Universitat de Lleida

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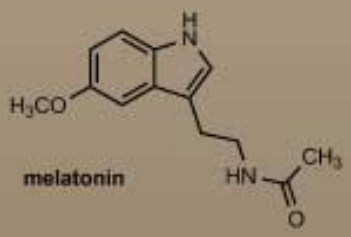

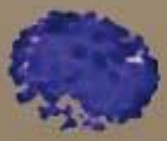

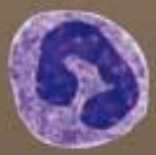

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DVM, MSc, PhD candidate

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2013

…

… …

“If we knew what it was we were doing, it would not be called research, would it?”

... Albert Einstein

...

... ...

Although one would say, “If we did it well the first time it would not be called re-search, would it?”

... Inspired from the previous quote

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2- *Reference*: **AGL2010-21273-C03/GAN**

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   Principal investigator: FERNANDO LÓPEZ GATIUS

   Title: NEOSPOROSIS BOVINA: INTERACIONES MATERNO-FETAL Y MECANISMOS ASOCIADOS CON LA PROTECCION FRENTE AL ABORTO EN GESTACIONES DE RAZAS CRUZADAS EN CONDICIONES DE CAMPO.

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Summary

Dairy cows are an essential element of our life; they provide us with milk and various milk by-products. In fulfilment to that objective they are subjected to rigorous management, nutrition and reproductive controls in order to obtain the most benefit out of them. They are threatened by elimination from the herd throughout their productive life; in the event of poor health and, subsequently, performance. Therefore, this thesis (by means of five studies) aimed at investigating factors that have potential effect on the maternal immune-health during gestation and the early postpartum, plus considering melatonin as a promising molecule in improving cows' health and performance.

In the first study, factors of potential influence on maternal peripheral leukocytes, as an indicator to immune status, throughout gestation (between Days 90-210) were determined. Of significant importance was the interaction between cows' age and *Neospora caninum*-seropositivity. Wherein, primiparous *Neospora*-seropositive cows had lower total leukocytes and neutrophils and higher monocytes. Season, twin-pregnancy, milk production and time were also related to changes in peripheral blood leukocytes.

Pursuing the maternal immune system further in its most critical phase, the second and third studies summarize different factors affecting maternal immune system during the peripartum period (from gestation Day 220 till 30 days postpartum). Artificial inseminating bull, plasma pregnancy associated glycoproteins (PAGs), *N. caninum-Coxiella burnetii* interaction, season, age, twin-pregnancy and time were found to significantly affect maternal peripheral leukocytes during that stage. Some bulls and higher plasma PAGs level were related to higher maternal leukocyte numbers throughout the peripartum period (these results

have not been previously published in dairy cattle). Different maternal leukocyte numbers were observed in response to infection with *N. caninum* or *C. burnetii* or both; compared to a group of cows seronegative to both, demonstrating different maternal immune responses towards each type of infection. It is to be noted that, aged (with 3 lactations or more) or twin-carrying cows had lower peripheral leukocytes throughout gestation and during the postpartum.

Proper melatonin dose in dairy cattle had to be evaluated first; which was the aim of the forth study in this thesis, in order to evaluate its possible effects in enhancing maternal health during the peripartum period. Different melatonin doses (0, 83, 166, 249 or 332 µg/kg body weight) were given as subcutaneous implants to lactating cows and melatonin pharmacokinetics were evaluated. Only, melatonin dose of 332 µg/kg was found to increase melatonin levels throughout the study period (50 days). In addition, this does did not decrease milk production of the studied cows.

Finally, possible melatonin effects on enhancing maternal health during the peripartum were evaluated in the fifth study of this thesis. Melatonin treated cows were found to have less likelihood of repeat breeding syndrome and pregnancy loss, and, therefore, less days open. Such effects have not been previously published in dairy cattle.

Resumen

El ganado vacuno de aptitud lechera es un elemento esencial de nuestra vida, nos proporciona leche y sus diversos subproductos. En cumplimiento de este objetivo se somete a unas condiciones rigurosas de manejo, nutrición y controles reproductivos para lograr un máximo beneficio. La vaca siempre está bajo el riesgo de eliminación de la explotación, tanto en caso de enfermedad como por un bajo rendimiento productivo y/o reproductivo. Por lo tanto, esta tesis (mediante los cinco estudios que se realizaron) se destinó a investigar los factores que poseen un efecto potencial sobre la salud de la vaca durante la gestación y el posparto temprano. Se evaluó además el efecto de la melatonina exógena como un producto con expectativas para mejorar el estado sanitario y productivo de la vaca lechera.

En el primer estudio, se evaluaron los factores que podían influir sobre la población de leucocitos maternos, como un indicador de su estado inmunológico, a lo largo de la gestación (entre los días 90 a 210). De gran importancia fue la interacción entre la edad de la vaca y la seropositividad a Neospora caninum. En este estudio, las vacas primíparas y Neospora-seropositivas presentaron menor número de leucocitos totales y neutrófilos, pero altos números de monocitos. También, la estación, la gestación gemelar, la producción de leche y el tiempo de muestreo se relacionaron con cambios en los leucocitos de la sangre periférica materna.

Siguiendo el sistema inmune materno más allá en su fase más crítica, el segundo y el tercer estudios resumen los diferentes factores que afectan al sistema inmunológico materno durante el período periparto (de 220 días de gestación hasta 30 días después del parto). El toro usado en la inseminación artificial, las glicoproteínas asociadas a la gestación (PAG), la

interacción entre N. caninum y Coxiella burnetii, la estación, la edad, la gestación gemelar y el tiempo de muestreo afectaron significativamente los leucocitos maternos durante esa etapa. Algunos toros y un mayor nivel plasmático de PAGs se relacionaron con un mayor número de leucocitos maternos durante el período periparto (resultados que no han sido publicados anteriormente en las vacas lecheras). Diferentes valores de leucocitos maternos se observaron en respuesta a la infección por N. caninum o C. burnetii o ambos, en comparación con un grupo de vacas seronegativas a ambas enfermedades, demostrando una diferente respuesta inmune materna frente a cada tipo de infección. Cabe señalar que a una edad mayor (con 3 o más lactaciones), o en el caso de gestaciones dobles, las vacas presentaron menor contaje de leucocitos periféricos a lo largo de la gestación y durante el posparto.

Con el objetivo de evaluar la posible utilidad de la aplicación de la melatonina en la mejora de la salud materna durante el período periparto se determinó la dosis adecuada de melatonina para administrar en la vaca lechera. Este fue el objetivo del cuarto estudio de esta tesis. Se evaluó la farmacocinética de diferentes dosis de melatonina (0, 83, 166, 249 o 332 mg/kg peso) que se administraron como implantes subcutáneos en vacas en lactación. Sólo la dosis de melatonina de 332 mg/kg fue capaz a aumentar el nivel de la melatonina natural durante todo el período de estudio (50 días). Además, esta dosis no disminuía la producción de leche de las vacas estudiadas.

Por último, los posibles efectos de la melatonina en la mejora de la salud materna durante el periparto fueron evaluados en el quinto estudio de esta tesis. Las vacas tratadas con melatonina tenían menos probabilidad de sufrir el síndrome de vaca repetidora y pérdida de la gestación, y, por lo tanto, menos días abiertos (resultados que tampoco han sido publicados anteriormente en las vacas lecheras).

Resum

Les vaques lleteres són un element essencial de la nostra vida, ens proporcionen la llet i els seus diversos subproductes. En compliment d'aquest objectiu se sotmeten a unes condicions rigoroses de maneig, nutrició i controls reproductius per aconseguir el màxim benefici d'ells. Estan amenaçats per l'eliminació del ramat al llarg de la seva vida productiva, en cas de mal estat de salut i, consecutivament, rendiment productiu i/o reproductiu. Per tant, aquesta tesi (mitjançant els cinc estudis que es van realitzar) va ser destinada a investigar els factors que tenen un efecte potencial sobre la salut-immune de la mare durant la gestació i el postpart temprà, tenint en compte la melatonina com una molècula prometedora per millorar la salut i el rendiment de la vaca lletera.

En el primer estudi, s'han valorat els factors amb influència potencial sobre els leucòcits materns, com un indicador del seu estat immunològic, al llarg de la gestació (entre els dies 90-210). De gran importància va ser la interacció entre l'edat de la vaca i la seropositivitat a *Neospora caninum*. En aquest estudi, les vaques primípares i *Neospora*-seropositives van presentar menys nombres de leucòcits totals i neutròfils, però alts nombres de monòcits. També, l'estació, la gestació gemelar, la producció de llet i el temps de mostreig van estar relacionats amb canvis en els leucòcits de la sang perifèrica materna.

Seguint el sistema immune matern més enllà en la seva fase més crítica, el segon i el tercer estudis resumeixen els diferents factors que afectaven el sistema immunològic matern durant el període peripart (de 220 dies de gestació fins a 30 dies després del part). El toro de la inseminació artificial, les glicoproteïnes associades a la gestació (PAG), la interacció entre *N. caninum-Coxiella burnetii*, l'estació, l'edat, la gestació gemel·lar i el temps de mostreig

afectaven significativament els leucòcits materns durant aquesta etapa. Alguns toros i un major nivell de plasma PAGs van estar relacionats amb un major nombre de leucòcits materns durant el període peripart (resultats que no han estat publicats anteriorment a les vaques lleteres). Diferents nombres de leucòcits materns es van observar en resposta a la infecció per *N. caninum* o *C. burnetii* o ambdós, en comparació amb un grup de vaques seronegatius a ambdós, demostrant diferents respostes immunes maternes davant de cada tipus d'infecció. Cal assenyalar que, a una edat major (amb 3 o més lactacions) o en el cas de gestacions dobles les vaques van presentar menys nombres de leucòcits perifèrics al llarg de la gestació i durant el postpart.

S'havia de avaluar primer la dosi adequada de melatonina per administrar a les vaques lleteres, la qual va ser l'objectiu del quart estudi d'aquesta tesi, per tal d'avaluar la seva possible utilitat en la millora de la salut materna durant el període peripart. S'ha avaluat la famacocinética de diferents dosis de melatonina (0, 83, 166, 249 o 332 mg / kg pes) que es van administrar com implants subcutànies en les vaques lleteres. Es va trobar que només la dosi de melatonina de 332 mg/kg va ser capaç a augmentar el nivell plasmàtic de la melatonina durant tot el període d'estudi (50 dies). Més a més, aquesta dosi no disminuïa la producció de llet de les vaques estudiades.

Per últim, els possibles efectes de la melatonina en la millora de la salut materna durant l'peripart van ser avaluats en el cinquè estudi d'aquesta tesi. Les vaques tractades amb melatonina tenien menys probabilitat de repetició d'inseminació i pèrdua de la gestació, i, per tant, menys dies oberts. Aquests efectes no han estat publicats anteriorment a les vaques lleteres.

ملخص الرسالة

الأبقار الحلوب هي عنصر أساسي من حياتنا اليوميه، فهي توفر لنا متطلباتنا من الحليب و بالتالي تساعد أيضا علي توفير منتجات الألبان المختلفة. و إستكمالاً لهذا الهدف فإن صناعة الأبقار الحلوب تتعرض لإدارة و رعاية أكثر حزماً و صرامه في التغذية و تنظيم التناسل للخروج من هذه الأبقار بأكبر قدر من الفائدة. و في ضوء هذا فهي تحت تهديد مستمر طوال حياتها الإنتاجية بالإستبعاد من القطيع؛ و بخاصة عند سوء حالتها الصحيه و ما يترتب عليه من تدهور في الإنتاج. و لذلك، هذة الرسالة (من خلال خمس أبحاث علمية) هدفت إلى تحديد و دراسة العوامل التي من المحتمل أن تؤثر على الصحة المناعية للأبقار أثناء فترتي الحمل و ما بعد الولادة، أخِذة في الإعتبار إستخدام هرمون الميلاتونين كمادة واعدة في تحسين صحة الأبقار و من ثم إنتاجها و بقائها في القطيع؛ و بالتالي تلافي الخسارة الإقتصادية المترتبه علي إستبعاد بعض الأبقار من القطيع.

في البحث الأول من هذة الرسالة، تمت دراسة العوامل المؤثرة علي مستويات كريات الدم البيضاء؛ بإعتبارها مؤشر لقدرة الجهاز المناعي، في الدوره الدموية الطرفيه للأبقار خلال فترة الحمل (تحديدا بين الأيام 90 و حتي 210 من الحمل). من العوامل ذات اللأهميه كان التفاعل بين العدوي بطفيل نيوسبورا كانينوم (*Neospora caninum*) و سن الأبقار، حيث وجد أن كريات الدم البيضاء و الخلايا العدله (النيوتروفيل) في دم الأبقار ذات الولاده الأولي و المريضه بطفيل النيوسبورا كانينوم كانت في أدني مستوياتها بينما إحتوي دم هذه الأبقار علي مستويات مرتفعه من الخلايا وحيدات النوايا (المونوسايت). أيضا قد وجد أن الموسم و حمل التوائم و كمية إنتاج اللبن و أخيراً الوقت كانوا من العوامل التي كان لها تأثير ملحوظ علي مستويات كريات الدم البيضاء في ذات البحث.

إستكمالاً للبحث الأول كان من الضروري تتبع حاله الجهاز المناعي للأبقار الحوامل و دراسة العوامل التي قد تؤثر عليه خلال فترة ما قبل الولاده و ما بعد الولاده مباشرة (بالأخص بين الأيام 220 من الحمل و حتي ثلاثون يوماً بعد الوضع) و هي تعتبر من أكثر فترات حياة الأبقار حرجاً و أهمية، و قد تم هذا من خلال البحثين الثاني و الثالث. و من نتائج هذين البحثين قد وجد أن الثور المستخدم في عملية التلقيح الصناعي و مستوي الجليكو بروتينات المصاحبه للحمل (PAGs) في البلازما و التفاعل ما بين العدوي بطفيل نيوسبورا كانينوم (*Neospora caninum*) و بكتريا كوكسييلا بيرنيتي (*Coxiella burnetii*) و الموسم و السن و حمل التوائم و أخيراً الوقت من العوامل التي كان لها تأثير مهم علي

مستويات كريات الدم البيضاء في الأبقار المدروسة. و في ضوء هذه النتائج و لأول مرة في هذا المجال من البحث العلمي كان من المفاجئ أن بعض الثيران المستخدمه في عملية التلقيح الصناعي أو وجود مستويات مرتفعه في بلازما الدم من الجليكو بروتينات المصاحبه للحمل كان مصاحباً بزيادة ملحوظة في مستويات كريات الدم البيضاء طوال فترة الدراسة. بالإضافة إلي ذلك، لقد كان هناك إختلافاً ملحوظاً في مستويات كريات الدم البيضاء في الأبقار المريضه بطفيل نيوسبورا كانينوم أو ببكتريا كوكسييلا بيرنيتي أو بهما معا؛ مقارنة بمجموعه من الأبقار الصحيحه غير المريضه بأي منهما، مما دل علي قدرة الجهاز المناعي للأبقار الحوامل (في خلال فترة الدراسه) علي الإستجابه و التصدي للعدوي بطريقة مختلفه في كل حاله منها. كان أيضا من الملاحظ أن أدني مستويات كريات الدم البيضاء قد وُجدت في الأبقار المسنه (التي قامت بالوضع ثلاث مرات فما فوقهم) أو الأبقار حاملة التوائم و ذلك خلال فترة الحمل و حتي فترة ما بعد الوضع (فترة الأبحاث الثلاثه السابقه مجتمعين؛ أو من اليوم 90 في الحمل و حتي الثلاثون بعد الوضع).

لمعرفة ما إذا كان لهرمون الميلاتونين (هرمون الفتره الضوئيه) من فوائد علي صحه الأبقار الحلوب الحوامل بصفه عامه خلال فترتي ما قبل و مابعد الوضع مباشرة (و علي جهازها المناعي بصفه خاصه)، كان لابد من دراسه فارماكولوجيا الميلاتونين و ذلك لتحديد جرعة الميلاتونين المناسبه لهذه الأبقار؛ و هو كان هدف البحث الرابع في هذه الرسالة. و بالتالي تم إختبار عدة جرعات من الميلاتونين التي تم إعطائها بواسطة الزرع تحت الجلد (صفر أو 83 أو 166 أو 249 أو 332 مايكروجرام لكل كيلوجرام من وزن البقره) في عدد من الأبقار. و من خلال هذا البحث و جد أن الجرعة الوحيدة التي نتج عنها زياده ملحوظة في مستويات الميلاتونين في بلازما الدم لمدة 50 يوم علي الأقل هي جرعة 332 مايكروجرام لكل كيلوجرام من وزن البقره. و بالإضافة إلي ذلك لم ينتج أي أثر سلبي لهذه الجرعه علي إدرار أو إنتاجيه الحليب في الأبقار محل الدراسة.

دراسة فوائد الميلاتونين (بالجرعة المختارة من نتائج البحث السابق) علي صحه الأبقار الحلوب الحوامل بصفه عامه في فترتي ما قبل و مابعد الوضع مباشرة، مثَّل البحث الأخير في هذة الرسالة. حيث وجد أن الأبقار التي أعطيت الميلاتونين كزرع تحت الجلد كانت من أقل الأبقار عودةً للتلقيح مرة أخري و من أقلها فقدا للحمل بعد التلقيح و حدوث التخصيب و بالتالي كانت من الأبقار ذات أقل فارق زمني بين الوضع و الوضع الذي يليه. و كانت هذة هي أول مرة يتم فيها نشر مثل هذة النتائج في الأبقار.

Chapter 1: General Introduction

1. Chapter 1

General introduction

Pregnancy is a state of challenge for the maternal immune system. While it must be harmless to an in-*utero* growing foetus (which is antigenically half-foreign), paradoxically it must also be capable of defending the mother, and subsequently the foetus, against infections (either latent, as in case of *N. caninum* or *C. burnetii*, or new).

