=0.06$) to increase from pre to postpartum. Expression of *ACADVL* mRNA tended ($P=0.06$) to be affected by the TREAT by DPP interaction, being greater in G2 than TMR cows at +55 DPP (1.18, 1.50 and 2.19 $\pm$ 0.2 for TMR, G1 and G2, respectively). The *CPT1A* mRNA was greater ($P=0.03$) in G1 than G2 cows (1.83 and 1.30 $\pm$ 0.3 for G1 and G2, respectively) and *HMGCOA* mRNA was greater ($P=0.05$) in G2 than TMR cows (0.90 and 1.35 $\pm$ 0.2 for TMR, and G2, respectively) during the postpartum period. Hepatic *AOX*, *PDK*, *PDH1A* and *CS* mRNA were not affected by TREAT. Results indicate that hepatic expression of fatty acid oxidation and ketogenesis genes increased

HEPATIC EXPRESSION OF GENES RELATED WITH FATTY ACID METABOLISM IN HOLSTEIN COWS WITH DIFFERENT FEEDING STRATEGIES IN EARLY LACTATION



A.L. ASTESSIANO¹, P. CHILIBROSTE², M. FAJARDO², D.A. MATTIAUDA¹, A. MEIKLE³, M. CARRIQUIRY¹

#T392

¹SCHOOL OF AGRONOMY, UDELAR, URUGUAY, ²EEMAC, PAYSANDÚ, URUGUAY ³SCHOOL OF VETERINARY, UDELAR, URUGUAY

INTRODUCTION

The onset of lactation in dairy cows is accompanied by negative energy balance, which is characterized by mobilization of body fat, protein, and mineral stores to satisfy the requirements for milk production and maintenance. The liver plays a key role in the metabolic adaptation, through coordination and interconversion of nutrients to support pregnancy and lactation (Seifert and England, 1994; Reynolds et al., 2003).

OBJECTIVE

To study the effect of nutritional strategy (grazing vs. TMR) during the first 60 days postpartum in multiparous dairy cows on hepatic expression of genes associated with gluconeogenesis and fatty acid oxidation.

MATERIALS AND METHODS

✓ Location: EEMAC, Paysandú, Uruguay (Latitude 32.5°S, Longitude 58°W)

✓ Multiparous cows (n=25) were used in a randomized block design and assigned to 3 treatments (TREAT):

- TMR) total mixed ration (30 kgDM/d offered; 45% forage, 55% concentrate);
- G1) 50% pasture in one (am) grazing session (6 h; pasture allowance = 15 kgDM/d) + 50% TMR (15 kgDM/d offered) and
- G2) 50% pasture in two (am/pm) grazing sessions (9 h; pasture allowance = 15 kg DM/d) + 50% TMR (15 kgDM/d offered).

✓ Determinations:

- BCS (scale 1-5, Elanco Animal Health) registered every 14 d
- Liver biopsies obtained at -40, -20, 10 and 55 DPP to quantify by SYBR-Green real time RT-PCR, mRNA expression of:
 - pyruvate dehydrogenase (PDH1A)
 - pyruvate dehydrogenase kinase (PDK),
 - acyl-CoA dehydrogenase, very long chain (ACADVL),
 - acyl-CoA oxidase (ACoAox),
 - carnitine palmitoyltransferase-1A (CPT1A),
 - β-hydroxy-β-methylglutaryl-coenzyme A (HMGCoA)
 - citrate synthase (CS)

✓ Statistical analyses:

- Data were analyzed in a mixed model with repeated measures using PROC MIXED (SAS Institute, 2001).
- Means were considered to differ when P < 0.05

RESULTS

➢ Milk energy output did not differ among TREAT (27.8, 26.4 and 23.4 ± 1.4 Mcal/d for TMR, G1 and G2, respectively) while BCS decreased during the postpartum and at +55 DPP was greater in TMR than G1 and G2 cows.

Gene ¹	Treatments ²				se	PER	P-value		
	Days	TMR	G2	G1			DOL (PER)	TREAT (PER)	TREAT (DOL*PER)
ACADVL									
-40	1.00	1.00	1.00	1.00	0.13	<.0001	0.815	0.129	0.058
-20	0.96	0.96	0.96	0.96	0.13				
10	1.59	1.57	1.29	1.47	0.13				
55	1.18	2.19	1.50	1.62	0.13				
ACoAox									
-40	1.00	1.00	1.00	1.00	0.18	<.0001	0.599	0.316	0.515
-20	0.92	0.92	0.92	0.92	0.18				
10	1.47	1.55	1.42	1.48	0.18				
55	1.42	2.26	1.47	1.72	0.18				
CPT1A									
-40	1.01	1.01	1.01	1.01	0.15	<.0001	0.893	0.080	0.623
-20	1.06	1.06	1.06	1.06	0.15				
10	1.72	1.15	1.70	1.52	0.15				
55	1.53	1.30	1.83	1.55	0.15				
HMGCoA									
-40	1.00	1.00	1.00	1.00	0.17	0.0245	0.587	0.049	0.102
-20	0.85	0.84	0.85	0.85	0.18				
10	1.12	1.16	1.21	1.16	0.17				
55	0.72	1.57	1.64	1.31	0.18				
CS									
-40	1.00	1.00	1.00	1.00	0.13	0.281	0.435	0.843	0.385
-20	0.92	0.92	0.92	0.92	0.14				
10	0.93	1.16	0.90	1.00	0.14				
55	1.09	1.07	1.33	1.16	0.14				
PDH									
-40	1.01	1.01	1.01	1.01	0.21	0.357	0.360	0.281	0.485
-20	1.02	1.02	1.02	1.02	0.22				
10	0.92	1.04	1.14	1.03	0.21				
55	0.75	0.66	1.14	0.85	0.22				
PDK									
-40	1.00	1.00	1.00	1.00	0.25	0.069	0.753	0.726	0.688
-20	1.23	1.23	1.23	1.23	0.28				
10	1.65	1.50	1.51	1.55	0.25				
55	1.02	1.43	1.65	1.37	0.24				

² Nutritional treatments from calving (day 0) to 60 days postpartum

➢ Hepatic expression of ACADVL, ACoAox, and CPT1A and HMGCoA mRNA increased while PDK mRNA tended to increase from pre to postpartum.

