UNIVERSIDAD DE LA REPÚBLICA

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Programa de Posgrados

# MARCADORES ENDÓCRINO-METABÓLICOS DURANTE EL PERÍODO DE TRANSICIÓN Y SU ASOCIACIÓN CON SALUD Y REPRODUCCIÓN EN VACAS LECHERAS

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# APÉNDICE

## Artículos I a III

La presente Tesis está basada en los siguientes artículos, a los cuales se referirá en el texto por su numeración romana (I-III):

**I.** **Ruprechter G**, Noro M, Chilibroste P, Meikle A, Adrien ML. The nutritional and management strategy affects the metabolic and endocrine profiles of healthy dairy cows. Artículo en formato borrador para ser enviado para su publicación a la revista Animal.

**II**. **Ruprechter G**, Adrien ML, Larriestra A, Meotti O, Batista C, Meikle A, Noro M. Metabolic predictors of peri-partum diseases and their association with parity in dairy cows. Research in Veterinary Science 2018, 118: 191-198.

**III.** **Ruprechter G**, Noro M, Meotti G, Batista B, Adrien ML, Meikle A. Endocrine and reproductive parameters in sick and healthy primiparous and multiparous dairy cows. Theriogenology 141 (2020) 173-179

# Índice

# Resumen

La primera hipótesis de esta tesis fue que la estrategia nutricional (estabulación con suministro de ración totalmente mezclada o pastoreo junto con suministro de dieta parcialmente mezclada) modifica las concentraciones de metabolitos, minerales, enzimas y hormonas durante el período de transición de vacas lecheras sanas. Luego se hipotetizó que las concentraciones preparto de algunos metabolitos (NEFA, BHB, colesterol, albúmina y calcio) son predictivas (en su conjunto o individualmente) de las enfermedades (metritis, retención de placenta, mastitis, cetosis, hipocalcemia entre otras) luego del parto, independiente de la paridad. Finalmente que el estado de salud afecta el ambiente endocrino-metabólico que se asocia al desempeño productivo y reproductivo de vacas primíparas y multíparas.

En el primer trabajo (**Artículo I**) se encontró que la estrategia nutricional (ración totalmente mezclada, TMR vs dieta parcialmente mezclada, DPM, y dos sesiones de pastoreo) afectó la producción láctea de vacas sanas a favor del grupo TMR (n=10), en comparación al PASTORIL+DPM (n=10). Esto fue consistente con las mayores concentraciones de NEFA, BHB y cociente NEFA/colesterol en el grupo PASTORIL+DPM que reflejaron un déficit energético. Por otro lado, el cociente Ca/P fue menor en el grupo TMR lo que pudo estar asociado a la dieta así como también a la mayor producción láctea (**Artículo I**).

Para evaluar el valor predictivo de los metabolitos se realizó un estudio de cohorte prospectivo observacional en 308 vacas Holstein (primíparas, PP=126 y multíparas, MP=182) (**Artículo II**). La metritis fue la enfermedad de mayor incidencia (26,6%), seguida de retención de placenta (17,2%) y mastitis (15,2%). Las vacas MP presentaron una mayor concentración de NEFA que las PP (mayor movilización grasa) asociado a una mayor partición de nutrientes para la lactancia. Las vacas MP enfermas presentaron mayores concentraciones de NEFA que las vacas sanas al parto, mientras que las PP sanas presentaron mayores concentraciones de NEFA una semana postparto. Todas las vacas sanas tuvieron mayores colesterolemias en la segunda y tercer semana postparto. La calcemia fue afectada por la paridad, siendo menor en MP y manteniendo las MP sanas mayores calcemias al parto que las MP enfermas. La concentración de albúmina y colesterol preparto (-2 o -1 semana) resultaron predictivas de metritis, retención de placenta y mastitis en vacas MP, sugiriendo que ambos son marcadores económicos y precoces de riesgo de enfermedad a ser utilizados en programas de monitoreo de salud de rodeos. Las vacas con uno o dos eventos produjeron menos leche que las sanas, y no difirieron entre sí (7.547, 7.098 vs 8.165 L respectivamente).

Al investigar el impacto del estado de salud sobre el ambiente endocrino (insulina, IGF-I, adiponectina y leptina) y el desempeño reproductivo de vacas y vaquillonas (**Artículo III**), las mayores concentraciones de IGF-I e insulina observadas en las vacas PP durante el período de transición, apoyan una diferente adaptación metabólica acorde a la paridad. Mientras que las vacas MP presentaron una disminución de IGF-I posparto independientemente del estado de salud, las vacas PP sanas mantuvieron las concentraciones de IGF-I, pero las PP enfermas presentaron una pronunciada bajada de IGF-I. Esto se asoció con el efecto del estado de salud sobre el reinicio de ciclicidad ovárica, ya que en vacas PP fue más marcado que en MP (35% enfermas vs 66% sanas en PP y 59% vs 69% en MP). Sin embargo, las tasas de concepción fueron similares en vacas PP sanas y enfermas (85% vs 80%) mientras que en las MP sanas fueron mayores que en las MP enfermas (74%

vs 60%). Los datos sugieren que cuando las vacas PP superan el evento clínico de salud y comienzan a ciclar, su fertilidad es mayor a la de vacas MP.

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# Abstract

The first hypothesis of this Doctoral thesis was that the nutrition strategy modifies the concentration of metabolites, minerals, enzymes and hormones during the transition period in healthy dairy cows. Then, it was hypothesized that the prepartum concentration of some metabolites were predictive of diseases after calving, independent of parity. Finally, that health status affects the metabolic and endocrine environment that is associated with the productive and reproductive performance in primiparous and multiparous cows.

In the first study (**Paper I**) it was found that nutritional management (total mixed ration TMR vs partial mixed ration and two grazing sessions (GRAZING+PMR) affected milk production in healthy multiparous cows, being greater TMR cows (n=10) when compared to GRAZING+PMR (n=10). This was consistent with the greater concentrations of NEFA, BHB and the ratio NEFA/cholesterol in the GRAZING+PMR group that reflected an energy deficit. On the other hand, the ratio Ca/P was lower in TMR cows that could be associated with the diet and/or the greater milk production in this group (**Paper I**).

To evaluate the predictive value of the metabolites, a prospective cohort study was performed in 308 Holstein cows (primiparous, PP=126 and multiparous, MP=182) (**Paper II**). Metritis was the disease with a greater incidence (26.6%), followed by retained placenta (17.2%) and mastitis (15.2%). MP cows presented greater NEFA concentration (lipid mobilization) than PP cows associated with a greater nutrient partitioning towards lactation. The MP sick cows had more NEFA concentrations than healthy cows. All healthy cows had more cholesterol during the second and third week postpartum. The concentration of calcium was affected by parity, being lower in MP cows, and having MP healthy cows greater Ca concentrations at calving than sick MP cows. Albumin and cholesterol concentrations before calving (-2 or -1 weeks) were predictive for metritis, retained placenta and mastitis in MP cows, suggesting that both are good economic early markers for disease that can be used in health programs in the herds. The cows with one or two events produced less than healthy cows, and did not differ among them (7,547, 7,098 vs 8,165 L respectively).

Investigating the impact of health status on the endocrine environment (insulin, IGF-I, adiponectin and leptin) and the reproductive performance in primiparous and multiparous cows (**Paper III**), the IGF-I and insulin concentrations observed in PP cows during the transition period, supports the different metabolic adaptation according to parity. While MP cows presented an IGF-I decrease around calving independent from health status, PP healthy cows maintained them but PP sick cows had a sharp decrease in IGF-I concentrations. This was associated with the effect of the health status on ovarian cyclicity as in PP cows this was more marked than in MP cows (35% sick vs 66% healthy in PP and 59% vs 69% in MP cows). On the other hand, conception rates were similar in healthy and sick PP cows (85% vs 80%), while in MP healthy cows were greater than in sick MP cows (74% vs 60%). Data suggest that when PP cows overcome the clinical event and start to cycle, their fertility is greater than MP cows.


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# Introducción

El incremento de la producción láctea en Uruguay ha sido constante en los últimos años, pasando de producir 1.494 millones de litros por año en 2003-2004, a 2.049 millones de litros por año en 2016-2017. En la última década ha existido un aumento sostenido de la producción total de leche en Uruguay y por unidad de superficie, explicado esto, por un incremento en la producción por vaca (4.255 litros anuales/vaca masa vs 4.768 litros anuales/vaca masa) y aumentos en la carga (742 mil animales en 879,9 mil hectáreas vs. 767 mil animales en 764 mil hectáreas (DIEA, 2017). Históricamente el sistema productivo Uruguayo ha tenido una base pastoril, sin embargo, la estrategia de intensificación de la producción lechera en Uruguay se basó en un incremento significativo del uso de concentrados y reservas forrajeras, disminuyendo la cosecha directa de forraje por parte de los animales (Chilibroste et al., 2015), lo que permitió el salto productivo visualizado en la última década.

Dicha intensificación productiva exige un correcto balance nutricional, ya que aumenta los requerimientos para la síntesis de calostro y eventos asociados al parto (Drackley, 1999; Ingvartsen y Andersen, 2000) al tiempo que ocurre una depresión del consumo voluntario (Grummer, 1995) durante el período de transición (pasaje del estado “preñada no lactante” al “no preñado lactante”). Esto, implica cambios dramáticos para la vaca, induciendo un estado de balance energético negativo (BEN), considerado éste un mecanismo adaptativo para enfrentar la exigencia metabólica. El aumento de la gluconeogénesis hepática, la menor utilización de glucosa por parte de los tejidos periféricos (resistencia a la insulina), el desacople del eje somatotrófico: hormona de crecimiento (GH), factor de crecimiento similar a insulina (IGF-I) y la lipomovilización, con el aumento de ácidos grasos no esterificados (NEFA), son adaptaciones homeoréticas, para aumentar la disponibilidad de sustratos energéticos (Bell, 1995; Drackley, 1999). Sin embargo, este mecanismo adaptativo, aumenta la susceptibilidad de la vaca a enfermar durante el período de transición jugando el sistema inmune un rol clave, activando mecanismos locales y sistémicos de defensa que inducen inflamación. Se ha propuesto que la interrelación entre el sistema endócrino, metabólico e inmune de la vaca lechera induce un sistema inmune deprimido alrededor del parto y mayor susceptibilidad a enfermedades metabólicas e infecciosas (Esposito el al., 2014), ya que los mecanismos homeoréticos pueden ser insuficientes para optimizar las funciones que demanda la producción (Ingvartsen, 2006). Además, existiría mayor riesgo de enfermedades inflamatorias, que aumentan el estrés metabólico y comprometen la defensa inmunitaria (Trevisi et al., 2011) sumado a mayores problemas reproductivos (Bertoni et al., 2009; Pinedo et al., 2016; Esposito et al., 2014), disminución de las tasas de preñez y aumento de las tasas de descarte (Smith et al., 2000; Butler et al, 2003). Van Radan et al., (2011) argumentan que dicho declive reproductivo se ha frenado en los últimos años, luego de la incorporación de índices reproductivos en los sistemas de selección genética. No obstante, el estatus reproductivo sigue siendo una de las causas más importantes de las altas tasas de descarte involuntario (Gröhn et al., 2003) y en nuestro país, un estudio basado en 200 mil lactancias indicó que el intervalo parto concepción aumentó de 131 a 150 días de 1997-2001 a 2001-2005 (Rovere et al., 2007).

Las enfermedades del periparto impactan negativamente en la rentabilidad de las empresas lecheras ya que disminuyen la producción láctea y el desempeño reproductivo y acortan la vida útil de la vaca (Gröhn et al., 2003). Es importante considerar que la enfermedad disminuye la producción láctea no solo durante el proceso de enfermedad sino que continúa su impacto negativo luego de la remisión durante toda la lactancia (Wallace et al., 1996;  Fourichon et al., 1999; Gröhn et al., 2003). Se demostró un impacto negativo de la cetosis sobre la producción, reportándose pérdidas de 1,1 kg/día en el primer mes de lactancia (Carson, 2008) o retrasos en el reinicio de la actividad ovárica postparto y menor probabilidad de preñez a la primera inseminación asociado a cetosis subclínica (Walsh et al., 2007). Huzzey et al. (2007) reportan 8 kg menos de leche por día durante las 3 primeras semanas de lactación en vacas con metritis severa después del parto y una disminución láctea de 2,6 kg/día en los primeros controles lecheros en vacas multíparas con retención de placenta. Similares pérdidas reportan Dubuc et al. (2011) debido a metritis y retención de placenta (259 kg y 753 kg en 305 días en leche, respectivamente).

El progreso genético a favor de la alta producción de leche está asociado a las hormonas que regulan el metabolismo y fue demostrado que vacas de alto mérito genético presentan mayores concentraciones de GH y menores de insulina e IGF-I que vacas de bajo mérito genético (Gong et al., 2002). Las hormonas anabólicas, insulina e IGF-I se encuentran disminuidas alrededor del parto para favorecer el catabolismo periférico y soportar la lactancia (Meikle et al., 2013). Pero esta adaptación endocrino metabólica caracterizada por la secreción de insulina alterada, resistencia a insulina en los tejidos periféricos, e hipercetonemia en vacas durante el periparto, puede convertirse en un problema para la vaca. En este sentido, un BEN severo se ha relacionado con la aparición de hígado graso (Bauchart et al., 1998) y cetosis (Ingvartsen et al., 2006) siendo la medición de NEFA, beta-hidroxibutirato (BHB) y colesterol, útil para evaluar la capacidad adaptativa de la vaca, reflejando el éxito o no de dicha adaptación (Herdt, 2000). El hígado graso clínico o subclínico induce disfunción hepática (Turk et al., 2004) pudiendo llegar a ser éste, un problema para más del 50% de las vacas al inicio de la lactancia (Jorritsma et al., 2001). En Uruguay se ha reportado pérdida de peso y condición corporal en vacas durante el periparto cursando con altas concentraciones de NEFA y BHB, como resultado del aumento en la cetogénesis (Meikle et al., 2004; Cavestany et al., 2005).

Por otro lado, varios componentes del sistema de defensa (función de los neutrófilos, capacidad de respuesta linfocitaria a la estimulación por mitógenos entre otros) están alterados durante el período de transición (Lacetera et al., 2005; Goff, 2006) y se postula que sea una de las principales causas de la alta prevalencia de enfermedades como metritis o mastitis, durante el posparto temprano (Kimura et al., 2002). Por lo tanto, la inmunosupresión junto a marcados cambios en el estatus endocrino, metabólico y nutricional, estarían favoreciendo el desarrollo de la infección en la vaca después del parto (Burton et al., 2005; Goff, 2006; Spears y Weiss, 2008).

El tejido adiposo juega un rol clave en la presentación de las enfermedades del periparto ya que los adipocitos además de almacenar o movilizar los triglicéridos, sintetizan y liberan una amplia variedad de moléculas bioactivas conocidas como adipocinas. Éstas permiten la comunicación del tejido adiposo con otros órganos del cuerpo (hígado, músculos, cerebro, órganos reproductores), así como dentro del tejido adiposo (Ouchi et al., 2011). Se propone que las disfunciones metabólicas pueden deberse en parte a un desequilibrio en la expresión de adipocinas pro-inflamatorias (leptina, interleuquinas y factor de necrosis tumoral- α) y adipocinas antiinflamatorias (adiponectina), siendo el equilibrio adecuado entre ellas crucial para mantener la homeostasis en el cuerpo (Ouchi et al., 2011). Las citoquinas pro-inflamatorias, promueven una respuesta local y sistémica para contribuir con el sistema de defensa del organismo, determinando cambios metabólicos y endocrinos que inducen anorexia, fiebre y lipólisis (Elsasser et al., 1995). Además, estimulan la síntesis hepática de proteínas de fase aguda positiva (pAPP) como la haptoglobina (Hp) (Chan et al., 2004; Huzzey et al., 2009).

La leptina, ha sido vinculada a la regulación del consumo y metabolismo energético y fue determinada en rumiantes en el año 2000 (Delavaud y col., 2000). Su concentración varía acorde a los cambios de reservas corporales (tejido adiposo subcutáneo y mesentérico) (Ouchi, 2011) y en trabajos realizados en Uruguay en vacas lecheras se la reporta como buen indicador de variaciones de condición corporal durante el preparto (Meikle et al., 2018). Por otro lado, la adiponectina, secretada principalmente por el tejido adiposo pero que también se expresa en células del musculo cardíaco y esquelético (Jortay et al., 2012), posee importantes funciones metabólicas, incluyendo la mejora de la sensibilidad de la insulina, estimulación del apetito y la promoción de la beta - oxidación de ácidos grasos en hígado y músculo (Kadowaki et al., 2005). Bajas concentraciones de adiponectina en el periparto de la vaca lechera, podrían predisponer a una respuesta inflamatoria monocítica descontrolada. Este hecho podría estar relacionado con los potentes efectos anti-inflamatorios de las adipocinas en los macrófagos y otras células inmunes (Kabara et al., 2014).

Si bien no se conocen todos los factores de riesgo para la presentación de las enfermedades del periparto, se sabe que las enfermedades frecuentemente están interrelacionadas (Mulligan y Doherty, 2008). Además, se postula que la mayor producción láctea está relacionada con la incidencia de mastitis, quistes ováricos o mayor riesgo a cetosis (Igvartsen et al., 2003). Sin embargo, el mismo grupo reporta en trabajos posteriores, que las vacas de mayor producción no presentan mayor riesgo de enfermedades periparto, como ser hipocalcemia clínica, cetosis, metritis y retención de placenta, lo que sugiere que la patogénesis no está directamente relacionada con la producción de leche *per se*, sino con otras variables (Ingvartsen et al., 2006). De todos modos, las enfermedades metabólicas e inflamatorias son una manifestación de la incapacidad de la vaca para hacer frente a las demandas metabólicas de la producción de leche (Mulligan y Doherty, 2008).

Se estima que en sistemas intensivos de producción, bajo estabulación, cerca del 75 % de todas las enfermedades se producen dentro del primer mes posparto (Grummer, 1995; Goff y Horst, 1997; Ingvartsen et al., 2003; Ingvartsen, 2006), ya que el 30 a 50 % de las vacas son afectadas por algún tipo de enfermedad metabólica o infecciosa durante este período (Le Blanc, 2010). Factores de riesgo como el estado corporal o la paridad son importantes. Una excesiva condición corporal o variaciones de balance energético preparto predisponen a cetosis y lipidosis hepática (Ingvartsen et al., 2006) y la paridad tiene efecto diferencial en la incidencia de enfermedades siendo las vacas primíparas más susceptibles a metritis que las multíparas (Galvão et al., 2012) o las multíparas más susceptibles a hipocalcemia

(Reinhardt et al., 2011). Varios autores documentan menor incidencia de trastornos de la salud en los sistemas de producción a pastoreo (Bendixen et al., 1986; Washburn et al., 2002; White et al., 2002; Bruun et al., 2002, Ribeiro et al., 2011), sin embargo, Sepúlveda et al. (2015) reportan similares incidencias en sistemas pastoriles. Igualmente, se puede afirmar que tanto en sistemas extensivos como intensivos, dichos trastornos de salud, llevan a pérdidas económicas importantes para los productores (von Keyserlingk et al., 2009).

Con el fin de realizar medicina preventiva a nivel individual o de rodeo, se han determinado variables sanguíneas para predecir riesgo de enfermedad durante el período de transición en vacas lecheras en sistemas estabulados, alimentadas con dietas totalmente mezclada (TMR) (Van Saun, 2009). Concentraciones de NEFA > 0,4 mmol/L de 7 a 10 días preparto han sido asociadas con aumento de riesgo en 2-4 veces de presentación de desplazamiento de abomaso (Le Blanc et al., 2005) y en 2 veces aumento de riesgo de retención placentaria (Quiroz-Rocha et al., 2009). En Chile, se observó que vacas con concentración de NEFA al parto de 1,2 mmol/L, presentaron mayor incidencia de mastitis clínica y de fiebre de leche que vacas con valores <1,2 mmol/L (Meléndez et al., 2009). Para confirmar la cetosis (consecuencia del severo BEN), la prueba de oro, es la determinación de BHB en sangre, ya que en ausencia de oxalacetato, la producción de BHB es mayor a su utilización, siendo la causa de su aumento en sangre. Se ha establecido un límite de 1,2 mmol/L para el diagnóstico de cetosis subclínica y de 2,6 mmol/L para el de cetosis clínica (Oetzel, 2004). Se ha reportado, que concentraciones > 1,2 mmol/L aumentan el riesgo en 3-8 veces de presentación de desplazamiento de abomaso (Duffield et al., 2005) y 3 a 6 veces el riesgo de metritis, endometritis, cetosis clínica y mastitis (Duffield et al., 2009). También se ha demostrado la asociación de la cetosis con la disminución de la performance reproductiva, reportando que vacas con BHB en leche > 1,0 mmol/L en la primera semana postparto tienen 1,5 veces más probabilidad de no tener ovulación incluso por 9 semanas postparto y menor probabilidad de preñez a la primera inseminación (Walsh et al., 2007). Estudios recientes han indicado que el colesterol es un predictor del riesgo de presentación de enfermedades periparto en las vacas, reportando Sepulveda et al., (2015) que vacas con metritis leve y severa presentaron menores concentraciones de colesterol que vacas sanas, durante la 2° y 3° semana postparto.

En cuanto al balance mineral, se ha definido hipocalcemia subclínica a las concentraciones sanguíneas de Ca < 2,0 mmol/L (Oetzel, 2004). Dicha afección se reporta en vacas lecheras, principalmente multíparas (Reinhardt et al., 2011) y además que se asocia con mayor riesgo de presentación de mastitis, desplazamiento de abomaso, retención placentaria y cetosis (Curtis et al., 1985; Van Saun et al., 2005). El calcio también afecta la función de los neutrófilos que participan en la primera línea de defensa contra la infección, conduciendo a un estado de inmunosupresión (Kehrli et al., 2006). Al respecto se sabe que vacas con metritis puerperal exhiben marcada disminución en la capacidad fagocítica de los neutrófilos (Hammon et al., 2006).

El estatus inflamatorio sistémico asociado a las enfermedades periparto, puede ser monitoreado por marcadores inflamatorios; proteínas de fase aguda positivas (pPFA) y negativas (nPFA). Se ha señalado a la Hp que es una pPFA como buen marcador, para el diagnóstico de metritis; Huzzey et al. (2009) reportan que vacas con concentraciones de Hp ≥ 1g/L en el día 3 posparto presentaron 6,7 veces más riesgo de desarrollar metritis severa o leve, indicando que una respuesta inflamatoria de fase aguda precede a los eventos clínicos.

Por lo tanto, la determinación de las variables sanguíneas anteriormente mencionadas, puede ser una herramienta útil para monitorear la salud del rodeo e identificar enfermedades precozmente, colaborando con la medicina preventiva de la vaca lechera en transición y la optimización de una buena gestión de salud en las empresas lecheras. Si bien varios marcadores sanguíneos han sido determinados, en general los trabajos presentan indicadores parciales, lo que dificulta la comprensión holística de la problemática ya que se desconoce como se relacionan dichas variables entre sí, con la enfermedad en vaquillonas y vacas adultas. Tampoco se ha investigado que efecto tienen las enfermedades del periparto sobre las variables productivas, reproductivas o sobre la toma de decisión de descartar vacas. En trabajos realizados en nuestro país se ha demostrado el impacto de la paridad y el estado corporal del periparto sobre parámetros productivos y reproductivos, basados en la evaluación de metabolitos y hormonas (Meikle et al., 2004, Adrien et al., 2012, Meikle et al., 2013), caracterizando el metabolismo y el ambiente endócrino de vacas lecheras en sistemas pastoriles. Además, se demostró que vacas en sistema TMR en comparación a pastoril mas suplementación tenían mayor condición corporal y mayores concentraciones de albúmina, proteína, insulina e IGF-I, durante el postparto, consistente con la mayor densidad energética de la dieta (Meikle et al., 2013). Sin embargo, poco se conoce sobre la susceptibilidad de presentar enfermedades en el periparto o como éstas afectan, la producción o reproducción de vacas primíparas (PP) y multíparas (MP).

En suma, las vacas lecheras en el periparto son más susceptibles de presentar enfermedades metabólicas-nutricionales así como enfermedades inflamatorias, estando dichas enfermedades asociadas a marcadas alteraciones en el metabolismo energético y hormonal durante el período de transición. La tasa de movilización grasa y la actividad del tejido adiposo son responsables de los trastornos que afectan negativamente la función inmune y hepática (Rosen y Spiegelman, 2006; Deng y Scherer, 2010). Por otro lado, la estrategia nutricional puede afectar la concentración de algunas variables metabólicas y endócrinas y cierta información fue reportada comparando sistemas de manejo intensivos vs sistemas pastoriles mixtos (Adrien et al., 2012; Meikle et al., 2013; Astessiano et al., 2015). Si bien existe abundante literatura sobre marcadores metabólicos y enfermedades del periparto en vacas lecheras, no hemos encontrado estudios realizados en predios comerciales sobre el efecto de la paridad (PP vs MP) y el estado de salud sobre los perfiles metabólicos (por ej. NEFA, BHB, colesterol, albúmina y calcio) determinados durante el período de transición. Además, el conocimiento de la evolución de dichos marcadores en vacas sanas y enfermas durante el periparto, nos permitirá establecer momentos estratégicos de sangrado para monitorear salud y predecir riesgo de enfermedad en rodeos lecheros. Por otro lado, no hemos encontrado reportes sobre perfiles endocrinos (insulina, IGF-I, leptina y adiponectina) en vacas durante el período de transición acorde a la paridad (PP y MP) y al estatus de salud (sanas y enfermas) vinculado al éxito reproductivo.