1.1.1 The foetal-maternal dialogue and maternal immune system response.

The foetus is considered as a semi-allograft (due to its half-dam/half-sire origin) (Druckmann, 2001), thus the maternal immune system is a threat to the continuation of pregnancy. A dialogue between the dam and its foetus occurs at the foeto-maternal frontiers (the endometrium and the placenta), and it is essential for the gestation to be sustained. The dialogue starts already following the process of fertilisation. Immediately, embryonic signals of presence (in particular interferon-τ) to the maternal side prevent corpus luteum lysis and, therefore, maintain the production of progesterone (P4) (reviewed in Bazer *et al.*, 1994), (Spencer *et al.*, 2007). As a continuation, progesterone stimulates maternal lymphocytes to produce Progesterone-Induced Blocking Factor (PIBF) (Szekeres-Bartho *et al.*, 2001). PIBF has a several immune-modulatory functions and is responsible for shifting the dam's immunity from T-helper 1 (T_{h1}) or cellular type to T-helper 2 (T_{h2}) or the humoral type which is harmless to the foetus (Kelemen *et al.*, 1998, Szekeres-Bartho *et al.*, 2001, Druckmann and Druckmann 2005) (see Fig. 1.1).

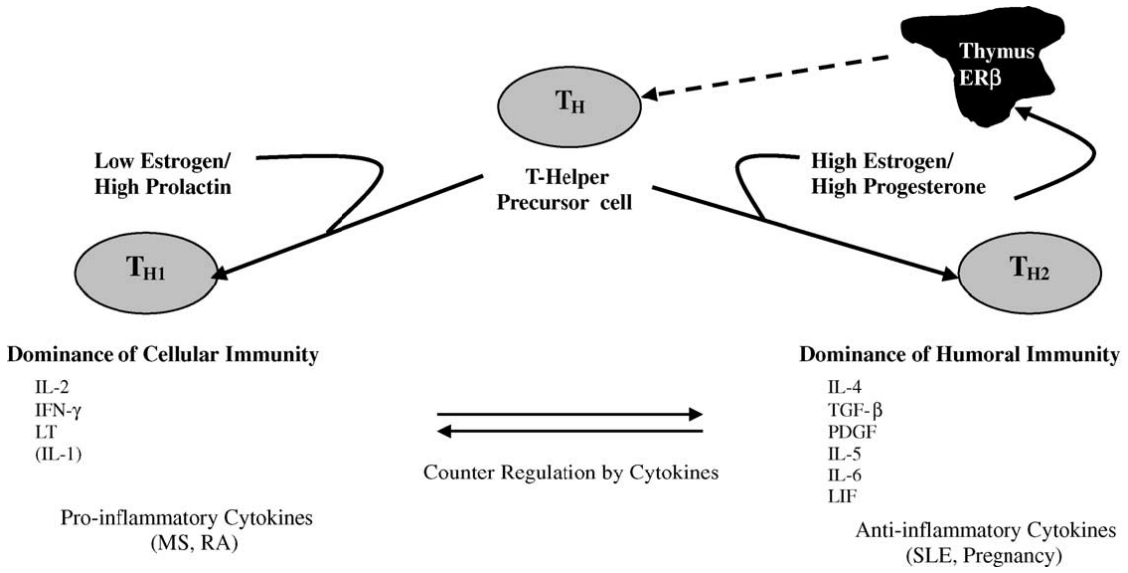

Figure 1.1. A diagram showing sex hormones control on T_{h1}/T_{h2} lymphocytes (Aschkenazi *et al.,* 2000 cited in Druckmann and Druckmann 2005). ER: oestrogen receptor; IL: interleukin; IFN: interferon; LT: lymphotoxin; MS: multiple sclerosis; RA: rheumatoid arthritis; TGF: transforming growth factor; PDGF: platelet derived growth factor; LIF: leukaemia inhibitory factor; SLE: systemic lupus erythematosus.

At the foetal side, down-regulation of MHC-1 on the trophoblastic cells (at the site of foetal-maternal connection, the placentome) was found during pregnancy (Ellis *et al.,* 1998, Bainbridge *et al.,* 2001), this should favour the immune-invisibility of the foetus, and consequently its survival inside the mother. However, a degree of incompatibility between the mother and her foetus is essential for the recognition, continuation and outcomes of pregnancy. In fact, complete foetal immune-invisibility, in other words full immune-compatibility with the dam, is not ideal; neither incompatibility is (because it will provoke the maternal immune system leading eventually to abortion). In effect, poorer outcomes and higher early embryonic mortalities were found in the compatible pregnancies than in the incompatible pregnancies, in addition proliferative responses of maternal lymphocytes were different between compatible and incompatible pregnancies (Aguilar *et al.*, 1997).

1.1.2 Bovine PAGs

Bovine pregnancy associated glycoproteins (PAGs) are a group of glycoproteins produced by the placental binucleate cells and poured directly into the maternal circulation. Although they belong to Aspartic protease family, their proteolytic function is disabled due to a mutation in their active site (Xie *et al.,* 1991, Xie *et al.,* 1994). Their functions are still unknown. However, researchers have been using them as indicators for foetal-placental wellbeing and foetal number (Patel *et al.,* 1997, Kornmatitsuk *et al.,* 2002, López-Gatius *et al.,* 2007, Serrano *et al.,* 2009). In addition, since they are produced early around implantation, they have been efficiently used on Day 29-30 as an early pregnancy diagnosis tool (Humblot 1988, Szenci *et al.,* 1998, Ayad *et al.,* 2007). PAGs levels and production will continue to increase throughout gestation till they reach the peak 1-5 days prior to calving (Zoli *et al.,* 1992). Although, direct immune suppressive effect of PAGs have not been confirmed till now, PAGs peak prepartum was claimed to be related to the immune-functions failure of the peripheral blood polymorph-nuclear cells just after calving (Dosogne *et al.,* 1999). The immune suppressive properties of bovine PAGs need further clarification.

1.1.3 Factors affecting cows' leukocyte counts

In the healthy dairy cow, factors such as physiological stressing conditions (Wegner *et al.,* 1976, Lacetera *et al.,* 2005, Lacetera *et al.,* 2006), breed (Greatorex 1957a, Greatorex 1957b), age (Mohri *et al.,* 2007, Graham *et al.,* 2009), pregnancy and pospartum status (Newbould 1976, Kehrli Jr. *et al.,* 1989a, Kehrli Jr. *et al.,* 1989b, Detilleux *et al.,* 1995, Johnson *et al.,* 1990, Meglia *et al.,* 2005, Nazifi *et al.,* 2008), parity (Newbould 1976,

Mehrzad *et al.,* 2002, Mehrzad *et al.,* 2009) and milk yield (Newbould 1976, Detilleux *et al.,* 1995), have been demonstrated to affect peripheral white blood cells profiles.

1.1.4 *Neospora caninum* infection

Neosporosis is a recently emerged disease affecting most warm-blooded animals (Dubey and Lindsay 1993, Dubey 2003); caused by the intracellular protozoan parasite *Neospora caninum*. After its discovery in dogs in 1984 (Bjerkas *et al.,* 1984) and its description in 1988 (Dubey *et al.,* 1988), neosporosis has been related to abortion in cattle worldwide (Anderson *et al.,* 1991, Dubey 2003). In pregnant cows, higher *Neospora*-abortion rates are mainly observed in the second trimester of gestation; however cows may abort any time between 3 months and full term (Anderson *et al.,* 2000, Jenkins *et al.,* 2000, López-Gatius *et al.,* 2004a, López-Gatius *et al.,* 2004b). Congenital infection is the major route of infection (Schares *et al.,* 1998, López-Gatius *et al.,* 2004a) reaching as high as 95% (Davison *et al.,* 1999), although, less frequently, infection may occur through ingestion of food and water contaminated with the sporulated oocysts (McAllister *et al.,* 1998). If no abortion occurs, the majority of the foetuses will remain infected for life and maintain the infection in the herd (Anderson *et al.,* 1997). Aspects of host-parasite relationships have been carefully addressed in non-pregnant and pregnant cows (Fig. 1.2; reviewed in, Innes *et al.,* 2002). In our region of study (North-east Spain), *N. caninum*-seropositivity was found to be 40% of the pregnant dairy cattle, with abortion rates reaching 44% of the seropositive ones; the latter had 12–19 times higher risk of abortion than the seronegative ones (López-Gatius *et al.,* 2004a, López-Gatius *et al.,* 2004b).

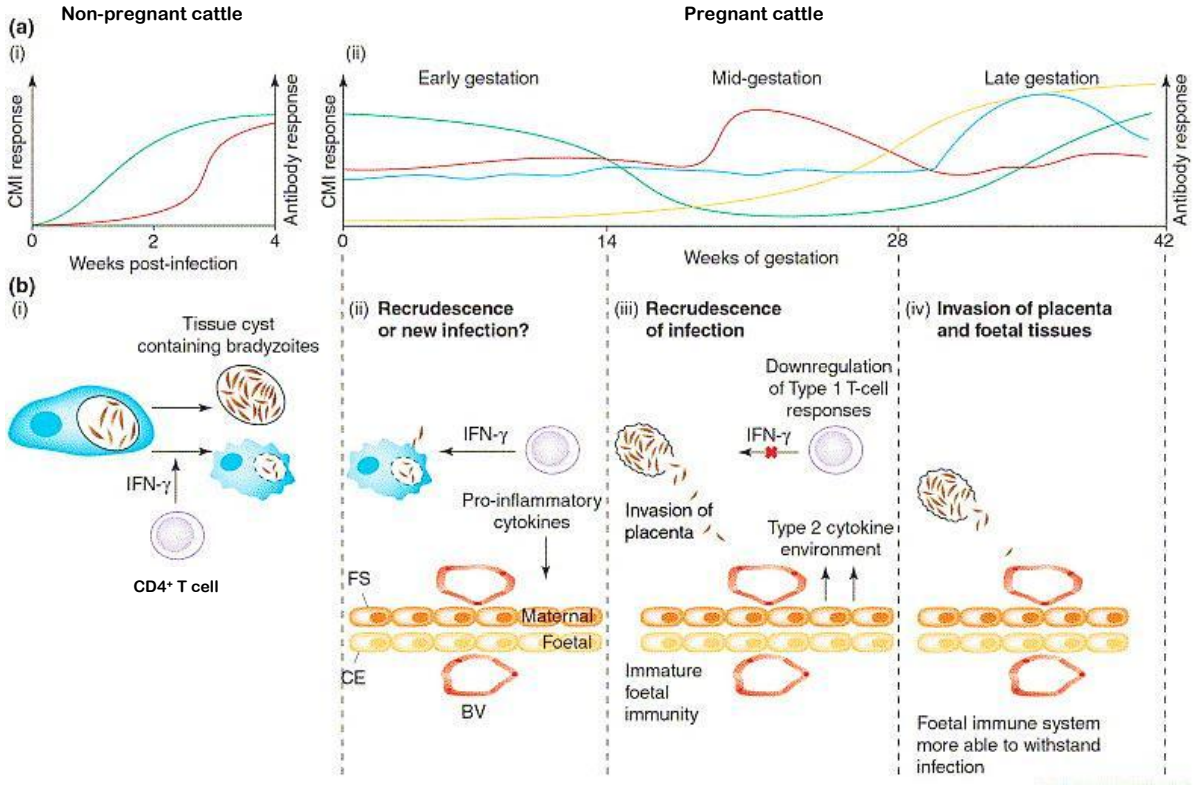

Figure 1.2. Illustration of the different aspects of the host-parasite relationship in non-pregnant (i a&b) and pregnant (ii a&b, iii and iv) cattle (adapted from Innes *et al.*, 2002). BV, blood vessel; CE, chorionic epithelium; FS, foetal-maternal syncytium. (ai) Green line: cell mediated immunity; Red line: antibody responses. (aii) Green line: cell mediated immunity; Red line: antibody responses when parasitaemia occurs in mid-gestation; Blue line: antibody responses when parasitaemia occurs in late-gestation; Yellow line: maturity of foetal immune response.

1.1.5 *Coxiella burnetii* infection

Coxiellosis is a worldwide zoonotic disease, also known as Q fever or Query fever in human, caused by the obligate-intracellular pleomorphic gram-negative bacteria *Coxiella burnetii* (Arricau-Bouvery and Rodolakis 2005, Guatteo *et al.*, 2011). The bacterium is very resistant and can survive for long time in the environment (Arricau-Bouvery and Rodolakis 2005), thus causing a substantial source of infection between animals and to human. Infected livestock, especially ruminants, and pets are the most common source for human infection,

the bacterium is shed in birth fluids and vaginal discharges, urine, milk and faeces, and the most common route of human infection is by inhalation of contaminated aerosols. *C. burnetii* has two antigenic phases (phases I and II) and in human the disease can be manifested either acutely or chronically (for more information on sources and routes of infections, antigenic phases and clinical manifestations in both human and animals, please refer to Arricau-Bouvery and Rodolakis 2005, Parker *et al.,* 2006, Angelakis and Raoult 2010). In animals, in contrast to human, Angelakis and Raoult (2010) described coxiellosis as strikingly asymptomatic. Nonetheless, abortion has been attributed to *C. burnetii* infection, mainly in small ruminants (Berri *et al.*, 2002). In addition, increased placental retention incidence in dairy cows was related to *C. burnetii* seropositivity (López-Gatius *et al.,* 2012). Coxiellosis in ruminants received worldwide attention recently, due to the 2007 human outbreak in the Netherlands in which the source of infection was neighbouring infected small ruminants farms (Karagiannis *et al.,* 2009, Roest *et al.,* 2011). In our geographical region, *C. burnetii* seroprevalence rates of more than 50% have been found in dairy cattle farms (López-Gatius *et al.,* 2012, Nogareda *et al.,* 2012).

1.1.6 Melatonin

Melatonin (*N-acetyl-5-methoxytryptamine*) is a small lipophilic indoleamine mainly synthesized in the pineal gland, it was first isolated from the bovine pineal gland in the late 1950s (Lerner *et al.,* 1958, Lerner *et al.,* 1959). In all studied mammal species, circulating levels of the hormone fluctuate in a circadian manner with higher levels observed at night (Tamura *et al.,* 2008). Pineal melatonin serve as a photoperiod signal transducer, in other words it provide the brain with information about the length of light/dark phases of the day. Such information is important for the orchestration of the circadian and seasonal rhythms and

their related biological processes, for example, the reproductive function in small ruminants (Arendt 1998, Weaver and Lockley, 2009). In addition to providing the brain with photoperiod information, melatonin was also found to have immune-regulatory functions and oncostatic effects, it is also a potent antioxidant and free radical scavenger (for more details please refer to: Maestroni 1993, Reiter 2003, Esquifino *et al.*, 2004) (Figure 1.3). Scarce information exists on the possible benefits of melatonin to dairy cows, and it seems to be a promising molecule in dairy industry.

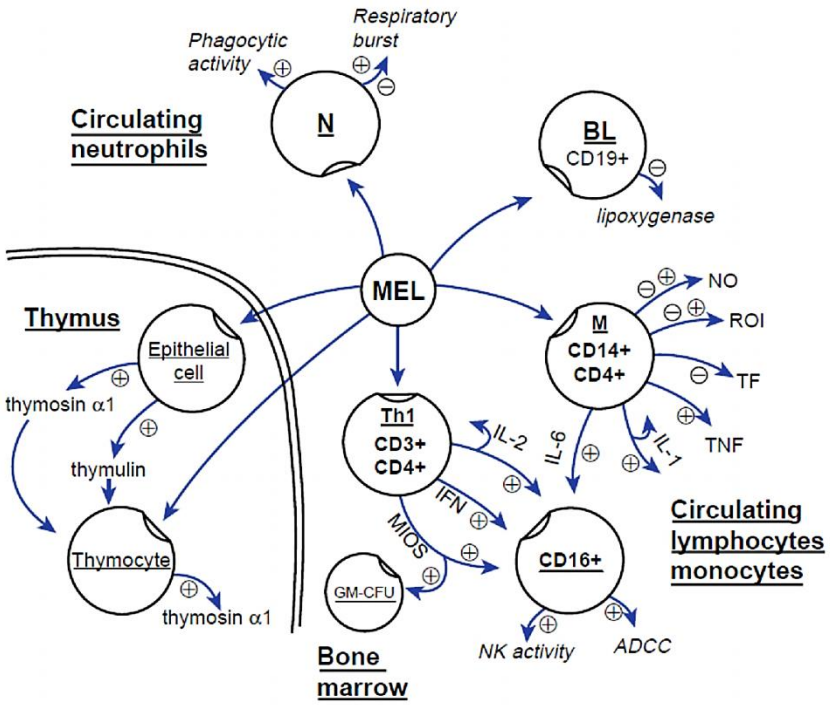

Figure 1.3. A diagram showing possible actions of melatonin on immune cells (Reiter 2003). MEL, melatonin; MIOS, melatonin-induced opioid system; GM-CFU, granulocytes and macrophages progenitor cells; NK, natural killer cell; ADCC, antibody-dependent cellular cytotoxicity; M, monocyte ($CD14^+/CD4^+$ cells); ROI, reactive oxygen intermediates; NO, nitric oxide; TF, tissue factor; BL, B-lymphocytes; N, neutrophils.

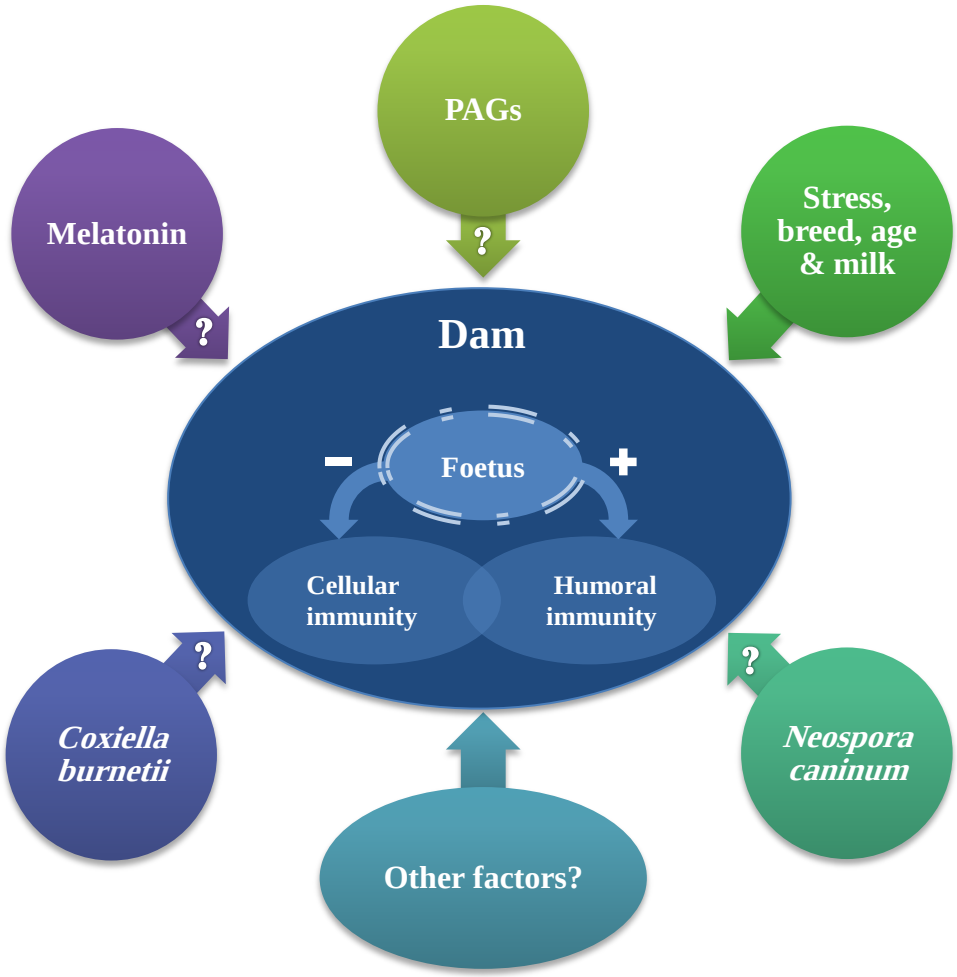

Figure 1.4. A diagram summarizing different factors with substantial/possible effects on cow's immune system. (? = still need further investigation, + = upregulation, - = downregulation).