➢ Expression of ACADVL mRNA tended to be affected by the TREAT by DPP interaction, being greater in G2 than TMR cows at +55 DPP

➢ The CPT1A mRNA was greater in G1 than G2 cows

➢ HMGCoA mRNA was greater in G2 than TMR cows during the postpartum period

➢ Hepatic AOX, PDK, PDH1A and CS mRNA were not affected by TREAT

Results indicate that hepatic expression of fatty acid oxidation and ketogenesis genes increased in early lactation to cope with negative energy balance. This increase was greater in G2 than TMR cows which could be associated to the greater energy requirement for maintenance due to the activity of walking and grazing in the former ones.

Global gene expression in the endometrium of primiparous dairy cows during the early-luteal phase of the estrous cycle

A.L. Astessiano¹, F. Peñagaricano³, J. Laporta³, A. Meikle², M. Carriquiry¹.

¹School of Agronomy, UDELAR, Montevideo, Uruguay, ²School of Veterinary Medicine, UDELAR, Montevideo, Uruguay, ³University of Florida, Gainesville, Florida, United States.

The uterine endometrium plays a central role in early conceptus-maternal communication for the establishment and maintenance of pregnancy. Primiparous Holstein dairy cows were used in a randomized block design to evaluate gene expression changes in the endometrium during the early-luteal phase of the estrous cycle induced by two different feeding strategies: total mixed ration (TMR) vs. pasture + TMR applied during early lactation. In particular, during the first 65 days of lactation, cows were fed either [i] TMR *ad libitum* (17 kgDM/d offered; 70% forage, 30% concentrate; **T1**) or [ii] grazing of alfalfa (*Medicago sativa*; 6-h am grazing in 3-d strips; pasture allowance=20 kgDM/d) plus TMR (70% of *ad libitum* TMR; 12 kgDM/d offered; **T2**). At 45 ± 1 d, cows were synchronized and at d 7 of the estrous cycle (d 0 = estrous) endometrial biopsies were obtained. A total of 10 endometrium samples (5 cows per treatment) were analyzed using RNA sequencing. Sequence reads were mapped to the bovine reference genome (bosTau7) using Tophat and the resulting alignments were used to reconstruct transcript models using Cufflinks. Gene expression differences were tested using edgeR package. From the 14,753 genes detected in the transcriptome, 102 genes were differentially expressed (FDR = 0.10; fold change ≥ 2) between T1 vs. T2. Specifically, 20 genes were significantly upregulated in T1 while 82 genes were upregulated in T2. Many of these genes are involved in biological processes such as regulating enzymatic activity (e.g. phospholamban, secretoglobin family 1A member 1), protein binding (e.g. caveolin 3, alpha-2-HS-glycoprotein) and immune response (e.g. immunoglobulin heavy constant epsilon, major histocompatibility complex class II DQ beta, myelin protein P0-like). Functional enrichment analysis, using both Gene Ontology and KEGG databases, revealed significant terms associated with cell and tissue development, cell adhesion, endopeptidases, calcium ion transport, calcium signaling, tryptophan metabolism, among others, with most of the genes being upregulated in T2 compared to T1. Overall, this study characterized genes and pathways expressed in the endometrium of dairy cows at day 7 of the estrous cycle, and evidenced a differential endometrial environment according with a nutritional management during early lactation in which most of the genes differentially expressed were upregulated in grazing cows.

Key words: dairy cows, nutrition, transcriptome



GLOBAL GENE EXPRESSION IN THE ENDOMETRIUM OF PRIMIPAROUS DAIRY COWS DURING THE EARLY-LUTEAL PHASE OF THE ESTROUS CYCLE

A.L. ASTESSIANO¹, F. PEÑAGARICANO³, J. LAPORTA³, M. CARRIQUIRY¹, A. MEIKLE²

¹SCHOOL OF AGRONOMY, UDELAR, MONTEVIDEO, URUGUAY, ²SCHOOL OF VETERINARY MEDICINE, UDELAR, MONTEVIDEO, URUGUAY, ³UNIVERSITY OF FLORIDA, GAINESVILLE, FLORIDA, UNITED STATES.



INTRODUCTION

In grazing systems, dairy cows do not get sufficient dry matter intake to sustain the high milk production that could be achieved by their genetic potential (Koer and Muller, 1998) impacting also their uterine functionality at breeding time (end of the voluntary waiting period). During the estrous cycle, the synthesis and excretion of histotroph by the endometrium is crucial as a nutritious fluid that provides a means of communication between conceptus and maternal environment through signaling molecules. Therefore, the physiological status of the endometrium just before the moment of blastocyst hatching (uterus at day 7) is key in the development and future elongation of the embryo, also known as a critical period of early embryo loss (Dickin and Morris 2008; Walsh et al., 2011).

OBJECTIVE

Provide information by RNA-Seq, on changes in the endometrial gene expression induced by the combined use of TMR and grazing during an important period of the estrous cycle: the early luteal phase.