# Hipótesis

El sistema de manejo y alimentación modifica las concentraciones sanguíneas de los metabolitos y hormonas durante el período de transición de vacas lecheras. Los metabolitos sanguíneos (NEFA, BHB, colesterol, albúmina y Ca), en su conjunto o individualmente, durante el preparto son buenos indicadores predictivos de las enfermedades postparto, independientemente de la paridad. Las hormonas metabólicas (insulina, IGF-I, adiponectina y leptina) son afectadas por el estado de salud de vacas y vaquillonas, estando asociadas con la reducción en la producción de leche y el desempeño reproductivo.

# Objetivos

### General

Evaluar la inter-relación entre el metabolismo, la endocrinología y los parámetros productivos y reproductivos de vacas lecheras primíparas y multíparas con diferente status de salud.

### Específicos

**Objetivo 1.** Investigar en vacas multíparas Holstein sanas, el efecto de dos estrategias nutricionales diferentes; Estabulación con dieta totalmente mezclada (TMR) y Pastoril +dieta parcialmente mezclada (DPM) sobre los parámetros metabólicos incluidos normalmente en rutinas de monitoreo y sobre concentraciones endócrinas asociadas con funciones metabólicas e inmunes (**Artículo I**).

**Objetivo 2.** Determinar si el estado de salud (vacas sanas vs vacas enfermas) interactúa con la paridad para afectar la condición corporal y los perfiles metabólicos y endócrinos durante el período de transición y el desempeño productivo y reproductivo (**Artículos II y III**).

**Objetivo 3**. Determinar y comparar el valor predictivo de marcadores metabólicos (NEFA, BHB, colesterol, albúmina y calcio) en el preparto para predecir riesgo de enfermedad en vacas Holstein primíparas (PP) y multíparas (MP) **(Artículo II)**.

**Objetivo 4**. Determinar la asociación de marcadores hormonales (IGF-I, insulina, adiponectina y leptina) durante el período de transición con la performance reproductiva de vacas PP y MP **(Artículo III)**.

## Materiales y Métodos

Diseños experimentales

Todos los estudios fueron aprobados por los Comités de Ética de las Facultades de Veterinaria de la Universidad de Montevideo, Uruguay y Unipampa, Brasil.

Para el **Artículo I,** el estudio se llevó a cabo en un predio lechero comercial desde agosto a noviembre 2015. Se seleccionaron de un rodeo de 1.400 vacas, 20 vacas Holstein MP que permanecieron sanas durante todo el período de estudio (-30 hasta +60 días en relación al parto) con parición agosto-octubre, número de lactancia promedio (media±ES) 3,1±1,2, peso corporal de 660 ± 82,1 kg y promedio de producción en lactancia anterior de 6.345 ± 1.463 L. Los animales fueron bloqueados acorde al peso, la condición corporal (CC), el número de lactancia y la fecha prevista de parto. Durante preparto se manejaron en conjunto en un potrero con agua y sombra y alimentadas con TMR una vez al día (12 kg/MS/vaca/d) (**Artículo I**). En el post parto, las vacas fueron asignadas al azar a dos estrategias de manejo nutricional hasta los 60 dpp. Las estrategias de manejo fueron: TMR (n=10); vacas estabuladas con dieta TMR y PASTORIL+DPM (n=10); vacas con doble pastoreo (7:30–11:30 AM y 6:00 PM–6:00 AM) y suplementadas con dieta parcialmente mezclada (DPM) desde 11:30 AM a 3:00 PM permaneciendo durante este tiempo las vacas en un potrero con sombra y agua *ad libitum*. Al grupo TMR se le ofreció 27 kg/MS/vaca/d, una vez al día (composición en **Artículo I**) y al grupo PASTOREO+DPM se le asignó una pradera de *Festuca arundinacea* y *Dactylis perseo*, con pastoreo en franjas diarias y alta asignación de forraje (40 kg MS/vaca/d) y suplementación con 25% de la misma TMR del grupo estabulado (la composición química de las dietas se detalla en **Artículo I**). La condición corporal (CC) se registró cada 15 días desde -30 a +60 días post parto (dpp) utilizando la escala de 5 puntos (Ferguson et al., 1994). Al mismo tiempo se obtuvieron muestras de sangre por punción de la vena coccígea, utilizando tubos secos al vacío para la determinación de NEFA, BHB, colesterol, urea, proteína total, albúmina, Ca, fósforo, magnesio, aspartato amino transferasa (AST) y gama glutamil transpeptidasa (GGT).

Para los **Artículos II y III,** el estudio se llevó a cabo en un predio lechero comercial estabulado localizado en Rio Grande do Sul, Brasil desde octubre 2014 a setiembre 2015 siguiendo un estudio de cohorte prospectivo observacional. Se seleccionaron vacas Holstein (n=126 PP y n= 182 MP) de un rodeo de 700 vacas, que se encontraban sanas al inicio del estudio con producción láctea promedio de 8.000 kg/lactancia y con dos estaciones de parto (primavera y otoño). Las vacas fueron evaluadas semanalmente desde -3 semanas a + 4 semanas en relación al parto. Durante el preparto, las vacas fueron manejadas en lotes separados por paridad en potreros (sin aporte de pasturas) alimentadas con TMR (13,3 kg MS/vaca/d) dos veces al día (8:00 y 16:30 hs) (composición de la TMR en **Artículo II**). Post parto fueron asignadas a estabulación con cama caliente hasta día 3 postparto recibiendo TMR (21,5 kg/MS/d en primavera y 28,9 kg/MS/d en otoño), 3 veces al día en comederos colectivos y ordeñadas 2 veces al día (composición de dietas pre y postparto en **Artículo II**). Al parto las vacas recibieron 300 mL de propilenglicol, 250 g de propionato de Ca diluido en 1L de agua vía oral y 40 g gluconato de Ca subcutáneo. A partir del día 3 las vacas se asignaron a un *free-stall*, fueron alimentadas con la misma TMR y ordeñadas 3 veces al día. El registro de producción láctea a 305d se obtuvo con DairyPlan C-21 software (GEA). Se registró la CC (Ferguson et al., 1994) y al mismo tiempo se obtuvieron muestras de sangre por punción de vena coccígea con tubos al vacío heparinizados una vez por semana desde -2 a +4 semanas en relación al parto (**Artículo II)** y hasta semana +7 (**Artículo III)**. En el plasma se determinó, NEFA, BHB, colesterol, albúmina, Ca, Hp (**Artículo II**) e insulina, IGF-I, leptina, adiponectina y progesterona (P4) (**Artículo III**). Los eventos de salud (hipocalcemia clínica, mastitis clínica, retención de placenta, metritis, cojeras, desplazamiento de abomaso) fueron registrados por un veterinario entrenado previamente. El diagnóstico de cetosis subclínica e hipocalcemia subclínica se realizó en muestras de sangre en el laboratorio.

**Criterio de diagnóstico de enfermedades clínicas:** *Retención placentaria*: el diagnóstico de la retención de placenta se realizó cuando las membranas fetales se encontraron visibles en la vulva 24 horas después del parto. *Metritis:* a partir del día 3 postparto, todas las vacas fueron monitoreadas 2 veces por semana hasta el día 21 post parto +/- 1 día mediante examen manual vaginal por un veterinario entrenado. El diagnóstico de metritis se realizó, mediante la presencia de secreción vaginal anormal durante el postparto temprano, sumado a muestras sanguíneas con concentración de Hp > 1 mg/dL, según Huzzey et al. (2011). *Mastitis clínica:* leche visiblemente anormal, con o sin cambios anormales de la glándula mamaria determinado durante el ordeñe por el ordeñador. *Desplazamiento de abomaso*: mediante percusión-auscultación. *Hipocalcemia puerperal:* vaca en decúbito dentro de las 72 horas después del parto exhibiendo anorexia, síntomas nerviosos, diferentes grados de pérdida de consciencia y la buena respuesta al tratamiento con calcio por vía intravenosa (recuperación del estado de postración y síntomas nerviosos).

**Criterio de diagnóstico de las enfermedades subclínicas:** se consideró *Cetosis subclínica* cuando la concentración de BHB ≥1,2 mmol/L (Oetzel, 2004) e *Hipocalcemia subclínica* cuando la concentración de Ca < 2,0 mmol/L (Goff, 2008).

**Retorno a ciclicidad ovárica:** El reinicio de la ciclicidad se determinó mediante análisis de las concentraciones de P4 plasmáticas, en muestras sanguíneas obtenidas desde la 2ª hasta la 8$^{va}$ semana posparto, 1 vez por semana. Se definió actividad luteal cuando la progesterona fue ≥ 1 ng/mL.

**Manejo reproductivo:** A 50 dpp las vacas fueron manejadas con protocolo de sincronización de inseminación artificial (IA) a tiempo fijo, consistiendo de: 2 mL de benzoato de estradiol (Sincrodiol, Ourofino Saúde Animal Ltda, Brazil), 1 mL de hormona liberadora de gonadotrofina (GnRH Gestran, ARSA S.R.L, Buenos Aires, Argentina) y un implante de P4 intra vaginal (CIDR, Zoetis), seguido a los 7 días por 2 mL prostaglandina inyectable (Estron Agener Uniao, Brazil), removiendo al mismo tiempo el implante intravaginal y administrando 0,5 mg de cipionato de estradiol inyectable (ECP, Zoetis). Todas las vacas fueron inseminadas 48 h más tarde (47 - 50 h). El diagnóstico de preñez se llevó a cabo al día 45 por ultrasonografía (DVU60vet, Oxson Trechnology). Vacas no preñadas ingresaron nuevamente al mismo protocolo.

Determinaciones en el laboratorio

Los metabolitos, minerales y enzimas se determinaron por espectrofotometría en auto analizadores automáticos: A25, Biosystems, España para el **Artículo I** y en Vitalab Selectra II, Vital Scientific, Dieren, The Netherlands para el **Artículo II** utilizando kits comerciales, en el Laboratorio de endocrinología y metabolismo animal, Facultad de Veterinaria, Montevideo Uruguay. Los kits utilizados fueron: NEFA, Wako NEFA-HR(2), Wako Pure Chemical Industries Ltd., Osaka, Japan; BHB, Randox Laboratories Limited, 55 Diamond Road, Crumlin, Country Antrim, BT29 4QY, United Kingdom. Albumina, calcio y colesterol: Wiener Lab S.A.I.C. Riobamba, Rosario, Argentina (**Artículo II**). Para el **Artículo I**, se utilizaron kits comerciales dedicados para A25, de la empresa Biosystems. Haptoglobina, se determinó por ELISA utilizando un kit comercial, (Tridelta Diagnostics Ltd., Morris Plains,NJ). Para todos los casos, el CV inter ensayo de los sueros control comercial fueron ≤ al 10%.

Para el **Artículo III**, se determinó P4 en 288 vacas (n=116 PP y n= 172 MP) semanalmente por radioinmunoanálisis (RIA) en fase sólida, con kit comercial (MP, Biomedicals, INC California). La sensibilidad del ensayo fue de 0.01 ng/mL y los CV intra e inter ensayo fueron menores al 10%. Para analizar los perfiles endócrinos y su relación con los parámetros reproductivos, se seleccionó al azar un sub grupo de 120 vacas incluyendo 57 PP (sanas n=28, enfermas n=29) y 63 MP (sanas, n=28, enfermas n=35). Se utilizaron las muestras correspondientes a las semanas: -2, -1, +1, +3 y +5 en relación al parto para su análisis. Adiponectina se determinó por RIA utilizando un kit comercial en fase liquida (Millipore, LA, USA) previamente descrito por Raddatz et al. (2008). La sensibilidad del ensayo fue de 1.5 ng/mL. Los CV intra e inter-ensayo para control 1 (8,4 ng/mL) fueron 9.1% y 8.5% respectivamente y para control 2 (81,5 ng/mL) 7,9 % y 11,1 % respectivamente. Insulina e IGF-I se determinaron por ensayo inmunoradiométrico (IRMA), con kits comerciales (Insulina: Diasource Immuno Assays S.A, Nivelles, Belgica) e (IGF-I: IGF1-RIACT Cis Bio International, GIF-SUR-YVETTE CEDEX, France). La sensibilidad del ensayo de insulina fue de 1,3 uIU/mL. Los CV intra e inter-ensayo para control 1 (19.4 uIU/mL) fueron 5,5% y 8,6% respectivamente y para control 2 (65,6 uIU/mL) 4,4 % y 4,9 % respectivamente. La sensibilidad del ensayo de IGF-I fue de 16 ng/mL. Los CV intra e inter-ensayo para control 1 (47,5 ng/mL) fueron 8,2% y 8,5% respectivamente y para control 2 (429 ng/mL) 9,9 % y 11,9 % respectivamente. La leptina fue determinada por RIA en fase líquida, con kit comercial (Multi- Species, Millipore, USA). La sensibilidad del ensayo fue de 2,8 ng/mL. Los CV intra e inter-ensayo para control 1 (8,3 ng/mL) fueron 9% y 10,2% respectivamente y para control 2 (32 ng/mL) fueron 8,7 % y 11 % respectivamente.

Análisis Estadístico

Para el análisis de los datos obtenidos se utilizó el paquete estadístico SAS (Sistema de Análisis Estadístico, SAS Institute, Cary, NC, EE.UU.). Se realizó un análisis univariado de los datos en todas las variables para identificar valores atípicos y para verificar la normalidad de los residuales.

Para el **Artículo I**, los datos de CC, producción y composición láctea, metabolitos, enzimas, minerales y hormonas fueron evaluados como medidas repetidas utilizando procedimiento mixto y la estructura de covarianza auto regresiva de primer orden. La estrategia nutricional (TMR vs PASTOREO+DPM), días y sus interacciones fueron considerados efectos fijos y la CC al parto utilizada como covariable. Se ajustaron los grados de libertad con Kenward-Rogers.

Para el **Artículo II,** las vacas fueron clasificadas retrospectivamente en 3 categorías acorde a su estatus de salud: Vacas "sanas" (sin eventos clínicos), "un evento" (un evento clínico) y "dos eventos” (más de un evento clínico). La asociación entre paridad y estado de salud se analizó con tablas de contingencia (2x2) utilizando PROC FREQ. La CC y variables metabólicas fueron analizadas como variables continuas utilizando PROC MIXED. El modelo consideró la paridad, el estado de salud, la semana y estación de parto como efectos fijos y sus interacciones. Se examinó la normalidad y homogeneidad de varianzas. La producción láctea a 305 días fue analizada por PROC MIXED y el modelo incluyó paridad, estado de salud y estación de parto como efectos fijos y las interacciones. Posteriormente los datos fueron evaluados por regresión logística múltiple (RLM) por semana (-2, -1 y parto) para estado de salud (sanas vs enfermas) y para cada enfermedad: metritis, retención de placenta y mastitis considerando la paridad y la estación de parto en el modelo. Las variables que no fueron significativas ($P>0,05$) fueron eliminadas manualmente del modelo. Los Odds ratio (OR) y su correspondiente intervalo de confianza (95%) fueron determinados por RLM para describir el nivel de asociación entre el metabolito de interés y el evento de salud postparto (considerando el evento de salud binomial). Para los metabolitos que permanecieron en el modelo se realizó la curva ROC y se determinó el área bajo la curva (AUC). El punto de corte se estimó por índice de Youden y valores de sensibilidad, especificidad y máxima verosimilitud positiva y negativa fueron determinados utilizando MedCalc V.17.6. (MedCalc®, Ostend, Belgica). La sensibilidad fue la proporción de animales diagnosticados con metritis, RP o mastitis que estaban por encima del punto de corte dado para un determinado metabolito, mientras que la especificidad fue la proporción de animales sin el evento clínico que estuvo por debajo del punto de corte dado (Dohoo et al., 2003).

Para el **Artículo III,** las vacas fueron clasificadas retrospectivamente en 2 categorías acorde a su estatus de salud: Vacas "sanas" (sin eventos clínicos) y vacas "enfermas" (uno o más de un evento clínico). Las concentraciones de insulina, IGF-I, leptina, y adiponectina fueron analizadas como medidas repetidas. El modelo incluyó la paridad, estado de salud y semana como efectos fijos y sus interacciones. La asociación entre paridad, estado de salud, estación de parto y las variables reproductivas: reinicio de ciclicidad ovárica, IA y tasa de preñez, fueron analizada por PROC FREQ utilizando tablas de contingencia 2x2. Las variables reproductivas fueron analizadas por Proc Genmod, incluyendo paridad y estado de salud en el modelo. Se realizó el análisis de sobrevida de Kaplan-Meier para analizar la probabilidad de reinicio de ciclicidad ovárica y preñez. Los datos fueron luego evaluados por RLM para el reinicio de la ciclicidad ovárica y tasa de preñez y se utilizó OR y IC 95% para describir el nivel de asociación entre las variables hormonales, el estado de salud, paridad y los parámetros reproductivos. Los datos fueron reportados como promedios ± SEM. Para todos los análisis estadísticos $P<0,05$ fue considerado significativo y $P\leq 0,1$ tendencia.

## Resultados principales

En el **Artículo I,** al estudiar el efecto de la estrategia nutricional (manejo y alimentación) sobre la CC y la producción láctea, se encontró efecto sobre la producción láctea, siendo mayor en el grupo TMR que en el grupo PASTORIL+DPM (39,2 ± 1,3 vs 28,7 ±1,1 L). Además, la CC tendió a ser diferente entre tratamientos (P=0,1), las vacas del grupo PASTORIL+DPM perdieron CC mientras las vacas del grupo TMR la mantuvieron, presentando una mayor CC a los +45 y +60 dpp que el grupo PASTORIL+DPM (P=0,05) (Fig. 1 del **Artículo I**).

Al estudiar el efecto de la estrategia nutricional sobre las concentraciones de los metabolitos se encontró que, las concentraciones de NEFA fueron mayores en el grupo PASTORIL+DPM que en el TMR, al igual que las concentraciones de BHB (Fig.1 A, B).

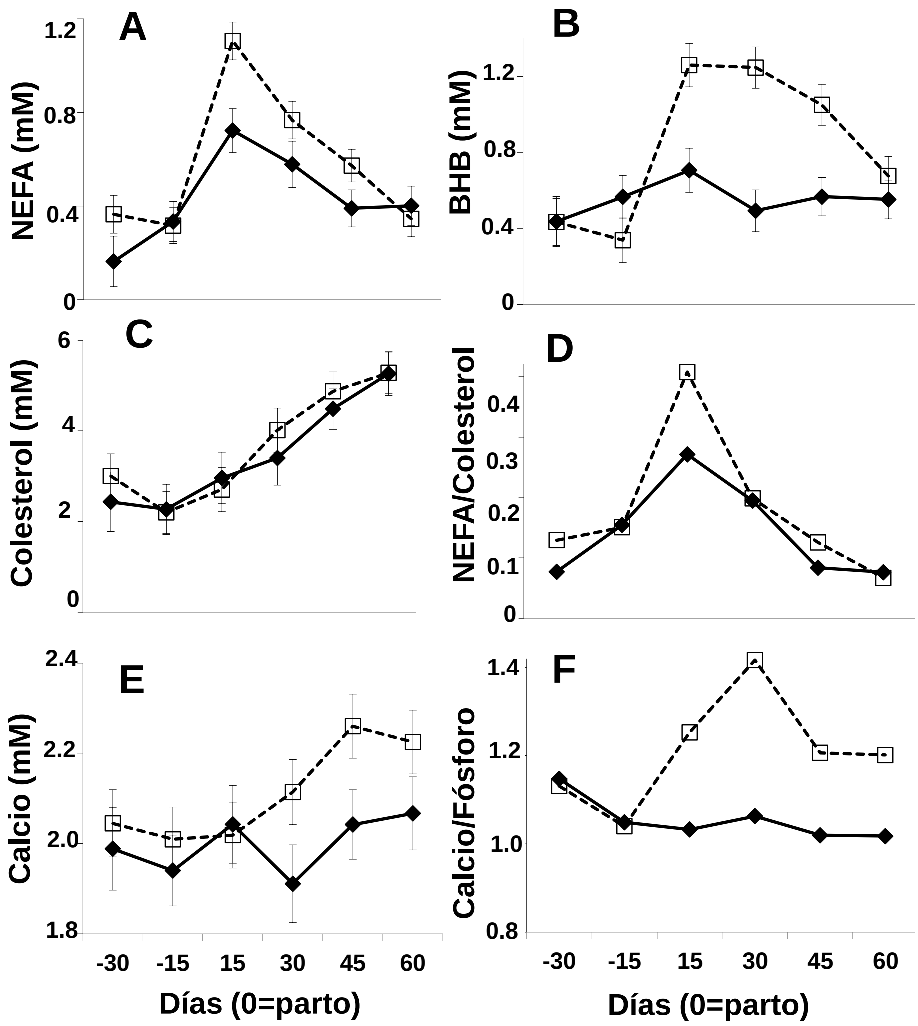


Figura 1. Concentraciones de NEFA (A), BHB (B), colesterol (C), Ca (E) y de los ratios NEFA/colesterol (D) y Calcio/fósforo (F) en vacas Holstein MP desde -30 a +60 dpp en sistema TMR (línea continua) y PASTORIL+DPM (línea punteada).

Independiente de la estrategia nutricional, las concentraciones de NEFA aumentaron hacia el día+15. Sin embargo, el grupo TMR mantuvo constantes sus concentraciones de BHB, mientras que las vacas del grupo PASTORIL+DPM aumentaron el BHB postparto.

Si bien las concentraciones de colesterol no difirieron acorde a la estrategia nutricional y aumentaron postparto hacia el día +60, el índice NEFA/colesterol tendió a ser afectado por la estrategia nutricional y fue mayor en el grupo PASTORIL+DPM a los +15 dpp (Fig. 1 C, D). Las concentraciones de Ca y P tampoco fueron afectadas por la estrategia nutricional, pero si lo fue el cociente Ca/P siendo mayor en el grupo PASTORIL+DPM a los 30dpp (Fig. 1E, F).

En el estudio de cohorte prospectivo observacional en vacas primíparas y multíparas (**Artículo II**), se encontró que de las 308 vacas evaluadas, 46,7% (n=144) enfermaron con al menos un evento clínico y 1,9% (n=6) fueron descartadas o murieron dentro de los primeros 30 días en leche. Una mayor incidencia de vacas enfermas se visualizó en la parición de otoño en comparación con la de primavera (55,3% vs 30,6%, P <0,0001). La metritis fue la enfermedad de mayor incidencia (26,6%, n= 82), seguida de retención de placenta (RP) (17,2 %, n=53) y mastitis (15,2%, n=47), sin efecto de paridad para ninguna de ellas. Las enfermedades de baja incidencia fueron evaluadas en conjunto como “otras” con una incidencia de 8,1% (n=25). Para hipocalcemia subclínica, se encontró efecto de la paridad, siendo mayor en MP que en PP (43% |n=77| vs 9,5% |n=12|, respectivamente, P<0,0001). El número y porcentaje de vacas acorde al estado de salud (sanas, 1 evento y 2 eventos) y enfermedades individuales (metritis, RP, mastitis e hipocalcemia subclinica) estratificados por paridad y estación de parto se muestran en la Tabla 1.

Tabla 1. Incidencia de eventos clínicos y subclínicos durante el período de transición por paridad [primíparas (PP), n=126; multíparas (MP), n = 182] y estación de parto.

| | **Primavera** | | Fisher | **Otoño** | | Fisher |
|---|---|---|---|---|---|---|
| | **PP** | **MP** | | **PP** | **MP** | |
| **Estado de salud y enfermedades** | n (%) | n (%) | P | n (%) | n (%) | P |
| Sanas | 31 (73,81) | 46 (66,67) | NS | 38 (45,24) | 49 (43,36) | NS |
| 1 evento | 10 (23,81) | 16 (23,19) | NS | 29 (34,52) | 37 (32,74) | NS |
| 2 eventos | 1 (2,38) | 7 (10,14) | NS | 17 (20,24) | 27 (23,89) | NS |
| Metritis | 2 (4,76) | 6 (8,70) | NS | 33 (39,29) | 41(36,28) | NS |
| RP | 4 (9,52) | 10 (14,49) | NS | 16 (19,05) | 23 (20,35) | NS |
| Mastitis clínica | 3 (7,14) | 7 (10,14) | NS | 13 (15,48) | 24 (21,24) | NS |
| Otros* | 3 (7,14) | 9 (13,0) | NS | 1 (2,56) | 12(19,67) | 0,01 |
| Hipocalcemia subclínica | 6 (14,0) | 27 (39,7) | 0,005 | 6 (7,4) | 50 (45,0) | <0,0001 |
| N° total de PP y MP | 42 | 69 | | 84 | 113 | |

* Otros: hipocalcemia clínica, cetosis, desplazamiento de abomaso, cojeras.

La evolución de la CC y las concentraciones de NEFA y BHB durante el período de transición acorde a paridad y estado de salud se muestran en Fig. 2.