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Principal objectives

Principal objectives

Dairy cows are subjected to high risk of elimination from the dairy herds; due to poor health conditions and/or performance during gestation and especially in the postpartum. Immune-health represents an important aspect of the broader cow's health term. This thesis aimed at studying cows' immune-health throughout gestation and the early postpartum, considering possible aspects of improvement of cows' health in general. Given the importance of gestational phases in dairy cattle, our main objective was divided into the following sub-objectives (using high-producing Holstein-Friesian dairy cattle),

1- Determining possible factors that affect peripheral leukocyte subpopulations throughout gestation, with special emphasis to *Neospora caninum*.
2- Underlining potential factors that cause changes in the peripheral leukocyte populations during the peripartum period (Late gestation and Early postpartum), considering some,
    a. Cow-related factors (ex. age, twin-gestation), foeuts-related factors (ex. pregnancy associated glycoproteins), environment-related factors (ex. season and time) or management-related factors (ex. inseminating bull).
    b. Chronic diseases existing in our geographical region (*N. caninum* and *C. burnetii*).
3- Determining the appropriate melatonin dose for long-term administration in dairy cows.
4- Determining the possible effects of long-term melatonin administration on cows' health during the peripartum period.

Chapter 2: Peripheral white blood cell counts throughout pregnancy in non-aborting *Neospora caninum*-seronegative and seropositive high-producing dairy cows in a Holstein Friesian herd

2. Chapter 2

Peripheral white blood cell counts throughout pregnancy in non-aborting *Neospora caninum*-seronegative and seropositive high-producing dairy cows in a Holstein Friesian herd

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Abstract

Pregnancy is characterized by transient changes in the maternal immune system, also evident at peripheral level. The present study analyses the kinetics and possible factors affecting peripheral white blood cell populations throughout pregnancy in a herd of high-producing dairy cows chronically infected or not with *Neospora caninum*. We examined 54 pregnant parous cows: 29 *Neospora*-seronegative and 25 *Neospora*-seropositive cows. Blood samples were collected on Days 90, 120, 150, 180 and 210 of gestation. General Linear Model (GLM) repeated measures analysis of variance showed that the interaction *Neospora*-seropositivity x parity significantly affected total leukocyte, neutrophil and monocyte counts with lower levels of total leukocytes, lower neutrophil and higher monocyte counts recorded in primiparous *Neospora*-seropositive cows. In addition, *N. caninum*-seropositive cows had significantly increased monocyte counts on Day 180 of gestation compared to seronegative ones. Other factors significantly associated with changes in total and/or differential leukocyte profiles were period of pregnancy, season, twin pregnancy and milk production. In conclusion, a parity-associated effect of chronic *N. caninum* infection was observed on peripheral blood cell profiles in dairy cattle during gestation.

Key words: *Neospora caninum*, Dairy cows, Pregnancy, White blood cells.

2.1 Introduction

The peripheral white blood cell profiles of healthy dairy cows are known to be affected by factors such as physiologically stressful conditions (Lacetera et al., 2005, Lacetera et al., 2006), breed (Greatorex, 1957a, b), age (Mohri et al., 2007, Graham et al., 2009), postpartum and pregnancy status (Kehrli et al., 1989, Johnson et al., 1990, Detilleux et al., 1995, Nazifi et al., 2008), parity (Mehrzad et al., 2002, Mehrzad et al., 2009) and milk yield (Detilleux et al., 1995). Peripheral immune responses have been used to assess the health status of an animal. As in other species, the peripheral immune cell populations of cows during pregnancy change as a result of conceptus-maternal crosstalk established through placentomes (Gifford et al., 2007, Oliveira and Hansen, 2008).

Neospora caninum, an obligate intracellular protozoan parasite closely related to *Toxoplasma gondii*, is a significant cause of abortion in cattle worldwide (Anderson et al., 2000, Dubey et al., 2007). In fact, in our geographical area of study (North-East Spain), the risk of abortion has been reported as 12–19 times higher in *N. caninum*-seropositive dairy cows than in their seronegative counterparts, and abortion rates range from 30% to 44% in seropositive animals (López-Gatius et al., 2004a, b). The most common route of *Neospora* infection in cow herds is endogenous transplacental transmission during pregnancy (Schares et al., 1998, López-Gatius et al., 2004b). Parasites cross the placenta and infect the foetus causing abortion or congenital infection. In naturally infected cattle, the rate of endogenous transplacental transmission has been estimated to be as high as 91–95% (Moen et al., 1998, López-Gatius et al., 2004b) and this will lead to persistence of the infection in the herd generation after generation (Dubey et al., 2007). In *Neospora*-infected non-aborting cows, clear immunomodulation of gestation occurs such that antibody levels against *N. caninum*

increase during the second half of gestation (López-Gatius et al., 2007a, Nogareda et al., 2007).

The objective of the present study was to examine the kinetics and possible factors affecting peripheral white cell blood populations throughout pregnancy in non-aborting, high-producing Holstein–Friesian dairy cows, including chronically *Neospora*-infected cows. To our knowledge, the possible effect of chronic *Neospora*-infection on peripheral white blood cell profiles has not been previously addressed.

2.2 Materials and Methods

2.2.1 Cattle and herd management

The study was performed over a 13-month period (July, 2007 to August, 2008) on a commercial Holstein–Friesian dairy herd in north-east Spain with previously confirmed cases of *N. caninum* infection in aborted foetuses. During the study period, the seroprevalence of *N. caninum* infection for the herd was 19% based on the yearly analysis of *N. caninum* seroprevalence included in the health management of this herd. The herd comprised a mean of 570 parous cows, with a mean annual milk production of 11070 kg per cow and a mean culling rate of 28% (percentage of cows culled over the total parous cows) in the herd during the study period. The cows reared within the herd, calved all year round, were milked three times per day, kept in open stalls and fed complete rations. Feeds consisted of cotton-seed hulls, barley, corn, soybean and bran, and roughage, primarily corn, barley or alfalfa silages and alfalfa hay. Rations were in line with NRC recommendations (US National Research Council, 2001). From a health standpoint, the herd was closed and well managed. All the animals were bred by artificial insemination. The semen doses used in the study population

were provided by 18 sires of proven fertility. All the animals were free of tuberculosis and brucellosis, as shown by yearly tests from 1985 to 2009. Strict vaccination programs were undertaken for the prevention of bovine virus diarrhoea (BVD) and infectious bovine rhinotracheitis (IBR). Modified live vaccines were used for animals 6–8 months old. Pregnant animals were given killed vaccines during the 7th month of each gestation period. Only cows free of clinical disease were included in the study. Exclusion criteria were abortion, mastitis, lameness, and digestive disorders. Based on these parameters, 61 cows were selected for the study. Of those, a seronegative cow was culled before parturition and six seropositive cows that aborted during the study (from Days 133 to 258 of gestation) were also excluded. The final analysed cows included 54 non-aborting parous cows, 29 *N. caninum*-seronegative and 25 *N. caninum*-seropositive. Serological tests for *Neospora* infection were performed during annual screening for brucellosis. *Neospora* seropositivity was defined as a positive blood test obtained 0–365 days before pregnancy diagnosis on Day 90 of gestation.

2.2.2 Pregnancy diagnosis and blood sample collection

Pregnancy diagnosis was performed on Days 28–34 post-insemination by transrectal ultrasonography, and by palpation per rectum on Days 90, 120, 150, 180 and 210. Since chronic *N. caninum* infection does not affect early pregnancy (López-Gatius et al., 2004b), the study population was comprised only of animals confirmed pregnant on Day 90. As indicated above, only non-aborting cows were included in the study. The outcome of pregnancy was recorded for all the animals. Blood samples were collected from each animal into two EDTA vacuum tubes (BD Vacutainer™, Becton-Dickenson and Company, Plymouth, UK) immediately before each pregnancy diagnosis. One tube was centrifuged (10

min, 1600 g) and the plasma stored at -20°C until analysis, whereas the blood in the second tube was used for haematological analysis.

2.2.3 Haematology

Total leukocyte counts were performed using an automated blood analyser (HemaVet Cell counter 850, CDC Technologies, Inc., Centerville, USA) standardized for the analysis of bovine blood. Total leukocyte numbers, together with differential counts for neutrophils, lymphocytes, monocytes, eosinophils, and basophils were expressed as counts per microlitre of blood. Manual counts were also conducted on a randomly selected percentage of samples. Briefly, thin blood smears stained with Wright-Giemsa stain were prepared, and 400 cells differentiated and counted using the battlement technique (Schalm et al., 1975) under the light microscope. Results were found to be comparable using both techniques by linear regression analysis (R^2=0.71; P=0.016).

2.2.4 *N. caninum* IgG antibodies

Plasma samples from each cow were tested for antibodies against N. caninum using a commercial enzyme-linked immunosorbent assay (ELISA) kit (CIVTEST anti-*Neospora*, Hipra, Girona, Spain) based on the whole tachyzoite lysate of *N. caninum* NC-1. This test, previously validated by the present authors (López-Gatius et al., 2004a), was performed according to the manufacturer's instructions and a value of P=6.0 relative index units taken to denote seropositivity. Seropositive and seronegative classification of the cows was based on the results of annual screening for neosporosis. This classification was confirmed using the plasma samples obtained during gestation. A cow was considered *Neospora*-seronegative

when no sample yielded a positive result during this period. Endogenous transplacental transmission was the predominant transmission pattern of N. caninum in the herd confirmed by pedigree analysis. However, if an animal was initially *Neospora*-seronegative and *Neospora*-seropositive in any of the subsequent analysis an avidity test was performed (*Neospora*–Ab SVANOVIR1, Svanova, Sweden) to distinguish between recent or exogenous transplacentally transmitted infection, and chronic or endogenous transplacental infection.

2.2.5 Data collection and statistical analysis

In the geographical area of study, there are only two clearly differentiated climate periods: warm (May–September) and cold (October–April). Given that reproductive parameters may be significantly impaired in the warm period (Labernia et al., 1996, López-Gatius, 2003), we studied the effect of season (warm versus cold) on peripheral white blood cell counts. The following data were recorded for each animal: parity, milk production at pregnancy diagnosis (Day 40), season, twin pregnancy, blood counts (total leukocyte, lymphocyte, neutrophil, monocyte, eosinophil, and basophil counts) and N. caninum seropositivity. The effects of parity (primiparous, multiparous), milk production at pregnancy diagnosis (<40 kg, ≥P40 kg), season (cold = more than three samples taken in the cold period, warm = more than three samples taken in the warm period), twin pregnancy, day of gestation (90, 120, 150, 180, 210), N. caninum seropositivity (seropositive vs. seronegative), and possible interactions of paired factors on total and differential leukocyte counts were assessed by General Linear Model (GLM) repeated measures analysis of variance using the SPSS computer package, version 14.0 (SPSS Inc., Chicago, IL, USA). GLM for repeated measurements was used to identify the effects of factors on the measured total and differential leukocyte counts. Period of gestation was considered the within-subject effect,

and the rest of factors were included as the between-subject effects. Parity, milk production, season, twin pregnancy and *N. caninum* seropositivity were considered dichotomous variables (0 or 1). Values are expressed as the mean ± standard deviation.

Table 2.1. Possible variables affecting white blood cell counts throughout pregnancy in non-aborting *N. caninum*-seronegative (N-; n = 29) and seropositive (N+; n = 25) high-producing dairy cows.

Factor	**N classes**	**Class description (n)**	**Mean ± SD (range)**	
			N-	**N+**
Non-aborting *N. caninum*-seropositive and seronegative cows				
Days of gestation	5	90, 120, 150, 180, 210		
Parity	2	Primiparous (14), Multiparous (40)		
Milk production	2	< 40 Kg (35), ≥40 kg (19)		
Season	2	Cold (40), Warm (14)		
Twin pregnancy	2	Single (45), Twin (9)		
Neospora caninum seropositivity	2	Seronegative (29), Seropositive (25)		
Mean leukocyte counts* (Days 90-210)	Continuous		6.3 ± 0.8 (5.0-7.7)	6.2 ± 0.8 (4.4-8.8)
Mean neutrophil counts* (Days 90-210)	Continuous		2.6 ± 0.8 (1.6-4.7)	2.5 ± 0.5 (1.6-3.3)
Mean lymphocyte counts* (Days 90-210)	Continuous		2.9 ± 0.4 (2.2-3.9)	3.0 ± 0.6 (2.2-4.7)
Mean monocyte counts* (Days 90-210)	Continuous		0.44±0.1 (0.25-0.7)	0.47 ± 0.12 (0.21-0.74)
Mean eosinophil counts* (Day 90-210)	Continuous		0.26 ± 0.2 (0.06-1.16)	0.25 ± 0.11 (0.06-0.48)
Mean basophil counts* (Day 90-210)	Continuous		0.01 ± 0.01 (0.0-0.04)	0.01 ± 0.01 (0.00-0.02)

*10^3 cells/μL

2.3 Results

The initial classification of cows as *Neospora*-seropositive or negative based on the annual screening for *N. caninum* infection was confirmed in the analysis of the blood samples collected during the gestation period in all the cows analysed. The possible variables affecting white blood cell counts throughout pregnancy in non-aborting *N. caninum*-seronegative (n = 29) and seropositive (n = 25) high-producing dairy cows included in the GLM analysis are provided in Table 2.1. Basophils were not included in the statistical analysis since they were observed in very small numbers during pregnancy and in similar levels in seropositive and seronegative animals (Table 2.1). GLM analyses revealed several factors affecting both within (over time) and between subject changes in total and differential white blood cells counts throughout gestation (Table 2.2). Total leukocyte counts were significantly affected by the interaction *Neospora*-seropositivity x parity (within-subject effects) with increased leukocyte counts being recorded in primiparous *Neospora*-seronegative cows at mid pregnancy (at Days 120–180 of gestation), compared to similar total leukocyte patterns observed in the other groups (Fig. 2.1). Neutrophil counts were also significantly affected by *Neospora*-seropositivity x parity (within-subject effects) due to a transient decrease on Day 180 in primiparous *Neospora*-seropositive cows (Fig. 2.2). While monocyte counts were significantly affected by both *Neospora* seropositivity (within and between subject effects) with higher levels recorded in *Neospora*-seropositive animals on Day 180 of gestation (Fig. 2.3A) and *Neospora*-seropositivity x parity (between subjects effect) (Fig. 2.3B).

Other factors affecting total and differential leukocytes were days of gestation, season, milk production and twin pregnancy. Total leukocytes, neutrophils and lymphocytes

Table 2.2. Factors affecting total leukocyte, neutrophil, lymphocyte, monocyte and eosinophil counts throughout gestation (Days 90-210) in non-aborting dairy cows (n = 54) given by GLM repeated measures analysis of variance.

*Subject effect**	*Factor*	*Df*	*F*	*P-value*
Total leukocytes				
Within	Neosporosis x Parity	1	9.91	0.003
	Days of gestation	1	12.71	0.001
	Season	1	47.61	<0.001
	Twins	1	4.43	0.041
Between	Milk production	1	4.13	0.048
Neutrophils				
Within	Neosporosis x Parity	1	11.32	0.002
	Days of gestation	1	8.70	0.005
	Season	1	54.37	< 0.001
Eosinophils				
Within	Twins	1	4.19	0.046
	Milk production	1	4.61	0.037
Lymphocytes				
Within	Days of gestation	1	6.73	0.013
	Season	1	10.53	0.002
Monocytes				
Within	Neosporosis	1	4.35	0.042
	Days of gestation	1	4.95	0.031
	Season	1	5.40	0.024
Between	Neosporosis	1	5.21	0.027
	Neosporosis x Parity	1	8.12	0.006

* Factors were considered as the 'between-subject effect' and days of gestation were considered as the 'within-subject effect'.

were significantly affected by days of gestation (within-subject effects), following a similar kinetics with increasing counts Day 90-150 of gestation, and, then decreasing towards the end of the study period. Cows sampled in warm period exhibited rising concentrations of total leukocytes, neutrophils, lymphocytes and monocytes (within-subject effects), compared to those sampled in the cold season, which did not show major changes during pregnancy. Higher milk production was related to lower levels of total leukocytes throughout pregnancy (between subject effects) while lower milk production was related to decreasing eosinophil counts on Day 90 (within-subject effects). Lastly, twin pregnancy was related to a transient

decrease in total leukocytes (within-subject effects), and a trend of decrease in lymphocytes (P=0.074) on Day 180 of gestation.