MATERIALS & METHODS

Location: EEMAC, Paysandú, Uruguay (Latitude 32.5°S, Longitude 58°W)

Experimental design and treatments:

- Primiparous Holstein dairy cows calving in fall (n = 20, 518 ± 47 kg BW, 2.7 ± 0.3 BCS) were used in a randomized block design
- During the first 65 days of lactation, cows were fed either
 - TMR *ad libitum* (17 kgDM/d offered; 70% forage, 30% concentrate; T1: control) or
 - grazing of alfalfa (Medicago sativa; 6-h am grazing in 3-d strips; pasture allowance=20 kgDM/d) plus TMR (60% of *ad libitum* TMR; 12 kgDM/d offered; T2)
- At 45 ± 1 d, cows were synchronized and at d 7 of the estrous cycle (d 0 = estrous) endometrial biopsies were obtained. A total of 10 endometrium samples (5 cows per treatment) were analyzed using RNA sequencing.
- Sequence reads were mapped to the bovine reference genome (bosTau7) using Tophat and the resulting alignments were used to reconstruct transcript models using Cufflinks.
- Gene expression differences were tested using edgeR package

RESULTS

- From the 14,753 genes detected in the transcriptome, 102 genes were differentially expressed (FDR = 0.10; fold change ≥ 2) between T1 vs. T2. Specifically, 20 genes were significantly upregulated in T1 while 82 genes were upregulated in T2.

- Many of these genes are involved in biological processes such as regulating enzymatic activity, protein binding and immune response.

List of 25 most significant genes that showed differential expression in endometrium at day 7 of estrus cycle between Holstein cow grazing pastures and supplemented with 60% TMR (T2) and cows fed TMR *ad libitum* (T1).

Gene	Name	logFC	P-value
MSMB	microsminoglycan, beta	-7.6	3.1 × 10 ⁻⁴
BOLA-DQB	major histocompatibility complex, class II, DQ beta	-6.4	3.8 × 10 ⁻⁴
SCGB1A1	secretoglobin, family 1A, member 1 (uteroglobulin)	-5.7	1.3 × 10 ⁻⁴
SEPPIN10	serpin peptidase inhibitor, clade B (ovalbumin), memb	-5.5	3.2 × 10 ⁻⁴
SPINK1	serine peptidase inhibitor, Kazal type 1	-5.0	1.5 × 10 ⁻⁴
FGG	fibrinogen gamma chain	-4.5	3.8 × 10 ⁻⁴
CDK5R2	cyclin-dependent kinase 5, regulatory subunit 2 (p39)	-3.7	4.7 × 10 ⁻⁴
CESA4	carboxylesterase 4A	-3.6	8.0 × 10 ⁻⁴
IGHE	immunoglobulin heavy constant epsilon	-3.5	4.2 × 10 ⁻⁴
IGJ	immunoglobulin J polypeptide, linker protein for immu	-3.0	4.9 × 10 ⁻⁴
LOC79008	myelin protein PL-like	6.4	1.8 × 10 ⁻⁴
CYP11A1	cytochrome P450, subfamily 1 (aromatic compound-induc	6.4	6.2 × 10 ⁻⁴
AOX2	aldehyde oxidase 2	6.0	4.1 × 10 ⁻⁴
HMGN2	hemicentin 2	6.0	5.0 × 10 ⁻⁴
MAMRN1	multimerin 1	6.0	8.6 × 10 ⁻⁴
TMEFF2	transmembrane protein with EGF-like and two follistat	4.8	3.5 × 10 ⁻⁴
MTF1	metallothionein 1	4.6	7.1 × 10 ⁻⁴
CD200R1L	CD200 receptor 1-like	4.3	5.3 × 10 ⁻⁴
CAV3	caveolin 3	4.1	7.2 × 10 ⁻⁴
IDO1	indoleamine 2,3-dioxygenase 1	3.5	3.4 × 10 ⁻⁴
PI3OC	protein C (inactivator of coagulation factors Va and	3.4	6.8 × 10 ⁻⁴
NOS2	nitric oxide synthase 2, inducible	3.0	3.3 × 10 ⁻⁴
AHG5	alpha 2-HS glycoprotein	3.0	6.0 × 10 ⁻⁴
CLDN11	claudin 11	2.8	5.5 × 10 ⁻⁴
ACTG2	actin, gamma 2, smooth muscle, enteric	2.8	6.0 × 10 ⁻⁴



GLOBAL GENE EXPRESSION IN THE ENDOMETRIUM OF PRIMIPAROUS DAIRY COWS DURING THE EARLY-LUTEAL PHASE OF THE ESTROUS CYCLE

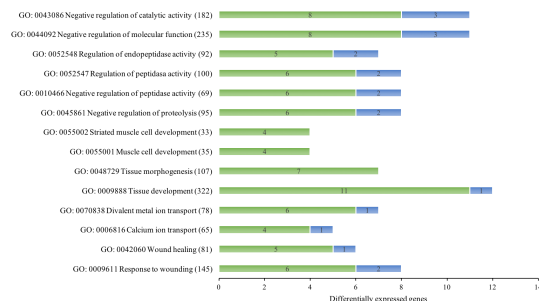
A.L. ASTESSIANO¹, F. PEÑAGARICANO³, J. LAPORTA³, M. CARRIQUIRY¹, A. MEIKLE²,

¹SCHOOL OF AGRONOMY, UDELAR, MONTEVIDEO, URUGUAY, ²SCHOOL OF VETERINARY MEDICINE, UDELAR, MONTEVIDEO, URUGUAY, ³UNIVERSITY OF FLORIDA, GAINESVILLE, FLORIDA, UNITED STATES.