A pesar de que las vacas MP presentaron menor CC que PP, el perfil de la evolución de CC fue similar en ambas categorías, siendo mayor en preparto, disminuyendo hacia la 3° semana postparto y no recuperando la CC inicial. El perfil de las concentraciones de NEFA acorde a la paridad y el estado de salud durante el período de transición fue diferente y se muestra en Fig. 2 (C, D). Si bien todas las vacas MP aumentaron la concentración de NEFA desde la semana-1 a la semana +1, las MP enfermas presentaron mayores concentraciones de NEFA al parto (P<0,05). Incluso, vacas MP con 2 eventos presentaron mayores concentraciones de NEFA que MP sanas y con 1 evento en semana +1 (P<0,05). Además, vacas MP sanas, lograron bajar las concentraciones de NEFA hacia la semana +3, a diferencia de las MP enfermas que las mantuvieron elevadas (P<0,05). En las vacas PP, aunque todas aumentaron su concentración de NEFA desde semana-1 a semana+1, las PP sanas presentaron mayor concentración de NEFA que las PP enfermas a la semana+1 (P<0,05), no existiendo diferencias en concentraciones de NEFA a la semana +3 acorde al estado de salud. El perfil de BHB fue similar en PP y MP durante el período de transición. Las vacas PP con 2 eventos presentaron mayor concentración de BHB que las PP sanas y con 1 evento al parto (P<0,05), mientras que las vacas MP con 2 eventos tuvieron mayor concentración de BHB que las vacas MP sanas en la semana +3 (P<0,05).

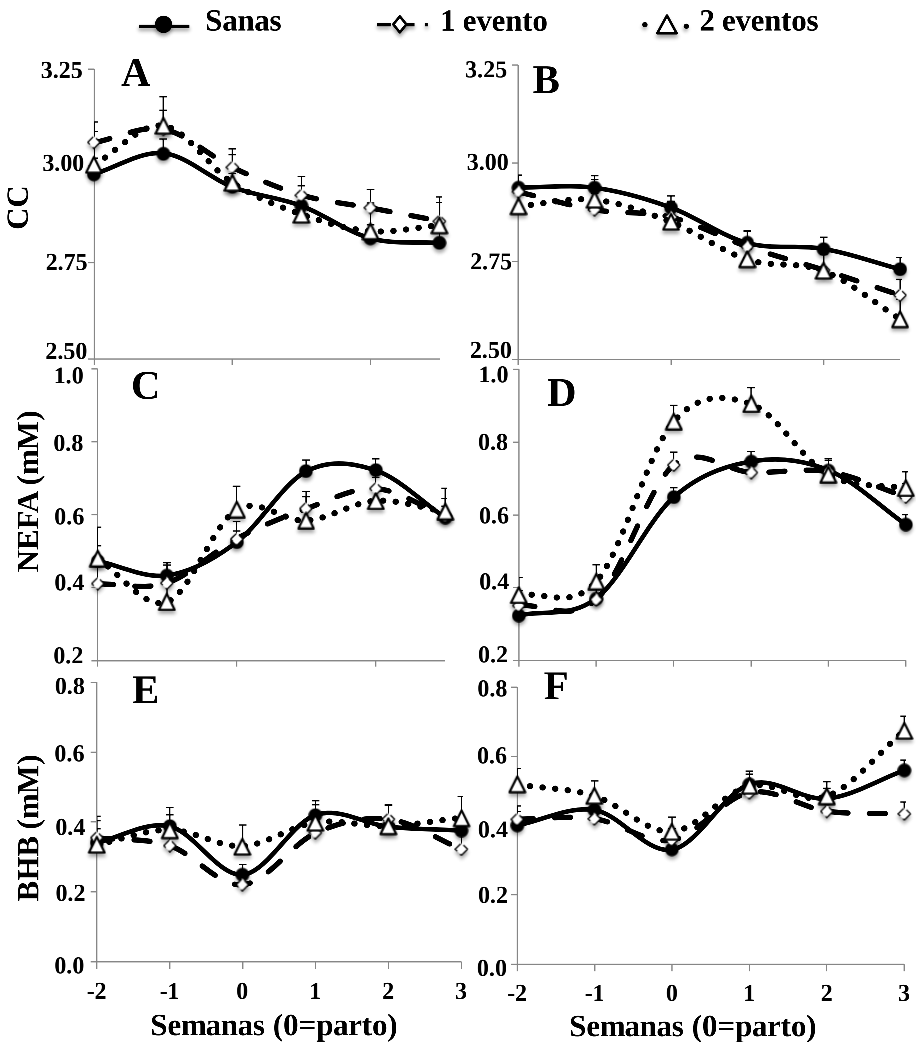


Figura 2. Evolución de la condición corporal (CC) (A, B) y las concentraciones de NEFA (C, D) y BHB (E, F) en vacas Holstein primíparas (A, C, E) y multíparas (B, D, F) sanas, con 1 evento y 2 eventos.

Los perfiles de las concentraciones de colesterol, albúmina y Ca durante el período de transición acorde a paridad y estado de salud se muestran en Fig. 3. Independiente de la paridad o del estado de salud, las concentraciones de colesterol disminuyeron al parto y se recuperaron postparto superando incluso las concentraciones visualizadas al inicio del preparto. Las vacas sanas (PP y MP) presentaron mayores concentraciones de colesterol que las enfermas (PP y MP) en las semanas+2 y +3 (P<0,05) y las vacas MP sanas mayor concentración que las MP enfermas en semana -1 (P<0,05).

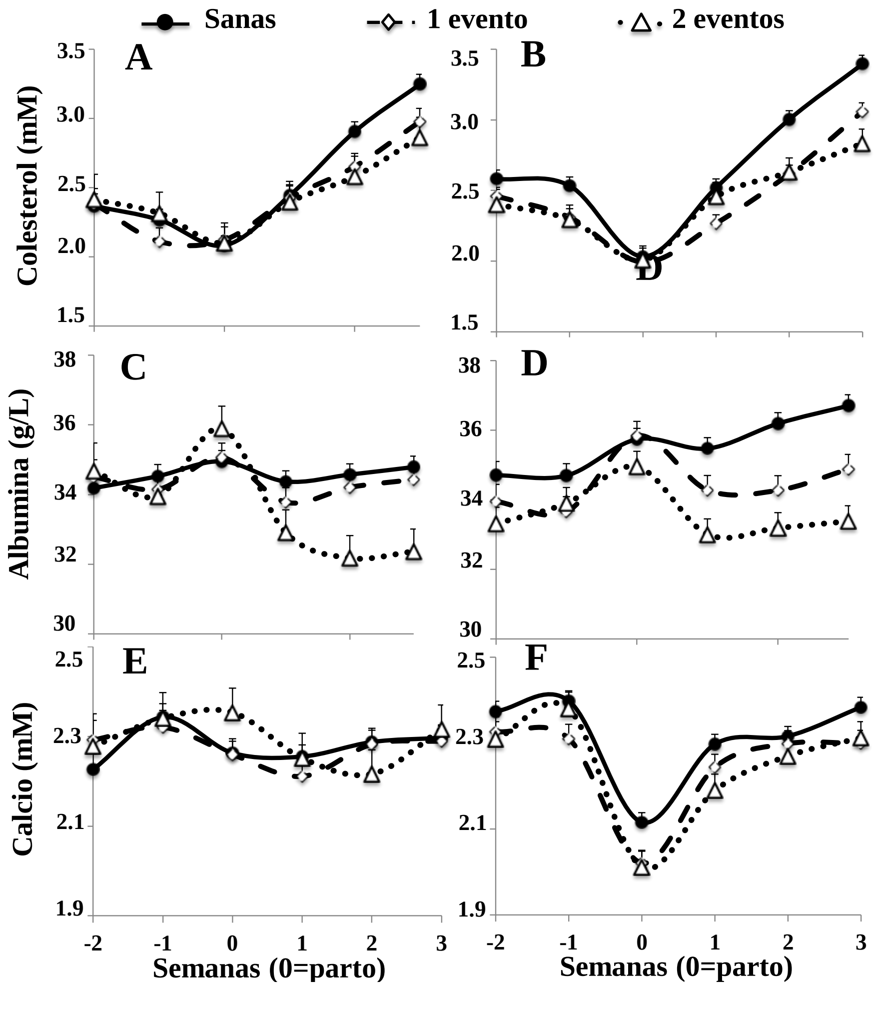


Figura 3. Concentraciones de colesterol (A, B), albumina (C, D) y calcio (E, F) en vacas Holstein primíparas (A, C, E) multíparas (B, D, F) sanas, con 1 evento y 2 eventos.

Además, las concentraciones de albúmina en vacas MP sanas fueron superiores que en las MP enfermas durante el preparto y el post parto (P<0,05, Fig.3 C, D). Incluso las vacas MP con 1 evento, presentaron mayores concentraciones de albúmina que las MP con 2 eventos durante el postparto (P<0,05). En vacas PP, si bien no hubo diferencias en las concentraciones de albúmina, entre vacas sanas y con 1 evento durante todo el período de estudio, ambas categorías de salud presentaron mayores concentraciones que las vacas PP con 2 eventos durante el postparto (P<0,01). Las concentraciones de Ca disminuyeron de forma marcada al parto en todas las vacas MP, no siendo así en PP, reflejando el efecto de la paridad en este momento (Fig.3

E, F). A pesar de que todas las vacas MP bajaron el Ca al parto, las MP sanas mantuvieron mayores concentraciones de Ca que las MP enfermas (P<0,05).

Al realizar la regresión logística incluyendo PP y MP y ambas estaciones de parto, la concentración de albúmina preparto (semana -2) fue predictiva de enfermedad postparto en vacas con 2 eventos en comparación a vacas sanas (OR [IC]); 1,27 [1,01 a 1,60] (P=0,03) y similares resultados mostró el colesterol para mastitis; 2,24 [1,06 a 4,74] P=0,03).

Debido a que la estación de parto y la paridad afectaron la concentración de metabolitos durante el preparto y el parto (P<0,01), se realizaron RLM para cada cohorte y paridad. En la cohorte de parición de primavera, no hubo predictores metabólicos de enfermedad para ninguna de las dos categorías. En la cohorte de parición de otoño, se encontraron predictores de enfermedad pero solamente en MP. Las concentraciones de albúmina en la semana -2, resultaron predictivas de metritis (OR [IC]); 1,31 [1,05-1,62] (P<0,01) y de RP; 1,38 [1,04-1,81] (P<0,05) y en la semana -1 predictivas de RP; 1,26 [1,01-1,57] (P<0,05). Además las concentraciones de colesterol resultaron predictivas de mastitis; 4,29 [1,26-14,61] en la semana -2, 6,32 [1,81-22,08] en la semana -1 y 9,01 [1,01-42,28] al parto (P<0,01). Los puntos de corte obtenidos para los metabolitos por semana y los porcentajes de sensibilidad y especificidad y los valores positivos y negativos de máxima verosimilitud se reportan en Tabla 4 del **Artículo II**.

La producción láctea fue afectada por la paridad, la estación de partos y el estado de salud (P<0,05). Las vacas PP produjeron menos que las MP (6.866 ± 272 vs 8.341 ± 213 L, P<0,0001) y la producción fue mayor en otoño que en primavera (8.360 ± 188 vs 6.789 ± 278 L, P<0,0001). Además, las vacas sanas produjeron más que las vacas enfermas (P<0,05), no difiriendo la producción entre vacas de 1 y 2 eventos (8.165 ± 199, 7.547 ± 276 y 7.098 ± 387 L para sanas, 1 o 2 eventos respectivamente).

En el **Artículo III**, se estudió la asociación entre el estado de salud y los perfiles endócrinos (IGF-I, insulina, adiponectina y leptina) con el desempeño reproductivo. La evolución de los perfiles de IGF-I e insulina acorde a la paridad y la semana fueron diferentes y para IGF-I, una tripe interacción paridad*estado de salud*semana fue significativa. Las concentraciones de insulina tendieron a ser mayores en vacas PP que en MP (19,5 ± 0,7 vs 17,9 ± 0,6 uUI/mL, P= 0,07). En vacas PP, las concentraciones de insulina permanecieron constantes, mientras que en vacas MP, disminuyeron desde el preparto hacia la semana+1 (P<0,003), aumentando posteriormente hacia el final del período de estudio sin diferencias acorde al estado de salud (Fig 4 A, B). También se encontró un efecto de paridad para IGF-I, presentando las vacas PP mayores concentraciones de IGF-I que vacas MP (136,2 ± 5,3 vs 103,6 ± 5,0 ng/mL, P <0,0001). Independiente del estado de salud, las concentraciones de IGF-I aumentaron en PP desde la semana -2 a la semana -1 (P<0,01) y disminuyeron posteriormente hacia la semana +1 (P<0,01) con una disminución pronunciada en las PP enfermas (Fig. 4 C). En cambio en las MP, si bien las concentraciones de IGF-I bajaron en todas las vacas hacia la semana+1, las MP sanas tendieron a tener mayor concentración de IGF-I que MP enfermas (P=0,1). Posteriormente, la concentración de IGF-I tendió a aumentar hasta la semana+5 pero no recuperando las concentraciones visualizadas en preparto (Fig. 4 D).

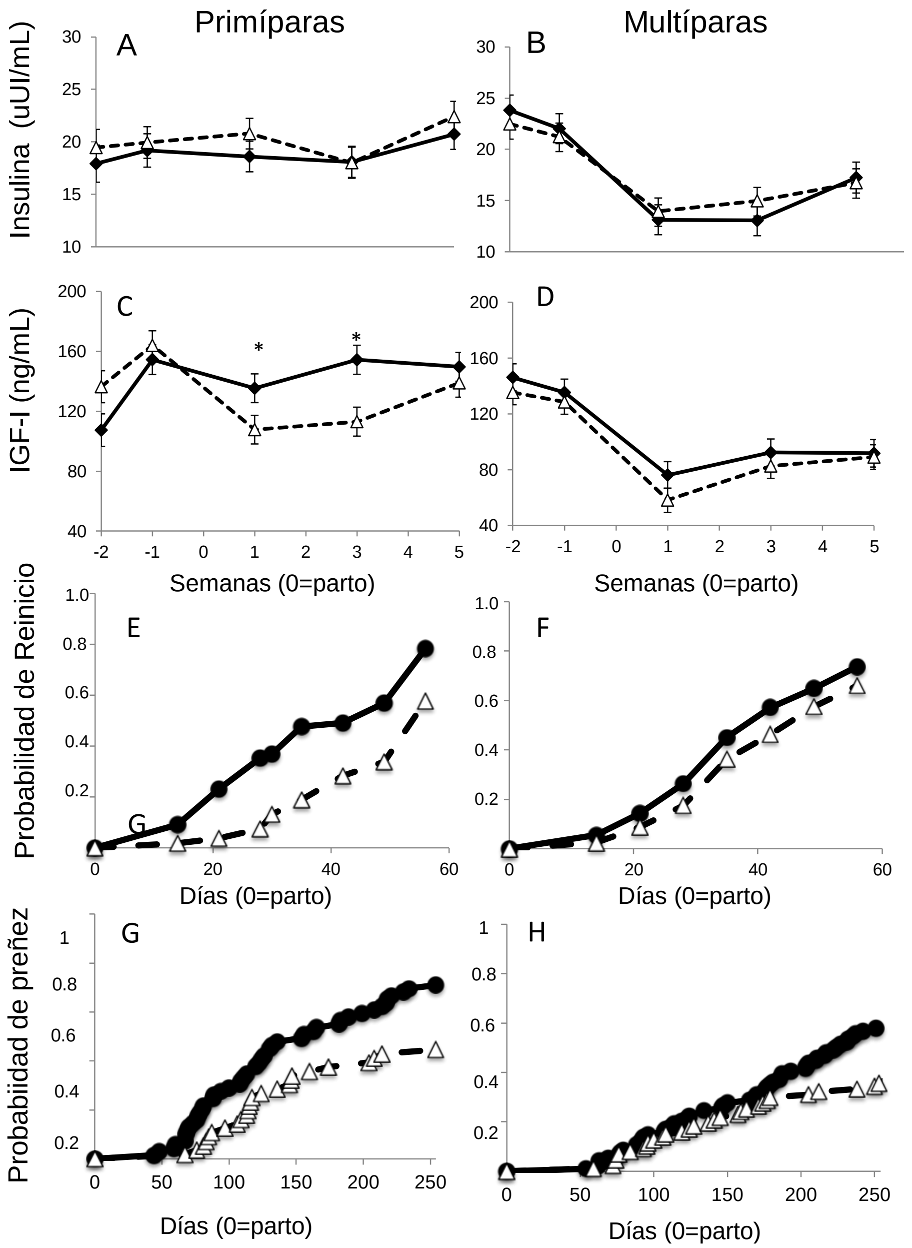


Figura 4. Concentraciones de insulina (A, B) e IGF-I (C, D) en vacas Holstein primíparas (PP, n=57) y multíparas (MP, n=63) desde -2 a +5 semanas en relación al parto y la probabilidad de reinicio ovárico (E,F) y preñez (G,H) en PP (n=116) y MP (n= 172), acorde al estado de salud [vacas sanas (línea continua) y vacas enfermas (línea punteada)].

Durante las primeras siete semanas postparto, 59,7% de un total de 288 vacas, reiniciaron la ciclicidad ovárica, mientras que el 40,3% no reinició. El estado de salud y la paridad afectaron el reinicio a la ciclicidad ovárica ($P<0,05$), no así la estación de parto. Un mayor porcentaje de vacas sanas reiniciaron la actividad ovárica en comparación a las vacas enfermas (67,9% [106/156] vs 50% [66/132], P=0,002). Además, un mayor porcentaje de vacas MP reiniciaron la ciclicidad ovárica en comparación a las PP; 64% [110/172] vs 53% [62/116], P=0,01). Sin embargo, esta diferencia estuvo dada por las vacas enfermas **(Artículo III)**, ya que no se encontraron diferencias entre vacas sanas (PP y MP), pero considerando las vacas enfermas, las PP presentaron menor probabilidad de reinicio ovárico que las vacas MP (Fig.4 E, F). En ambas categorías, un mayor porcentaje de inseminación se observó en vacas sanas, en comparación a vacas enfermas (94% vs 76,5%, P<0,01), al igual que fue mayor para PP que MP (90,4 vs 82,7, P<0,05) (**Artículo III**). La probabilidad de preñez también fue afectada por el estado de salud y la paridad (P<0,004, Fig. 4 G, H): más vacas sanas quedaron preñadas en comparación con enfermas (75% vs 54 %, P <0,01) y más PP que MP (74% vs 56%, P= 0,04). La tasa de concepción también fue mayor en PP que en MP (P=0,01) y en vacas sanas mayor que en vacas enfermas (P=0,04). Pero cabe destacar, que mientras que no se encontró efecto del estado de salud sobre la tasa de concepción de vacas PP, las vacas MP enfermas tuvieron una menor fertilización y/o mayor mortalidad embrionaria que vacas MP sanas (**Artículo III**).

# Discusión general

La estrategia nutricional afectó algunos metabolitos en vacas MP, reflejando la magnitud del BEN al inicio de lactancia. Las concentraciones de NEFA se encontraron por encima del punto de corte establecido para vacas frescas (0,6 mmol/L) en ambos grupos; niveles > 0,6 mmol/L de NEFA se han asociado a mayor susceptibilidad de enfermar (LeBlanc et al., 2005). El grupo TMR logró mantener las concentraciones de BHB dentro de los rangos de referencia (preparto: <0,5 mmol/L; vacas frescas: <0,8 mmol/L, Wittwer, 1996), consistente con la tendencia visualizada de una mayor CC de dichas vacas. Esto sugiere una mayor ingesta de MS y una mejor adaptación metabólica a la transición. Sin embargo, las vacas del grupo PASTORIL+DPM, desarrollaron un BEN de mayor magnitud, visualizado por mayores concentraciones de NEFA y BHB, presentando incluso valores de cetosis subclínica (concentración de BHB ≥1,2 mmol/L) a los 15 y 30 dpp. Más aún, considerando la presencia de cetosis subclínica del grupo PASTORIL+DPM, ésta podía ser la causa de la menor producción láctea como fuera reportado por otros (Duffeild et al., 2009; Ospina et al., 2010b). Sin embargo, aspectos nutricionales de la dieta (mayor % de FDN en el grupo PASTORIL+DPM) (**Artículo I**) que influencian negativamente el consumo voluntario de MS (Mertens, 2002) podrían estar contribuyendo a la menor producción láctea visualizada en este grupo.

Las bajas concentraciones de colesterol durante la transición fueron asociadas a un BEN más severo, un estado de salud comprometido y una alteración de la función hepática (Van Saun, 2004; Bionaz et al., 2007; Sepulveda et al., 2015; Ruprechter et al., 2018, **Artículo II**). Se encontraron datos contradictorios en referencia al colesterol en la bibliografía. Algunos reportan bajas concentraciones de colesterol como un efecto positivo de vacas a pastoreo en comparación a las estabuladas (4,53 vs 4,60 mmo/L respectivamente) a los 120 días de lactancia (Radkowska y Herbut, 2014) mientras otros reportan mayores concentraciones de colesterol en las vacas a pastoreo durante la transición (Meikle et al., 2013). Sin embargo, en nuestro trabajo no se encontraron diferencias entre tratamientos, manteniéndose en ambos grupos, las concentraciones de colesterol dentro de los rangos de referencia durante el período de estudio (preparto: 2,0-4,0 mmol/L; vacas frescas: 2,7-5,3 mmol/L, Lager et al., 2012), reflejando el incremento del colesterol postparto la recuperación del consumo voluntario de MS como fuera reportado previamente (Janovick Guretzky et al., 2006; Quiroz-Rocha et al., 2009). No obstante, la mayor concentración de NEFA del grupo PASTORIL+DPM, afectó el cociente NEFA/colesterol, siendo mayor en dicho grupo en comparación al TMR (0,40 vs 0,26, respectivamente) a los 15 días postparto siendo incluso mayor al punto de corte establecido para vacas frescas (<0,30, Van Saun 2004). Estos autores han reportado que NEFA/colesterol > 0,3 al inicio de lactancia está asociado a mayor riesgo de enfermedad.

Las concentraciones de Ca y P no se vieron afectadas por la estrategia nutricional, y el P se mantuvo siempre dentro de la rangos de referencia (1,10- 2,30 mmol/L, Merck, 2011) en ambos grupos. A su vez, solamente el grupo PASTORIL+DPM a los 30, 45 y 60 dpp, logró mantener las concentraciones de Ca dentro de los rangos de referencia (2,1 – 2,6 mmol/L, Merck, 2011). El grupo TMR presentó durante todo el período de estudio, concentraciones de Ca por debajo del límite inferior de referencia (≤ 2,14 mmol/L, Rodriguez et al., 2017), estando en hipocalcemia subclínica. Este hecho fue consistente con el menor cociente Ca/P en el grupo TMR (1,0 vs 1,4) para TMR y PASTORIL+DPM respectivamente, a pesar de estar ambos grupos dentro del rango de referencia (1,0-1,6, Wittwer, 1996). Las bajas concentraciones de Ca postparto y el menor cociente Ca/P visualizado en el grupo TMR, podría ser explicado por un desbalance entre Ca y P de la dieta consumida por este grupo (errores de mezclado de la ración), exacerbado por una mayor salida de Ca en leche (mayor producción láctea en grupo TMR).

En el **Artículo II**, la incidencia de enfermedad (46,4%) fue similar a lo reportado para vacas lecheras por otros investigadores (58%, Van Saun, 2004; 30 a 50%, Le Blanc, 2010). La mayor incidencia de enfermedad encontrada en la parición de otoño, se asoció a la mayor producción láctea, siendo atribuible a una mayor tasa de aceleración en el aumento diario de la producción, más que al aumento de leche “per se” según lo reportado (Ingvartsen, 2006). Además, la mayor producción visualizada en otoño, es consistente con la mayor oferta de MS asociado a un mejor índice de temperatura y humedad (ITH) que en la parición de primavera, en que fuera superado el umbral de confort para la producción láctea (ITH >72) (Polsky y von Keyserlingk, 2017).

La incidencia de metritis (26,6%) fue similar a lo reportado (21%) por Hammon et al. (2006) pero menor (40%) que lo reportado por Giuliodori et al. (2013) y mayor (0,7 a 2,2%) que lo reportado por otros (Rajala y Gröhn, 1998; Bruun et al., 2002). En la bibliografía se encontró disparidad en relación a la incidencia de metritis y se ha argumentado, que los criterios de diagnóstico no bien definidos entre los diferentes trabajos de investigación dificultarían su comparación (Huzzey et al., 2007). La incidencia de RP (17,2%) fue levemente superior a lo reportado (3 a 13%) por Ingvartsen et al. (2003). Varios factores de riesgo se asocian a la presentación de RP incluyendo abortos, distocia, parto de mellizos, terneros nacidos muertos, fiebre de leche u otros (Correa et al., 1993; Grohn y Rajala-Schultz, 2000) y es considerada de causa multifactorial.

La incidencia de mastitis clínica encontrada (15,2%) estuvo dentro de lo reportado (7 a 44%) por Ingvartsen et al. (2003) o similar en vacas a pastoreo (11 a 24%) (Bargo et al., 2009; Ribeiro et al., 2013; Sepúlveda et al., 2015). La hipocalcemia clínica fue muy baja (1,3%) por lo que fue considerada junto con DA, cojeras y cetosis clínica como "otras". Además, la incidencia encontrada de hipocalcemia subclínica y su mayor incidencia esperable en vacas MP fue similar a la reportada por otros (Goff et al., 2006; Reinhardt et al., 2011) y es consistente con el manejo preventivo para hipocalcemia que se realizó en el predio (**Artículo II**). La muy baja incidencia de cetosis clínica y subclínica y DA (0,3%, 3,3% y 2% respectivamente) reflejan el manejo preventivo realizado en el predio (**Artículo II**). Se ha reportado una incidencia de 8,9 a 34% de cetosis clínica (Ingvartsen, 2006; Rushen et al., 2008), 30% de cetosis subclínica (Duffield et al., 2009) y 5 - 7% de DA a la izquierda (LDA) (LeBlanc et al., 2005). Más aún, la cetosis clínica y subclínica han sido asociadas al desarrollo del LDA (LeBlanc et al., 2005), por lo que nuestros resultados son consistentes con esta observación, considerando la baja incidencia de ambas patologías.