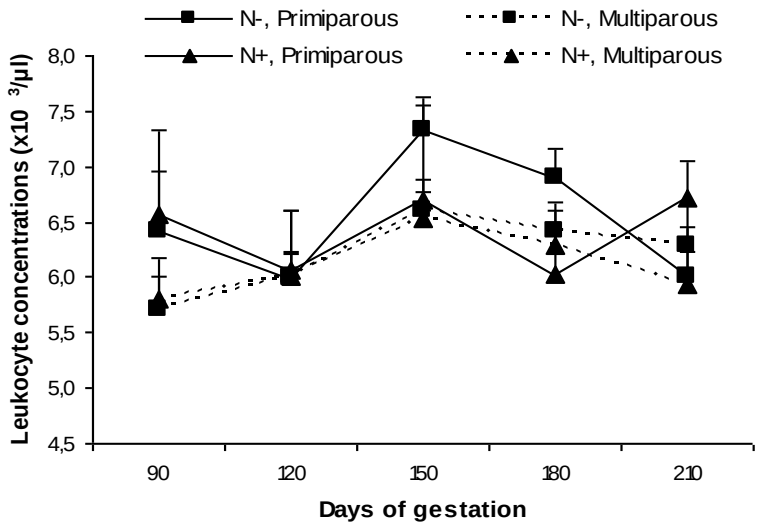


Figure 2.1. Mean total leukocyte concentrations ($x10^3/\mu L$) recorded during gestation in non-aborting lactating dairy cows (*n* = 54) by the interaction between *Neospora* seropositivity (*Neospora*-seronegative cows, n=29; *Neospora*-seropositive cows, n=25) and parity (primiparous, n=14; multiparous, n=40).

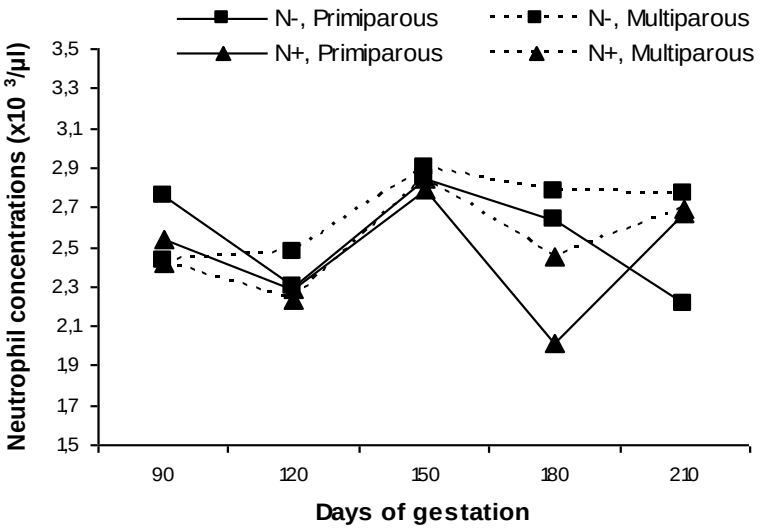
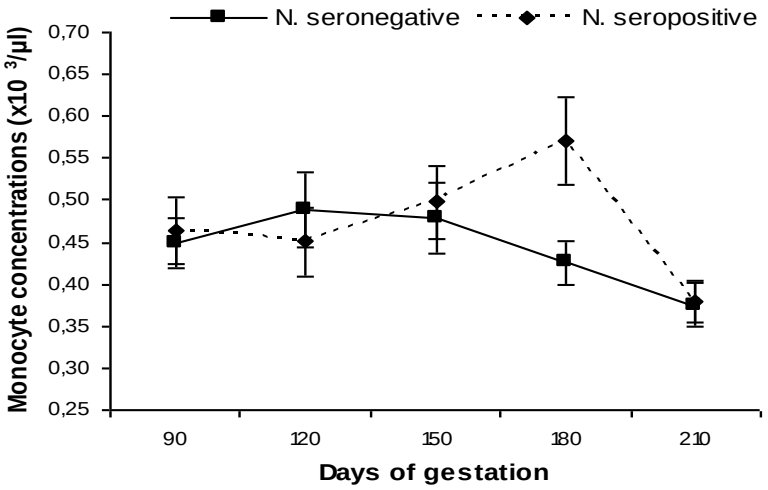


Figure 2.2. Mean neutrophil concentrations ($x10^3/\mu L$) recorded during gestation in non-aborting lactating dairy cows according to the interaction between *Neospora* seropositivity (*Neospora*-seronegative cows, n=29; *Neospora*-seropositive cows, n=25) and parity (primiparous, n=14; multiparous, n=40).

A)



B)

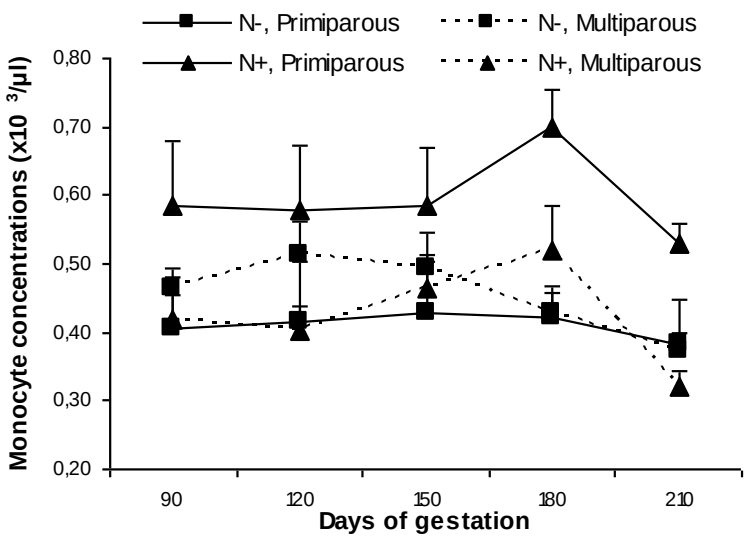


Figure 2.3. Mean plasma monocyte concentrations ($x10^3/\mu L$) recorded during gestation in non-aborting lactating dairy cows (*n* = 54) by *Neospora* seropositivity (*Neospora*-seronegative cows, n=29; *Neospora*-seropositive cows, n=25) (A) and the interaction between *Neospora* seropositivity (*Neospora*-seronegative cows, n=29; *Neospora*-seropositive cows, n=25) and parity (primiparous, n=14; multiparous, n=40) (B).

2.4 Discussion

In the present study, the kinetics and possible factors affecting white blood cell counts throughout pregnancy in non-aborting *N. caninum*-seronegative and seropositive high-producing dairy cows were analysed. Despite the fact that both overall and differential white blood cell counts remained within the reference ranges reported for healthy cattle (Smith, 2001), factors such as the interaction *Neospora*-seropositivity x parity, *Neospora* seropositivity, days of pregnancy, season, twin pregnancy and milk production showed a significant effect on total and/or differential peripheral white blood counts during the gestation period.

Several studies have established that total and/or differential peripheral leukocytes show a negative correlation with parity in cows (Mehrzad et al., 2002, Mehrzad et al., 2009), with higher levels in primiparous cows which seems to make them less vulnerable to some infectious diseases (Mehrzad et al., 2002, Mehrzad and Zhao, 2008). In the present study, no significant effect of parity, primiparous versus multiparous cows, on peripheral white cell counts was observed. However, total leukocyte, neutrophil and monocyte counts were significantly affected by the interaction *Neospora*-seropositivity and parity, which could have masked the expected parity differences in the total of the analysed animals. Increased leukocyte counts were recorded in primiparous *Neospora*-seronegative cows at mid pregnancy, while lower neutrophil and higher monocyte counts were recorded around Day 180 of pregnancy in primiparous *Neospora*-seropositive cows. These results points out to a parity-associated differential maternal immune response to neosporosis in chronically infected cows. A different immune response against *N. caninum* between primiparous and

multiparous cows has been previously suggested (Thurmond and Hietala, 1997, Yániz et al., 2010).

In the present study significant changes in total leukocytes, neutrophils, lymphocytes and monocytes were observed during pregnancy. Previous studies have shown differences in peripheral blood populations due to postpartum and pregnancy status (Detilleux et al., 1995, Nazifi et al., 2008). Total leukocytes and lymphocytes reached a maximum at Day 150 of gestation and then decreased towards the end of the study period. During pregnancy an increase in the proportion of specific lymphocyte subsets such as $CD4^+$ cells has been observed at different time points (Oliveira and Hansen, 2008), which would explain the peak of total leukocytes and lymphocytes noted. In addition, as indicated above, significantly higher monocyte levels were recorded around Day 180 of pregnancy in primiparous *Neospora*-seropositive cows. In nonaborting cows, as calves are usually born clinically healthy but infected, it is tempting to conclude that the vertical transmission is particularly likely to occur around the second half of gestation when antibody levels against *N. caninum* increase (López-Gatius et al., 2007a, Nogareda et al., 2007). The possible recrudescence of *N. caninum* infection around that time could have determined the peripheral monocyte peak, and this response could have been especially noticeable in primiparous dams, where recrudescence and transmission to the progeny will take place for the first time. The increased levels of monocytes, however, decreased soon afterwards and reached similar levels as those observed in seronegative animals.

Late pregnancy is characterized by a significant drop in peripheral monocytes ($CD68^+$), which are directed towards the endometrial stroma (Oliveira and Hansen, 2008) under the regulation of placental trophoblast cells (Fest et al., 2007). In *N. caninum*

experimental infections in pregnant dams, peripheral blood mononuclear cells subpopulations (PBMC) (in particular $CD3^+$, $CD4^+$, $CD8^+$ and activated $IL\text{-}2R^+$) decreased after infection compared to uninfected control animals, while at the same time, these subpopulations increased in internal organs (Almería et al., 2003). Similarly, the lower peripheral neutrophil counts in primiparous *Neospora*-seropositive on Day 180 may have been due to neutrophil recruitment towards the focus of infection in an attempt of the dam to control parasite recrudescence at the placental level. Neutrophils play an important role during *T. gondii* tachyzoite replication (Bliss et al., 2001, Bennouna et al., 2003) and during the invading stage of *T. gondii* and *N. caninum* tachyzoites, neutrophils suffers an enhanced molecule gene transcription and adhesion function towards infected endothelial cells (Taubert et al., 2006). The differences in neutrophil and monocyte kinetics observed in primiparous *Neospora*-seropositive cows may be explained by their different responses under chemotactic and inflammatory signals, particularly in the qualitative and quantitative expression of adhesion molecules (Wagner and Roth, 2000).

Peripheral cell subpopulation profiles also differed in cows in which more than three blood samples were collected in the warm season compared to the cold season. In the warm period, cows showed increased total leukocyte, neutrophils, lymphocyte and monocytes counts compared to those in the cold season, with the latter animals showing no major changes during pregnancy. These results partly agree with results from previous studies in which increased numbers of circulating leukocytes (Wegner et al., 1976) and neutrophils (Lee et al., 1976) were detected in heat-stressed cows.

The total leukocytes counts were also significantly affected by milk production. The fact that higher milk production was related to a decrease in total peripheral white blood cells

could be an indication of recruitment of leukocyte populations into mammary tissues in higher producing cows (Newbould, 1976), without compromising immune function traits (Detilleux et al., 1995, Kornalijnslijper et al., 2003).

Twin pregnancies were related to a transient decrease in total leukocytes on Day 180 of gestation and eosinophils at the end of the study period. In pregnant cattle, progesterone is first produced in the *corpus luteum* and then this role is taken over by placental binucleate cells (Shemesh, 1990). Maternal plasma oestrone sulphate (Dobson et al., 1993), pregnancy-associated glycoprotein (PAG) (Dobson et al., 1993, Echternkamp et al., 2006, López-Gatius et al., 2007b, Serrano et al., 2009), and progesterone concentrations (Echternkamp et al., 2006) have all been positively correlated with foetal number. Given the inhibitory effect of progesterone on T-lymphocyte proliferation (Cannon et al., 2003), the placental relay in progesterone production may lead to temporary blockade of total leukocytes driven by lymphocyte counts, and eosinophils in twin-bearing cows in late pregnancy. In addition, several studies have attributed these immunosuppressive properties to PAGs in vitro (Dunbar et al., 1990, Hoeben et al., 1999).

In conclusion, our results indicate that factors such as the interaction *Neospora*-seropositivity x parity, *Neospora* seropositivity, period of pregnancy, season, twin pregnancy and milk production have a significant effect on total and/or differential counts of peripheral white blood cells during the gestation period in high-producing dairy cows. A parity dependent effect of chronic *N. caninum* infection on peripheral white blood cells was observed. Primiparous *Neospora*-seropositive cows showed different patterns of total leukocytes, neutrophils, and monocytes, particularly around Day 180 of gestation.

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Chapter 3: The Inseminating Bull and Plasma Pregnancy-Associated Glycoprotein (PAG) Levels Affect Peripheral Leukocyte Counts during the Late Pregnancy/Early Postpartum Period in High-Producing Dairy Cows

3. Chapter 3

The Inseminating Bull and Plasma Pregnancy-Associated Glycoprotein (PAG) Levels Affect Peripheral Leukocyte Counts during the Late Pregnancy/Early Postpartum Period in High-Producing Dairy Cows

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Abstract

It has been established that the immunological and endocrine status of the peripartum dairy cow determines the animal's subsequent productive and reproductive performance. Thus, at parturition reduced immune functions of peripheral blood polymorphonuclear cells (PMN) has been observed after a peak in pregnancy-associated glycoproteins (PAGs), and, more recently, the inseminating bull was linked to plasma levels of bovine PAGs in pregnant Holstein-Friesian dairy cows. The present study sought to determine whether changes in leukocyte counts during the peripartum period, indicative of the animal's immune status, could be related to the inseminating bull and to plasma PAG levels. Ninety six clinically healthy, single pregnant cows in a commercial dairy herd were selected. Four samples were collected before parturition (on gestation Days 220–226, 234–240, 248–254, and 262–268) and two samples after parturition (on Days 14–21, and 28–34 postpartum) to analyse total and differential blood cell counts. Based on GLM analysis of variance procedure for repeated measures, the inseminating bull was found to affect counts of total leukocytes and lymphocytes ($P<0.001$; between-subjects effects) throughout the peripartum period. In addition, cows with high plasma PAG levels (>900 ng/mL) on Day 262-268 of gestation had higher numbers of total leukocytes and neutrophils throughout the peripartum ($P<0.001$; between-subjects effects). Young animals (≤ 1 lactation) had higher total leukocyte and lymphocyte counts than older cows (two or more lactations) throughout the study period. These results reveal a clear relationship between the inseminating bull or plasma PAG levels and peripheral leukocyte counts during the peripartum period in dairy cows.

Key words: Sire effect; Pregnancy-Associated Glycoproteins; White Blood Cell Counts; Peripartum Immunity; Dairy Cattle.

3.1 Introduction

Gene selection for milk production is essential in the dairy industry. Indeed, the bulls used in artificial insemination (AI) centres have been attributed a key role in the milk production increase produced in the past decades [1,2]. However, effects other than milk production could be related to the use of a given bull. For example, the sire has been found to affect the establishment and maintenance of pregnancy in different breeds [3-6], the occurrence of placental retention [7] and to determine both gestation length and calf birth-weight (and consequently dystocic parturition) [4,8-10]. In effect, the sire line used to inseminate dams has been linked to a number of factors such as: the immune status of the progeny [10-12]; somatotropin concentrations in the milk-yielding progeny [13,14]; and the levels of several hormones in the inseminated dam including progesterone, oestrogen, placental lactogen and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in pregnant Ayrshire dams [8] and peripartum 17β-estradiol and postpartum $PGF_{2\alpha}$ in pregnant Brahman cows [15]. Recently, the inseminating bull was also found to affect plasma levels of bovine pregnancy-associated glycoproteins (PAGs) in pregnant Holstein-Friesian dairy cows [16,17].

PAGs synthesized by trophoblastic binucleate cells have lately attracted the attention of researchers since, despite their structure indicating they belong to the aspartic proteinase family (a group of proteolytic enzymes related to pepsin, cathepsin D and cathepsin E) [18], their functions are not fully understood and they tend to be enzymatically inactive due to a mutation in their active site [19]. Bovine PAGs have been efficiently used in pregnancy diagnosis as early as on Days 29-30 post-insemination [20-22] and are also often used as indicators of foetal-placental well-being, foetal loss, foetal number and foetal sex [16,23,24].

Some immune-suppressive properties of bovine PAGs and PAG-like molecules (PSPB) have been suggested in cattle [25-27]. In particular, diminished immune functions of peripheral blood polymorphonuclear cells (PMN) have been detected after PAGs peak at parturition [28]. However, this immune suppression effect has yet to be confirmed.

During pregnancy, in response to progesterone, the immune status of the pregnant dam shifts from a T-helper 1 (T_h1) or cellular type to a T-helper 2 (T_h2) or humoral type immunity [29,30]. As a result, the foetus, considered as a semi-allograft, survives inside the mother's uterus [31]. Bearing in mind that white blood cell counts seem to mirror the immunological state of an animal [32-35], the objectives of the present study were: (1) to find out whether the inseminating bull has an effect on total and/or differential leukocyte counts in the inseminated dam during the peripartum period; and (2) to test the relationship between plasma PAG levels and these leukocyte counts in peripartum pregnant dams.

3.2 Materials and methods

3.2.1 Study population and management

The study was performed on 96 high milk-producing non-aborting Holstein-Friesian cows during the peripartum period (last two months of gestation and first month after parturition). All pregnancies were single pregnancies and the dams gave birth to live calves. The commercial dairy herd to which the cows belonged was from north-east Spain, and included a mean of 562 lactating cows during the study period. The animals included in this study conceived from October 2008 to January 2009. Mean annual milk production was 11020 kg per cow. The animals were reared within the herd, milked three times per day

before drying-off and fed complete rations according to NRC recommendations [36]. All animals were bred by AI using semen from six bulls of proven fertility, five Holstein-Friesian bulls (bulls number 1 to 5) and one Limousin breed bull (bull number 6). The bulls were randomly allocated to younger cows or heifers and old cows. The mean annual culling rate for the study period was 28%. All animals were tuberculosis and brucellosis free, as shown by yearly tests from 1985 to 2011.

3.2.2 Pregnancy diagnosis and blood sample collection

Pregnancy was diagnosed by transrectal ultrasound between Days 28-34 postinsemination and confirmed on Day 60. Thereafter, pregnancy was followed by transrectal palpation on Days 90, 120, 150, 180 and at dry-off on Day 210 of gestation. Only confirmed pregnant animals on Day 210 were included in the study. Parturition data were recorded for all animals.

During the study period, six blood samples were collected from each animal. Four samples were collected before parturition (on gestation Days 220-226, 234-240, 248-254 and 262-268) and two samples after parturition (on Days 14-21 and 28-34 postpartum). It was not possible to obtain blood samples at parturition due to farm management policy. The blood samples (10 ml) were collected from the coccygeal vein (or artery) of each animal into EDTA vacuum tubes (BD Vacutainer®, Becton, Dickinson and Company, Plymouth, UK) and 1 ml of the EDTA-anticoagulated whole blood was separated for haematological analysis. The remaining blood was rapidly centrifuged (within 30 min of collection) at 2500 rpm for 10 min, and the plasma stored at -84˚C until serological analysis.