RESULTS

List of biological process of Gene Ontology terms that were significantly enriched with genes differentially expressed in endometrium at day 7 of estrus cycle. The graph shows the GO ID, term name, total number of genes within each term, and the number of genes up-regulated (green) and down-regulated (blue) in Holstein cow grazing pastures and supplemented with 60% TMR (T2) and cows fed TMR *ad libitum* (T1).



RESULTS

List of KEGG pathways term that were significantly enriched (P-value <0.01) with genes differentially expressed in endometrium at day 7 of estrus cycle in Holstein cow grazing pastures and supplemented with 60% TMR (T2) and cows fed TMR *ad libitum* (T1).

Functional Characterization ¹	DEG	Genes	P-value
Cell adhesion molecules (95)	6	PD-L1, CDH5, PECAM1, CLDN11, MIP2, BOLA-DQB	0.005
Calcium signaling pathway (113)	6	INOS, P2RX2, CHP2, PLN, MYLK, CACNA1F	0.012
Tryptophan metabolism (30)	3	CYP1A1, IDO1, AOX2	0.014

¹The table shows the term name and the total number of genes within each term. Gene symbols that are underlined denote genes down-regulated in endometrium in Holstein cow grazing pastures and supplemented with 60% TMR (T2; Treatment) and cows fed TMR *ad libitum* (T1; Control).

CONCLUSIONS

Overall, this study characterized genes and pathways expressed in the endometrium of dairy cows at day 7 of the estrous cycle, and evidenced a differential endometrial environment according with a nutritional management during early lactation in which most of the genes differentially expressed were upregulated in grazing cows

7.4. GLOBAL GENE EXPRESSION IN ENDOMETRIUM OF PRIMIPAROUS DAIRY COWS INDUCED BY THE COMBINED USE OF TMR AND GRAZING DURING THE EARLY-LUTEAL PHASE OF ESTROUS CYCLE. Astessiano A.L, Peñagaricano F, Laporta J, Meikle A, Carriquiry M, *se encuentra en la etapa final de discusión.*

El objetivo es estudiar el efecto de diferentes estrategias nutricionales, pastoreo + DTM vs. DTM *ad libitum* durante la lactancia temprana, sobre el transcriptoma global en el útero de vacas lecheras cíclicas, identificando genes y vías metabólicas diferencialmente expresados, al final del período de espera voluntario

La hipótesis es que las vacas alimentadas con dietas que permitan maximizar la ingesta de nutrientes y la energía durante la lactancia temprana, presentan un perfil de expresión génica favorable al crecimiento y a la implantación embrionaria, que reduciría la mortalidad embrionaria temprana.

GLOBAL GENE EXPRESSION IN ENDOMETRIUM OF LACTATING PRIMIPAROUS DAIRY COWS INDUCED BY THE COMBINED USE OF TMR AND GRAZING DURING THE EARLY-LUTEAL PHASE OF ESTROUS CYCLE

INTRODUCTION

In dairy cows, the reproductive success after calving is limited by the resumption to ovarian cyclicity and the early embryo mortality which are both affected by the typical negative energy balance of early lactation (Santos et al., 2004). In grazing systems, dairy cows do not get sufficient dry matter intake (DMI) to sustain the high milk production that could be achieved by their genetic potential (Kolver and Muller, 1998). Moreover, sward herbage allowance impacted in both, cow ovarian cyclicity (Meikle et al., 2013) and uterine gene expression at the end of the voluntary waiting period (day 60 after calving, Astessiano et al., 2017). Thus, total mixed ration (TMR) feeding

in early lactation to grazing dairy cows has increased in the last decade in order to meet cow DMI and nutrient requirements (Chilibroste et al., 2012, Wales et al., 2013).

Many of the functional and morphological changes that occur throughout the estrous cycle and pregnancy, are regulated by different hormones (Spencer et al., 2004). During the estrous cycle, the endometrium undergoes a locally and timely synchronized reorganization to meet the needs of the early developing conceptus. The synthesis and excretion of histotroph by the endometrium is crucial as a nutritious fluid that provides a means of communication between conceptus and maternal environment through signaling molecules. It has been observed that the endometrial transcriptome does not change due to the presence of an embryo until approximately day 16 (Shimizu et al. 2010; Forde and Lonergan 2012).

Several transcriptome studies in beef cattle attempted to research the uterine receptivity before the window of embryonic recognition (day 16, Forde and Lonergan., 2012; day 14, Minten et al., 2013). However, the physiological status of the endometrium just before the moment of blastocyst hatching is key in the development and future elongation of the embryo, determining as a critical period of early embryo loss (Walsh et al., 2011, Spencer et al., 2015). Binelli et al. (2015) studied the endometrial characteristics of the uterus at day 6 post insemination in pregnant versus non-pregnant beef cows, and found that pathways related to steroid biosynthesis and cell proliferation were up-regulated in pregnant cows indicating a more receptive state of the endometrium in these animals.

Studies that researched the effect of nutrition in early lactation on the endometrium gene expression at the end of the voluntary waiting period, are lacking. Recent results of candidate genes at day 7 of estrous cycle in the endometrium of dairy cows, showed a greater expression of genes related to embryo growth and uterine function (IGF system and progesterone and adiponectin receptors) in cows fed with diets allowing increased energy intake (Astessiano et al., 2017). Data evidenced the importance of nutritional management on uterine functionality, which may be associated with the

greater early embryo losses found in lactating vs dry cows (Santos et al. 2004). Next-Generation-Sequencing-Technologies is a tool that can be used to analyze the dynamic transcriptomes to understand the expression and regulation of the genome. Our hypothesis was that cows fed diets that allow maximizing nutrient intake and energy during early lactation, present a uterine profile of gene expression favorable to growth and embryo development. Therefore, our objective was to provide information about RNA-Seq, on changes in the endometrial gene expression induced by the combined use of TMR and grazing during an important period of the estrous cycle: the early luteal phase.