Ingvartsen (2006) reporta como principales factores de riesgo para la presentación de cetosis e hígado graso, la alta CC al parto, la excesiva lipomovilización, el bajo consumo de nutrientes y factores de manejo asociados a las dietas. Además, elevadas concentraciones de NEFA preparto y de BHB y NEFA postparto han sido usadas para detectar vacas a riesgo de enfermedad (LeBlanc et al., 2005; Ospina et al., 2010a). En nuestro trabajo, las concentraciones de NEFA y BHB preparto no resultaron predictivas, pudiendo ser una posible explicación el ambiente confortable y de bajo estrés en el que se encontraban las vacas, como fuera discutido por otros (Drackley et al., 2001; Ospina et al., 2010b). Por lo tanto, considerando las bajas concentraciones de NEFA y BHB en preparto y la adecuada CC visualizada durante el período de transición, podemos sugerir un buen manejo de la dieta de transición (del punto de vista energético) y un buen manejo preventivo de cetosis al parto, experimentando las vacas un leve BEN. Esta pudo ser la causa de la baja incidencia de cetosis y DA en nuestro trabajo y que las variables BHB y NEFA no resultaran predictivas de enfermedad en contraste con la bibliografía citada.

Por otro lado, la concentración de albúmina preparto (-2 o -1 semana) resultó predictiva de metritis y RP en vacas MP, sugiriendo a la albúmina como un marcador económico y precoz de riesgo de enfermedad a ser utilizado en programas de monitoreo de salud a nivel de rodeo. Existen pocos reportes al respecto, pero este hallazgo fue consistente con Van Saun (2004) quien reportó que vacas con concentraciones de albúmina < 32,5 g/L preparto tenían mayor riesgo de enfermar (OR: 1,46; IC 95%: 1,04-2,04) o que bajas albuminemias reflejan una función hepática alterada (Bionaz et al., 2007), ya que la albúmina es una proteína de fase aguda negativa sintetizada por el hígado. También el colesterol resultó predictivo de mastitis, siendo la concentración inversamente proporcional al riesgo a medida que se acerca el parto (**Artículo II**). En este sentido se ha reportado menores concentraciones de colesterol preparto asociado a mayor riesgo de mastitis, metritis o RP. Además, la disminución del colesterol en el preparto se asocia a la disminución del consumo de MS (Janovick Guretzky et al., 2006) y esto a su vez se asocia con mayor riesgo de enfermedad; vacas enfermas comieron menos que vacas sanas durante la transición (Huzzey et al., 2007).

La paridad influenció fuertemente la adaptación a la transición viéndose reflejado en los perfiles metabólicos y la CC, como fuera citado previamente (Wathes et al., 2007; Adrien et al., 2012). En el presente trabajo, las vacas PP presentaron mayor

CC que las MP, pero encontrándose ambas categorías con una óptima CC, como fue establecido por Garnsworthy (2006). A pesar de que ambas categorías incrementan la concentración de NEFA debido al BEN, las vacas MP presentaron un mayor grado de movilización, probablemente debido a la mayor demanda energética para la producción láctea visualizada en este estudio. Sin embargo, a pesar de que no se encontraron diferencias en concentraciones de NEFA en las vacas PP (sanas o enfermas), las vacas MP enfermas tuvieron mayores concentraciones al parto que las vacas MP sanas. Incluso, en vacas MP con 2 eventos la concentración de NEFA permaneció elevada durante la semana +1 a diferencia de las MP sanas y con 1 evento. Esto concuerda con lo reportado; altas concentraciones de NEFA se asociaron a disfunción inmune alrededor del parto (Kehrli et al., 1989; Hammon et al., 2006).

Los perfiles de colesterol y albúmina fueron afectados por el estado de salud en ambas categorías hacia el final del estudio, presentando las vacas enfermas menores concentraciones de ambos metabolitos. Esto refleja el mayor desafío metabólico que enfrentan las vacas que se enferman durante la transición, siendo peor para vacas con más de un evento clínico, reflejando la no recuperación del consumo de MS. Al respecto Huzzey et al. (2007) reportaron que las vacas enfermas tienen menor consumo que las vacas sanas durante todo el período de transición. Un hallazgo interesante fue que el colesterol y la albúmina preparto resultaron predictivos de enfermedad pero solo para vacas MP. Este hallazgo de que las vacas MP enfermas - pero no PP- que se encontraban sanas durante el preparto, tuvieran menor concentración de estos metabolitos en el preparto, puede estar asociado a una peor recuperación de la lactancia anterior, y/o a un inadecuado manejo durante el período seco. Además, esto es consistente con los perfiles de NEFA, ya que las vacas MP enfermas – especialmente vacas con 2 eventos – tuvieron menores concentraciones de albúmina y mayores de NEFA, en acuerdo con lo reportado por Van Saun (2004). Más aún, en vacas enfermas, el peor perfil metabólico (NEFA, colesterol y albúmina) visualizado durante el período de vacas frescas, es consistente con la menor producción láctea encontrada en comparación a las vacas sanas, como fuera reportado por Ospina et al. (2010b).

Como era esperable, la paridad también afectó los perfiles de calcio; se reporta una mayor incidencia de hipocalcemia clínica y subclínica en vacas MP (Reinhardt et al., 2011). Las vacas MP presentaron menores calcemias al parto, debido a la mayor producción láctea y a un envejecimiento del mecanismo homeostático de respuesta a la hipocalcemia, resultando en una peor o más prolongada hipocalcemia, a medida que aumentan las lactancias (Reinhardt et al., 2011). Además, la hipocalcemia afecta el consumo, llevando a mayor lipomovilización en lactancia temprana (Goff, 2008). En el presente trabajo, si bien no hubo diferencias en la calcemia de las vacas PP acorde al estado de salud, en las vacas MP enfermas se encontraron menores concentraciones de Ca y mayores de NEFA al parto. Más aún, la hipocalcemia al parto se asoció a la presentación de RP (Melendez et al., 2004) y aumenta la susceptibilidad de presentar metritis y mastitis (Curtis et al., 1983, 1985), y en este sentido se encontraron menores calcemias en las vacas MP con estas enfermedades. Sin embargo, esto no fue observado en las vacas PP, y no encontramos una explicación para este hecho. También debe ser considerado que la activación del sistema inmune resulta en menores niveles de calcio (Waldron et al., 2003).

Las mayores concentraciones de IGF-I e insulina encontradas en las vacas PP durante el período de transición, han sido reportadas previamente (Wathes et al., 2007); la producción láctea se encuentra subordinada al crecimiento en vacas PP, y estas hormonas anabólicas revelan la diferente adaptación metabólica acorde a la paridad. Más aún, la pronunciada disminución de IGF-I encontrada en las vacas MP post parto a diferencia de las PP, refleja un mayor desacople del eje somatotrófico a favor de la producción como fuera reportado previamente (Wathes et al., 2007). Además, las bajas concentraciones de insulina, tienen un rol clave en la regulación de este mecanismo, ya que la insulina estimula la secreción hepática de IGF-I, modificando la expresión de receptores hepáticos para la hormona de crecimiento y de proteínas de unión a IGF-I (Zhou et al., 2005).

El estado de salud interactuó con la paridad y la semana, afectando los perfiles de IGF-I; mientras las vacas PP sanas mantuvieron mayores concentraciones de IGF-I durante el primer mes postparto, las PP enfermas presentaron una intensa caída de IGF-I en respuesta a la enfermedad; este hecho no fue observado en las vacas MP. La pronunciada bajada de IGF-I alrededor del parto en vacas enfermas, estuvo probablemente asociado a una disminución en el consumo de MS como fuera reportado previamente (Huzzey et al., 2007; Roche et al., 2011). Los datos sugieren una diferente adaptación metabólica a la enfermedad y lactancia acorde a la paridad. Cabe destacar que, mientras las vacas MP enfermas presentaron mayores concentraciones de NEFA que MP sanas, esto no fue visualizado en las vacas PP. Por lo tanto, la razón de las bajas concentraciones de IGF-I en vacas PP enfermas podría ser el resultados del estrés del primer parto, asociado a la adaptación a la nueva rutina de ordeñe y manejo (Eicher et al., 2007), y/o a un BEN más leve asociado a la menor producción láctea en ésta categoría en relación a las vacas MP enfermas.

Las variables reproductivas (reinicio de ciclicidad ovárica, tasas de preñez y concepción) fueron afectadas por la paridad. Hasta el día 50 postparto, las vacas PP presentaron un menor porcentaje de reinicio de ciclicidad ovárica en acuerdo con reportes previos (Santos et al., 2009). En vacas lecheras, la primera ovulación postparto se ha ligado a la severidad y duración del BEN (Butler, 2000). En varios estudios (Villa-Godoy et al., 1988; Meikle et al., 2004; Wathes et al, 2007; Santos et al., 2009;) el BEN en PP se reporta como más severo una semana previa al parto e inmediatamente después. Sin embargo, las bajas concentraciones de NEFA y mayores de IGF-I encontradas en las vacas PP (**Artículo III**), reflejan un mejor balance energético en esta categoría, de modo que factores asociados al parto como los mencionados anteriormente y reportados previamente (Eicher et al., 2007), podrían explicar el retardo en el reinicio de la ciclicidad ovárica en las vacas PP.

Sin embargo, la proporción de vacas preñadas fue mayor en PP que en MP. De forma similar, Santos et al. (2009) reportan que las vacas MP reinician antes que PP pero que el porcentaje de preñez al día 30 post IA fue mayor en vacas PP. En nuestro trabajo, la mayor producción láctea de las vacas MP pudo explicar la menor proporción de preñez, debido a un mayor estado catabólico, ya que la producción láctea fue negativamente asociada a la habilidad de vacas lactantes de concebir y mantener la preñez (Butler, 2003). Sin embargo, otros reportan que no hay asociación entre la producción láctea y la concepción o sobrevida embrionaria (Tenhagen et al., 2001; Santos et al., 2009). Un aumento del flujo hepático es requerido para sostener la mayor producción láctea en las vacas MP, llevando a un aumentado del clearance de P4; por lo tanto, las menores concentraciones de P4 podrían explicar una probable muerte embrionaria y la menor proporción de preñez en esta categoría (Lucy, 2001; Sangsritavong et al., 2002).

El estado de salud afectó el porcentaje de reinicio de ciclicidad ovárica durante las 7 semanas postparto, siendo menor en vacas enfermas (50% enfermas vs 67,9% sanas), lo cual se asoció con menores concentraciones de colesterol, albúmina e IGF-I en las vacas enfermas (**Artículos II y III**). Las vacas enfermas presentan menor consumo de MS y menores concentraciones de colesterol y albúmina (Van Saun, 2004; Janovick Guretzky et al., 2006). Como fuera mencionado previamente, IGF-I, estimula las células foliculares bovinas y la ovulación (Lucy, 2000). Más aún, la concentración de IGF-I una semana post parto se asoció con el reinicio de la ciclicidad ovárica y mayores concentraciones de IGF-I en la 2° semana postparto fueron reportadas en vacas que reiniciaron ciclicidad ovárica en comparación a vacas que no ovularon (Beam y Butler, 1999). Cabe destacar, que el efecto del estado de salud sobre el reinicio de ciclicidad ovárica en vacas PP fue más marcado que en MP (35% de enfermas vs 66% de sanas en PP y 59% vs 69% en MP). Esta diferente respuesta reproductiva a la enfermedad es consistente con los perfiles de IGF-I, ya que una pronunciada bajada de IGF-I fue visualizada en las vacas PP enfermas.

La menor probabilidad de reinicio de ciclicidad ovárica en vacas enfermas es consistente a su vez con el menor porcentaje de preñez visualizado en vacas enfermas (75% vacas sanas vs 54% enfermas). Esto puede ser explicado por el efecto negativo que las enfermedades inflamatorias producen a lo largo del tiempo, ya que éstas pueden causar fiebre y comprometer la reproducción al interrumpir el desarrollo del oocyto o del embrión o alterando la función uterina, meses después de cesada la fase aguda de enfermedad (Hansen, 2009). Otro hallazgo relevante fue que, la interacción de la paridad y el estado de salud con la tasa de concepción fue significativa. Mientras que no se encontraron diferencias en vacas PP acorde al estado de salud en la tasa de concepción (85% vs 80% para vacas PP sanas y enfermas respectivamente), las vacas MP enfermas tuvieron menor porcentaje de concepción que las vacas sanas (74% vs 60% para vacas MP sanas y enfermas respectivamente); esto pudo deberse a una menor fertilización y/o mayor mortalidad embrionaria en vacas MP enfermas. Los datos sugieren que cuando las vacas PP superan el evento clínico de salud y comienzan a ciclar, su fertilidad es mayor a la de vacas MP. En contraposición, en las vacas MP, se observó un mayor efecto negativo y/o efecto prolongado en el tiempo de las enfermedades sobre la reproducción, sugiriendo que la fertilización y el mantenimiento de la preñez son barreras importantes para el éxito reproductivo en esta categoría.

# Conclusiones

- La estrategia nutricional afectó los perfiles endócrino- metabólicos durante la transición, ya que las vacas en sistema PASTORIL+DPM presentaron menores concentraciones de insulina y mayor lipomovilización (mayor concentración de NEFA) y concentraciones de BHB indicativas de cetosis subclínica, asociado a una menor producción de leche en comparación con las vacas en sistema TMR (**Artículo I**).

- De todos los metabolitos determinados, solamente las concentraciones preparto de albúmina y colesterol resultaron predictoras de metritis, retención de placenta y mastitis en vacas multíparas, no así en primíparas (**Artículo II**).

- La evolución de los perfiles metabólicos y endócrinos en vacas sanas y enfermas durante la transición fueron diferentes acorde a la paridad, lo cual podría explicar parcialmente el observado desempeño productivo y reproductivo (**Artículos II y III**).

- La paridad interactuó con el estado de salud sobre el desempeño reproductivo, de manera que las vacas primíparas enfermas presentaron un retraso en el reinicio de la ciclicidad ovárica y menores concentraciones de IGF-I, mientras que las vacas multíparas enfermas presentaron menor porcentaje de preñez (**Artículo III**).

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# *Anexos*

## The nutritional and management strategy affects the metabolic and endocrine profiles of healthy dairy cows

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### Abstract

The aim of this study was to investigate the effect of two different nutritional strategies; total mixed ration (TMR) versus grazing + partial mixed ration (GRAZING+PMR) on the metabolic parameters usually included in herd monitoring programs and on endocrine concentrations associated with metabolic and immune functions in healthy multiparous Holstein cows. Twenty cows without clinical events during entire study period (-30 until +60 days related to calving) were selected from a commercial dairy herd. Before calving, cows were managed together and fed a TMR once a day (12 kg DM/cow/d) and after calving randomly assigned to two nutritional and management strategies until day 60: a) cows fed a total mixed ration (TMR), (27 kg DM/cow/d), housed in free-stall and b) pasture grazing with access to paddock in two grazing sessions with a high forage allowance (40 kg DM/cow/d) and supplemented with a partial mixed ration (GRAZING+PMR), (9 kg DM/cow/d), (n=10). Every 15 days BCS was determined and blood samples were obtained for non-esterified fatty acid (NEFA), Β-hydroxybutyrate (BHB), protein, albumin, cholesterol, urea, Calcium (Ca), phosphorus (P), aspartate aminotransferase, ɣ glutamyl transpeptidase, haptoglobin, insulin, IGF-I, leptin and adiponectin determination. Weekly milk production and milk composition was determinate. Cows in the GRAZING+PMR group presented greater NEFA and BHB concentrations and greater Ca/P ratio and lower insulin concentrations after calving than cows in the TMR group. Moreover, cows in the GRAZING+PMR group

presented subclinical ketosis and lower milk yield revealing that the nutritional and management strategy affected the cow adaptation to transition.

*Keywords:* Dairy cow, grazing, total mixed ration, metabolite, hormone

## 1. Introduction

The incidence of diseases in the dairy cow is high during the transition period (3 weeks before until 3 weeks after calving) (Ingvartsen, 2006; LeBlanc et al., 2006). Because of this, herd monitoring programs for transition dairy cows (at individual or herd levels), has become a need for a good health management, to minimize losses related to health disorders. For many years, several blood parameters have been investigated that are useful to evaluate metabolic pathways and cow adaptation during the transition period. Blood concentrations of Β-hydroxybutyrate (BHB), non-esterified fatty acid (NEFA) and cholesterol were reported as useful to evaluate the energy metabolism (Van Saun, 2004; Meikle et al, 2004; Ospina et al., 2010b); protein, albumin and urea concentrations to evaluate the protein metabolism (Van Saun 2004; Meikle et al., 2004; Puppel and Kuczynska, 2016), calcium (Ca) and phosphorus (P) for the mineral metabolism (Goff, 2008) and aspartate aminotransferase (AST) and ɣ glutamyl transpeptidase (GGT) activity for liver function (Kaneko et al., 2008). In a lesser extent, hormones like insulin, insulin-like growth factor-I (IGF-I) and leptin have been proposed as energy metabolic regulators, and adiponectin and haptoglobin (Hp) linked to immune response and inflammation during the transition period (Huzzey et al., 2011; Adrien et al., 2012; Kadivar et al., 2012; Piechotta et al. 2012; Kalamaras, 2012).

Additionally, the risk for different peripartum diseases has been associated with specific metabolite concentrations. Indeed, NEFA, BHB and cholesterol concentrations during close up and or fresh cow period have been related to subclinical ketosis, left displaced abomasum, mastitis, metritis or retained placenta (LeBlanc et al, 2005; Duffield et al 2009; Ospina et al 2010a, Ruprechter et al., 2018). Also, Ca, albumin and adiponectin concentrations around calving were related to depressed immune function, subclinical hypocalcemia, metritis, retained placenta or left displaced abomasum (Martinez et al., 2012; Reinhard et al 2015; Ruprechter et al., 2018). Nevertheless, most of the previous reviewed information was investigated in intensive management systems, [housed cows in free-stalls and fed a total mixed ration (TMR)]. Less information was found in grazing systems (Bargo, 2009; Ribeiro et al., 2011; Sepulveda et al., 2015) probably due to the reported lower incidence of peripartum diseases in these production systems (White et al., 2002; Ribeiro et al., 2011), and/or difficulties in diagnosis and record systems.

Moreover, nutritional management may affect some metabolite concentrations, and some information was reported comparing grazing systems vs intensive management systems (Adrien et al., 2012; Meikle et al., 2013; Astessiano et al., 2015). Additionally, grazing dairy production systems have become interesting due to their economic environmental and animal-welfare advantages (Dillon, 2006). Taken all this in consideration and in order to establish herd monitoring programs in grazing production systems, the aim of the present study was to investigate, the effect of two different nutritional strategies; total mixed ration (TMR) versus grazing + partial mixed ration (GRAZING+PMR) on the metabolic parameters usually included in herd monitoring programs and on endocrine concentrations associated with metabolic and immune functions in healthy multiparous Holstein cows.

## 2. Materials and Methods

### 2.1. Cows, herd management and treatments

The experiment was carried out at a commercial dairy farm in Uruguay from August to November 2015. Animal procedures were approved by the Animal Experimentation Committee of the University of Uruguay (Protocol number CEUAFVET-221).

Healthy multiparous Holstein cows (n= 20) during entire study period (-30 until +60 days related to calving) calved between August and October with lactation average of (mean±SD) 3.1±1.2, prepartum body weight of 660 ± 82.1 kg and previous lactation milk production average of 6,345 ± 1,463 L were selected from the herd (n= 1,400 dairy cows). Body condition score (BCS) was registered using the scale 1-5 (Edmondson et al., 1989) every 15 days from -30 until +60 days related to calving. One month before expected calving, the cows were blocked according to body weight, BCS, number of lactation and expected calving date. During close-up, cows were managed together in the same paddock with shadow and water and fed a TMR once a day (12 kg DM/cow/d), composed of canola seed (21%), corn silage (51%), corn grain (26%) and mineral and vitamin supplement (1%).

After calving, the cows were randomly assigned to two nutritional and management strategies until day 60: a) TMR (n= 10) cows housed in free-stall fed a TMR and b) GRAZING+PM (n=10) pasture grazing with access to paddock in two grazing sessions (7:30–11:30 AM and 6:00 PM–6:00 AM) and supplemented with a partial mixed ration (PMR) from 11:30 AM to 3:00 PM remaining at this time the cows in a paddock, with shadow and water *ad libitum*.

The TMR group was fed a TMR *ad libitum* according to requirements (27 kg DM/cow/d) once a day, composed of whole sorghum plant silage (33%), grain dry rolled of *Sorghum* (12,5%), citrus pulp (10%), canola seed (16,5%), sorghum burlanda (10%), soybean hull (16%), mineral and vitamin mix (1.3%) and urea (0.2%). The GRAZING+PMR group was assigned to a grazing paddock, composed of *Festuca arundinacea* and *Dactylis perseo*, grazing daily strips, with a high forage allowance (40 kg DM/cow/d) and supplemented with 25% of the same TMR referred before (9 kg DM/cow/d). Foods chemical composition is shown in Table 1.

## 2.2. Blood samples collection and analyses

Blood samples were obtained every 15 days from −30 to +60 days related to calving, immediately after the morning milking from the coccygeal vein with Vacutainer® serum tubes. Samples were centrifuged (3000×g for 10 min) within 2 h after collection and serum was stored at −20 °C until assayed. Cows were milked twice a day (6:30 AM and 6:30 PM) and individual milk production was measured weekly with commercial milk meter (Waikato®), every Monday in both milkings. Also individual milk composition (protein, fat, lactose) was determined in both samples weekly.

Serum variables (NEFA, BHB, protein, albumin, cholesterol, urea, Ca, P, AST, and GGT) were determined by colorimetric assays on A25 autoanalyzer (© Biosystems S.A., Barcelona, Spain) using commercial kits. Wako [NEFA-HR(2)], (Wako Pure Chemical Industries Ltd., Osaka, Japan) for NEFA and Biosystems for the rest of the variables (metabolites, minerals and enzymes). The inter-assay coefficient of variation (CV) for all commercial serum controls was less or equal than 10 %. Haptoglobin concentrations (Tridelta Diagnostics Ltd., Morris Plains, Ireland) were measured by ELISA (Thermo, Multiskan EX, USA). The inter-assay CV for all commercial serum controls was less or equal than 10 %.

Insulin and IGF-I concentrations were measured using immune radiometric 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). All samples were determined in a single assay for each hormone. For insulin, the assay detection limit was 0.6 μIU/mL, and intra-assay CV for control 1 (21.4 μIU/mL) and 2 (56.9 μIU/mL) were 8.0 and 9.3 %, respectively. For IGF-I, the assay detection limit was 0.3 ng/mL, and intra-assay CV for control 1 (42.3 ng/mL) and control 2 (519.8 ng/mL) were 9.1 and 9.9 %, respectively. Leptin concentrations were determined by a liquid phase radioimmunoassay (RIA) using a commercial Multi-Species Leptin kit (RIA kit, Millipore, USA). The sensitivity was 3.0 ng/mL and the intra-assay CV for control 1 (4.5 ng/mL) and control 2 (19.6 ng/mL) were 8.5 and 7.4 %, respectively. Adiponectin concentrations were measured using an RIA kit (HADP-61 HK, Millipore, USA) with undiluted serum samples. The sensitivity was 1.54 ng/mL, and the intra-assay CV were 9.1% and 11.1% for control 1 (8.4 ng/mL) and control 2 (81.5 ng/mL) respectively.

## 2.3. Statistical Analysis

Data of BCS, milk production, milk components, serum metabolite and hormones were analyzed by SAS as repeated measures using the MIXED procedure with days as an repeated effect and the first-order autoregressive [AR(1)] covariance structure. Nutritional strategies (TMR vs GRAZING+PMR), days and their interaction were considered as fixed effects and BCS at calving as covariable. The Kenward-Rogers procedure was used to adjust the denominator degree of freedom. For all statistical analysis $P < 0.05$ was considered a significant effect and $P \leq 0.1$ as a tendency.

## 3. Results

The nutritional strategy affected the milk production being greater in the TMR group than in the GRAZING+PMR group (39.2 ± 1.3 vs 28.7 ±1.1 L) (Table 2). Milk production increased until 60 days after calving in the TMR group, while the GRAZING+PMR group tended to show a plateau curve (Figure 1A). Also milk fat was affected by nutritional strategy and days, being lower in the TMR group than in the GRAZING+PMR group (3.4 ± 0.07 vs 3.7 ± 0.06 %), (Table 2 and Figure 1C). Milk protein tended to be affected by nutritional strategy and show a different profile at the beginning of lactation (Table 2, Figure 1B). BCS decreased from day -15 until +60 in the GRAZING+PMR group and was lower at days +45 and +60 than the TMR group. TMR cows maintained their BCS during the study period (Table 3 and Figure 1E).

The mean concentrations of NEFA were greater in the GRAZING+PMR group than the TMR group (0.57 ± 0.03 vs 0.43 ± 0.04 mmol/L) and also were BHB concentrations (0.83 ± 0.06 vs 0.55 ± 0.07 mmol/L) (Table 3). Independent of nutritional strategy, NEFA concentrations increased after calving until day +15 and were greater at this time in the GRAZING+PMR group than in the TMR group (1.1 ± 0.08 vs 0.7 ± 0.09 mmol/L), decreasing thereafter until end of study (Fig. 2A).

The TMR group maintained the BHB concentrations constant during the study period, while the GRAZING+PMR group increased BHB concentrations after calving being greater than the TMR group at +15, +30 and +45 days related to calving (Fig. 2 B).