3.2.3 Haematological analysis

Blood samples were automatically analysed on the day of collection using a HEMAVET® HV-950FS Multispecies Haematology System (Drew scientific, inc., Dallas, USA) for total and differential (neutrophils, lymphocytes, monocytes and eosinophils) leukocyte counts. The device was pre-adjusted for cow's blood as specified by the manufacturer.

3.2.4 PAG radioimmunoassay

Plasma PAG concentrations in the samples were determined by double antibody radioimmunoassay (RIA-706) [37,38]. Rabbit polyclonal antiserum AS#706 was raised against caprine $PAG_{55kDa+62kDa}$ (accession numbers P80935 and P80933) and prepared using the method described by Vaitukaitis et al. [39]. Briefly, plasmas (10 µl of each plasma sample) were incubated for 16 h (at room temperature) with 100 µl of tracer (28 000 cpm) and 100 µl of primary antiserum (AS#706 diluted to 1:240 000). The total assay volume was adjusted to 0.5 mL by using Tris-BSA buffer (pH 7.6).The end of the procedure was similar to that previously described [37,38]. Samples with high PAG concentrations (>50 ng/mL) were diluted (1/50, 1/100 and 1/200) and assayed again. Minimum detection limit (MDL) was 1.5 ng/mL. Intra-assay and inter-assay Coefficients of Variances (CV) were 7.8 (4.90 ± 0.38 ng/mL) and 16.0% (4.32 ± 0.69 ng/mL), respectively. Since plasma PAG levels start to vary dramatically close to parturition [23], we only used plasma PAG concentrations in the last prepartum sample (Day 262-268 of gestation) for statistical analysis.

3.2.5 Data collection and statistical analysis

The data obtained for each animal were: semen-providing bull (bulls one to six; from n=9 to n=29); plasma PAG concentration [classified as Low (≤140 ng/mL; n=16); Medium (141-899 ng/mL; n=64) or High (≥900 ng/mL; n=16)]; age (young, ≤ 1 lactation, n=52; and old, two or more lactations, n=44); and total (TLC) and differential leukocyte counts.

The effects of AI semen (Bull), plasma PAG level, age and time (sampling week) on total and differential leukocyte counts were examined using a general linear model (GLM) analysis of variance for repeated measures implemented in the SPSS computer pack v.17 (SPSS Inc., Chicago, IL, USA). The different blood counts were introduced as a within subject factor with six levels (repetitions), and the contrast specified was repeated [40,41]. Following this, Tukey's honestly significant difference (HSD) test was performed on plasma PAG levels and bull groups, whenever significant, for comparisons among the different groups.

Table 3.1. Changes in total and differential leukocyte counts produced over the peripartum period.

Factors	Within-subjects effects	V[a]	df	F	P
Time	TLC[b]	0.765	5	4.975	0.001
	Neutrophils	0.698	5	7.012	<0.001
	Lymphocytes	0.814	5	3.554	0.006
	Monocytes	0.864	5	2.514	0.036

[a] Corresponding *Wilks' Lambda* value in the multivariate analysis.

[b] Total leukocyte counts.

Table 3.2. Factors affecting total (TLC) and differential leukocyte counts during the peripartum period.

Factor	Between-subjects effect	df	F	P
Bull	TLC[a]	5	5.237	<0.001
	Lymphocytes	5	6.732	<0.001
PAG level	TLC[a]	2	8.301	0.001
	Neutrophils	2	5.639	0.005
	Eosinophils	2	4.063	0.021
Age	TLC[a]	1	4.123	0.045
	Lymphocytes	1	7.082	0.009

[a] Total leukocyte counts.

3.3 Results

Mean (±SD) lactation number and milk production on Day 50 postpartum for the entire study population were 2.61 ±1.54 lactations (range 1 to 8 lactations; counting in the lactation which has just started) and 42.8 ±8.8 kg (range 24 to 63 kg), respectively. Mean plasma PAG concentration on Day 262-268 of gestation was 539 ng/mL (ranging from 76 ng/mL to 3463 ng/mL).

Changes produced over time (within-subject effects) and factors affecting (between-subject effects) total and differential leukocyte counts during the peripartum period are shown in Tables 3.1 and 3.2, respectively. No interactions were found.

3.3.1 Effect of time

According to GLM repeated measures analysis of variance, numbers of total leukocytes, neutrophils, lymphocytes and monocytes varied significantly during the peripartum period (Table 3.1). Total leukocyte and neutrophil counts increased up to Weeks -4 and -2, respectively (Figs. 3.1a, 3.1b), lymphocytes gradually decreased throughout the peripartum period (Fig. 3.1b), whereas monocytes sharply increased between the 2nd and 4th postpartum weeks (Fig. 3.1c).

Table 3.3. Tukey's Post Hoc Honestly Significant Difference (HSD) multiple comparisons analysis of total leukocyte (TLC) and lymphocyte counts (means ± SD) for the different semen-providing bulls*.

	Bull 1 (*n*= 18)	Bull 2 (*n*= 11)	Bull 3 (*n*= 9)	Bull 4 (*n*= 12)	Bull 5 (*n*= 29)	Bull 6 (*n*= 17)
TLC	8.05[a] (±2.17)	7.26[a b] (±1.98)	7.7[a b] (±1.70)	6.17 [c] (±1.90)	6.67 [b c] (±1.64)	5.88 [c] (±1.64)
Lymphocytes	3.3[a] (±1.16)	2.48 [b] (±0.96)	2.99 [a] (± 0.94)	2.22 [b] (±0.78)	2.30 [b] (±0.76)	2.16 [b] (±0.73)

* Different within-row letters denote significant differences: (P≤0.01)

3.3.2 Inseminating-bull effect

Cows classified according to the inseminating bull showed significantly different TLC and lymphocyte counts during the study period (Table 3.2). Tukey's HSD test for multiple comparisons revealed that cows inseminated with semen from Bull 1 had a higher TLC than cows inseminated with semen from Bulls 4, 5 and 6 (P<0.001; Table 3.3). The overall; of the six samples, mean differences in TLC between Bull 1 and Bulls 4, 5 and 6 were found to be 1880, 1380 and 2170 cells/µL peripheral blood, respectively. Whilst, TLC of Bull 1 was not statistically different from Bulls 2 and 3. Similarly, lymphocyte counts in cows inseminated

with semen from Bull 1 were higher than in those inseminated with semen from Bulls 2, 4, 5 and 6 (P<0.01; Table 3.3). In addition, TLC in cows inseminated with semen from Bulls 2 and 3 were higher than in cows inseminated with semen from Bulls 4 and 6 (P<0.01). Figure 3.2 shows one example representing significant differences between two bulls. Number of inseminated animals with Bulls 1 to 6 were 18, 11, 9, 12, 29 and 17, respectively.

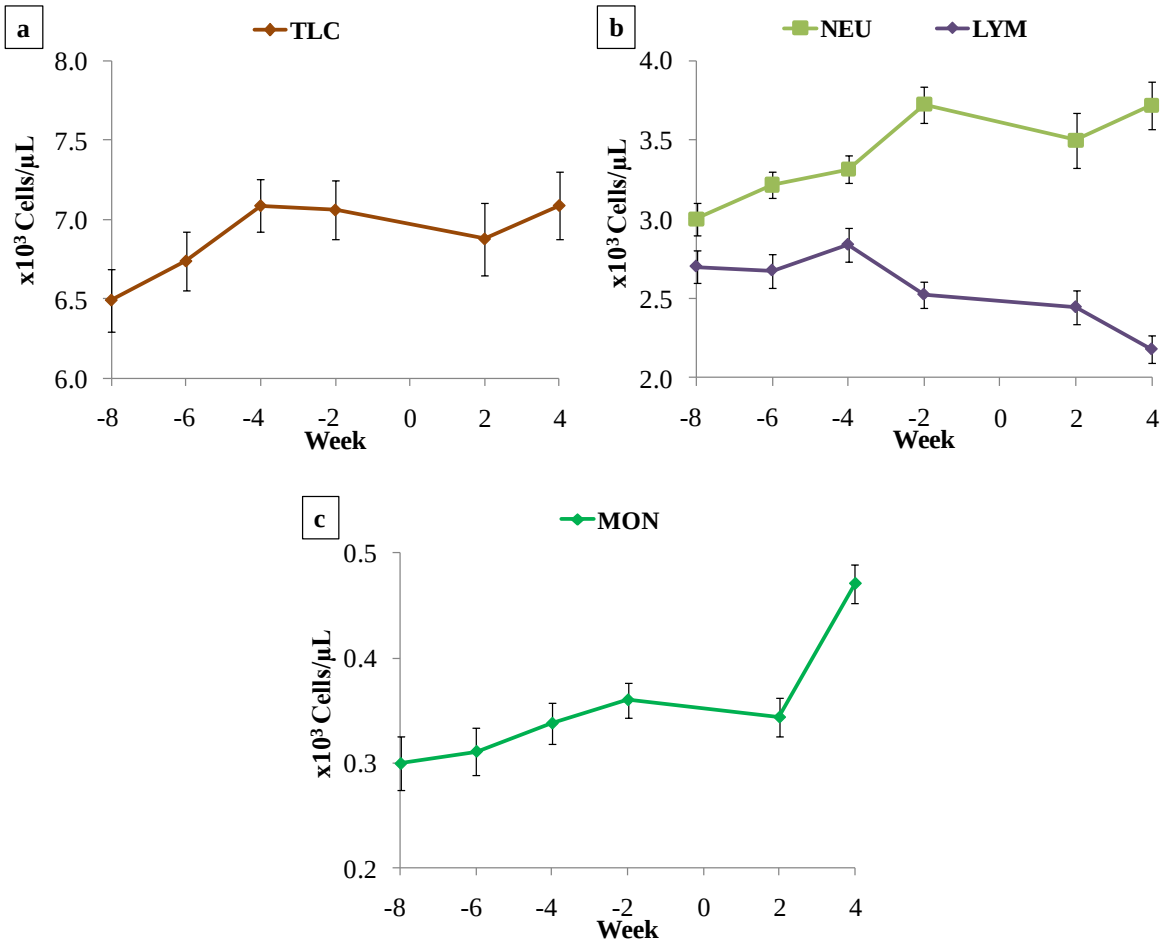

Figure 3.1. Means ±SEM of total leukocyte (TLC) (a), neutrophil (NEU) and lymphocyte (LYM) (b) and monocyte (MON) (c) counts throughout the peripartum period (0 = parturition).

3.3.3 Effects of plasma PAG levels

Animals grouped according to plasma PAG levels on gestation Day 262-268 showed significantly different TLC and neutrophil counts during the study period (P=0.001 and P=0.005, respectively; Table 3.2). Tukey's HSD test for multiple comparisons revealed that animals with high plasma PAG levels had higher TLC and neutrophil counts than those with medium or low plasma PAG levels (P<0.001 and P=0.01, respectively; Table 3.4 and Figure 3.3).

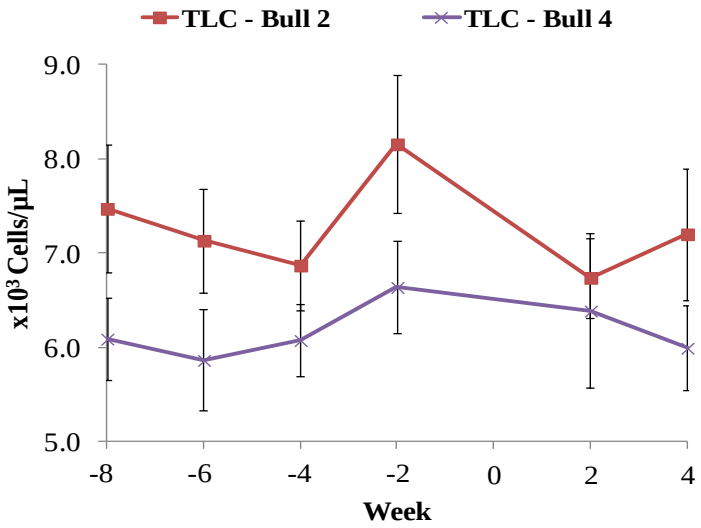

Figure 3.2. Example representing significant (P<0.01) differences of total leukocyte (TLC) counts between two bulls. Mean ± SEM TLC counts during the peripartum period (0 = parturition) for the bulls two and four. Number of inseminated animals for each bull were 11 and 12, with a mean (± SD) age of cows inseminated with each bull of 3.5 (±1.2) and 3.5 (±1.8) lactations, respectively.

3.3.4 Age effects

Throughout the study period, young cows had higher total leukocyte and lymphocyte counts than older cows (P=0.045 and P=0.009, respectively; Table 3.2 and Figure 3.4).

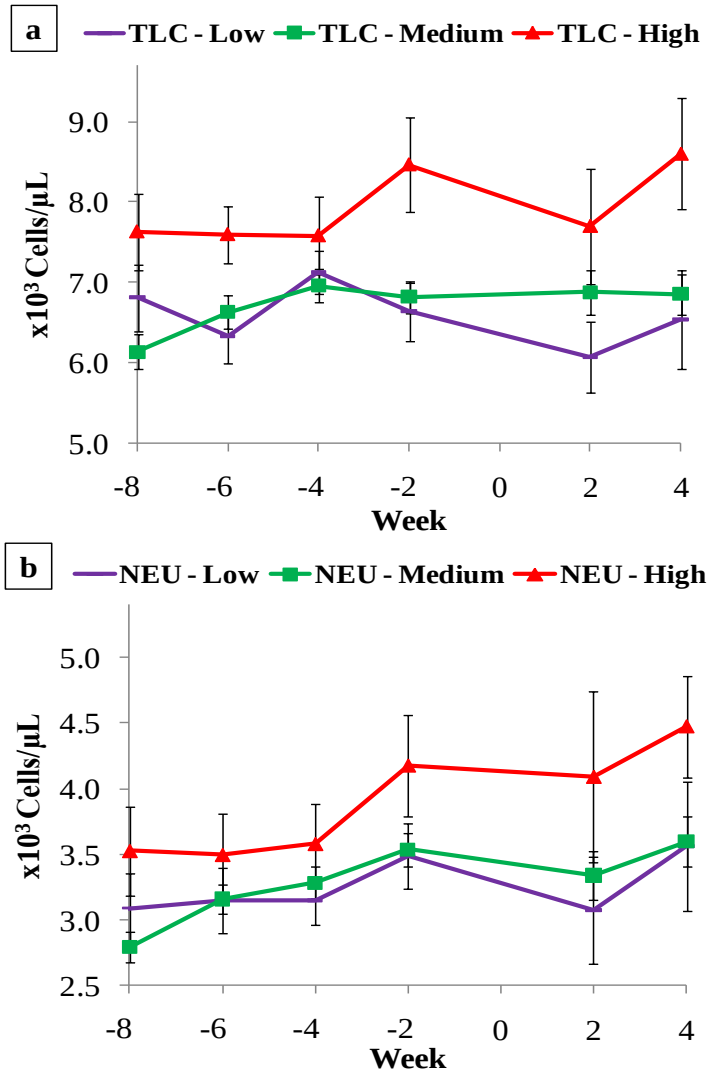

Figure 3.3. Means ±SEM of total leukocyte (TLC) (a), and neutrophil (NEU) (b) counts during the peripartum period (0 = parturition) for the different plasma PAG levels classified as Low (≤140 ng/mL; n=16); Medium (141-899 ng/mL; n=64) or High (≥900 ng/mL; n=16).

Table 3.4. Tukey's Post Hoc Honestly Significant Difference (HSD) multiple comparisons analysis for total leukocyte (TLC), neutrophil and eosinophil counts (means ± SD) for the different plasma PAG groups*.

	Low (≤140 ng/mL; n=16)	Medium (141-899 ng/mL; *n*=64)	High (≥900 ng/mL *n*=16)
TLC	6.58 (±2.08) [a]	6.71 (±1.83) [a]	7.93 (±2.11) [b]
Neutrophils	3.25 (±1.24) [c]	3.28 (±1.16) [c]	3.89 (±1.45) [d]
Eosinophils	0.43 (±0.32) [e]	0.51 (±0.44) [ef]	0.64 (±0.49) [f]

* Different within-row letters denote significant differences: a vs. b, $P < 0.001$; c vs. d, $P=0.01$ and e vs. f, $P < 0.05$.

3.4 Discussion

Our study shows for the first time that the inseminating bull can affect peripheral leukocyte and lymphocyte counts in the inseminated dam both during late gestation and the early postpartum. Also, groups of animals established according to plasma PAG level on Day 262-268 of gestation were related to changes in peripheral leukocyte counts throughout the peripartum period.

Different bulls were associated with different levels of circulating white blood cells. This finding implies that, depending on the heterogeneity between sire and dam, some sires are capable of stimulating the mother's immunity during gestation and even beyond after parturition. It could also mean that immunity during pregnancy is not solely dependent on the dam but rather depends on both dam and foetus, whereby the sire plays an indirect role. Hence, although PAG-bull interactions could not be detected in the present study, the effect of the bull on PAG levels could explain the bull effect on white blood cells. In effect, the sire

of foetus has been identified as an important factor affecting levels of plasma PAGs in AI cows [16,17], and our results showed a clear relationship between high plasma PAG levels and elevated total leukocyte and neutrophil counts. Thus, some bulls and high plasma PAGs were related to higher leukocyte counts. Whether the herein reported PAGs effect is associated with activated immunity or not needs further investigation. In disagreement, other studies have suggested that PAGs could participate in the peripartum immune-suppression state [25-28].