MATERIALS AND METHODS

The experiment was carried out at the EEMAC Research Station, School of Agronomy, Uruguay (30°S, 53 W). Animal procedures were approved by the Animal Experimentation Committee of University of Uruguay (UdelaR, Montevideo, Uruguay).

Animals and Experimental Design

Primiparous Holstein dairy cows calving in fall ($n = 18$, 528 ± 40 kg of body weight (BW), and, 3.2 ± 0.2 of body condition score (BCS)) were used in a randomized block design with two treatments. Cows were blocked by expected calving date, BW and BCS. At calving cows were randomly assigned to two treatments during the first 65 days postpartum (DPP): TMR fed *ad libitum* (**T0**; Control, 17 kgDM/d offered; 70% forage, 30% concentrate; $n = 9$) or grazing of alfalfa (*Medicago Sativa*; 6-h am grazing in 3-d strips; pasture allowance=20 kgDM/d) pasture and supplemented with TMR (70% of *ad libitum* TMR; 12 kgDM/d offered; **T1**; Treatment, $n = 9$) and all cows consumed 2.7 kgDM/day of a commercial concentrate at each milking. During the prepartum period, from -40 to -21 DPP, all cows were managed together as a single group and fed on maize silage, moha hay and a concentrate according to your requirements. From -21 DPP to calving cows were managed in individual stalls (10 x 4 m) with water *ad libitum* and shade available and were fed TMR of corn silage of a prepartum diet to prevent BCS loss.

Cows were milked twice a day (5:00 and 15:00) and milk yield was registered daily and weekly milk samples were obtained for the analysis of protein, fat and lactose. Cow BCS (scale 1 = emaciated, 5 = fat) by one trained observer using the scale of Edmonson et al. (1989) and BW were registered every two weeks from -30 to +65 DPP.

At $+45 \pm 1$ day cows were synchronized. Hormonal treatment involved placing at day -11 (45 DPP) a natural progesterone intravaginal device (Cattle Intravaginal device SYNTEX - DIB®) with an intramuscular injection of 2 mg of Estradiol benzoate (Synthetic analogue of 17β estradiol, 1 mg/mL); at day -8 the implant was removed, 400 UI of equine chorionic gonadotropin (eCG, NOVORMON® 5000) and Cloprostenol (500 µg de Cloprostenol/cows), were also injected intramuscularly. That same day, patches were placed in the sacro-coccygeal region to aid in heat detection for 3 days after implant removal, observed the animals twice daily (after each milking) for 40 minutes. All animals used in the present study were clear of obvious infection at the time of sample collection.

Blood and tissue collection

At day 7 of the estrous cycle (day 0 = estrous), endometrial biopsies from a subset of 5 heifers of each group were obtained, as described by Meikle et al. (2001). Endometrial samples were immediately frozen in liquid nitrogen and stored at -80°C until total RNA was isolated.

Blood samples were collected at day -3, 0 (estrous) and 7 (day of biopsy) of the estrous cycle via jugular venipuncture using Vacutest® tubes (8 mL, Vacutest Kima, Arzergrande, Italy) that contained clot activator gel. Samples were centrifuged (2000 X g for 15 min at 4°C) within 1 h after collection and serum was stored at -20°C until assayed for progesterone analysis.

Progesterone and estradiol 17 β determinations

Progesterone and estradiol 17 β concentrations were assessed at day -3, 0 (estrous), and 7 (day of biopsy) of the estrous cycle. Progesterone was determined to confirm ovulation after synchronization and was measured by a solid-phase RIA using a commercial kit ((MP Biomedicals, Inc. Solon, OH, USA). All samples were analyzed in a single assay; the sensitivity was 0.01 ng/mL, the intra-assay CV was 6.2%.

The concentration of estradiol 17 β was analyzed using radio immunoassay in liquid phase (MP Biomedicals, Inc. Solon, OH, USA). For estradiol 17 β concentration the RIA had a sensitivity of 3.5 pg/ml and the intra-assay coefficients of variation for 10 pg/ml were 12.5%.

RNA extraction

A subsample of 10 cows ($n = 5$ per treatment) were selected based on calving date for RNA-Seq analysis. Isolation of total RNA from endometrial tissue and synthesis of cDNA by reverse transcription was performed according to Astessiano et al. (2011). The RNA integrity was assessed using Agilent Bioanalyzer chips ($RIN = 7 \pm 0.5$).

Mapping sequence reads and assembly of transcripts

Sequence reads were mapped to the reference bovine genome (bosTau7) using the software package Tophat (v2.0.13) (Trapnell et al., 2009; Kim et al., 2013). Two rounds of alignments were performed: firstly, novel splice junctions were discovered in each sample, and then these novel splice junctions plus known splice junctions from Ensembl annotation were combined and supplied to Tophat for a second alignment. This strategy allows for a full utilization of the novel junctions identified in each of the samples (Peñagaricano et al., 2013a; Peñagaricano et al., 2014). The resulting alignments were then used to reconstruct transcript models using the software package Cufflinks (v2.2.1) (Trapnell et al., 2010). This program uses graph theory to find a parsimonious set of transcript models that satisfy with the alignments. The tool *cuffmerge* was used for merging together each of the assemblies with the reference bovine annotation file in order to combine novel transcripts with known annotated transcripts. This strategy maximizes the quality of the final assembly (Peñagaricano et

al., 2013a; Peñagaricano et al., 2014). Finally, for each sample, the number of reads that mapped to each gene was obtained with the script *htseq-count* (Anders et al., 2015).