Independent of the nutritional strategy, mean cholesterol concentrations increased after calving until the end of study, reaching at +60 days greater cholesterol concentrations than at -15 days related to calving (Fig. 2 C).
NEFA/cholesterol index tended to be affected by nutritional strategy and was greater in the GRAZING+PMR group than the TMR group (0.40 vs 0.27) at 15 days after calving (Table 3; Figure 2D).

The activity of AST and GGT was affected by days, being fluctuant and with high variability during the study period independent of nutritional strategy (Table 3; Fig. 2 E, F). Mean protein concentrations were affected by days while albumin and urea concentrations tended to be affected, being urea concentrations greater in the TMR group than in the GRAZING+PMR group at 60 days after calving (Table 3, Fig. 3 A, B, C).

Although mean Ca and P concentrations were not affected by nutritional strategy (Table 3, Figure 3D,E), the Ca/P relation was affected by it being greater at +30 days in the GRAZING+PMR group in comparison to the TMR group (Table 3, Fig. 3F).

Insulin concentrations were affected by nutritional strategy and days, being greater in the TMR group than in the GRAZING+PMR group (10.9 ± 0.9 vs 7.4 ± 0.7 uUI/mL). Although IGF-I was not affected by nutritional strategy, both hormones independent of nutritional strategy decreased their concentrations from -30 to +15 days, increasing thereafter until the end of study, but not recovering the GRAZING+PMR group the close-up IGF-I concentrations. (Table 3, Fig. 4 A, B). Adiponectin concentrations were not affected by nutritional strategy and remained constant during the study period. Nevertheless, although leptin concentrations were not affected by nutritional strategy, days tended to affect this hormone concentration as lower concentrations were observed 15 days before calving (Table 3, Figure 4 C, D). Haptoglobin concentrations were not affected by nutritional strategy and remained constant during the study period (Table 3).

## 4. Discussion

This study demonstrated that the nutritional strategy affected several metabolic and endocrine profiles of healthy multiparous dairy cows. The low Hp concentrations visualized during the entire study period in all cows confirmed the health status of them, since Hp > 1ng/mL has been related to inflammation or tissue damage (Huzzey et al., 2009).

The GRAZING+PMR treatment was designed to maximize herbage intake by offering a very high forage allowance in two grazing sessions and by the inclusion of 25% of TMR. Nevertheless, despite the similar genetic merit of the cows, the cows in the TMR group produced in average 10.5 L/day more than cows in the GRAZING+PMR group (e.g., 27% reduction in GRAZING+PMR cows). Data is consistent with previous reports: Vibart et al. (2008) with 32% of DMI of pasture and the rest of TMR reported no differences in milk production; Bargo et al. (2002) reported a 16% reduction in milk production when pasture herbage allowance was 50% of the diet and White et al. (2002) showed a 30% milk reduction when pasture vs TMR diets were compared. Although comparisons are difficult to perform as results depends on type and quality of the feed and actual DMI was not measured, our results are more in the range of the latter study, suggesting that the lower milk yield on grazing conditions is a result of a lower DMI and increased energy demands for waking and grazing activity (Kolver and Muller, 1998; Bargo et al., 2002; Meikle et al., 2013). Indeed, when estimated by NEL, cows in the GRAZING+PMR consumed 35% DM less than the TMR group (26.5 kg vs 17.2 kg of estimated DM for TMR and GRAZING+PMR respectively).

Interestingly, although all cows were healthy, they developed a NEB at the beginning of lactation, visualized by their NEFA concentrations being both groups above the cutoff point established for fresh-cows (0.6 mmol/L, LeBlanc et al., 2005) and therefore more predisposed to get sick. Nevertheless, cows in the GRAZING+PMR group developed a greater one, reflected by their higher NEFA and BHB concentrations, as reported by others (Adrien et al., 2012) and consistent with the lower DM intake and NEL of this group. Moreover, GRAZING+PMR cows presented even BHB concentrations ≥1.2 mmol/L at 15 and 30 days after calving, reported as subclinical ketosis (LeBlanc et al., 2005), while the TMR group maintained BHB concentrations between reference ranges (close-up:<0.5 mmol/L; fresh-cow:<0.8 mmol/L, Wittwer, 1996). This is consistent with a trend on higher BCS, a higher estimated DMI, and a better metabolic adaptation to transition period. Indeed, considering the subclinical ketosis of the GRAZING+PMR group, this could be also a reason for the lower milk production as reported previously (Duffield et al., 2009). Also, the greater lipomovilization of the GRAZING+PMR group could favor the greater milk fat visualized in this group (Eicher, 2004), but on the other hand, nutrition aspects (higher FDN % of the GRAZING+PMR group) could also influence the milk fat content and the lower milk yield because of the negative effect on voluntary DMI (Mertens, 2002).

Low cholesterol concentration during close-up or fresh-cow period has been associated with a pronounced negative energy balance and also poor health status and altered liver function (Van Saun, 2004; Bionaz et al., 2007; Sepulveda et al., 2015; Ruprechter et al., 2017). Nevertheless, lower cholesterol has been reported as a positive effect of grazing condition in cows around 120 days of lactation (4.53 vs 4.60 mmol/L for grazing and TMR cow respectively) (Radkowska and Herbut, 2014) and Meikle et al., (2013) reported greater cholesterol concentrations in cows on grazing conditions during transition period. In the present study, cholesterol concentrations were similar between treatments and always between reference ranges (close-up: 2.0-4.0 mmol/L; fresh-cow: 2.7-5.3 mmol/L, Lager et al., 2012) during the entire study period. Indeed, the increase in cholesterol concentrations after calving reflected the recovery of voluntary DMI (Janovick Guretzky et al., 2006; Quiroz-Rocha et al., 2009). Nevertheless, the greater NEFA concentrations of the GRAZING+PMR group affected the NEFA/cholesterol index, being higher in the later (0.40 vs 0.26; for GRAZING+PMR and TMR group respectively) at 15 days after calving and greater than the reference cutoff point (< 0.30, Van Saun, 2004) established for fresh cows been more susceptible to get sick.

Considering enzyme activity, it has been reported higher AST and GGT activity associated to displaced abomasum and also to fatty liver, together with elevated NEFA and BHB concentrations after calving (Sejersen et al., 2012). Limited information about these enzymes was found in grazing conditions, but higher AST activity was reported on grazing conditions because of the higher nitrogen content of pasture forage that could influence hepatic nitrogen metabolism (Radkowska and Herbut, 2014). Nevertheless, in our study there were no differences between nutritional strategies and interestingly although the high NEFA and BHB concentrations visualized in the GRAZING+PMR group, liver function was not affected as both enzymatic activities were always lower than the upper limit of reference that could indicated cytosolic or canalicular damage (AST < 110 UI, GGT< 39 UI, Kaneko 2009).

In the present study, independent of nutritional strategy the concentrations of protein, albumin and urea were always between reference ranges (Protein: 66 - 90 g/L, albumin: 29 – 41 g/L, urea: 2.6 - 7.0 mmol/L, Kaneko 2008; Lager et al., 2012), arguing for a correct metabolic protein balance in accordance with de CP% of the TMR ration and the GRAZING+PMR.  Indeed, urea concentrations were reported to be very fluctuant and influenced by factors as daily protein intake and carbohydrate availability for the ruminal microorganisms, and liver and kidney function, among others (Van Saun, 1997). It has been also reported, lower serum protein and albumin concentrations around calving because of their utilization for immunoglobulin synthesis and decreased DMI (Cavestany et al., 2009; Adrien et al., 2012) and after calving because they have been used to provide amino acids for milk production (Prodanovic et al., 2012). Also, albumin, a negative acute phase protein, synthesized by the hepatocytes is reported to be low during liver injury, kidney diseases, inflammatory conditions or malnutrition (Lager and Jordan 2012; Ruprechter et al., 2018).

Calcium and P concentrations were not affected by nutritional strategy and although P concentrations were between reference ranges (1.10- 2.30 mmol/L, Merck, 2011), only the GRAZING+PMR showed mean Ca concentrations inside reference ranges (2.1 – 2.6 mmol/L, Merck, 2011) but only at 30, 45 and 60 days after calving. The TMR group presented Ca concentrations below reference range and Ca concentrations reported as subclinical hypocalcemia (≤ 2.14 mmol/L, Rodriguez et al., 2017) during the entire study period. This is also consistent with the lower Ca/P relation of the TMR group (1.0 vs 1.4) for TMR and GRAZING+PMR respectively, although both were always ≥1.0, considered inside the reference range (1.0 - 1.6) (Wittwer, 1996). The lower calcium concentrations after calving in the TMR cows and the inappropriate Ca/P ratio could be explained by an incorrect Ca/P ratio of the consumed diet in this group (errors in mixture of the ration) exacerbated by the greater output of Ca in milk in this group.

Independent of the nutritional strategy, insulin and IGF-I concentrations were low around calving reflecting the metabolic adaptation to the NEB of the cows as reported previously (Butler 2000; Adrien et al., 2012) and consistent with the elevated NEFA concentrations at this time in the present study. As expected, insulin concentrations were greater and NEFA lower in the TMR group, consistent with the greater energy density of the TMR diet, and no extra energy requirements for grazing activities or daily waking from the grazing paddock to the milking parlour. The lower estimated DMI and energy availability of the GRAZING+PMR group visualized through the lower insulin and greater NEFA and BHB concentrations, were also reflected on the lower milk yield of these cows, consistent with previous reports (Meikle et al., 2013; Asstesiano et al., 2015). Moreover, although IGF-I concentrations were not affected by nutritional strategy, IGF-I tended to be greater after calving in the TMR group, arguing for a better and faster recovery of de NEB and consistent with the greater insulin concentrations in this group. Indeed, stimulatory action of insulin and/or nutrient availability were reported on the hepatic IGF-I synthesis (Butler, 2000; Rhoads et al., 2004).

Adiponectin and leptin concentrations were not affected by nutritional strategy in contrast with Asstesiano et al., (2015) who reported greater adiponectin and leptin in cows on grazing conditions. Also very low adiponectin concentrations (<7ng/mL) were associated with placenta retention (Aye et al., 2014), but in the present study, mean adiponectin concentrations were almost greater, and similar between groups (22 ± 1.5 and 25 ± 1.2 ng/mL, for TMR and GRAZING+PMR respectively).

## Conclusion

The nutritional strategy affected the metabolic and endocrine adaptation to transition, so that cows on GRAZING+PMR systems could achieve the challenge of not presenting clinical disease but resenting milk production and developing subclinical ketosis.

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**Table 1.** Nutrient composition of the total mixed ration (TMR) and Pasture.

| Nutrient profile | TMR | Pasture |
|---|---|---|
| Dry mater % | 86.6 | 29.5 |
| CP% | 18.2 | 12.2 |
| NDF% | 36.0 | 55.3 |
| ADF% | 21.9 | 27.8 |
| Ether extract % | 3.2 | 1.8 |
| NEL (Mcal/kg) | 1.52 | 1.50 |
| Ca% | 0.45 | . |
| P% | 0.47 | . |

CP% (percentage of Crude Protein), NDF% (percentage of neutral detergent fiber), NDA%(percentage of acid detergent fiber), NEL (Net Energy Lactation), Ca% (percentage of calcium), P% (Percentage of phosphorus). Ca% and P% was not determined for Pasture.

**Table 2.** F-tests of fixed effects for milk production, 4% fact corrected milk (FCM) and milk components from healthy Holstein cows from calving until 60 days after (*n*=20). Fixed effects were nutritional treatment (Treatment), days (related to calving) and the interaction (Treatment*days).

| | Treatment | Days | Treatment*days |
|---|---|---|---|
| Milk | <.0001 | <.0001 | 0.002 |
| 4% FCM | <.0001 | 0.04 | <.0001 |
| Protein | 0.11 | <.0001 | 0.05 |
| Fat | 0.00 | <.0001 | 0.81 |
| Lactose | 0.07 | 0.005 | 0.74 |

**Table 3.** F-tests of fixed effects for metabolite, hormones and inflammatory marker concentrations in healthy Holstein cows from 30 days before until 60 days after calving (*n*=20). Fixed effects were nutritional treatment (Treatment), days (related to calving) and the interaction (Treatment*days).

| | Treatment | Days | Treatment*days |
|---|---|---|---|
| BCS | 0.10 | <.0001 | 0.09 |
| BHB | 0.01 | <.0001 | 0.00 |
| NEFA | 0.01 | <.0001 | 0.08 |
| Cholesterol | 0.46 | <.0001 | 0.62 |
| NEFA/Chol | 0.10 | <.0001 | 0.03 |
| AST | 0.83 | 0.02 | 0.65 |
| GGT | 0.81 | <.0001 | 0.13 |
| Protein | 0.67 | <.0001 | 0.56 |
| Albumin | 0.33 | 0.14 | 0.46 |
| Urea | 0.20 | 0.07 | 0.06 |
| Ca | 0.19 | 0.02 | 0.21 |
| P | 0.51 | 0.28 | 0.81 |
| Ca/P | 0.01 | 0.54 | 0.47 |
| Insulin | 0.01 | <.0001 | 0.92 |
| IGF-I | 0.37 | <.0001 | 0.13 |
| Leptin | 0.19 | 0.07 | 0.14 |
| Adiponectin | 0.25 | 0.13 | 0.98 |
| Haptoglobin | 0.16 | 0.73 | 0.48 |

NEFA/Chol (Ratio NEFA/Cholesterol), AST (Aspartate Transaminase), GGT (Gamma-Glutamyl Transferase), Ca (Calcium), P (Phosphorus), Ca/P (Calcium/Phosphorus).

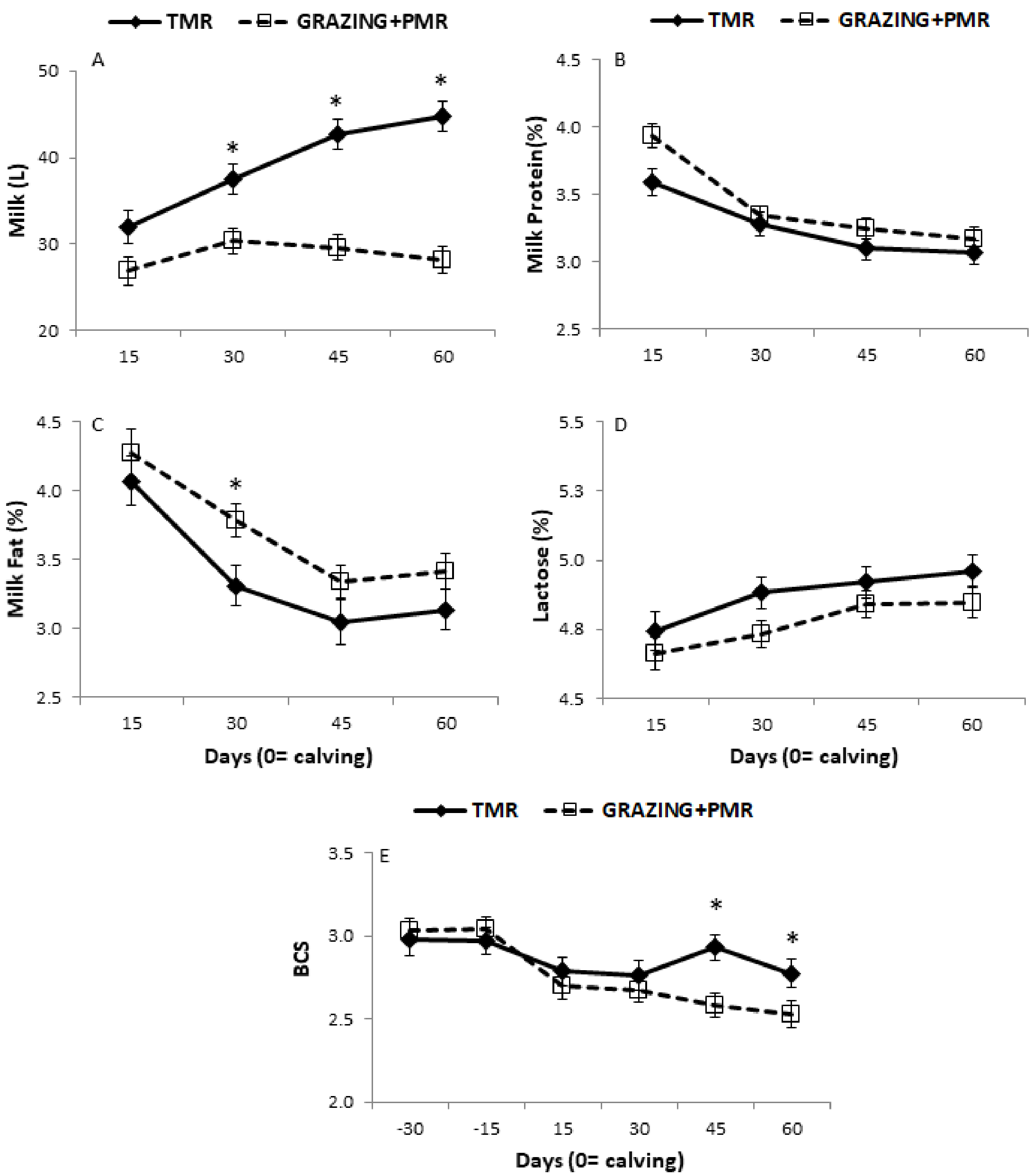


**Fig.1.** Milk production and milk composition (protein, fat and lactose) from calving until 60 days after (A,B,C,D) and body condition score (BCS) from -30 days until +60 days in relation to calving (E) for TMR group (continuous line) and GRAZING+PMR group(dotted line).

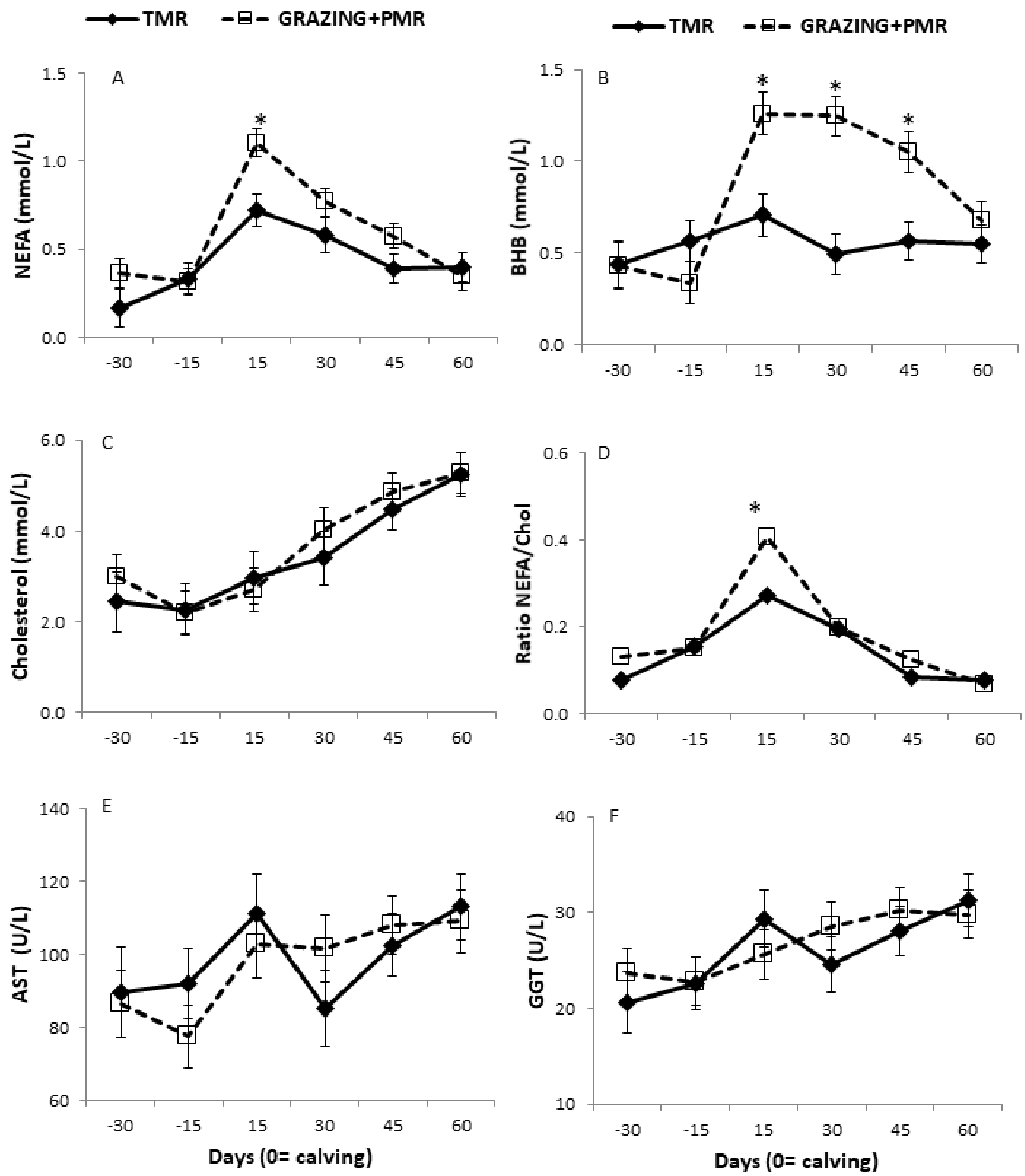


**Fig.2.** Concentrations of NEFA (A), BHB (B), cholesterol (C), ratio NEFA/cholesterol (D) and AST (E) and GGT (F) activity in multiparous Holstein cows belonging to TMR group (continuous line) and GRAZING+PMR group (dotted line), from -30 days until +60 days related to calving.

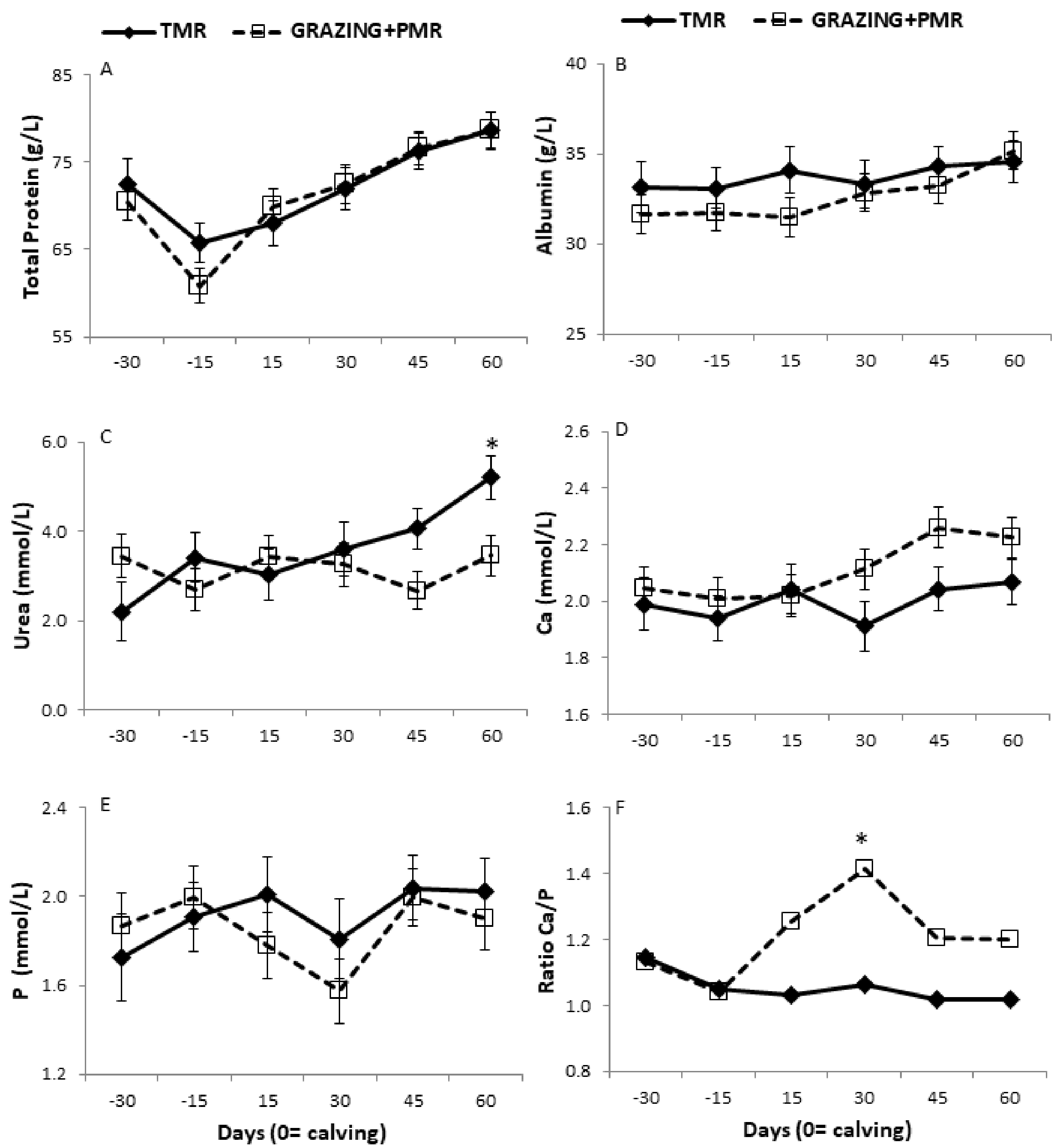


**Fig.3.** Concentrations of total protein (A), albumin (B), urea (C), Ca (D), P(E) and ratio Ca/P (F) in multiparous Holstein cows belonging to TMR group (continuous line) and GRAZING+PMR group (dotted line), from -30 days until +60 days related to calving.