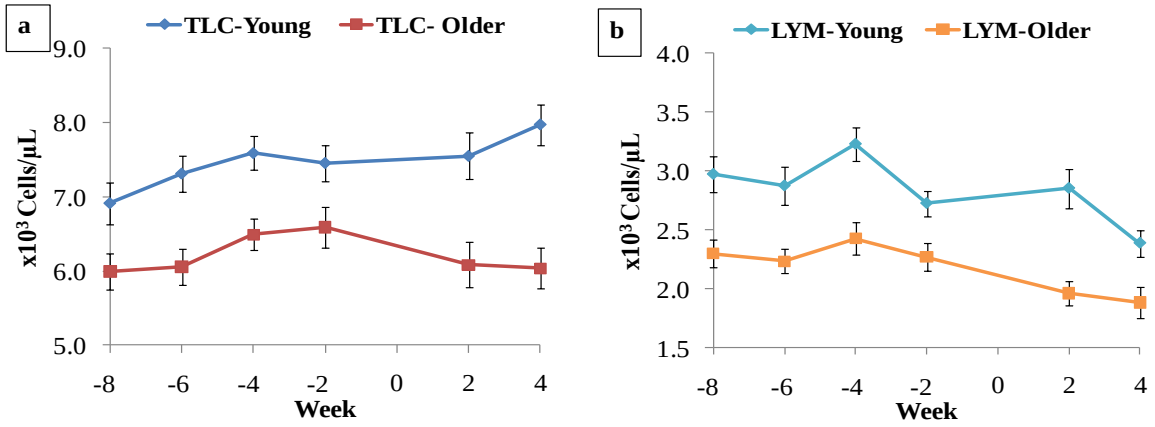

Figure 3.4. Means ±SEM of total leukocyte (TLC) (a) and lymphocyte (LYM) (b) counts during the peripartum period (0 = parturition) for young (≤ 1 lactation) and older cows (two lactations or more).

Although not examined in the present study, heterogeneity between maternal and paternal major histocompatibility complex (MHC) molecules (indirectly presented through the fetus) could be another possible explanation for the observed bull effect. MHC molecules are cell-markers through which the immune system is able to differentiate self cells from non-self cells. In fact, down-regulation of MHC-1 on foetal trophoblast cells (at the site of foetal-maternal connection, the placentome) occurs during pregnancy [42,43], and this is likely to promote the immune-invisibility of the foetus and consequently its survival inside the mother. This down-regulation occurs in all mono-nucleated trophoblast cells (accounting for most

trophoblast cells) [44,45]. However, some bi-nucleated trophoblast cells might still express a mixture of paternal and maternal MHC-1 molecules [42,43], probably establishing different levels of foetal exposure to the mother's immune system, eventually leading to different leukocyte counts. Further investigations are needed to clarify the nature of the bull effect on leukocyte counts.

The age-related difference observed here in total leukocytes, with lower numbers found in older cows, is in agreement with previous reports [46,47]. Similarly, the augmented patterns of total leukocyte, neutrophil and monocyte counts, and decreased lymphocyte counts detected over time are similar to those reported previously [48-51].

In conclusion, we show here, for the first time, that maternal peripheral total and differential blood cell counts during the peripartum are affected by the inseminating bull and that higher maternal plasma PAG levels are linked to higher peripheral total leukocyte and neutrophil counts.

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Chapter 4: Peripheral leukocyte counts in dairy cows chronically infected with *Coxiella burnetii* and/or *Neospora caninum* during the peripartum period

4. Chapter 4

Peripheral leukocyte counts in dairy cows chronically infected with *Coxiella burnetii* and/or *Neospora caninum* during the peripartum period

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Abstract

Coxiellosis and neosporosis are two different diseases caused by intracellular microorganisms, *C. burnetii* and *N. caninum*. Both agents are associated with reproductive problems and abortion in cattle, and, in addition, *C. burnetii* is zoonotic (causing human Q fever). For both diseases the host-immune relationships during gestation are still under investigation. The present work aimed at analysing the peripheral leukocyte counts in the peripartum period (from 8th prepartum to 4th postpartum week) in *C. burnetii* and/or *N. caninum* infected dairy cows in field conditions. A total of 121 pluriparous dairy cows were randomly selected from a high-milk producing herd. Of those 41 were control, 15 were *N. caninum* infected, 55 were *C. burnetii* infected and 10 were both *N. caninum* and *C. burnetii* infected. Blood samples were withdrawn, every two weeks, and total and differential leukocyte counts were analysed using an automatic haematology system. Effects of *N. caninum-C. burnetii* interaction on total and differential leukocytes were analysed using mixed models procedure. The effects of time, age, parturition season, twin pregnancy and inseminating bull were also evaluated. Significant changes of blood total leukocytes, neutrophils (numbers and percentages) and lymphocytes (percentages) were observed due to *N. caninum-C. burnetii* interaction, while eosinophils were only affected by *N. caninum* infection. In addition, none of the cows aborted which indicated that although the immune system acted in different ways towards the existing infections, this immune response did not provoke abortion. Earlier leukocyte changes (increased neutrophils numbers and percentages and decreased eosinophils numbers), around Day 234 of gestation, were noted in *N. caninum* infected cows, suggesting a recrudescence at that time, and hypothesizing that co-infection with *N. caninum* could have activated the immune system against existing *C. burnetii* infection (in cows infected with both *N. caninum* and *C. burnetii*). The present work provides

an insight to the peripartum cellular immune responses taken place in cattle infected with *N. caninum* and/or *C. burnetii*. Although both are intracellular organisms, different peripheral cellular immune responses were observed towards infection with *N. caninum*, *C. burnetii* or both.

Key words: Coxiellosis, Neosporosis, Intracellular Microorganisms, Immunity, Peripheral Blood Cell Counts, Haematology.

4.1 Introduction

Coxiellosis in animals (Q fever in humans) is a worldwide zoonotic disease, caused by *Coxiella burnetii*; an intracellular gram-negative bacteria (Arricau-Bouvery and Rodolakis, 2005; Guatteo et al., 2011). *C. burnetii* is highly resistant and it survives harsh environmental conditions (Arricau-Bouvery and Rodolakis, 2005), thus causing a significant source of infection between animals and to human [For more information on sources and routes of infection, antigenic phases and clinical manifestations in human and animals, please refer to (Arricau-Bouvery and Rodolakis, 2005; Parker et al., 2006; Angelakis and Raoult, 2010)]. Abortion, mainly in small ruminants, and increased incidence of retained placenta, in dairy cows, have been attributed to *C. burnetii* infection (Berri et al., 2002; López-Gatius et al., 2011), although it is frequently asymptomatic in most animals (Angelakis and Raoult, 2010). Recently, the human Q fever outbreak in 2007 in the Netherlands has drawn more attention, both public and scientific, towards coxiellosis in ruminants; as the source of infection was nearby infected dairy ovine and caprine flocks (Karagiannis et al., 2009; Roest et al., 2011). *C. burnetii*, as an intracellular microorganism, typically activates the cell-mediated immune response, however antibody-mediated type immunity also plays an important role in protection against *C. burnetii* (Shannon and Heinzen, 2009; Chen et al., 2011). In mice, it was demonstrated that the pregnancy-caused suppression of cell-mediated immunity led to reactivation of persistent *C. burnetii* infection (Sidwell and Gebhardt, 1966 cited in Shannon and Heinzen, 2009). The existing information of the host immune-responses toward *C. burnetii* infection is still incomplete.

Neosporosis is a disease affecting most warm-blooded animals caused by the intracellular protozoan parasite *Neospora caninum*, first described in the 80´s, that is now

considered as a main cause of abortion in cattle worldwide (Anderson et al., 1991; Dubey and Lindsay, 1993; Dubey, 2003). Higher abortion rates, due to neosporosis, in pregnant cows are observed mainly in the second trimester of gestation (Anderson et al.; 2000, Jenkins et al.; 2000, López-Gatius et al.; 2004a, López-Gatius et al., 2004b). In cows, congenital infection is the major route of infection (Schares et al., 1998; López-Gatius et al., 2004a) reaching as high as 95% (Davison et al., 1999). If no abortion occurs, the majority of the foetuses will remain infected for life and maintain the infection in the herd (Paré et al., 1996; Anderson et al., 1997). Effective immunity against *N. caninum* is carried out by the cellular type (T_{h1} mediated) (Boysen et al., 2006; Klevar et al., 2007). In addition, IFNγ played an important role in protection against *N. caninum*-caused abortion in dairy cows (Lopez-Gatius et al., 2007; Williams et al., 2007; Almeria et al., 2009). Moreover, T_{h1} (IFNγ, TNFα and IL-12p40) and T_{reg} (IL-10) cytokines genes were up-regulated in *Neospora*-seropositive pregnant cows (Almeria et al., 2012), underlining their importance in immunity against *N. caninum*.

During gestation there is a clear immunomodulation and the immune response against pathogens can be different than that in non-pregnant animals. In fact, attenuated cell-mediated immunity (T_{h1}) during pregnancy is indispensable for foetal-survival inside the mothers' uterus. However, such weakened immune system gives opportunities for the reactivation of dormant infections, especially those controlled via cell-mediated immunity such as neosporosis or coxiellosis. Due to the gestation-effect on the immunity and the incomplete information on both *N. caninum* and *C. burnetii* host-immune relationships, the aim of the present study was to evaluate changes in peripheral leukocyte counts, as an indicator for immune status, in *C. burnetii* and/or *N. caninum* naturally infected dairy cows during the peripartum period.

4.2 Materials and Methods

4.2.1 Study population and management

The study was performed on 121 high milk-producing Holstein-Frisian cows during the peripartum period (from 8th prepartum week to 4th postpartum week). The study cows were clinically healthy animals that belonged to a commercial dairy herd from north-east Spain. The herd included a mean of 562 lactating cows during the study period. Mean annual milk production per cow and culling rate in the herd were 11020 kg and 28%, respectively. The cows were reared within the herd, milked three times per day and fed complete rations according to NRC recommendations (Subcommittee on Dairy Cattle Nutrition, Committee on Animal Nutrition et al. 2001) (NRC, 2001). All animals were bred by AI using semen from bulls of proven fertility. Pregnancy was diagnosed by trans-rectal ultrasound between Days 28-34 post-insemination and confirmed on Day 60. Afterwards, pregnancy was followed-up by trans-rectal palpation on Days 90, 120, 150, 180 and at dry-off on Day 210 of gestation. Only confirmed pregnant animals on Day 210 were included in the study. Parturition data were recorded for all animals; even if they abort (no abortion-based exclusion from the study was done). All animals were tuberculosis and brucellosis free, as shown by yearly tests from 1985 to 2011. The herd had previously confirmed cases of *Neospora caninum* infection in aborted foetuses, though dogs have no access to the farm. Mean *N. caninum* and *C. burnetii* seroprevalence in the herd were 10% and 50% during the study period, respectively.

4.2.2 Blood samples collection

Six blood samples were collected during the peripartum period, four samples were collected on gestation Days 220-226, 234-240, 248-254 and 262-268, and two samples after parturition on Days 14-21 and 28-34 postpartum (representing weeks -8, -6, -4, -2 ,2 and 4, respectively). However, it was not possible to obtain blood samples at parturition due to farm management instructions. The blood samples (10 ml) were collected from the coccygeal vein/artery of each animal into EDTA vacuum tubes (BD Vacutainer®, Plymouth, UK) and 1 ml of the EDTA-anticoagulated whole blood was separated for haematological analysis. The remaining blood was rapidly centrifuged (within 30 min of collection) at 2500 rpm for 10 min, and the plasma was stored at -26˚C until serological analyses.

4.2.3 Haematology

Blood samples were automatically analysed (as previously described in Abdelfatah-Hassan et al., 2012) on the day of collection using a HEMAVET® HV-950FS Multispecies Haematology System (Drew scientific, inc., Dallas, USA) for total and differential (neutrophils, lymphocytes, monocytes and eosinophils) leukocyte counts. The device is adjusted for cow's blood analysis as specified by the manufacturer. Total leukocytes results are obtained in numbers (thousand cells/ml), while differential leukocytes are obtained in both numbers (thousand cells/ml) and percentages of the total leukocytes.

4.2.4 Serology

Serological tests for *Neospora* and *Coxiella* infection were performed during annual screening for brucellosis. *N. caninum* and *C. burnetii* seropositivity were defined as a positive blood test obtained 0–365 days before pregnancy diagnosis on Day 90 of gestation.

4.2.4.1 Anti-Coxiella burnetii antibodies detection

Antibodies against *C. burnetii* in the plasma samples were analysed by an indirect ELISA using CoxLS kit (LSIVET RUMINANT Milk/Serum Q FEVER from Laboratoire Service International, Lissieu, France) which was performed following the manufacturers' instructions. A mixture of both *C. burnetii* antigenic phases (phases I and II; isolated from domestic ruminants by INRA, Nouzilly, France) was coated on the ELISA plates for the detection of total anti-*C. burnetii* immunoglobulin G antibodies (IgG), as specified by the kit manufacturer. The resulting optical densities (ODs) were read at 405 nm wave-length filter (with 620 nm as a reference filter) on a Multiskan® FC plate reader (Thermo Fisher Scientific, Vantaa, Finland). For each sample, the sample-to-positive (S/P) ratio was calculated as follows: $(OD_{sample} - OD_{negative\ control})/(OD_{positive\ control} - OD_{negative\ control})$, and then expressed as titers (titer = S/P × 100). A Sample was considered to be *C. burnetii* positive when a titer > 40 was obtained.

4.2.4.2 Anti-Neospora caninum antibodies detection

Samples were tested for the presence of *N. caninum* antibodies using an indirect ELISA kit (CIVTEST® NEOSPORA; Hipra, Girona, Spain) based on the whole tachyzoite lysate of *N. caninum* (NC-1). This test was previously validated (López-Gatius et al., 2004b)

and the procedure followed the manufacturers' instructions. The resulting optical densities (ODs) were read at 405 nm wave-length filter (with 620 nm as a reference filter) on a Multiskan® FC plate reader. For each sample, the titration was calculated as follows: [(OD $_{\text{sample}}$ - OD $_{\text{negative control}}$)/(OD $_{\text{positive control}}$ - OD $_{\text{negative control}}$) x 100]. A Sample was considered to be *N. caninum* positive when a titer > 9 was obtained.

4.2.5 Statistics

The data obtained for each animal were: *Neospora*-seropositivity, *Coxiella*-seropositivity and total and differential leukocyte counts. Also, data related to other factors that was previously shown to affect peripheral leukocyte counts (Serrano et al., 2011, Abdelfatah-Hassan et al., 2012) were analysed: in particular, Age (Young = 2 lactation; n=36, and Older ≥ 3 lactations; n=85, based on the lactation which starts after parturition), season of parturition (Cold, from October to April; n=18, and Warm, from May to September; n=103), twin-pregnant or not (Twin; n=13, and Single; n=108) and artificial-insemination (AI) bull (8 groups). Four classes (groups) were considered in relation to *N. caninum*-*C. burnetii* interaction: seronegative to both *N. caninum* and *C. burnetii* (serve as a Control group), *N. caninum*-seronegative and *C. burnetii*-seropositive, *N. caninum*-seropositive and *C. burnetii*-seronegative and seropositive to both *N. caninum* and *C. burnetii*.

The data was analysed via mixed models procedure for repeated measures using PASW statistics 18 package (SPSS Inc., Chicago, IL, USA). Different models were fitted, using different covariance structures, and the best model was selected based on the best AIC and BIC information criterions. The final mixed model included the effects of time (sampling weeks), neosporosis, coxiellosis and their interaction (as factors) on total and differential

leukocyte counts throughout the study period (as dependent variables). The remaining factors (age, season, twins and AI bull) were also included as factors in the same mixed model analysis to account for their effects. The level of significance was set at P-value ≤0.05.

4.3 Results

Of the 121 study cows 33.9% (n=41) were seronegative for both *N. caninum* and *C. burnetii*, 12.4% (n=15) were only *Neospora*-seropositive, 45.5% (n=55) were only *Coxiella*-seropositive and 8.3% (n=10) were seropositive for both *N. caninum* and *C. burnetii*. (20.7% of the study cows were *N. caninum* seropositive; n=25, and 53.7% of the study cows were *C. burnetii* seropositive; n=65).

4.3.1 Effects of Neosporosis and/or Coxiellosis

Total leukocyte counts (TLC) shows significant changes during the peripartum period among the different groups (F=4.36, P=0.038; figure 4.1a). TLC of cows seropositive only to *C. burnetii* showed the highest levels throughout the study, while cows seropositive to *N. caninum* showed the lowest number of TLC, with the exception of a punctual peak on the 2nd postpartum weak in which this group showed the highest levels in that study week. Meanwhile, TLC of cows seronegative for both *N. caninum* and *C. burnetii* (Control) were between the previous two groups, though were slightly higher than in *N. caninum* seropositive cows. Finally, TLC of cows seropositive for both *N. caninum* and *C. burnetii* were comparable to those of the Control cows.

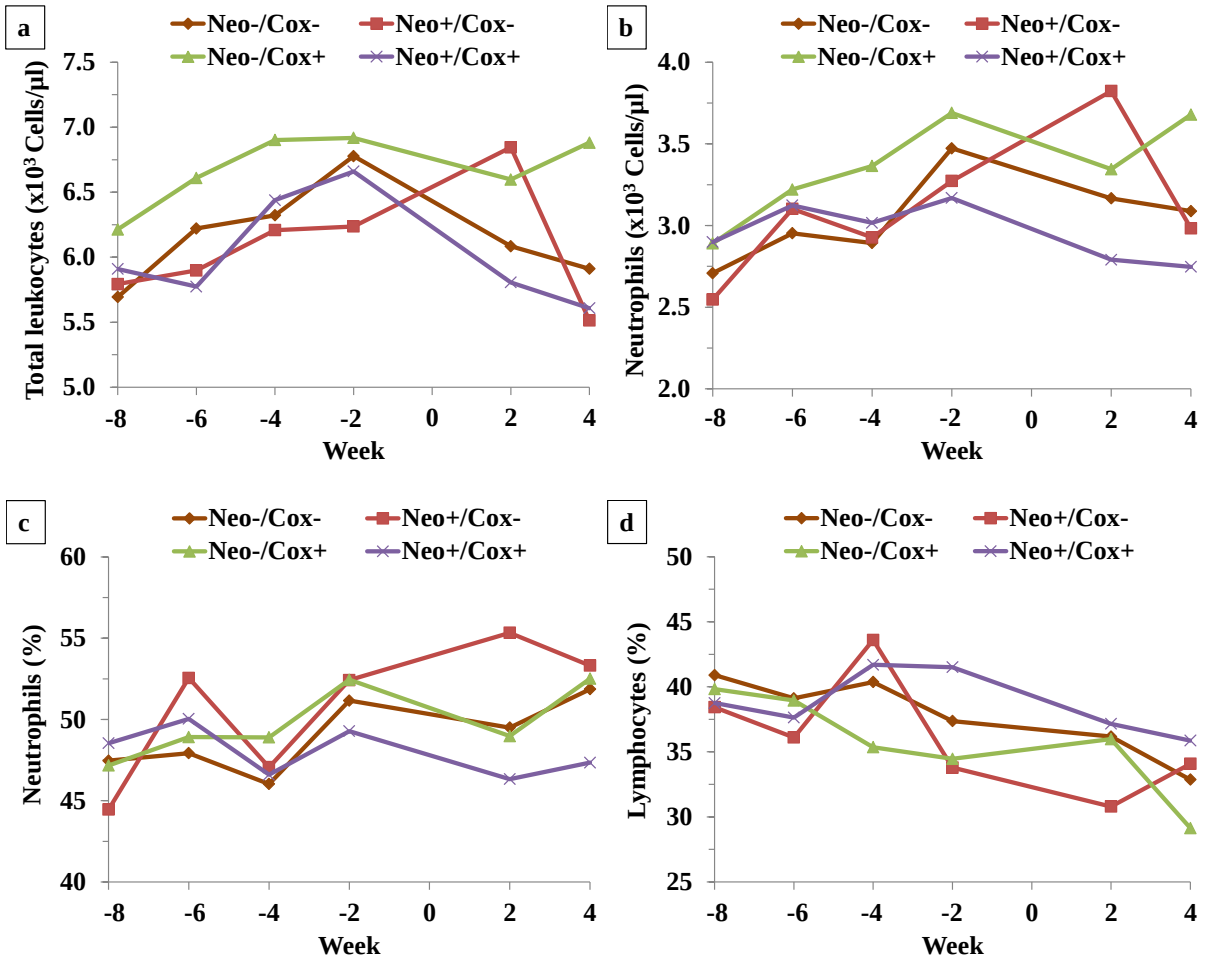

Figure 4.1. Changes in a) total leukocytes, b) neutrophil numbers, c) neutrophil percentages and d) lymphocyte percentages in response to *N. caninum* (Neo) and *C. burnetii* (Cox) interaction throughout the peripartum period in dairy cows. (+ = seropositive, - = seronegative, week 0 = parturition).