Overall gene expression analysis

Differentially expressed genes were detected using the *edgeR* package (v.3.12.0) (Robinson et al., 2010). This package combines (i) the application of the trimmed mean of M-values as normalization method of the RNA-Seq data, (ii) an empirical Bayes procedure for estimating the genewise negative binomial dispersion parameters, and (iii) an exact test for assessing differential gene expression between treatments. To account for multiple hypothesis testing and control the false discovery rate, the *P*-values reported by the exact test were corrected using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995).

Gene set enrichment analysis

The enrichment of Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Medical Subject Headings (MeSH) functional terms with significant genes was analyzed using a test of proportions based on the cumulative hypergeometric distribution (Peñagaricano et al., 2013b). Specifically, differentially expressed genes that showed a *P*-value ≤ 0.01 and had Ensembl annotations were tested against the background set of all genes with Ensembl annotations. These enrichment analyses were performed using *goseq* R package (Young et al., 2010) and *meshr* R package (Tsuyuzaki et al. 2015). In addition, the semantic similarities among GO functional terms were calculated using the R package *GOSemSim* (Yu et al., 2010).

RESULTS

Reproductive hormones concentrations

Plasma P4 and E2 concentrations were not affected by nutritional treatment and average were 1.2 ± 0.3 ng/mL and 8.1 ± 0.8 pg/mL, respectively. However, plasma P4 concentrations and the ratio P4 to E2 increased ($P < 0.01$) from -3 to 7 days of the estrous cycle (0.4 to 2.8 ± 0.1 ng/mL and 0.06 to 0.4 ± 0.01 , respectively).

Sequencing of the transcriptome of Bovine endometrial tissue

To evaluate the changes in the endometrial gene expression induced by the combined use of TMR and grazing during an important period of the estrous cycle: the early-luteal phase (day 7), endometrial samples were analyzed using RNA-Seq. Table 1 shown the summary of sequencing read alignments to the bovine reference genome. More than 20 million reads were sequenced for each sample and 94% were mapped to unique genomic regions.

Table 1. Summary of sequencing read alignments to the reference genome

Sample ID	Total Mapped Reads	Uniquely Mapped Reads	Percent Uniquely Mapped	Reads Mapped to Exons
T1 ₁	37,152,210	35,111,280	94.5	29,295,040
T1 ₂	26,922,904	25,481,582	94.6	21,335,122
T1 ₃	22,462,835	21,250,385	94.6	17,768,605
T1 ₄	33,235,442	31,262,281	94.1	26,509,439
T1 ₅	31,327,295	29,268,416	93.4	25,702,485
T2 ₁	34,847,186	32,325,388	92.8	28,008,295
T2 ₂	37,863,861	35,888,851	94.8	29,281,865
T2 ₃	34,836,140	32,932,124	94.5	28,143,180
T2 ₄	33,573,261	31,389,456	93.5	26,537,166
T2 ₅	34,307,798	32,552,382	94.9	27,169,562

T0: Cows were feed TMR fed *ad libitum* and T1: cows grazing pastures and supplemented with 60% TMR (T1). A total of 10 cows were used (5 per treatment). 1-5: biological replicates.

Differential gene expression in endometrial tissue between treatments

From a total of 14,753 genes were detected in the transcriptome, 102 genes were differentially expressed (FDR = 10% and fold change ≥ 2) when comparing endometrium of T1 vs T0 cows. More specifically, 82 genes were significantly up-regulated in T1 and 20 genes were up-regulated in T0 cows. A list with the 25 most differentially expressed genes can be see in Table 2 (All of genes DEG are provide in Table S1). In order to identify the most common functions and processes in which these genes are involved, we characterized them using the annotation provided by Gene Ontology (GO), InterPro and the Protein Families database (PFAM) terms. Only the most relevant functions and processes were selected, and only terms with 3 or more significant genes are displayed.

Table 2. List of 25 most significant genes that showed differential expression in endometrium at day 7 of estrus cycle between Holstein cow grazing pastures and supplemented with 60% TMR (T1; Treatment) and cows fed TMR *ad libitum* (T0; Control).