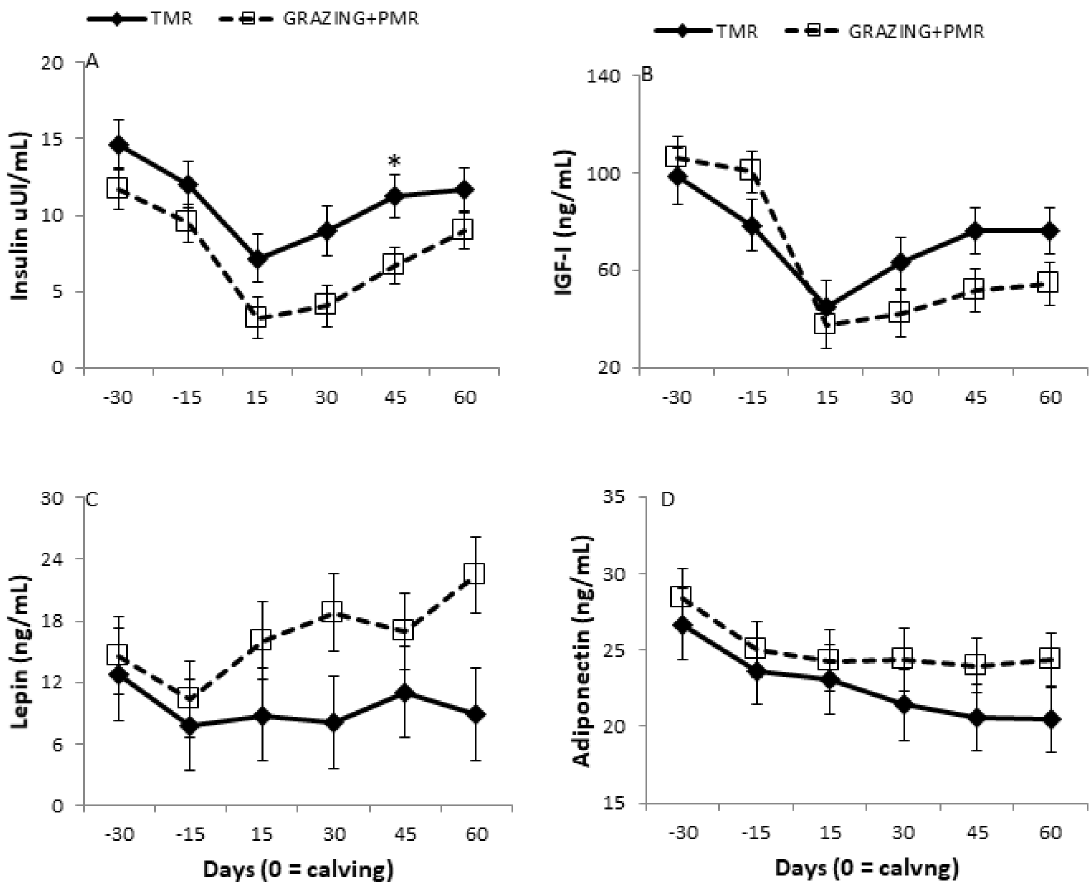


**Fig.4.** Concentrations of insulin (A), IGF-I (B), leptin (C) and adiponectin (D), in multiparous Holstein cows belonging to TMR group (continuous line) and GRAZING+PMR group (dotted line), from -30 days until +60 days related to calving.

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# Metabolic predictors of peri-partum diseases and their association with parity in dairy cows

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ABSTRACT

The predictive values of plasma non-esterified fatty acid (NEFA), beta-hydroxybutyrate (BHB), cholesterol, albumin and calcium to predict risk of peripartum diseases in primiparous (PP) and multiparous (MP) Holstein cows was investigated. Besides it was assessed if the health status interacted with parity on body condition score and metabolic profiles during the transition period. Dairy cows (126 PP and 182 MP) from a commercial dairy *free stall* herd (loose-housing system) were weekly body condition scored and tail bled for metabolites determination from −3 to +4 weeks relative to calving. Peripartum diseases were diagnosed by a single trained veterinarian, while subclinical diseases (ketosis and hypocalcemia) were diagnosed at the laboratory. Cows were classified as healthy cows, cows with one event, or cows with two clinical events following a prospective observational cohort study, with only healthy cows enrolled at the beginning of the study. The largest incidence was for metritis (26.6%) followed by retained placenta (17.2%) and mastitis (15.2%) with no effect of parity, while subclinical hypocalcemia incidence was greater in MP than PP cows (43% vs 9.5%) respectively. In MP cows albumin concentrations were predictive for metritis at week −2 and for retained placenta at weeks −2 and −1, while cholesterol was predictive for mastitis at week −2, −1 and at calving. The interaction between health status and parity affected all metabolites during the transition period. This study showed a different evolution of metabolic profiles in healthy and sick cows during the transition according to parity, pointing out albumin and cholesterol as diseases predictors before calving.

## 1. Introduction

Over the past decades dairy cows have undergone intensive genetic selection, which has increased milk yield to a level where the demands for nutrients from the diet and body tissue reserves often results in ill-health and infertility (Mulligan and Doherty, 2008). The relationship of 'the transition cow' metabolism and the pathogenesis of peri-partum diseases has increased, and maintaining health and productivity is one of the most difficult tasks for dairy herds. Approximately 75% of diseases in dairy cows typically happen in the first month after calving (LeBlanc et al., 2006). These problems are increasingly known to be rooted in immune function and feed intake 2 to 3 wks before calving, arguing for the importance of nutritional management of the transition dairy cow (LeBlanc et al., 2006).

The negative energy balance (NEB) that takes place during this period is associated with the development of many of these diseases (Herdt, 2000). Body condition score (BCS) during the transition period and the risk of peri-partum diseases has been established; e.g., cows with BCS ≥ 3.75 have a higher risk to develop clinical ketosis or displacement of the abomasum (DA) (Seifi et al., 2011). Moreover, a direct effect of BCS on feed intake was suggested (Garnsworthy, 2006); e.g., cows with BCS ≤ 3.25 at calving lost less BCS after calving and reached their maximum dry matter intake (DMI) earlier than cows with higher BCS. Parity is also a well-known risk factor for some diseases; e.g., multiparous cows are more likely to develop ketosis and hypocalcemia (Seifi et al., 2011; Reinhardt et al., 2011). As NEB is reflected in some metabolites, many metabolic markers can be used for evaluating the cow adaptation to the NEB and the risk of peri-partum diseases (Van Saun, 2009). Elevated serum non-esterified fatty acid (NEFA) concentrations 7 to 10 days pre-partum is a risk factor for DA (LeBlanc

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et al., 2005). Also elevated serum post-partum β-hydroxybutyrate (BHB) concentrations were reported as a risk factor for ketosis (Duffield et al., 2005; LeBlanc et al., 2005), metritis and mastitis (Duffield et al., 2009). Moreover, subclinical hypocalcemia may make cows more susceptible to secondary diseases (Reinhardt et al., 2011), as it reduces the ability of immune cells to respond to stimuli (Kimura et al., 2006). This could contribute to infections, such as mastitis or metritis (Martinez et al., 2012). In the first week post-partum, confined cows with an altered hepatic function had lower albumin and cholesterol concentrations (Bionaz et al., 2007) and grazing dairy cows with severe metritis or having more than one clinical disease after calving presented lower post-partum cholesterol concentrations (Sepúlveda et al., 2015). Few reports on associations of lower prepartum cholesterol or albumin concentrations and postpartum diseases were found (Kaneene et al., 1997; Van Saun, 2004). Also Trevisi et al. (2010) reported lower prepartum cholesterol concentrations in multiparous cows classified by their liver activity index as low, but the authors concluded that the lack of association with udder health was probably due to the limited number of cows included in the study. An earlier prediction of the probabilities for disease may enable the farmer to modify the herd management to improve its health status.

Although there is abundant literature on metabolic markers and peripartum diseases in dairy cows, we have not found field trial reports of the effect of parity (primiparous vs multiparous cows) and health status on metabolic profiles (e.g NEFA, BHB, cholesterol, albumin and calcium concentrations) determined weekly during the transition period. Moreover, the evolution of these metabolic markers during the transition period of healthy and sick cows will allow us to select the strategic moment for bleeding in order to monitor and predict disease risk in dairy herds. Thus, the aims of this study were: a) assess whether the health status (healthy vs sick cows) interacted with parity to affect BCS and metabolic profiles during the transition period and milk production, b) determine and compare the predictive value of metabolic markers (NEFA, BHB, cholesterol, albumin and calcium) to establish disease risk during the transition period in primiparous (PP) and multiparous (MP) Holstein cows.

## 2. Materials and methods

### 2.1. Cows and herd management

The study was conducted from October 2014 to September 2015 in a commercial dairy *free stall* (loose-housing system) herd in Rio Grande do Sul, southern Brazil. All procedures were carried out in accordance with regulations of the Animal Experimentation Committee (SIPPEE 10.059.15 CEUA 0522017 UNIPAMPA, Brazil).

Holstein dairy cows ($n = 126$ PP and $n = 182$ MP) from a 700-cow herd were selected, with an approximate milk yield of 8000 kg per lactation. The calving seasons were spring/summer (October 2014 to January 2015) and autumn/ winter (May to August 2015). The average temperature-humidity index (THI, Mader et al., 2006) in the autumn calving season was 72, while in spring calving season was 82. Cows were evaluated from 3 weeks before calving, until 4 weeks after calving.

From day −21 until calving, cows were kept on paddock separately by parity without significant pasture allowance and were offered 13.3 kg/d of DM as total mixed ration (TMR) including anionic salts in both calving seasons (2014 y 2015) according to close-up requirements (NRC, 2001) twice a day at 8:00 and 16:30 h (Table 1). After calving, all cows were hosted together in a compost barn until day 3 in milk. They were fed the diet 3 times daily as TMR for ad libitum intake, without access to pasture according to fresh cows requirements (NRC, 2001) in collective feeders, at 5:30, 10:00 and 15:0 h, and milked 2 times daily. The total daily offer of DM was 21.5 kg/d in the spring season (2014) and 28.9 kg/d in the autumn calving season (2015). Close-up and fresh cow diet composition is shown in Table 1. Cows had ad libitum access to water and they received 300 mL of propylene glycol, 250 g of calcium propionate diluted in 1 L water orally and 40 g Calcium gluconate subcutaneously at calving. After day 3 in milk, cows were hosted in a *free-stall* and were fed the same TMR, but milked 3 times daily (DeLaval rotary switch 80 stations). Data for 305-d milk yield were obtained from DairyPlan C-21 software (GEA).

**Table 1**
Ingredient and nutrient composition of close-up and fresh cow (calving seasons 2014 and 2015) of the total mixed diets (DM basis).

| Item | Diet | | |
|---|---|---|---|
| | Close-up | 2014 | 2015 |
| Ingredient, % | | | |
| Corn silage | 57 | 29 | 29 |
| Ryegrass haylage | – | 11 | 11 |
| Oat hay/ryegrass | 20 | 6 | 3 |
| Ground corn grain | – | 19 | 21 |
| Soybeans hull | – | – | 18 |
| Solvent-extracted soybean meal | 10 | 14 | 16 |
| Ground wheat grain | 10 | – | – |
| Citrus pulp | – | 12 | – |
| Close-up mineral supplement | 3 | – | – |
| Fresh cow mineral supplement | – | 2 | 2 |
| Nutrient profile | | | |
| Dry mater % | 39.84 | 46.08 | 56.35 |
| CP % | 12.8 | 13.4 | 15.2 |
| NDF % | 51.8 | 37.2 | 39.8 |
| NFC % | 26.7 | 40.6 | 37.3 |
| Starch % | 16.1 | 21.3 | 23.7 |
| Ether extract % | 3.8 | 3.4 | 3.4 |
| NEL, Mcal/kg | 1.47 | 1.68 | 1.67 |
| DCAD mEq/kg | −50 | 80 | 100 |
| Ca % | 0.63 | 0.88 | 0.63 |
| P % | 0.44 | 0.37 | 0.37 |
| Mg % | 0.35 | 0.20 | 0.21 |
| K % | 1.27 | 1.20 | 1.20 |
| S % | 0.32 | 0.22 | 0.22 |
| Na % | 0.10 | 0.22 | 0.16 |
| Cl % | 0.74 | 0.57 | 0.43 |

### 2.2. Study design, diseases recording and sample collection

This study followed a prospective observational cohort study and only healthy cows were enrolled at the beginning of the study. Peripartum diseases were diagnosed by a single trained veterinarian using the following criteria: Clinical hypocalcemia was defined as any recumbent cow within 72 h after parturition exhibiting anorexia, nervous symptoms, staggering, varying degrees of unconsciousness, and good response to intravenously administered calcium (Duffield et al., 1999). Clinical mastitis was characterized by the presence of abnormal milk or by signs of inflammation in 1 or more quarters, evaluated by the milking person from calving to 30 days in milk (DIM) at the start of milking (Duffield et al., 1999). Retained placenta (RP) was defined as failure to expel the placenta within 24 h after parturition (LeBlanc, 2008). Starting at d 3 until d 21 ± 1 after calving, all cows were monitored for metritis twice weekly, every Tuesday and Friday by a veterinarian, using a manual vaginal examination. Presence of abnormal vaginal secretion associated with blood Haptoglobin (Hp) concentration > 1 mg/dL, was used for diagnosis of metritis following Huzzey et al. (2011). Lameness was diagnosed weekly using the scale from 1 to 5, where grade 1 had no alteration in gait and grade 5 the cow was severely limping, without supporting the member on the floor. Cows with lameness were considered those with locomotion score ≥ 3 (Bicalho et al., 2007). Left displaced abomasums (LDA) was diagnosed based on an auscultation of a “ping” during percussion of the left side of the abdomen. During these health checks, presence of any other clinical disease(s) was recorded, and also deaths and culling because of health problems were recorded. Subclinical diseases were diagnosed at the laboratory based on specific metabolite determination. Subclinical

**Table 2**
Incidence of clinical and subclinical event during the transition period by parity [primiparous (PP), n = 126; multiparous (MP), n = 182] and calving season.

| Disease and health status | Spring | | Fisher | Autumn | | Fisher |
|---|---|---|---|---|---|---|
| | PP | MP | | PP | MP | |
| | n (%) | n (%) | P | n (%) | n (%) | P |
| Healthy | 31 (73.81) | 46 (66.67) | NS | 38 (45.24) | 49 (43.36) | NS |
| 1 event | 10 (23.81) | 16 (23.19) | NS | 29 (34.52) | 37 (32.74) | NS |
| 2 events | 1 (2.38) | 7 (10.14) | NS | 17 (20.24) | 27 (23.89) | NS |
| Metritis | 2 (4.76) | 6 (8.70) | NS | 33 (39.29) | 41(36.28) | NS |
| RP | 4 (9.52) | 10 (14.49) | NS | 16 (19.05) | 23 (20.35) | NS |
| Clinical mastitis | 3 (7.14) | 7 (10.14) | NS | 13 (15.48) | 24 (21.24) | NS |
| Others[a] | 3 (7.14) | 9 (13.0) | NS | 1 (2.56) | 12 (19.67) | 0.01 |
| Subclinical hypocalcemia | 6 (14) | 27 (39.7) | 0.005 | 6 (7.4) | 50 (45) | < 0.0001 |
| Total n of PP and MP | 42 | 69 | | 84 | 113 | |

[a] Others include: clinical hypocalcemia, ketosis, DA and lameness.

ketosis was defined by BHB > 1.2 mmol/L (Duffield et al., 2009) and subclinical hypocalcemia by calcium ≤ 2.0 mmol/L (Reinhardt et al., 2011).

Body condition score (BCS) was determined weekly during the transition period, using a 5- point scale (Ferguson et al., 1994). In the same period, blood samples were collected weekly from all cows, from the coccygeal vessel into 10-mL sterile heparinized tubes, centrifuged at 3000 ×*g* for 20 min. Plasma was stored frozen (−20 °C) until further analysis for BHB, NEFA, cholesterol, albumin, Hp and calcium concentrations at the animal endocrine and metabolism laboratory, Veterinary faculty, Montevideo, Uruguay. Metabolites were measured by colorimetric assays on Vitalab Selectra II autoanalyzer (Vital Scientific, Dieren, The Netherlands) using commercial kits: NEFA, Wako NEFA-HR (2), Wako Pure Chemical Industries Ltd., Osaka, Japan; BHB, Randox Laboratories Limited, 55 Diamond Road, Crumlin, Country Antrim, BT29 4QY, United Kingdom. Albumin, calcium and cholesterol: Wiener Laboratories S.A.I.C. Riobamba, Rosario, Argentina. Haptoglobin concentrations (Tridelta Diagnostics Ltd., Morris Plains, Ireland) were measured by ELISA (Thermo, Multiskan EX, USA). The inter-assay coefficient of variation (CV) for all commercial serum controls was less or equal to 10%.

### 2.3. Statistical analysis

Cows were classified according to their health status in 3 categories as "healthy cows", "1 event" (one clinical event) and "2 events" (more than one clinical event). The associations between parity (MP and PP) and health status, and the individual disease outcomes were analyzed using 2 × 2 contingency tables generated by the PROC FREQ statement in SAS (version 9.2; SAS Institute, 2009). From these tables, the Mantel-Haenszel chi-square test was used to determine the type 1 error risk of the relationship.

Descriptive statistics were calculated for NEFA, BHB, cholesterol, albumin and Ca concentrations by week, during close-up period and further evaluated as continuous outcomes using PROC MIXED, analyzing their differences between health status by period: "close-up" (−2 and −1 week related to calving), "calving" and "fresh cows" (1, 2 and 3 weeks post calving). Each period was considered an independent test because the concentrations of the metabolic markers of interest will change relative to time from calving and their association with health status may likely be influenced by time as reported previously (Huzzey et al., 2011). The model included the fixed effects of parity (PP vs MP), health status (healthy, 1 event or 2 events), week, calving season (spring 2014, autumn 2015) and the interaction (parity × health status, parity × health status × week). Milk production as 305-d milk yield, was also evaluated using PROC MIXED, considering fixed effects of parity, health status, calving season and their interactions in the model.

Data were further evaluated using multivariable logistic regression (MLR) analysis by week (−2, −1 and calving) for health status (healthy vs sick cows) and for individual outcomes: metritis, RP and mastitis, considering parity and calving season in the model. Variables that were not significant ($P > 0.05$) were removed by manual backward stepwise elimination. Those individual outcomes with low incidence were not evaluated, due to their small number. Odds ratio (OR) and their respective 95% confidence interval determined by MLR were used to describe the level of association between the metabolite of interest and the postpartum health outcome, considering the individual outcome as a binary classification; cows with the individual outcome and healthy cows (without any other individual outcome).

For metabolites that remained in the final models, receiver operator characteristic (ROC) curves were constructed to determine area under the curve (AUC). The cutpoint of the metabolites was estimated by Youden Index, and the values of sensitivity, specificity, positive and negative likelihood ratio (LR) were determined using MedCalc V.17.6. (MedCalc®. MedCalc Software. Acacialaan 22. B-8400 Ostend. Belgium). Sensitivity was the proportion of animals diagnosed with metritis, RP or mastitis that were at or above a given metabolite cutpoint, while specificity was the proportion of animals without clinical disease that were below a given cutpoint (Dohoo et al., 2003).

For all statistical analysis $P < 0.05$ was considered a significant effect and $P \leq 0.1$ as a tendency.

## 3. Results

From the 308 cows evaluated, 46.7% ($n = 144$) had at least one clinical event and 1.9% ($n = 6$) were discarded or died within the first 30 DIM. A greater incidence of sick cows was diagnosed in the autumn calving season compared to the spring season (55.3% vs 30.6%, $P < 0.0001$). No interaction was found among calving season and parity. The number and proportion of cows and their health status (Healthy, 1 event and 2 events), and individual outcomes (metritis, RP, mastitis and subclinical hypocalcemia) stratified by parity and calving season is shown in Table 2. The largest overall incidence was for metritis (26.6%, $n = 82$) followed by retained placenta (17.2%, $n = 53$) and mastitis (15.2%, $n = 47$), with no effect of parity in any disorder. The median time of diagnosis of metritis was 9 DIM (range of 5 to 19 DIM), and of mastitis 10 DIM (range of 1 to 25 DIM). Due to the low proportion of clinical ketosis ($n = 1$), lameness ($n = 13$), displaced abomasums ($n = 6$) and clinical hypocalcemia ($n = 4$), all conditions were considered as "others" with an overall incidence of 8.1% ($n = 25$). Also, subclinical ketosis had a low incidence (3.3%, data not shown). Considering subclinical hypocalcemia, MP presented greater incidence than PP cows, being 43% ($n = 77$) vs 9.5% ($n = 12$) respectively, $P < 0.0001$.

In Table 3, the analysis of BCS and metabolites according to parity, health status, week and their interactions during the close-up period, at

**Table 3**
Probabilities for fixed effects during close-up (weeks −2 and −1), Calving and Fresh cow (week +1, +2 and +3).

| | Effect | Albumin | Cholesterol | Calcium | NEFA | bHB | BCS |
|---|---|---|---|---|---|---|---|
| Close up | Parity | 0.1396 | 0.3274 | 0.0697 | 0.0004 | < 0.0001 | 0.0017 |
| | Health status | 0.4507 | 0.4023 | 0.8035 | 0.3447 | 0.5279 | 0.6181 |
| | Week | 0.6204 | 0.0012 | 0.0008 | 0.3577 | 0.3758 | 0.3233 |
| | Calving season | < 0.0001 | 0.0086 | 0.1759 | < 0.0001 | 0.1871 | 0.0334 |
| | Parity vs healthy | 0.166 | 0.4056 | 0.1668 | 0.2453 | 0.4322 | 0.4465 |
| | Parity vs healthy vs week | 0.124 | 0.726 | 0.0539 | 0.0619 | 0.4258 | 0.8068 |
| Calving | Parity | 0.6268 | 0.0828 | < 0.0001 | < 0.0001 | < 0.0001 | 0.0125 |
| | Health status | 0.7026 | 0.7858 | 0.2183 | 0.0024 | 0.0645 | 0.9051 |
| | Calving season | < 0.0001 | < 0.0001 | 0.0526 | < 0.0001 | 0.3657 | 0.0748 |
| | Parity vs healthy | 0.0775 | 0.7167 | 0.0944 | 0.3433 | 0.1448 | 0.6115 |
| Fresh cow | Parity | 0.0065 | 0.568 | 0.316 | 0.017 | < 0.0001 | < 0.0001 |
| | Health status | < 0.0001 | < 0.0001 | 0.1913 | 0.834 | 0.1972 | 0.5719 |
| | Week | 0.0086 | < 0.0001 | < 0.0001 | < 0.0001 | 0.2537 | < 0.0001 |
| | Calving season | < 0.0001 | 0.5844 | 0.286 | < 0.0001 | < 0.0001 | 0.7924 |
| | Parity vs healthy | 0.1785 | 0.6387 | 0.4932 | 0.1287 | 0.7334 | 0.2148 |
| | Parity vs healthy vs week | 0.1332 | < 0.0001 | 0.5265 | 0.0079 | 0.0127 | 0.2353 |

calving and fresh cow is shown. BCS and concentrations of NEFA and BHB were affected by parity during the entire transition period, while calcium tended to be affected or was affected during the close up or calving respectively. Cholesterol concentrations tended to be affected by parity at calving and albumin was affected by parity but only in fresh cows. Neither BCS nor the concentration of the different metabolites were associated with health status during the close-up period, but health status affected NEFA and BHB concentrations at calving and albumin and cholesterol concentrations in fresh cows (weeks +1, +2 and week +3 for albumin and weeks +2 and +3 for cholesterol).

The evolution of BCS, NEFA and BHB concentrations during the transition period is shown in Fig. 1. Although MP cows presented lower BCS than PP cows, they presented a similar profile, being greatest in the close up period, decreasing until the end of the experiment (week +3), not recovering the initial BCS. During the transition period, the

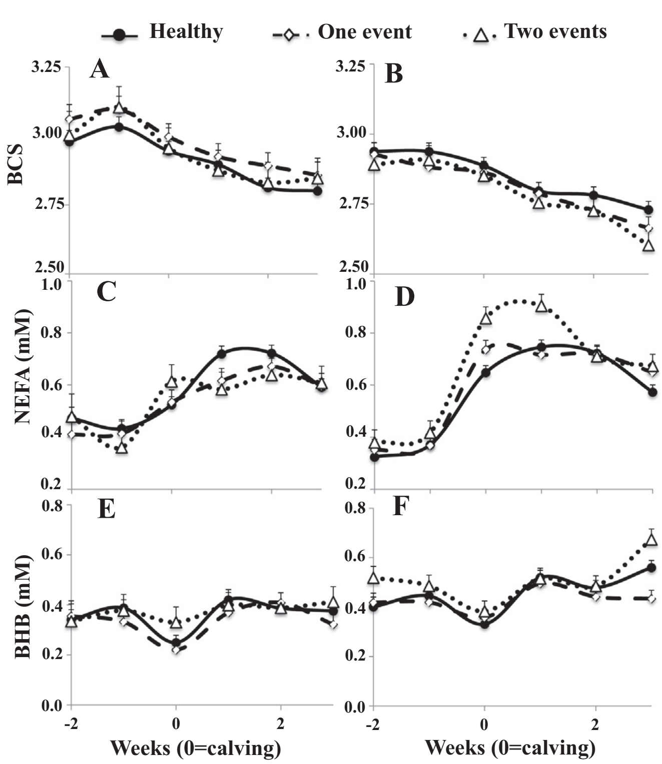


**Fig. 1.** Body condition score (BCS) evolution (A, B), non sterified fatty acid (C,D) and β hydroxibutirate (E,F) concentrations in primiparous (A, C, E) and multiparous (B, D, F) Holstein cows.

differential NEFA profiles according to health status and parity is shown in Fig. 1 (C and D). All MP cows increased their NEFA concentrations from week −1 to week +1, but sick MP cows presented higher NEFA concentrations than healthy MP cows at calving ($P < 0.05$). Also at week +1, MP cows with 2 events presented higher NEFA concentrations than healthy and 1 event MP cows ($P < 0.05$). In addition, healthy MP cows decreased their NEFA concentrations by week +3, while sick MP cows maintained higher NEFA concentrations ($P < 0.05$). In PP cows, although NEFA concentrations increased from week −1 to week +1, healthy PP cows presented the greatest NEFA concentrations at week +1 in comparison to sick PP ($P < 0.05$) and at week +3 there were no differences between the three health status categories. BHB profiles were similar in both parities during the transition period. While PP cows with 2 events presented higher BHB concentrations than healthy PP and 1 event cows at calving ($P < 0.05$), MP cows with 2 events had greater BHB values at week+3 than healthy MP cows ($P < 0.05$).