Infection with *N. caninum* and/or *C. burnetii* significantly affected neutrophil numbers and percentages (F=6.11, P=0.014 and F=5.87, P=0.016, respectively; figure 4.1b & 4.1c). Neutrophil numbers followed a similar pattern to that of total leukocytes, with slight differences. Again the highest levels were observed in *Coxiella*-seropositive animals during the study period, with exception of the 2nd postpartum sample in which a punctual increase of neutrophils (higher than those observed in *Coxiella*-seropositive cows) was observed in *Neospora*-seropositive animals. Prepartum neutrophil numbers were not significantly

different in *N. caninum*-seropositive cows compared to those of the seronegative cows or to those seropositive for both *N. caninum* and *C. burnetii*.

In contrast to neutrophil numbers, higher percentages of circulating neutrophils were found in *N. caninum*-seropositive cows, with two peaks on 6th prepartum and 2nd postpartum weeks. Neutrophil percentages of cows *C. burnetii*-seropositive or seronegative for both *N. caninum* and *C. burnetii* had fairly similar percentages of circulating neutrophils. Cows seropositive for both *N. caninum* and *C. burnetii*, had the lowest neutrophil numbers and percentages starting after the 4th prepartum till the 4th postpartum weeks.

Lymphocyte percentages also showed changes in the peripheral blood in relation to *N. caninum* and/or *C. burnetii* infection (F=5.20, P=0.024; figure 4.1d). Past the 6th prepartum week, higher lymphocyte percentages were observed in cows seropositive for both *N. caninum* and *C. burnetii*. Cows seropositive for *N. caninum* had lower percentages of circulating lymphocytes, except for a peak on the 4th prepartum week and increased later on the 4th postpartum week. Lymphocyte percentages were not drastically changed during the study period in cows seronegative for both *N. caninum* and *C. burnetii* and in cows seropositive only for *C. burnetii*, although the overall level was lower in the latter group. Lymphocyte numbers in the different groups followed a comparable pattern to that of lymphocyte percentages; however they failed to reach statistical significance.

Eosinophils were significantly changed considering only *N. caninum* infection (F=5.55, df=1, P=0.019; figure 4.2). Cows that were *N. caninum*-seropositive (n= 25, 20.66% of the study cows) had lower eosinophil numbers during the study period, which reached its lowest levels on the 6th prepartum week. Meanwhile, *N. caninum*-seronegative cows showed

slight increasing levels prepartumly which fell down approaching parturition and again increased postpartumly.

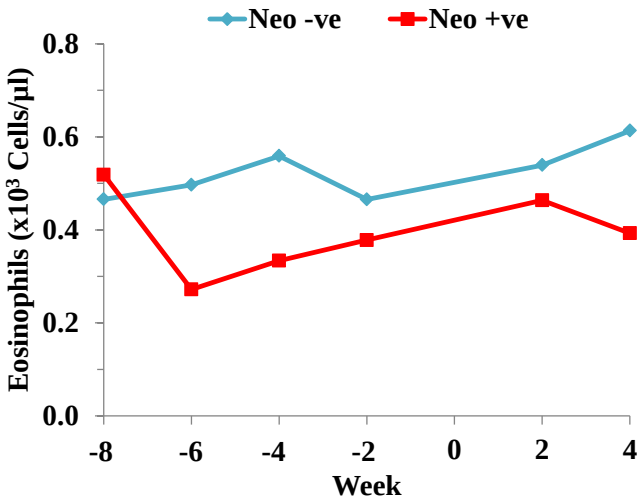

Figure 4.2. Changes in eosinophil numbers between *N. caninum* seropositive (Neo +ve) and seronegative (Neo -ve) dairy cows throughout the peripartum period. (week 0 = parturition)

4.3.2 Other factors included in the statistical analysis

Significant changes in relation to time were found in TLC, neutrophils (number and percentage), lymphocytes (number and percentage) and monocytes ($P \leq 0.01$). Numbers of TLC, neutrophils, monocytes, and eosinophils showed an increasing pattern throughout the study period, while lymphocytes showed a decreasing pattern (Data not shown). In addition, cows' age affected peripheral leukocytes, where lower numbers of TLC, neutrophils, lymphocytes and monocytes obtained in cows with 3 or more lactations ($P<0.01$, $P=0.03$ for monocytes). Moreover, cows calving during the cold season showed significant higher numbers of TLC, neutrophils, monocytes and eosinophils ($P \leq 0.01$), while they showed significant lower lymphocyte numbers and percentages ($P<0.05$ and $P<0.01$, respectively). Furthermore, twin pregnant cows had lower TLC, neutrophils numbers and percentages and

eosinophil numbers ($P \leq 0.01$ and $P=0.05$ for eosinophils), however they had higher lymphocyte percentages ($P<0.01$) compared to single pregnant animals. Maternal TLC, neutrophil numbers, lymphocyte percentages and numbers and percentages of monocytes were significantly affected by the different AI bulls used ($P<0.01$ and $P=0.02$ for lymphocytes %).

4.4 Discussion

The present study showed clear differences in the peripheral total and differential leukocyte counts, indicative of cow's immune status, in response to natural infection with *N. caninum* and/or *C. burnetii* during the peripartum period. Seropositive cows to *C. burnetii* showed the highest TLC during the study period, while *N. caninum* seropositive cows showed the lowest TLC during the study period with exception of a peak at 2nd postpartum week. Seronegative cows and seropositive cows to both pathogens showed intermediate levels between the other groups. Higher total leukocytes seen in cows seropositive only to *C. burnetii* was a reflection to higher neutrophils. In addition, past the 6th prepartum week and coming closer to parturition differential leukocyte counts started to change remarkably, especially in *N. caninum* infected cows (without or with *C. burnetii* co-infection).

Changes in differential leukocyte counts, and subsequently total leukocytes, in *N. caninum* infected cows starting after the 6th prepartum week (very low eosinophil numbers plus a peak in neutrophil percentages on the 6th prepartum week followed by a peak in lymphocyte numbers [not shown] and percentages on the 4th prepartum week) could only suggest a reactivation of *N. caninum* infection around that time point. On the 6th prepartum week in *N. caninum* infected cows eosinophil numbers were at their lowest level, which

undoubtedly contributed to the appearance of the neutrophil percentages peak of the 6th prepartum week. Later on and depended on whether the cows were only *N. caninum* or *N. caninum* and *C. burnetii* infected different subsequent immune responses were obtained (detailed in the following paragraph). Thus, hypothesizing that co-infection with *C. burnetii* and *N. caninum*, by some mechanisms, led to a co-activated immune response against either *C. burnetii* or *N. caninum* infection or both.

Higher percentages of lymphocytes and lower numbers and percentages of neutrophils were observed in cows seropositive to both *N. caninum* and *C. burnetii* but not in cows only seropositive to either of *N. caninum* (except the neutrophils % peak on the 6th prepartum week) or *C. burnetii*. Additionally, lymphocyte percentages peaked on the 4th prepartum week in *N. caninum* infected cows and afterwards fell down in only *N. caninum* infected cows, whereas continued at higher levels in both *N. caninum* and *C. burnetii* infected cows. Meanwhile, lymphocyte percentages in only *C. burnetii* infected cows were low throughout the study. Furthermore, neutrophil numbers and percentages were not different between the 8th and the 6th prepartum week between only *C. burnetii* infected and both *N. caninum* and *C. burnetii* infected cows. However, starting from the 6th prepartum week they increased in cows infected with only *C. burnetii* and decreased gradually in cows infected with both *N. caninum* and *C. burnetii* throughout the study. Taken together all of the above reinforce the assumption that co-infection with either pathogen changed the immune response against the other one during the peripartum period, yet it is not clear which infection was in charge.

As suggested above, a reactivation of *N. caninum* infection could have taken place around the 6th prepartum week (Days 234-240 of gestation). A possible explanation to the observed differences in leukocyte subpopulations would be that *N. caninum* reactivation was

the responsible for indirectly/accidentally launching the immunity against *C. burnetii* and resulted in different total and differential blood counts in cows infected with both *N. caninum* and *C. burnetii*. At the placental level, maternal immune system was induced by recrudescent *N. caninum* infection between gestation weeks 20-33 (140 to 230 days of gestation), and this activation was supposed to be non-detrimental to the foetus however essential for the control of placental parasitosis (Rosbottom et al., 2011). The present results are in agreement with those results, as *N. caninum* infected cows in this study were non-aborting and showed evidences of reactivated immunity around the same gestation days (Day 230 of gestation). They found, in addition, that 2-4 weeks after recrudescence a placental infiltration with lymphocytes ($CD4^+$ and $CD8^+$ T-cells), plus an up-regulated cytokine gene expression (such as IL-4 and IFNγ and to a lesser extent IL-12 p40, IL-10 and TNFα). Similarly, at the peripheral blood mononuclear cells (PBMC; i.e. lymphocytes) level, an elevated expression of IFNγ, TNFα, IL-12p40 and IL-10 has been found throughout gestation (at least from days 45 till 210) in non-aborting naturally *N. caninum*-infected cows in our same geographical zone (Almeria et al., 2012). The above results, together with our results, show that maternal immune system during pregnancy is able at least to control the reactivated persistent infection and not provoking abortion, if not at all controlling the rate of placental and foetal infection.

Virulent *C. burnetii* evades being killed within the mononuclear phagocytic cells by controlling important steps during the phagocytosis process (preventing phagolysosomal fusion), whilst addition of IFNγ restored their phagocytic activity; by means of phagosome maturation and phagosome alkalinization (Ghigo et al., 2004), and *N. caninum* reactivation proved to increase the expression of IFNγ (Rosbottom et al., 2011, Almeria et al., 2012). Moreover, Andoh and others (Andoh et al., 2007), using mouse as a model for acute Q fever infection, found that T-lymphocytes-deficient and IFNγ-knockout mice show increased

susceptibility to *C. burnetii* infection, and it was shown that *N. caninum* reactivation also resulted in increased T-lymphocytes at least as infiltration at the placental level (Rosbottom et al., 2011). Therefore, elevated expression of IFNγ, TNFα, and other T_{h1} cytokines, at the PBMC and placental levels, plus lymphocytes infiltration to the placenta in response to *N. caninum* reactivation (Rosbottom et al., 2011, Almeria et al., 2012), reinforce our hypothesis that *N. caninum* triggered the immune response against *C. burnetii* infection. Especially that, higher lymphocyte percentages were found in cows infected with both *N. caninum* and *C. burnetii* than in cows infected only with either. The triggering mechanism by which *N. caninum* indirectly/accidentally activated the immune responses against *C. burnetii* or both infections is yet to be investigated.

Levels of *C. burnetii* antibodies were lower throughout gestation in cows concurrently infected with *C. burnetii* and *N. caninum* than in only *C. burnetii* infected cows (Garcia-Ispierto et al., 2011), which suggested also a cross-protection in such animals. Such results are complementary to ours and once more provide support to our hypothesis.

Of interest was the punctual and elevated increase observed in TLC on the 2nd postpartum week in cows seropositive to *N. caninum*, related to increased neutrophils numbers and percentages at this time point. In an earlier study, lower peripheral neutrophil counts were observed in primiparous *Neospora*-seropositive cows on Day 180 which was hypothesized that it might have been due to neutrophil recruitment towards the focus of infection in an attempt of the dam to control parasite recrudescence at the placental level (Serrano et al., 2011). Neutrophils play an important role during the invading stage of *T. gondii* and *N. caninum* tachyzoites with enhanced molecule gene transcription and adhesion function towards infected endothelial cells during this process (Taubert et al., 2006).

Neutrophils could also play similar role in case of *N. caninum* infection, however this needs further investigation.

Finally, previous studies have shown differences in peripheral blood populations due to normal pregnancy and postpartum status in healthy cows (Detilleux et al., 1995; Nazifi et al., 2008). In addition, effects of artificial inseminating bull, season, age and sampling time observed in the present study were in accordance with the results previously evaluated by the present authors (Serrano et al., 2011, Abdelfatah-Hassan et al., 2012).

In conclusion, the present results demonstrated different maternal peripheral leukocyte subpopulations (indicator to maternal immune responses) towards infection with *N. caninum*, *C. burnetii* or both *N. caninum* and *C. burnetii* compared to control cows during the peripartum period. Such differences suggested a co-activated immune system by *N. caninum* towards *C. burnetii*. However such hypothesis needs further investigation. In addition, although the immune system seemed to be activated towards recrudescent infection of *N. caninum* and/or *C. burnetii*, abortion did not occur. Finally, taking in mind that efficient vaccinations against *N. caninum* and *C. burnetii* infections are still missing, the present results could provide ideas for researches interested in the development of effective vaccinations against such infections.

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Chapter 5: Pharmacokinetics of Exogenous Melatonin in Lactating Dairy Cows

5. Chapter 5

Pharmacokinetics of Exogenous Melatonin in Lactating Dairy Cows

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Abstract

This study addresses the pharmacokinetics (PK) of long-term melatonin treatment in lactating dairy cows. Melatonin doses of 0 (control), 83, 166, 249 or 332 µg/kg body weight were given as subcutaneous implants on Day 120 of gestation to 20 multiparous lactating dairy cows (4 cows/dose-group). Blood samples were obtained weekly from Day 120 of gestation for 8 weeks. Melatonin pharmacokinetics, for each dose, were assessed using non-compartmental PK and mixed model procedures. Cows in the 332 µg/kg group showed a higher peak plasma melatonin concentration (C_{max}) and area under the plasma concentration curve from treatment Day 0 to Day 49 ($AUC_{0–49d}$) than cows in the control, 83 µg/kg, 166 µg/kg and 249 µg/kg groups. Values of C_{max} for the groups 83 µg/kg, 166 µg/kg and 249 µg/kg did not differ significantly from the control group. The Time needed to reach the maximum plasma melatonin level (T_{max}) was seven days for all cows receiving 332 µg/kg, whereas T_{max} varied in the remaining groups. Further, melatonin half-life was longer in cows producing less than <31 kg/day milk one week before treatment than in higher producers. Our results suggest that a subcutaneous dose of 332 µg/kg is able to induce pharmacological levels of melatonin in lactating cows, and that milk production, at the time of treatment, correlates negatively with the subsequent melatonin half-life of the hormone in the bloodstream.

Key words: photoperiod manipulation, melatonin implants, milk production.

5.1 Introduction

Melatonin (*N-acetyl-5-methoxytryptamine*), a small lipophilic indoleamine mainly synthesized in the pineal gland, was first isolated from the bovine pineal gland in the late 1950s (Lerner et al. 1958, Lerner et al. 1959). In most mammal species, circulating levels of the hormone vary in a circadian manner, with levels being higher at night (Tamura et al. 2008). The main function of melatonin is to convey light-dark information to the brain to orchestrate its circadian and seasonal rhythms and related biological processes, such as reproductive function in small ruminants (Arendt, 1998; Weaver and Lockley, 2009). Pineal melatonin also was found to have immune-related functions and oncostatic effects as an antioxidant and free radical scavenger (Maestroni, 1993; Reiter, 2003).

The clinical applications of melatonin in humans such as the treatment of sleep disorders and its use to improve the reproductive performance of small ruminants has prompted studies designed to address the pharmacokinetics of melatonin, both in humans and several animal models. Such studies examining the pharmacokinetics (PK) of melatonin following different routes of administration have been conducted in humans (Lane and Noss, 1985; Mistraletti et al., 2010; Waldhauser et al., 1984), rats (Mo-Yin Chan et al., 1984), hamsters (Ferreira et al., 1996), dogs (Saaf et al., 1980) and small ruminants (English et al., 1987, Eriksson et al., 1998). In red deer, melatonin was found to cause seasonal changes in reproduction (Adam and Atkinson, 1984; Adam et al., 1989; Webster et al., 1991). Dairy products, especially milk, could be a good source of melatonin. In elderly subjects, drinking melatonin-enriched milk in the evening was found to improve sleep quality and increase day-time activity (Valtonen et al., 2003).

Only two studies have addressed the pharmacokinetics of melatonin in cows. In these studies, the hormone was administered intravenously at doses of 10 µg/kg (Berthelot et al., 1990) and 270 µg/kg (Eriksson et al., 1998). However, to the best of our knowledge, the PK of melatonin given via other routes of administration or at different doses has not been examined in dairy cows. The use of slow-release implants of a drug avoids the need for administration via cannula or frequent injections, and provides continuous fairly constant circulating levels of the drug allowing for assessment of drug effects in the long term. The present study was designed to determine the pharmacokinetics of different melatonin doses administered in the form of subcutaneous slow-release implants in lactating dairy cows. In addition, since melatonin is known to diminish prolactin secretion (Dahl et al., 2000), the possible relationship between melatonin treatment and milk production was also evaluated.