Gene	Name	logFC	P-value
<i>MSMB</i>	microseminoprotein, beta	-7.6	3.1×10^{-5}
<i>BOLA-DQB</i>	major histocompatibility complex, class II, DQ beta	-6.4	3.8×10^{-4}
<i>SCGB1A1</i>	secretoglobin, family 1A, member 1 (uteroglobin)	-5.7	1.3×10^{-4}
<i>SERPINB10</i>	serpin peptidase inhibitor, clade B (ovalbumin), memb	-5.5	3.2×10^{-5}
<i>SPINK1</i>	serine peptidase inhibitor, Kazal type 1	-5.0	1.5×10^{-4}
<i>FGG</i>	fibrinogen gamma chain	-4.5	3.8×10^{-5}
<i>CDK5R2</i>	cyclin-dependent kinase 5, regulatory subunit 2 (p39)	-3.7	4.7×10^{-4}
<i>CES4A</i>	carboxylesterase 4A	-3.6	8.0×10^{-6}
<i>IGHE</i>	immunoglobulin heavy constant epsilon	-3.5	4.2×10^{-4}
<i>IGJ</i>	immunoglobulin J polypeptide, linker protein for immu	-3.0	4.9×10^{-5}
<i>LOC790208</i>	myelin protein P0-like	6.4	1.8×10^{-8}
<i>CYP1A1</i>	cytochrome P450, subfamily I (aromatic compound-induc	6.4	6.2×10^{-6}
<i>AOX2</i>	aldehyde oxidase 2	6.0	4.1×10^{-5}
<i>HMCN2</i>	hemicentin 2	6.0	5.0×10^{-7}
<i>MMRN1</i>	multimerin 1	6.0	8.6×10^{-8}
<i>TMEFF2</i>	transmembrane protein with EGF-like and two follistat	4.8	3.5×10^{-5}
<i>MT3</i>	metallothionein 3	4.6	7.1×10^{-5}
<i>CD200R1L</i>	CD200 receptor 1-like	4.3	5.3×10^{-7}
<i>CAV3</i>	caveolin 3	4.1	7.2×10^{-6}
<i>IDO1</i>	indoleamine 2,3-dioxygenase 1	3.5	3.4×10^{-4}
<i>PROC</i>	protein C (inactivator of coagulation factors Va and	3.4	6.8×10^{-4}
<i>NOS2</i>	nitric oxide synthase 2, inducible	3.0	3.3×10^{-4}
<i>AHSG</i>	alpha-2-HS-glycoprotein	3.0	6.0×10^{-4}
<i>CLDN11</i>	claudin 11	2.8	5.5×10^{-4}
<i>ACTG2</i>	actin, gamma 2, smooth muscle, enteric	2.8	6.0×10^{-4}

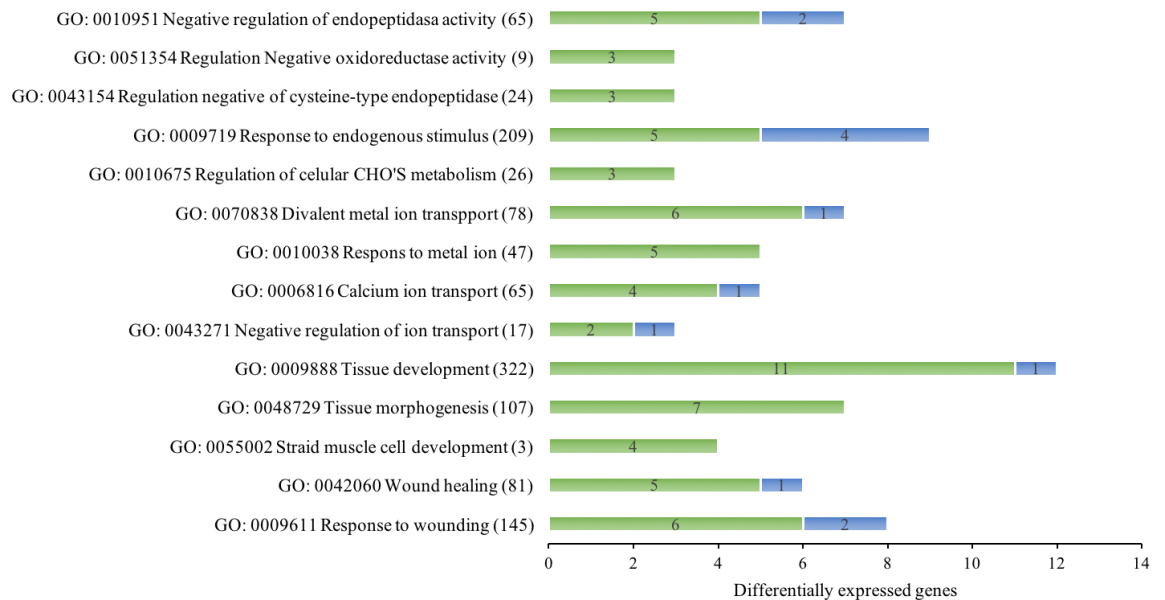
Many of these genes are involved in biological process regulating enzymatic activity: secretoglobin family 1A member 1 (*SCGB1A1*), serpin peptidase inhibitor clade B (*SERPINB10*) and serine peptidase inhibitor Kazal type 1 (*SPINK1*) were up-regulated in T0 cows, whereas metallothionein 3 (*MT3*), caveolin 3 (*CAV3*) and alpha-2-HS-glycoprotein (*AHSG*) were up-regulated in T1 cows. In addition, many of these genes are related to molecular function with protein binding, such as *SERPINB10*, *SPINK1*, and fibrinogen gamma chain (*FGG*) that were up-regulated in T0 cows when compared to T1 cows. Many other genes are directly involved with the immune response, such as major histocompatibility complex class II DQ beta (*BOLA-DQB*), *SCGB1A1*, *SERPINB10*, *SPINK1*, *FGG*, immunoglobulin heavy constant epsilon (*IGHE*), immunoglobulin J polypeptide (*IGJ*) were up-regulated in T0 cows when compared to T1 cows.