The profiles of cholesterol, albumin and calcium concentrations during the transition period are shown in Fig. 2. Cholesterol concentration decreased at calving and increased during postpartum, reaching to greater values than at the start of close-up period, regardless of parity or health status. Healthy cows (MP and PP) presented higher cholesterol concentrations than sick cows (MP and PP) at week +2 and +3 ($P < 0.05$), and healthy MP more cholesterol concentrations than sick MP at week −1 ($P < 0.05$). Healthy MP cows had higher albumin concentrations than sick MP cows during close-up and fresh period ($P < 0.05$, Fig. 2 C and D). Moreover, 1 event MP cows had also greater albumin concentrations than 2 events MP cows ($P < 0.05$) during the fresh period. In PP cows, no differences in albumin concentrations were found between healthy and 1 event PP cows during the experiment, but these two groups had greater albumin concentrations during the fresh period than 2 events PP cows ($P < 0.01$). Calcium concentrations declined sharply at calving in all MP cows, whereas it was not as evident in PP cows, reflecting the effect of parity at this time (Fig. 2 E and F). In spite of the decrease of calcium concentrations at calving in all MP cows, healthy MP cows had higher concentrations than sick MP cows ($P < 0.05$).

The logistic regression analysis including all data in PP and MP cows of both calving seasons showed that albumin concentrations on week −2 was predictive for disease, as the decrease in one unit of albumin was associated with an odd ratio (OR) of 1.27 (95% CI: 1.01 to 1.60, $P = 0.03$) in 2 events cows when compared to healthy cows. Similarly, cholesterol concentrations were predictive for mastitis being the OR 2.24 (95% CI: 1.06 to 4.74, $P = 0.03$).

As calving season and parity affected metabolite concentrations during close-up and calving (Table 3), MLR analysis was performed for

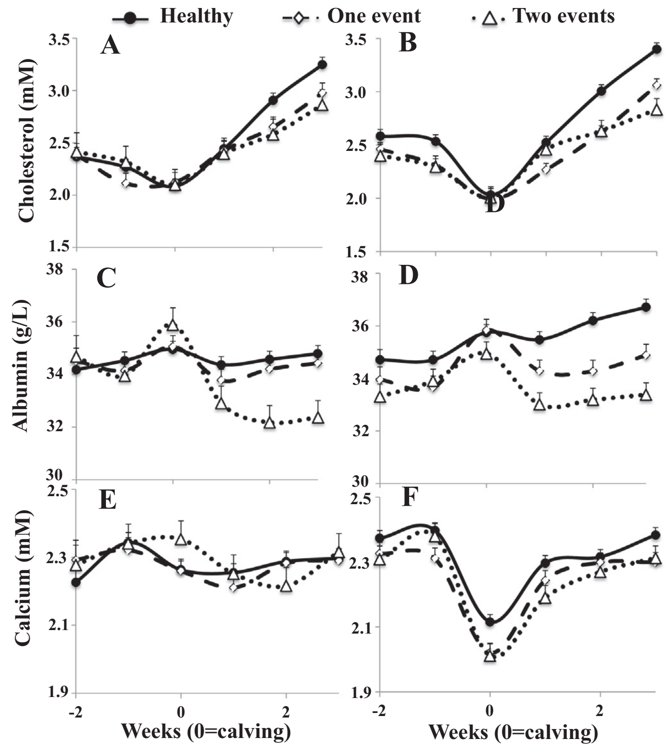


**Fig. 2.** Cholesterol (A, B), albumin (C,D) and calcium (E,F) concentrations in primiparous (A, C, E) and multiparous (B, D, F) Holstein cows.

each cohort and parity separately. In cohort 2014, there was no metabolite diseases predictor for PP or MP cows. In cohort 2015, significant effects were found only in MP cows. Albumin concentrations were predictive for metritis (OR [IC]); 1.31 [1.05–1.62] ($P < 0.01$) and RP; 1.38 [1.04–1.81] ($P < 0.05$) at week −2 and predictive for RP; 1.26 [1.01–1.57] ($P < 0.05$) at week −1. Also cholesterol was predictive for mastitis; 4.29 [1.26–14.61] at week −2, 6.32 [1.81–22.08] at week −1 and 9.01 [1.01–42.28] at calving ($P < 0.01$).

The AUC of the albumin and cholesterol plasma concentrations, as well as values of sensitivity, specificity, positive and negative likelihood ratio (LR) of the cutpoints are shown in Table 4. As shown in Fig. 3, the closer the ROC curve is to the upper left corner, the greater the accuracy of differentiation between cows with and without the heath outcome (metritis, RP or mastitis). Thus, the test is perfect to distinguish between two groups if AUC is 1 and less accurately if AUC is 0.5–0.7, as was the case of the present study.

Milk production was affected by parity, calving season and health status ($P < 0.05$). Primiparous cows produced less milk than MP (6866 ± 272 vs 8341 ± 213 L, $P < 0.0001$) and production was greater in the autumn calving season compared to the spring season (8360 ± 188 vs 6789 ± 278 L, $P < 0.0001$). Also, healthy cows produced more than sick cows ($P < 0.05$), but 1 or 2 events cows did not differ in milk production among them (8165 ± 199, 7547 ± 276 and 7098 ± 387 L for healthy, 1 or 2 events cows respectively).

## 4. Discussion

As far as we know this is the first study reporting that the evolution of metabolic profiles in healthy and sick animals during the transition period varies according to parity. Moreover, prepartum albumin and cholesterol concentrations were predictors for metritis, RP and mastitis but only in MP cows.

The overall incidence of illness in our study (46.4%) was similar to the reported for lactating dairy cows by others (58%, Van Saun, 2004; 30 to 50%, LeBlanc, 2010). A higher incidence of illness was found in the autumn calving season (55.3%) associated with the higher milk production in this calving season. It has been reported that this association is not due to the higher milk production per se, but probably because of a higher rate of acceleration in milk yield (Ingvartsen, 2006). The greater milk production in autumn calving is consistent with the total DM offer (although TMR was offered ad libitum 3 times daily, autumn cows had 7.4 kg DM more than spring cows) associated to the better THI than the spring season, as in the latter the THI exceeded the comfort threshold (> 72) for milk production (Polsky and von Keyserlingk, 2017). In our study, the metritis incidence was 26.6% similar to the reported (21%) by Hammon et al. (2006), been lower than (40%) reported by Giuliodori et al. (2013), but higher than found by others (0.7 to 2.2%, Rajala and Gröhn, 1998; Bruun et al., 2002). Indeed, the incidence of metritis varies among studies and as Huzzey et al. (2007) stated, the diagnostic criteria may be poorly described among studies, which makes it difficult to compare. Overall, incidence of RP in the present study was 17.2%, slightly higher than the reported (3 to 13%) by Ingvartsen et al. (2003). Many risk factors were associated with RP including abortion, dystocia or induced parturition, twins, stillborn calf, milk fever, and increasing age (Correa et al., 1993; Grohn and Rajala-Schultz, 2000) been therefore RP considered multifactorial. The clinical mastitis overall incidence found in this study (15.2%) was in the range (7 to 44%) reported by Ingvartsen et al. (2003) or similar to the reported (11 to 24%) in grazing dairy cows (Bargo et al., 2009; Ribeiro et al., 2013; Sepúlveda et al., 2015).

Clinical hypocalcemia was very low (1.3%), because of this, it was considered together with DA, lameness and clinical ketosis. The incidence of subclinical hypocalcemia and their higher presentation in MP as expected, was similar to the reported by others (Goff, 2006; Reinhardt et al., 2011), and is consistent with the management for hypocalcemia prevention at the herd as described in materials and methods. The very low incidence of clinical, subclinical ketosis and DA (0.3%, 3.3% and 2% respectively) in the present study reflects the preventive management of the herd, as all cows received 300 mL of propylene glycol at calving. Indeed, a high incidence of these diseases has been reported, 8.9 to 34% clinical ketosis (Ingvartsen, 2006;

**Table 4**
Test performance for albumin and cholesterol to diagnose metritis, RP or mastitis in close-up and calving.

| Test performance (CI 95%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Outcome | Metabolite | Week | AUC | Cutpoint | Se (%) | Sp (%) | +LR | −LR |
| Metritis | Albumin (g/L) | −2 | 0.66 (0.55–0.77)** | ≤33.1 | 42 (24–61) | 91 (79–97) | 4.72 | 0.70 |
| RP | Albumin (g/L) | −2 | 0.70 (0.55–0.80)* | ≤34.2 | 61 (36–83) | 80 (65–90) | 3.06 | 0.49 |
| | | −1 | 0.68 (0.54–0.78)* | ≤34.0 | 54 (32–76) | 83 (69–92) | 3.14 | 0.55 |
| Mastitis | Cholesterol (mmol/L) | −2 | 0.71 (0.60–0.77)** | ≤2.4 | 79 (54–94) | 60 (44–74) | 1.97 | 0.35 |
| | | −1 | 0.70 (0.60–0.81)** | ≤2.3 | 73 (50–89) | 61 (45–75) | 1.86 | 0.45 |
| | | 0 | 0.68 (0.56–0.78)* | ≤1.7 | 58 (37–78) | 72 (57–83) | 2.04 | 0.58 |

* $P < 0.05$.
** $P < 0.01$.

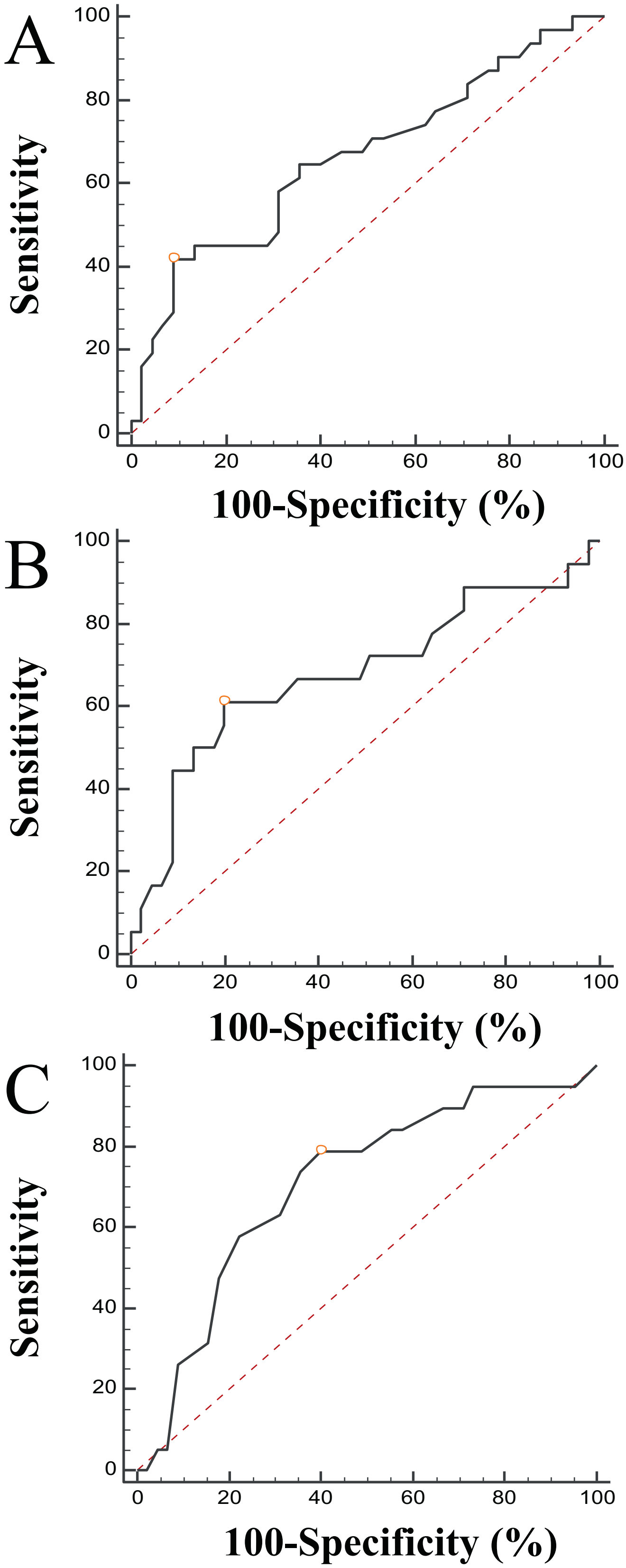


**Fig. 3.** Receiver operator characteristic (ROC) curve for albumin concentrations as predictor for Metritis (A) and Retained Placenta (B) and cholesterol as predictive for Mastitis (C) at week −2.

Rushen et al., 2008), 30% subclinical ketosis (Duffield et al., 1997) and increasing incidence of left DA (LDA) in the last decade, from between 1 and 2% to 5 to 7% (LeBlanc et al., 2005). Moreover, clinical and subclinical ketosis has been associated with development of LDA (LeBlanc et al., 2005) and thus, our findings agree with this observation, taken into account the low incidence of both diseases. Ingvartsen (2006) reported over conditioning at calving, excessive fat mobilization, low nutrient intake, diet specific factors or management as major factors increasing the risk of ketosis and fatty liver. Also, Seifi et al. (2011) reported in the first and second weeks postpartum, that fat cows were at significantly higher risk of developing clinical ketosis. Even more, elevated prepatum NEFA and postpartum NEFA and BHB concentrations were also used to identify cows at risk of developing diseases (LeBlanc et al., 2005; Ospina et al., 2010a). This was not the case of the present study as NEFA and BHB concentrations during the close-up period were not predictive. This could be the result of the cows being in a comfortable and low-stress environment, as discussed by Drackley et al. (2001) and Ospina et al. (2010b). Therefore, considering the low BHB and NEFA concentrations measured in close-up and low BHB concentrations and good BCS during the transition period, we can suggest a good energy management of transition diet and ketosis prevention at calving, experimenting cows a mild NEB. This may be the reason of the low incidence of ketosis and DA found in the present study and BHB and NEFA variables as not illness predictive in contrast with the cited literature.

Albumin concentrations were predictive in MP cows for metritis and RP at week −2 and for RP at week −1. There are very few reports on this metabolite and disease prediction. The data are consistent with Van Saun (2004) who reported that cows with albumin concentrations < 32.5 g/L during close-up (3 to 21 days prepartum) were 1.46 (95% CI: 1.04–2.04) times more likely to experience a postpartum disease event. Indeed, albumin concentrations reflect a good nutritional early dry and close-up management, as the use of blood chemistries (among others; albumin) in the form of metabolic profiles to determine nutritional status has been advocated (Payne et al., 1970; Van Saun, 2004). Also greater albumin concentrations reflect a healthy liver, as albumin is considered a negative acute phase protein, and decreases in cows with altered hepatic function (Bionaz et al., 2007). The association found among albumin concentrations at week −2 -when all cows were clinically healthy- with pospartum health output, and the fact that albumin determination is an easy and cheap technique, suggest that it could be a relevant tool to monitor herd health status. Also cholesterol concentrations resulted predictive for mastitis, during close-up and at calving, as cholesterol concentrations ≤ than 2.4, 2.3 or 1.8 mmol/L at weeks −2, −1 or calving respectively were 4.3, 6.3 or 9.0 times more likely to experience mastitis as calving approaches. In reference to this, we agree with Kaneene et al. (1997), who reported that cows with lower prepartum cholesterol concentrations are more likely to experience mastitis, metritis or RP postpartum. The decreases in cholesterol concentrations during the close-up period can be explained by the decrease in dry matter intake (DMI) during this time (Janovick Guretzky et al., 2006) and also DMI has been associated with disease development; sick cows consume less feed compared to healthy cows during the transition period (Huzzey et al., 2007). In contrast, Amadori et al. (2015) did not found that cholesterol concentrations were predictive for diseases, while Quiroz-Rocha et al. (2009) reported greater prepartum cholesterol concentrations in cows presenting RP. It should be taken in consideration that the former study included 75 multiparous cows from 26 different herds, while the latter included 1038 (primiparous and multiparous) cows from 20 herds but only RP was registered. Taking into account the performance of albumin and cholesterol to predict metritis, RP or mastitis, although AUC indicated median accuracy (0.66 to 0.71), data of sensitivity, specificity and LR are in the order of other metabolites (Geishauser et al., 1997b; LeBlanc et al., 2005). Indeed, no reports on albumin and cholesterol parameters were found, the sensitivity reported of BHB and NEFA to predict LDA was 48 and 56%

respectively, while specificity was 80 and 78% and LR = 2.4 and 2 respectively (LeBlanc et al., 2005).

The metabolic adaptation to the transition period was strongly influenced by parity as reflected by BCS and all metabolic profiles, as cited by others (Wathes et al., 2007; Adrien et al., 2012). In the present study, PP cows had higher BCS than MP cows, being their respective BCS in the target established as the optimum for both categories as cited by Garnsworthy (2006). Although both categories increased their NEFA concentrations, due to the NEB, MP cows had a greater degree of mobilization probably due to the greater energy demands for milk production as shown in this study. Interestingly, although no differences were found in NEFA concentrations in PP cows, sick MP presented higher NEFA than healthy MP cows at calving, and moreover, NEFA concentrations remained greater in 2 events cows than healthy and 1 event cows in the first week postpartum. Elevated NEFA was associated with immune dysfunction around calving (Kehrli et al., 1989; Hammon et al., 2006).

Cholesterol and albumin profile was affected by health status in both categories until the end of the study, as sick cows presented lower concentrations of both metabolites. This reflects the greater metabolic challenge faced by cows who become sick during the transition, being worse for cows with more than one clinical event reflecting the no recovering of their DMI. This agree with Huzzey et al. (2007) who stated that sick cows consume less feed compared to healthy cows during the transition period. Interestingly, cholesterol and albumin were the disease predictors detected in the close up but only in MP cows. The finding that those clinically healthy MP cows– but not PP cows- during the close up that will become sick later (during fresh period) had lower concentration of these metabolites could be associated to a worse recovery from previous lactation and/or inadequate management during far off dry period. Data is also consistent with NEFA profiles, as sick MP cows – especially cows with two events- had lower albumin and greater NEFA concentrations. These associations have been already reported (Van Saun, 2004). Overall, the worse metabolic profile (NEFA, cholesterol and albumin) found in sick cows during fresh period is consistent with the lower milk production found when compared to healthy cows, as reported by Ospina et al. (2010b).

Parity also affected calcium profile as expected. Reinhardt et al. (2011) reported that the incidence of subclinical and clinical hypocalcemia increases with age. The MP cows had lower calcium levels mainly at calving due to greater milk production and also because the normal homeostatic response to hypocalcemia is decreased with age resulting in greater or prolonged hypocalcemia in older animals (Reinhardt et al., 2011). Also hypocalcemia reduces feed intake so that greater body fat mobilization occurs in early lactation (Goff, 2008). In the present study while no differences were found in sick and healthy PP cows, sick MP cows presented lower Ca and higher NEFA concentrations at calving. Moreover, hypocalcemia significantly increases a cow's susceptibility to RP, metritis and mastitis (Curtis et al., 1983, 1985). Indeed, MP cows with these diseases had lower calcium concentration at calving, but this was not the case in PP cows and we have no obvious explanation for this finding. It should be also taken into account that the activation of the immune system could result in lower calcium levels (Waldron et al., 2003).

## 5. Conclusion

In summary, prepartum albumin and cholesterol concentrations were predictors for metritis, RP and mastitis in MP cows showing a mild negative energy balance in terms of NEFA, BHB and BCS profiles. Moreover, it is shown that the evolution of metabolic profiles in healthy and sick cows during the transition period varies according to parity and is associated with lower milk production.

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# Endocrine and reproductive parameters in sick and healthy primiparous and multiparous dairy cows

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To investigate the association of health status and parity with hormone profiles during the transition period and reproductive parameters in Holstein dairy cows, a prospective observational cohort study was carried out including only healthy primiparous (PP, n = 116) and multiparous (MP, n = 172) cows at the beginning of the study. A subset of 120 healthy and sick cows was randomly selected for insulin, IGF-I, leptin and adiponectin determination. Primiparous cows had greater IGF-I and adiponectin concentrations ($P < 0.05$) and tended ($P=0.07$) to have greater insulin concentrations than MP cows. While healthy and sick MP and sick PP cows presented a sharp decrease in IGF-I concentrations after calving, healthy PP cows maintained them. Postpartum adiponectin concentrations were lower in sick than in healthy MP cows. A greater percentage of healthy cows ovulated during the first 7 weeks after calving when compared to sick cows (67.9% vs 50%, $P=0.002$) and a similar trend was found for MP vs PP cows (64% vs 53%, $P=0.01$). More healthy cows were inseminated in comparison to sick cows (94% vs 76.5%, $P < 0.01$) and more PP than MP cows (90.4% vs 82.7%, $P < 0.05$). Similarly, healthy cows presented a greater proportion of pregnancy than sick cows (75% vs 54%, $P < 0.01$) and the proportion of pregnancy was higher in PP than in MP cows (74% vs 56%, $P=0.04$). Health status interacting with parity yielded different endocrine profiles, which may partially explain the differences in reproductive performance.


## 1. Introduction

Due to intensive genetic selection in addition to improvements in nutrition and management of dairy herds, milk production has increased substantially over the last decade [1]. This increase in milk production demands a correct nutritional balance in order to fulfill lactation requirements despite the decrease in voluntary dry matter intake (DMI) around calving [2,3]. The uncoupling of the somatotrophic axis (high growth hormone [GH] vs low insulin-like growth factor-I (IGF-I) concentrations), the increase in hepatic gluconeogenesis, lower glucose utilization by peripheral tissues, and lipolysis are homeorhetic adaptations for increasing energy substrates availability [4,5].

Also, the interrelationship of the endocrine, metabolic and immune system may induce a depressed immune system around calving by activating local and systemic defense mechanisms that induce inflammation [6]. This increases the chances of getting sick as it has been shown that 30%–50% of the cows are affected by some form of metabolic or infectious disease around calving [7–9] in addition to reproductive problems or/and increased culling rates [6,10,11]. Additionally, it is known that peripartum diseases are interrelated; e.g., retained placenta to hypocalcemia [12] (which is considered a gateway disease [13]) predisposing an animal to metritis and mastitis [14,15] or affecting reproductive performance [16,17]. Indeed, reproductive failure is still an important reason for involuntary culling in dairy herds [18].

Furthermore, parity affects metabolic adaptation to lactation; primiparous cows, which have not reached their adult body size

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and continue to grow during pregnancy and lactation, present metabolic differences compared to older cows [19]. The competing demands of the mammary gland are superimposed on growth requirements, and both insulin and IGF-I stimulate growth. The profiles of these anabolic hormones and metabolites (such as non-esterified fatty acids [NEFA]) during the transition period according to parity have been inconsistent [20,21]. IGF-I and insulin concentrations are low around calving (because of homeorhetic adaptation), affecting the reproductive axis at both central (hypothalamus-pituitary gland) and peripheral (gonads, reproductive tract, and embryo) level [22]. Indeed, a positive relation between IGF-I and the first postpartum ovulation has been demonstrated [23,24]. Moreover, low IGF-I concentrations were reported in cows developing postpartum diseases, but because of the limited number of cows used, the authors concluded that a reliable association could not be established [25].

Adiponectin and leptin, because of their insulin sensitizing actions and association with body condition, could be potential regulators of metabolism during the transition from pregnancy to lactation [26]. Even if no relationship between leptin and the first postpartum luteal activity could be demonstrated, higher leptin concentrations were associated with shorter intervals to the first observed estrus [27]. Adiponectin is not only a metabolic hormone, as has also been linked to inflammation. Indeed, low adiponectin concentrations around calving have been proposed to be linked to inflammatory disease pathophysiology [28] and could be another mechanism that contributes to poor fertility in dairy cows [26]. As far as we know, there are no reports on endocrine (insulin, IGF-I, leptin and adiponectin) profiles during the transition period in sick and healthy primiparous and multiparous cows and their association with reproductive success.

Taking these findings into account, the objectives of this study were to assess whether health status interacted with parity affecting hormone profiles (IGF-I, insulin, adiponectin, and leptin) during the transition period, and if they were associated with reproductive performance in primiparous (PP) and multiparous (MP) Holstein cows.

## 2. Materials and methods

### 2.1. Cows and herd management

The study was conducted in a commercial dairy free stall (loose-housing system) herd in Rio Grande do Sul, southern Brazil. All procedures were carried out in accordance with regulations of the Animal Experimentation Committee (SIPPEE 10.059.15 CEUA 0522017 UNIPAMPA, Brazil).

From a 700-cow herd, 288 Holstein dairy cows (n = 116 PP and n = 172 MP) were selected, with an approximate milk yield of 8000 kg per lactation and two calving seasons; spring/summer (October 2014 to January 2015) and autumn/winter (May to August 2015).

Cows were kept on paddocks without significant pasture allowance from day −21 until calving, separated by parity, and were offered 13.3 kg/d of DM as total mixed ration (TMR) including anionic salts according to close-up requirements (NRC, 2001) twice a day at 8:00 and 16:30 hs. After calving, all cows were kept together in a compost barn until 3 days in milk (DIM) and were fed a TMR 3 times daily for *ad libitum* intake in collective feeders, at 5:30, 10:00 and 15:00 hs, and were milked 2 times daily. They did not have access to pasture. The total daily offer of DM was 21.5 kg/d in the spring calving season (2014) and 28.9 kg/d in the autumn calving season (2015). Close-up and fresh cow diet composition has been reported previously [9]. Cows had *ad libitum* access to water and they received 300 mL of propylene glycol, 250 g of calcium propionate diluted in 1L water orally and 40 g Calcium gluconate subcutaneously at calving. After 3 DIM, cows were translated to a *free-stall* and were fed the same TMR, but milked 3 times daily (DeLaval rotary 80 stations). Data for 305-d milk yield were obtained from DairyPlan C-21 software (GEA).

### 2.2. Study design, disease recording and sample collection

This is a prospective observational cohort study and only healthy cows were enrolled at the beginning of the study. Peripartum diseases were diagnosed by a single trained veterinarian as reported before [9], using the following criteria: Clinical hypocalcemia was defined as any recumbent cow within 72 hs after parturition exhibiting anorexia, nervous symptoms, staggering, varying degrees of unconsciousness, and good response to intravenously administered calcium. Clinical mastitis was characterized by the presence of abnormal milk or by signs of inflammation in 1 or more quarters, evaluated by the milking person from calving to 30 DIM at the start of milking. Retained placenta (RP) was defined as failure to expel the placenta within 24 h after parturition. Between 3 and 21 ± 1 DIM, all cows were monitored for metritis twice weekly, every Tuesday and Friday by a veterinarian, using a manual vaginal examination. Presence of abnormal vaginal secretion associated with blood haptoglobin (Hp) concentration > 1 mg/dL, was used for diagnosis of metritis. Lameness was diagnosed weekly using the scale from 1 to 5, where grade 1 had no alteration in gait and at grade 5, the cow was severely limping, without supporting the member on the floor. Cows with lameness were considered those with locomotion score ≥3. Left displaced abomasum (LDA) was diagnosed based on an auscultation of a “ping” during percussion of the left side of the abdomen. During these health checks, the presence of any other clinical disease(s) was recorded, as well as deaths and culling because of health problems.

Cows were classified according to their health status in healthy cows (without clinical events) and sick cows (one or more clinical events) in order to analyze endocrine and reproductive variables. From the 288 cows evaluated, 45.8% (n = 132) became sick and 1.9% (n = 6) were culled or died within the first 30 DIM. Health status was not affected by parity and the proportions of healthy and sick cows were for MP: 52.9% and 47.0% respectively and for PP: 56.0% and 43.9% respectively.

### 2.3. Reproductive management

From 50 days after calving, cows were managed with a timed artificial insemination (AI) synchronization protocol consisting in 2 mL of estradiol benzoate (Sincrodiol, Ourofino Saúde Animal Ltda, Brazil), 1 mL of gonadotropin-releasing hormone (GnRH Gestran, ARSA S.R.L, Buenos Aires, Argentina) and an intravaginal progesterone (P4) device (CIDR, Zoetis, Brazil), followed seven days later by an injection of 2 mL prostaglandin (Estron Agener Uniao, Brazil), removing at this time the intravaginal device, and an injection of 0.5 mg estradiol cipionate (ECP, Zoetis, Brazil) [29]. All cows were inseminated 48 h later (47–50 h). Pregnancy diagnosis was performed on day 45 by ultrasound (DVU60vet, Oxson Technology, Brazil). Non-pregnant cows were submitted to the same synchronization protocol.

### 2.4. Blood sample collection and analyses

From week −2 until week +7, blood samples were collected from the coccygeal vein into 10-mL sterile heparinized tubes. Samples were centrifuged at 3000 x g for 20 min and plasma was stored frozen (−20 °C) until further analysis. In all cows (n = 288) plasma P4 concentrations were measured weekly after calving using a solid-phase $^{125}$I radioimmunoassay (RIA) kit (MP, Biomedicals, ICN Biomedicals, Inc. California). The sensitivity of the assay was 0.01 ng/mL, and the intra and inter-assay coefficients of variation (CVs) were not greater than 10.6%. Days to first ovulation (resumption of ovarian cyclicity) was defined as the interval between calving and the first day in which plasma P4 concentrations were ≥1 ng/mL.

To analyze the endocrine profiles and their relationship with reproductive parameters, a subset of 120 cows that included 57 PP cows (healthy n = 28, sick n = 29) and 63 MP cows (healthy n = 28, sick n = 35) was randomly selected. Insulin, IGF-I, leptin, and adiponectin concentrations were determined on weeks −2, −1, +1, +3, and +5 related to calving (a total of 600 samples for each hormone). IGF-I and insulin concentrations were measured using immunoradiometric assays (IRMA) with commercial kits. Plasma IGF-I concentrations were determined using the IGF-I RIACT kit (Cis Bio International, GIF SUR YVETTE CEDEX, France). The assay's sensitivity was 16 ng/mL, and the intra- and inter-assay CVs for control 1 (47.5 ng/mL) were 8.2% and 8.5%, respectively, and for control 2 (429 ng/mL) 9.9% and 11.9%, respectively. Plasma insulin concentrations were determined using the INS-IRMA kit (DIA Source Immune Assays S.A., Belgium). The assay's sensitivity was 1.3 μIU/mL, and the intra- and inter-assay CVs for control 1 (19.4 μIU/mL) were 5.5% and 8.6%, respectively, and for control 2 (65.6 μIU/mL) 4.4% and 4.9%, respectively. Leptin concentrations were determined with a liquid phase RIA using a commercial Multi-Species Leptin kit (RIA kit, Millipore, USA). The RIA had a sensitivity of 2.8 ng/mL, and the intra- and inter-assay CVs for control 1 (8.3 ng/mL) were 9% and 10.2% respectively and for control 2 (32 ng/mL) 8.7% and 11% respectively. Adiponectin concentrations were measured using an RIA kit (HADP-61 HK, Millipore, USA) with undiluted plasma samples. The assay's sensitivity was 1.54 ng/mL, and the intra and inter-assay CVs for control 1 (8.4 ng/mL) were 9.1% and 8.5% respectively and for control 2 (81.5 ng/mL) 7.9% and 11.1% respectively.

### 2.5. Statistical analysis

Insulin, IGF-I, leptin, and adiponectin concentrations were analyzed as repeated measures. The model included the fixed effects of parity (PP vs MP), health status (healthy and sick cows), week, and these factors' interactions. The associations between parity, health status, calving seasons and resumption of ovarian cyclicity, AI, and pregnancy rates were analyzed using 2 × 2 contingency tables generated by the PROC FREQ statement in SAS (9.2; SAS Institute, 2009). From these tables, the Mantel- Haenszel chi-square test was used to determine the relationship's type 1 error risk. Reproductive parameters were also evaluated by Proc Genmod, including parity and health status in the model. Also, Kaplan-Meier survival analysis for probability of resumption of ovarian cyclicity and pregnancy were performed. Data were further evaluated using multivariable logistic regression (MLR) analysis for resumption of ovarian cyclicity and pregnancy rates. Variables that were not significant ($P > 0.05$) were removed by manual backward stepwise elimination. Odds ratio (OR) and their respective 95% confidence interval (CI) determined by MLR were used to describe the level of association between the endocrine concentrations, health status, parity and reproductive parameters. Data are reported as least squares means and pooled standard errors. For all statistical analysis $P < 0.05$ was considered a significant effect, and $P \leq 0.1$ as a trend.

The present study was performed to investigate the role of health status and parity on hormone profiles during the transition period and their association with reproductive performance. The sample size calculation for hormone profiles, assuming a SD of 30%, power = 80%, P-value = 0.05, revealed that 24 animals per group are needed to detect a 25% difference in the mean between two compared groups. For percentage of resumption of ovarian cyclicity in the first 7 weeks after calving, assuming 50 vs 70%, with a power = 80% and P-value = 0.05, 93 animals per group are needed (considering a power = 65%, 66 animals are needed). For pregnancy proportion, assuming 65 vs 80%, with a power = 80% and P-value = 0.05, 138 animals per group are needed (the same with a power = 65%, 98 animals are needed). Thus, the sample size considered in this study for endocrine profiles was appropriate, while for reproductive parameters, only moderate differences could be detected.

## 3. Results

The significance of the fixed effects of the subset groups' hormonal concentrations (n = 120) is shown in Table 1. IGF-I and adiponectin concentrations were significantly affected by parity; insulin tended to be affected by this factor, while leptin did not reach significance. The evolution of IGF-I and insulin concentrations according to parity and week differed, and a triple interaction parity*status*week was found for IGF-I.

Insulin concentrations tended to be greater in PP than MP (19.5 ± 0.7 vs 17.9 ± 0.6 uUI/mL, $P = 0.07$). In PP cows, insulin concentrations remained constant from week −2 until week +5, while in MP cows, insulin concentrations decreased from the close-up period until week +1 ($P < 0.003$), increasing thereafter until the end of the study without differences according to health status (Fig. 1 A, B). Overall, IGF-I concentrations were higher in PP than MP cows (136.2 ± 5.3 vs 103.6 ± 5.0 ng/mL, $P < 0.0001$). Regardless of health status, IGF-I concentrations increased in PP cows from week −2 to week −1 ($P < 0.01$) and decreased thereafter until week+1 ($P < 0.01$) with a sharp decrease in sick PP cows (Fig. 1 C). Although concentrations decreased in all MP cows at week+1, healthy MP cows tended to have greater IGF-I concentrations than sick MP cows ($P = 0.1$). After week+1, IGF-I concentrations in MP cows tended to increase until week +5, but the close-up concentration was not recovered (Fig. 1 D).

Adiponectin concentrations were greater in PP than MP cows (21.9 ± 1.1 versus 19.0 ± 1.0 ng/mL, $P = 0.04$). In all cows, the concentration decreased from week −2 until the end of the study regardless of parity. Also, healthy MP cows tended to have greater adiponectin concentrations than sick MP cows throughout the fresh cow period ($P < 0.10$, Fig. 1F). Parity did not reach significance for leptin concentrations (16.4 ± 1.4 and 13.6 ± 1.4 ng/mL for MP

**Table 1**
F-tests of fixed effects for hormone concentrations in cows from week −2 until week +5 according to calving in primiparous (n = 57) and multiparous (n = 63) Holstein cows. Fixed effects were parity, health status (Health), week according to calving and their interactions.

| | Parity | Week | Health | Health*week | Parity*week | Health*parity*week |
|---|---|---|---|---|---|---|
| Insulin | 0.07 | <.0001 | 0.49 | 0.94 | <.0001 | 0.74 |
| IGF-I | <.0001 | <.0001 | 0.22 | 0.01 | <.0001 | 0.03 |
| Adiponectin | 0.04 | <.0001 | 0.17 | 0.19 | 0.18 | 0.64 |
| Leptin | 0.15 | <.0001 | 0.65 | 0.89 | 0.71 | 0.94 |

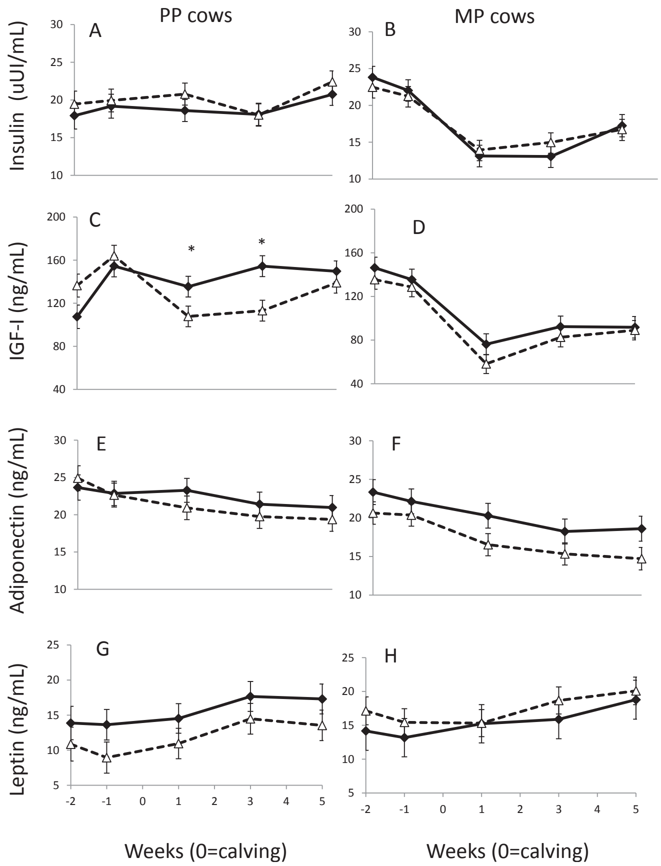


**Fig. 1.** Insulin (A, B), IGF-I (C, D), adiponectin (E, F) and leptin (G, H) concentrations in Holstein primiparous (PP, n = 57) and multiparous (MP, n = 63) cows from −2 to +5 weeks related to calving according to health status [healthy cows (●) and sick cows (Δ)].

and PP cows, respectively, $P = 0.15$), and concentrations increased after calving ($P < 0.05$).

During the first seven weeks after calving, from a total number of 288 cows, 59.7% became cyclic, while 40.3% did not. Health status and parity affected the resumption of ovarian cyclicity ($P < 0.05$) independent of the calving season. A greater percentage of healthy cows reinitiated ovarian cyclicity when compared to sick cows (67.9% [106/156] vs 50% [66/132], $P = 0.002$). Although a greater percentage of MP cows reinitiated ovarian cyclicity when compared to PP cows (64% [110/172] vs 53% [62/116], $P = 0.01$), this difference was due to sick cows (Table 2). Indeed, no differences according to parity were found in healthy cows, but sick PP cows presented a lower probability of resumption of ovarian cyclicity than sick MP cows (Fig. 2 A, B).

In both categories, a greater percentage of healthy cows were inseminated when compared to sick cows (94.0% vs 76.5%, $P < 0.01$) and this was also greater for PP than MP cows (90.4 vs 82.7, $P < 0.05$) (Table 2). The probability of pregnancy was also affected by health status and parity ($P < 0.004$, Fig. 2 C, D). More healthy cows became pregnant when compared to sick cows (75% vs 54%, $P < 0.01$) and more PP than MP cows (74% vs 56%, $P = 0.04$). Conception rate was higher in PP than MP cows ($P = 0.01$) and in healthy than in sick cows ($P = 0.04$). Interestingly, while no differences according to health status were found in conception rates of PP cows, sick MP cows had a lower fertilization and/or higher embryonic mortality than healthy MP cows (Table 2).

To analyze the endocrine variables that could explain the reproductive parameters, a logistic regression (LR) was performed including parity, health status, and hormone concentrations; only parity and health status remained in the model ($P < 0.05$) for AI and pregnancy variables. For resumption of ovarian cyclicity, parity, health status, insulin and IGF-I concentrations remained in the final model ($P = 0.01$). Insulin concentrations at week −1 were significant for resumption of ovarian cyclicity (OR [IC]); 1.09 [1.01–1.17]) and IGF-I concentration at week +1 (1.01 [1.00–1.02]) ($P < 0.05$).

## 4. Discussion

This is the first study associating health status and parity with insulin, IGF-I, leptin and adiponectin profiles during the transition period, and investigating their relation with reproductive parameters. A greater proportion of healthy cows reinitiated ovarian cyclicity during the first 7 weeks after calving compared to sick cows, and the findings for the proportion of insemination and pregnancy were similar. While a lower proportion of PP cows reinitiated their ovarian cyclicity, they presented a greater proportion of pregnancy. Moreover, the significant interaction of health status and parity with IGF-I profiles is novel, and may explain the different reproductive outcome found in healthy and sick primiparous and multiparous cows.

Due to their impact on the reproductive status, endocrine profiles during the transition period were determined [6,11,23]. As a consequence of the increased requirements for milk production and decreased DMI, insulin levels decreased [23,30]. The higher overall IGF-I and insulin concentrations found in PP dairy cows during the transition period have been reviewed previously [19]; milk production is subordinate to growth in PP cows, and these anabolic hormones reveal the difference in metabolic adaptation according to parity. Indeed, the sharp decrease in IGF-I concentrations found in MP cows after calving in contrast to PP cows is an argument for a greater uncoupling of the somatotrophic axis in favour of milk production as previously reported [19]. Adiponectin concentration was also affected by parity and was greater in PP cows. No reports of adiponectin concentrations according to parity in dairy cows have been found, but data are consistent with insulin and IGF-I profiles. Indeed, greater adiponectin concentrations have been related to insulin sensitivity [26]. The adiponectin decrease found after calving is consistent with its role in gluconeogenesis suppression [31], which may facilitate an increase in glucose supply to the mammary gland [32]. Leptin was not affected by parity and

**Table 2**
Reproductive parameters in healthy and sick lactating primiparous (PP) and multiparous (MP) dairy cows.

| Variable | PP (n = 116) | | MP (n = 172) | |
|---|---|---|---|---|
| | Healthy (n = 65) | Sick (n = 51) | Healthy (n = 91) | Sick (n = 81) |
| Resumption of ovarian cyclicity (%) | 66[a] | 35[b] | 69[a] | 59[c] |
| AI (%) | 97[a] | 82[b] | 92[a] | 71[b] |
| Pregnancy (%) | 82[a] | 66[b] | 69[b] | 43[c] |
| Conception (%) | 85[a] | 80[ab] | 74[b] | 60[c] |

Within row different letters differ $P < 0.05$.

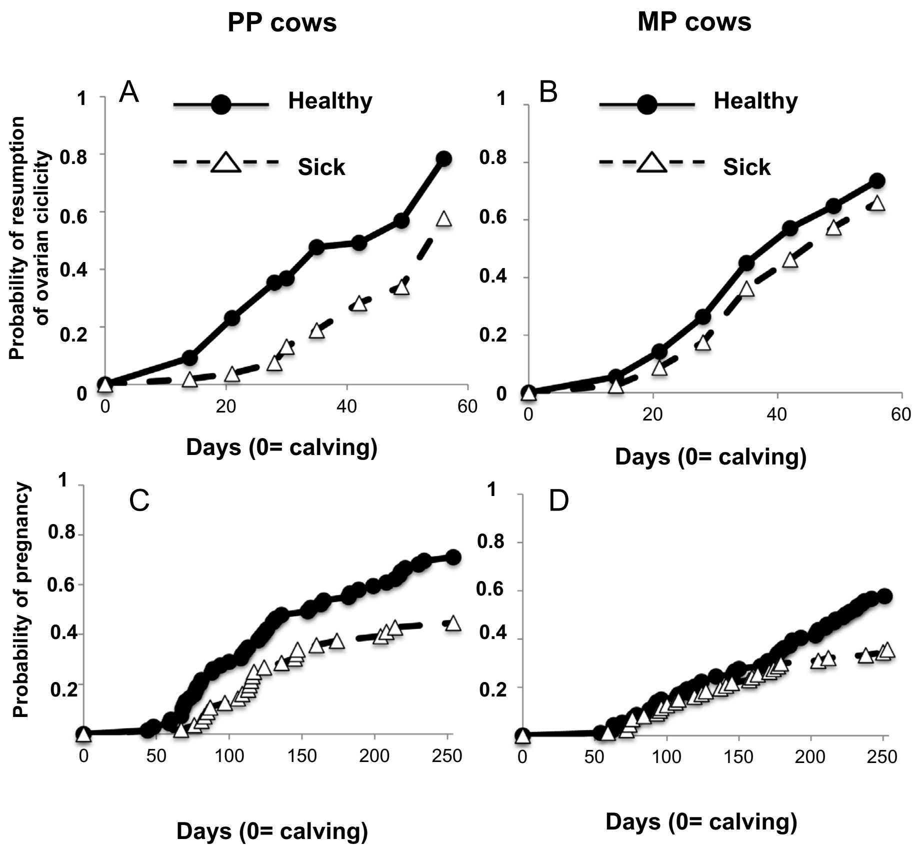


**Fig. 2.** Probability of resumption of ovarian cyclicity and pregnancy in Holstein primiparous (PP, n = 116) and multiparous (MP, n = 172) cows according to health status [healthy cows (●) and sick cows (Δ)].

concentrations increased after calving in all cows independent of parity, which could be explained by the high concentrate content of the diet in the present study as reported previously [33].

Healthy PP cows maintained greater IGF-I concentrations during the first month after calving, while sick PP cows presented a sharp decrease in response to illness, and this effect was not observed in MP cows. The sharper IGF-I decrease around calving in sick cows was probably associated with a decrease in DMI as previously reported [34,35]. Data suggest a different metabolic adaptation to illness and lactation according to parity. Interestingly, while sick MP cows had greater NEFA concentrations than healthy MP cows, this was not found in PP cows [9]. Thus, the reason for the lower IGF-I in sick PP cows could be the result of calving-induced stress in addition to management and milking routine adaptation at this time [36], and/or a milder NEB associated with a lower milk production when compared to sick MP cows.

Although there was no effect of health status on adiponectin and leptin profiles during the transition period, sick MP cows tended to have lower adiponectin concentrations than healthy cows after calving. Indeed, hypoadiponectinemia has been linked to inflammatory disease pathophysiology [37]. Also, the lower adiponectin concentrations found in sick MP cows after calving are consistent with the lower IGF-I concentrations in this group. Moreover, adiponectin, because of its insulin sensitizing actions, could be a metabolic regulating factor during the transition period [38].

Although the sample size of this study is limited, the reproductive parameters (resumption of ovarian cyclicity, pregnancy and conception rates) were affected by parity. Until day 50 after calving, PP cows presented a lower percentage of resumption of ovarian cyclicity in accordance with previous reports [39]. In dairy cows, the first ovulation after calving has been linked with the severity and duration of NEB [23]. In several studies [19,21,39,40] the NEB in PP cows has been reported to be at its worst one week before calving and early thereafter. However, the lower NEFA and greater IGF-I concentrations in PP cows (this study and [9]) reflect a better energy balance in this category, so that parity-associated factors mentioned before and reported previously [36] could explain the delay of resumption of ovarian cyclicity in PP cows. In contrast, the proportion of pregnant cows was greater in PP than MP cows. Similarly, Santos et al. [39] reported that MP cows were more likely to start ovarian cyclicity earlier than PP cows, but pregnancy rate was higher in PP cows. In our study, the greater milk production of MP cows could explain the lower proportion of pregnancy because of the increased catabolic state, as milk yield was negatively associated with the ability of lactating cows to conceive and maintain pregnancy [39]. However, others reported no association between milk yield and conception or embryonic survival [39,41]. An increase in liver flux is needed to support higher milk production in MP cows, leading to a greater P4 clearance; thus, lower circulating P4 concentrations could explain embryonic loss and the lower pregnancy proportion in this category [42,43].

Health status affected the percentage of resumption of ovarian cyclicity during the first 7 weeks after calving and was lower in sick cows, which is associated with the worse NEB (lower cholesterol, albumin and IGF-I concentrations) of them ([9] and the present study). Sick cows presented lower DMI and lower cholesterol and albumin concentrations [44,45]. As mentioned before, IGF-I stimulates bovine follicular cells and ovulation [46]. Indeed, IGF-I concentrations one week after calving were associated with resumption of ovarian cyclicity. Beam and Butler [47] also reported greater IGF-I concentrations two weeks after calving in cows that reinitiated ovarian cyclicity in comparison to non-ovulating cows. Interestingly, the negative effect of illness on resumption of ovarian cyclicity in PP cows was more marked than in MP cows, consistent with the sharper decrease in IGF-I profiles observed in sick PP cows. The lower probability of resumption of ovarian cyclicity in sick cows was consistent with the lower proportion of sick cows that became pregnant. This could be explained by the carryover effects of inflammatory diseases after calving, because disease can induce fever and compromise reproduction, by disrupting oocyte and embryonic development and uterine function which can last for months after the acute diseases has ceased [48]. Interestingly, the interaction of parity and health status with conception rates was significant. Despite a slight numerically difference, in favour of healthy cows, no differences according to health status were found in conception rates in PP cows, although it should be taken into account that for these small differences the sample size is underpowered. Nevertheless, in MP, differences were detected, sick cows had lower conception rates than healthy cows; this may have been due to lower fertilization and/or higher embryonic mortality in sick MP cows. Overall, data suggest that when PP cows overcome the clinical event and start to cycle, their fertility is higher than MP cows. In contrast, in MP cows, greater negative and/or carryover effects of diseases on reproduction were observed, suggesting that fertilization and maintenance of pregnancy are important barriers for reproductive success in this category. Nevertheless, the interpretation of the present data should be carefully performed due to the limited number of cows included for reproductive outcomes.

## 5. Conclusion

Healthy and sick cows had different endocrine profiles during the transition period, which may partially explain the observed reproductive performance. Parity interacted with health status on reproductive performance; sick PP cows showed a delay in the resumption of ovarian cyclicity and lower IGF-I concentrations, while sick MP cows showed a lower proportion of pregnancy.

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