Functional enrichment analysis and pathway analysis using Gene Ontology and KEGG

In order to gain more insight into key processes that may possibly explain functional differences between nutritional treatments in the endometrium, the significant enrichment of GO Biological Processes terms and KEGG pathways with genes differentially expressed (DEG) between treatments were analyzed using Fisher's exact test ($P\text{-value} < 0.01$), a test of proportions based on the cumulative hypergeometric distribution. Genes that are assigned to the same GO term can be considered members of a category of genes that are more related in terms of some aspect of their biology than any given random sets of genes. The GO categories (GO term names, number of genes within the term, total number of DEG) that showed significant enrichment with DEG detected between treatments are displayed in Fig 1A. Most of these genes are up-regulated in T1 compared to T0 cows, and a functional enrichment and pathway analyses revealed enriched expression of genes associated with cell and tissue development (peptidase inhibitor 16; *PII6*, actin gamma 2; *ACTG2*, *CAV3*, actin alpha 1; *ACTA1*), endopeptidase (*AHSG*, *CAV3*, *MT3*, *SNCA*, *SERPINB10*, *SPINK1*), calcium ion transport (*PLN*, *MT3*, *CAV3*, synuclein alpha; *SNCA* and *SPINK1*), and

related to the immune system (protein C; *PROC*, placenta-expressed transcript1protein; *PLET1*, villin 1; *VIL1*, *CAV3*, *AHSG*).

Figure 1. A) List of biological process of Gene Ontology terms that were significantly enriched with genes differentially expressed in endometrium at day 7 of estrus cycle. The graph shows the GO ID, term name, total number of genes within each term, and the number of genes up-regulated (green) and down-regulated (blue) in Holstein cow grazing pastures and supplemented with 60% TMR (T1; Treatment) and cows fed TMR *ad libitum* (T0; Control).



Some of the KEEG terms significantly enriched (P -value < 0.01) with DEG detected in the endometrium between T1 and T0 cows are presented in Table 3. These pathways are involved with cell adhesion, calcium signaling and tryptophan metabolism and many of these genes are up-regulated in T1 cows.

Table 3. List of KEEG pathways term that were significantly enriched (P -value < 0.01) with genes differentially expressed in endometrium at day 7 of estrus cycle in Holstein cow grazing pastures and supplemented with 60% TMR (T1; Treatment) and cows fed TMR *ad libitum* (T0; Control).

Functional Characterization ¹	DEG	Genes	P-value
Cell adhesion molecules (95)	6	<i>PD-L1</i> , <i>CDH5</i> , <i>PECAM1</i> , <i>CLDN11</i> , <i>MPZ</i> , <u><i>BOLA-DQB</i></u>	0.005
Calcium signaling pathway (113)	6	<i>INOS</i> , <i>P2RX2</i> , <i>CHP2</i> , <i>PLN</i> , <i>MYLK</i> , <u><i>CACNA1F</i></u>	0.012
Tryptophan metabolism (30)	3	<i>CYP1A1</i> , <i>IDO1</i> , <i>AOX2</i>	0.014

¹The table shows the term name and the total number of genes within each term. Gene symbols that are underlined denote genes down-regulated in endometrium in Holstein cow grazing pastures and supplemented with 60% TMR (T1; Treatment) and cows fed TMR *ad libitum* (T0; Control).

Functional enrichment analysis using Medical Subject Headings

Medical Subject Headings (MeSH) is a collection of descriptors that represent key topics discussed in the Journal Articles, each MeSH descriptor is clustered into many categories including, *Phenomena and Processes*, *Chemicals and Drugs*, and *Diseases* and are assigned to PubMed publications, therefore an additional step is required to associate MeSH to genes. Genes assigned to the same MeSH term can thus be regarded as members of a category of genes that are more closely related in terms of some aspect of their biology than are random sets of genes.

The significant enrichment of MeSH with genes differentially expressed between treatments was also analyzed using Fisher's exact test (P -value < 0.01). The most

relevant MeSH term from category *Chemicals and Drugs* that are were significantly enriched with DEG between treatment are associated with calcium phosphates, receptors calcitriol, insulin and progesterone, and from the category *Diseases* only cattle diseases was significantly enriched with DEG between treatments. The list of DEG corresponding to these categories is presented in Table 4 and all MeSH term (MeSH term name, total genes, DEG and *P*-value) that were significantly enriched with DEG between treatments are presented in Supplementary material, S2.

Table 4. Gene set enrichment analysis using Medical Subject Heading (MeSH) terms with genes differentially expressed in endometrium at day 7 of estrus cycle in Holstein cow grazing pastures and supplemented with 60% TMR (T1; Treatment) and cows fed TMR *ad libitum* (T0; Control).

MeSH Term Name ¹	Differentially expressed genes	P- value
Calcium Phosphates (2)	<i>AHSG, NOS2</i>	0.0003
Receptors, Calcitriol (2)	<i>NOS2, CYP27B1</i>	0.0003
Histamine (3)	<i>MYLK, MYH11</i>	0.0008
Muscle Protein (45)	<i>KCNMB1, DES, TAGLN, PECAM1, CAV3</i>	0.0009
Cystatins (7)	<i>AHSG, CSTA</i>	0.0055
Inhibins (8)	<i>INHBA, MMP9</i>	0.0073
Protein Kinase C (107)	<i>CDH5, ACR, PECAM1, PPARD, 5HTR1A, FGG</i>	0.0092
Chymotrypsin (50)	<i>AHSG, PROC, ACR, <u>CSN3</u></i>	0.0097
Insulin (56)	<i>INHBA, IGFBP3, GFAP, <u>OXT</u>, <u>CSN3</u></i>	0.0024
Progesterone (71)	<i>NOS2, SPADH1, MMP9, <u>OXT</u>, <u>CSN3</u></i>	0.0067
Cattle Diseases (139)	<i>IGFBP3, CCL11, NOS2, LCN15, HHIP, MMP9, IGLL1</i>	0.0089

¹The table shows the term name and the total number of genes within each term. Gene symbols that are underlined denote genes down-regulated in endometrium in Holstein cow grazing pastures and supplemented with 60% TMR (T1; Treatment) and cows fed TMR *ad libitum* (T0; Control).

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