5.2 Materials and Methods

5.2.1 Study cows

The study was performed on clinically healthy 20 multiparous Holstein-Friesian lactating pregnant dairy cows randomly selected from a high-milk producing herd in north-east Spain during the winter solstice. To homogenize the study population, only pregnant cows in their second term of gestation, which is when gestation is firmly established (López-Gatius and Garcia-Ispierto, 2010), were included in the study. All cows had undergone two or more lactations and their mean weight was approximately 650 kg. The cows were milked three times per day and were fed complete rations according to NRC recommendations (2001). Mean annual milk production during the study period was 11250 kg per cow in the herd. All cows were bred by AI using semen from bulls of proven fertility. All cows carried

singletons, as determined by ultrasound on Day 40 of gestation, and gestation was again confirmed before melatonin administration on Day 120 of gestation.

5.2.2 Blood sampling and melatonin administration

Blood samples were obtained on Days 120, 127, 134, 141, 148, 155, 162, and 169 of gestation (corresponding to treatment days 0, 7, 14, 21, 28, 35, 42 and 49, respectively) from cows in the early morning (between 8:00 and 10:00 h, sunrise at 7:30). These blood samples (5 ml) were collected from the coccygeal vein/artery of each animal into EDTA vacuum tubes (BD Vacutainer®, Plymouth, UK), and then centrifuged (10 min at 2500 rpm) within 30 min of collection and the plasma stored at -28˚C until tested.

Melatonin (Melovine®, CEVA Salud Animal, Barcelona) was administered as 3 to 12 slow-releasing subcutaneous implants on Day 120 of gestation, immediately after the first blood sample collection (Day 0 of treatment). Each implant contained 18 mg melatonin and was administered using an s.c. implanter (CEVA Salud Animal, Barcelona) to an area of free moving skin between the thighs, dorsal to the base of the udder. Unlike the base of the ears, the animal cannot rub this area against objects and thus affect the rate of melatonin release. The cows were divided into 5 dose (µg/kg body weight) groups (4 cows per group); control (0 µg/kg), 3 implants (83 µg/kg), 6 implants (166 µg/kg), 9 implants (249 µg/kg) and 12 implants (332 µg/kg).

5.2.3 Melatonin Assay

Plasma melatonin levels were determined using a Melatonin ELISA kit (IBL, Hamburg, Germany). The extraction and test procedures used were those described in the manufacturer's protocol and the resultant optical density was read at 405 nm (using 620 nm filter as a reference) on a Multiskan® FC photometer (Thermo Fisher Scientific, Vantaa, Finland). The assay's working principle is a basic competitive ELISA procedure in which the sample melatonin competes with a biotinylated-melatonin for its antibody-binding sites (polyclonal; rabbit). The amount of biotinylated-melatonin bound to the antibodies is inversely related to the amount of melatonin in the sample. Finally, the amount of melatonin in the sample is quantified by comparison with a standard melatonin dose-response curve (standards of concentration 3.6, 10, 30, 100 and 300 pg/ml, provided in the kit). The sensitivity of this melatonin assay was 1.6 pg/ml, and intra-assay and inter-assay coefficients of variation were 3-11% and 6-19%, respectively.

5.2.4 Pharmacokinetics analysis

The serial plasma melatonin concentration/time data obtained in the serological assay were used in the pharmacokinetic (PK) analysis. A non-compartmental PK model was constructed for individual cow data using PKsolver 2.0, a free add-in program for pharmacokinetic and pharmacodynamic data analysis in Microsoft Excel, designed in 2010 by Zhang et al. (2010). Peak plasma melatonin concentrations (C_{max}) and the time taken to reach C_{max} (T_{max}) were directly obtained from the data. The area under the plasma concentration versus time curve and area under the first moment curve from zero to 49 days ($AUC_{0–49d}$ and $AUMC_{0–49d}$) were calculated using the linear trapezoidal method. The terminal

elimination slope (λ_z) was set to be automatically calculated. Other variables including area under concentration-time curve and area under first moment curve from 49 days to infinity ($AUC_{49d-\infty}$ and $AUMC_{49d-\infty}$), half-life ($t_{1/2}$), mean residence time (MRT), clearance rate ($C_{l/F}$) and volume of distribution based on the terminal slope ($V_{z/F}$) were also calculated. For the control group, the only data that could be calculated (since they lacked a melatonin dose) were C_{max}, T_{max}, AUC_{0-49d}, $AUMC_{0-49d}$ and MRT. All PK data are provided as the mean ± SD.

5.2.5 Statistical analyses

Statistical analysis was performed on the original plasma melatonin concentration/time data and the PK data obtained in the non compartmental analysis. The effects of the different melatonin doses given to the study cows on the pharmacokinetics of melatonin were analysed by mixed models analysis using the PASW statistics 18 package (SPSS Inc., Chicago, IL, USA). The final model, selected according to the best Akaike's Information Criterion (AIC) and Schwarz's Bayesian Criterion (BIC), included the effect of melatonin administration (factor) on its different pharmacokinetic variables (dependent variable). In addition, lactation number, mean milk production one week before treatment and days in milk at treatment were introduced as covariates in the mixed model. Whenever appropriate, *post hoc* comparisons were performed on the estimated marginal means using the least significant difference (LSD) test.

Plasma melatonin concentration/time data were analyzed by the mixed models procedure for repeated measures using the PASW statistics 18 package. The final mixed model included the effects of time, treatment and their interaction (factors) on plasma

melatonin concentrations throughout the study period (dependent variable). Also, lactation number, milk production and days in milk were used as covariates in the mixed model. Different models were fitted using different covariance structures, and the best model was selected according to the best AIC and BIC criterions.

Milk production in the cows was recorded once weekly before the study and then on each study day. The effect of the different melatonin doses on milk production during the study period was assessed using the mixed models procedure for repeated measures (PASW statistics 18). Time, melatonin doses and their interaction were introduced as factors, while lactation number, milk production and days in milk were introduced as covariates. Different models were fitted using different covariance structures and the best model fitting the data was again selected based on the best AIC and BIC criteria.

In the two mixed model procedures for repeated measures conducted for the melatonin concentration/time data and milk data, *post hoc* comparisons were performed, whenever applicable, using Tukey's Honestly Significant Difference (HSD) test. Lactation number, milk production and days in milk data were collected from data for the current lactation. The level of significance was set at a P-value <0.05.

5.3 Results

Mean plasma melatonin concentrations (±SEM) as a function of dose and time for the cows examined are shown in Figure 5.1. One cow in the 166 µg/kg group aborted in the middle of the study and was excluded, leaving only three cows in this group. Mean (±SD)

lactation number, milk production of the lactation in progress (one week before the study) and days in milk for the different groups of cows are provided in Table 5.1.

Table 5.1. Mean (±SD) lactation number, milk production (mean for the week before treatment) and days in milk at treatment for the different treatment groups.

Melatonin treatment group (n)	Lactation n	Milk at treatment	Days in milk at treatment
Control (4)	3.00 (± 0.82)	37.43 (± 8.05)	380.50 (± 90.45)
83 µg/kg (4)	3.50 (± 1.91)	30.53 (± 7.08)	333.25 (± 73.98)
166 µg/kg (3)*	2.00 (± 0)	35.20 (± 4.66)	305.67 (± 24.70)
249 µg/kg (4)	2.25 (± 0.50)	28.18 (± 6.38)	395.25 (± 77.14)
332 µg/kg (4)	4.00 (± 1.83)	32.70 (± 7.79)	383.00 (± 85.86)

* One cow aborted during the study and was excluded.

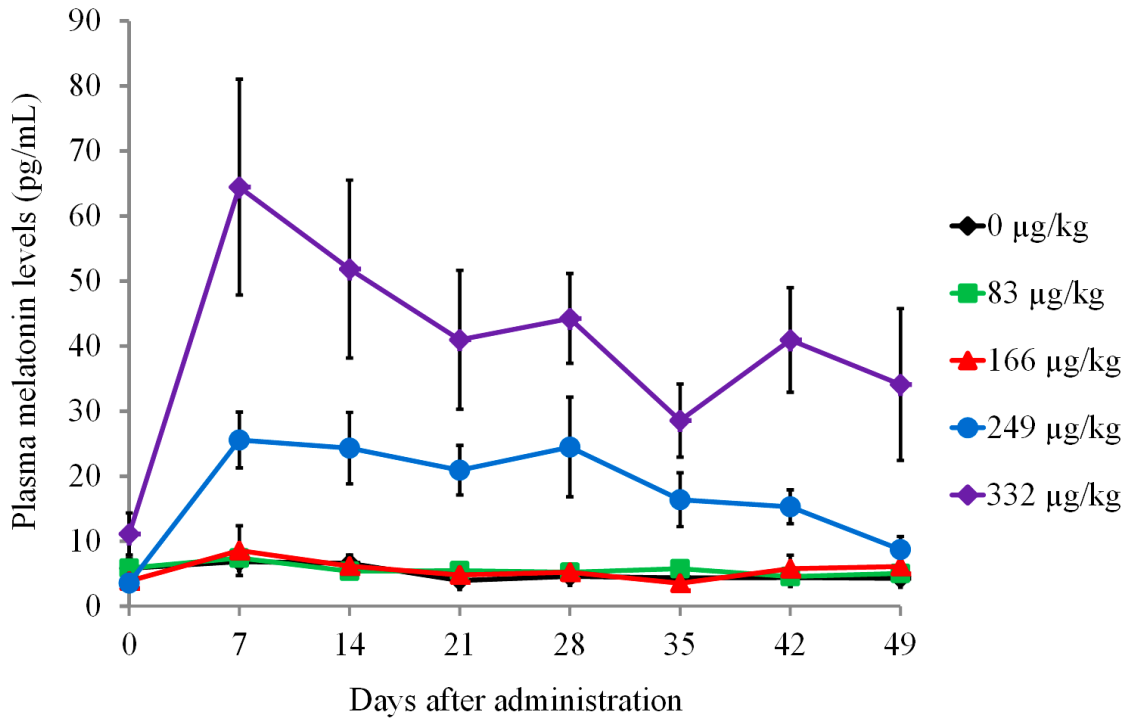

Figure 5.1. Plasma melatonin concentration (pg/ml; mean ±SEM) versus time for five different melatonin doses (µg/kg) administered subcutaneously on Day 120 of gestation

to lactating Holstein-Friesian dairy cows (4 cows/group; except 3 cows in the 166 µg/kg group).

5.3.1 Melatonin pharmacokinetics

Table 5.2 shows the mean (±SD) pharmacokinetic data obtained for the different melatonin doses. Significant differences between groups were detected in maximum plasma melatonin levels (C_{max}) post-treatment (df=4, F= 6.7, P=0.006). Thus, the 332 µg/kg group showed higher plasma melatonin concentrations than the control, 83 µg/kg, 166 µg/kg and 249 µg/kg groups (Table 5.2 and Fig. 5.1). C_{max} in the 83 µg/kg and 166 µg/kg groups did not differ to that recorded in the control group. In the 249 µg/kg group, C_{max} apparently increased (though was significantly lower than that observed in the 332 µg/kg group), however, it was not significantly higher than the values recorded in the control, 83 µg/kg or 166 µg/kg groups. The time taken to reach the maximum plasma melatonin level (T_{max}) was seven days (i.e. sample 2) for all cows treated with a melatonin dose of 332 µg/kg body weight, whereas T_{max} varied in the remaining groups, even among individual cows within the same group.

The area under curve from 0 to 49 days ($AUC_{0–49d}$) was significantly different among groups (df=4, F=12, P=0.001). According to the *post hoc* test, the AUC_{0-49d} was significantly greater for the cows receiving a melatonin dose of 332 µg/kg compared to the other groups, among which there were no significant differences in this variable (Table 5.2). Terminal elimination slope (or λ_z) values failed to vary significantly, whereas the volume of distribution ($V_{z/F}$) did vary significantly among the groups (df = 3, F = 5.2, P = 0.027) such that animals in the 166 µg/kg dose group showed a higher volume of distribution than those in the remaining groups (P<0.02). The remaining PK variables did not vary significantly according to the melatonin dose administered to the animals.

Significant effects were observed of milk production one week before treatment on melatonin half-life ($t_{1/2}$), the ratio C_{last}/C_{max}, $AUC_{0-\infty}$, $AUMC_{0-\infty}$ and $MRT_{0-\infty}$ (P=0.025, P=0.047, P=0.022, P=0.024 and P=0.025, respectively). These effects determined that cows producing less than 31 kg/day milk showed a longer $t_{1/2}$, higher C_{last}/C_{max} ratio, larger $AUC_{0-\infty}$, larger $AUMC_{0-\infty}$ and longer $MRT_{0-\infty}$ than cows producing more than this amount.

5.3.2 Effect of different melatonin doses on plasma melatonin levels

In agreement with our pharmacokinetic results, the mixed model tests for repeated measures revealed significant effects of both treatment (df=4, F=9.2, P=0.002; Fig. 5.1) and time (df=7, F=7, P<0.001; Fig.5.1). Hence, a melatonin dose of 332 µg/kg gave rise to 2- and 6-fold increases in C_{max} (P=0.006) or AUC_{0-48d} (P=0.002), compared to the 249 µg/kg group, or to the Control, 83 µg/kg and 166 µg/kg groups, respectively. The interaction treatment with time was also significant (df=28, F=3, P=0.005; Fig. 5.1). No significant effects however were observed of any of the covariates, lactation number, milk production and days in milk at treatment on plasma melatonin levels. The mixed model that best fitted the data had a covariance structure of the heterogeneous autoregressive type.

Based on Tukey's HSD, cows treated with a melatonin dose of 332 µg/kg showed significantly higher plasma melatonin levels than animals in the remaining groups (P<0.001). Moreover, the 249 µg/kg melatonin dose group showed significantly lower plasma melatonin levels than the 332 µg/kg group (P<0.001) yet significantly higher levels than those recorded in the control, 83 µg/kg (P=0.001) and 166 µg/kg (P=0.007) groups. However, no differences in plasma melatonin levels were detected among the control, 83 µg/kg and 166 µg/kg groups.

Table 5.2. Pharmacokinetics data [mean (±SD)] according to the different melatonin doses administered subcutaneously on Day 120 of gestation to 19 lactating dairy cows.

Melatonin treatment group (n)		Control (4)*	83 µg/kg (4)	166 µg/kg (3)**	249 µg/kg (4)	332 µg/kg (4)
Parameter	Unit					
Lambda_z (λ_z)	day^{-1}	-	0.02 [a] (±0.01)	0.01 [a] (±0.01)	0.06 [b] (±0.03)	0.02 [a] (±0.01)
$t_{1/2}$	day	-	90.73 (±114.10)	85.13 (±38.66)	19.53 (±18.97)	116.43 (±142.03)
T_{max}	day	12.25 (±11.95)	10.50 (±12.12)	14.00 (±7.00)	19.25 (±14.43)	7.00 (±0.00)
C_{max}	ng/ml	9.26 [c] (±3.68)	8.43 [c] (±2.32)	10.20 [c] (±5.53)	31.24 [c] (±9.94)	64.44 [d] (±33.15)
C_{last_obs}/C_{max}		0.44 (±0.14)	0.60 (±0.25)	0.70 (0.08)	0.29 (±0.14)	0.55 (±0.31)
$AUC_{0\text{-}49d}$	ng/ml·day	246.12 [e] (±94.41)	270.08 [e] (±86.62)	296.64 [e] (120.55)	919.11 [e] (±293.15)	1925.56 [f] (±778.78)
$AUC_{0\text{-}\infty}$	ng/ml·day	-	1259.86 (±1659.09)	1197.23 (±677.32)	1246.55 (±539.29)	9330.49 (±11713.36)
$AUC_{0\text{-}49d}/AUC_{0\text{-}\infty}$		-	0.46 (±0.27)	0.28 (±0.10)	0.79 (±0.22)	0.45 (±0.34)
$AUMC_{0\text{-}\infty}$	$ng/ml \cdot day^2$	-	379162.42 (±705995.88)	178524.10 (±126660.55)	55202.56 (±55973.54)	3408442.44 (±6307816.34)
$MRT_{0\text{-}49d}$	day	21.82 (±1.68)	21.78 (±1.69)	20.95 (±1.98)	23.05 (±1.13)	21.25 (±1.10)
$MRT_{0\text{-}\infty}$	day	-	138.21 (±165.09)	132.43 (±51.79)	38.47 (±22.96)	172.70 (±208.81)
$V_{z/F}$	mg/(ng/ml)	-	6.01 [g] (±1.50)	12.40 [h] (±3.88)	3.29 [g] (±1.79)	4.02 [g] (±2.28)
$C_{l/F}$	mg/[(ng/ml)/day]	-	0.11 (±0.07)	0.13 (±0.11)	0.16 (±0.09)	0.05 (±0.03)

Terminal elimination slope (λ_z), half life ($t_{1/2}$), maximum plasma melatonin concentration (C_{max}), time to reach C_{max} (t_{max}), last observed concentration (C_{last_obs}), area under the plasma concentration/time curve from zero to 49 days and from zero days to infinity ($AUC_{0–49d}$ and $AUC_{0–\infty}$), area under first moment curve from zero days to infinity ($AUMC_{0–\infty}$), mean residence time from zero to 49 days and

from zero days to infinity ($MRT_{0\text{-}49d}$ and $MRT_{0\text{–}\infty}$), volume of distribution based on the terminal slope ($V_{z/F}$) and clearance rate ($C_{l/F}$).

* Other PK data for the control group could not be calculated (as it lacks the dose)

** One cow aborted during the study and was excluded from the analysis.

Within-row letter differences indicate a significant difference (a-b <0.05, c-d <0.01, e-f <0.002, g-h <0.02).

Significances were calculated after correction by covariates such that all cows were assigned a lactation number = 3, mean milk production one week before the study = 32 kg/day, and days in milk at treatment = 360 days.

5.3.3 Effect of different melatonin doses on cows' milk production

Melatonin treatment had no effect on milk production. Neither were any effects observed of any of the covariates examined except milk production one week before the study (df=1, F=63.1, P<0.001). The only factor significantly affecting milk production was time (df=7, F=10.5, P<0.001), since milk production decreased with time. One cow from the 249 µg/kg group was dried-off three weeks before the end of the study, thus milk production for this cow was omitted from the statistical analysis.

5.4 Discussion

This study aimed to examine the pharmacokinetics of melatonin administered as subcutaneous implants in lactating cows. Significantly higher C_{max} and $AUC_{0\text{-}49d}$ were recorded compared to controls when the highest dose of melatonin was given, i.e. 332 µg/kg (216 mg/cow; 12 implants). When a reduced melatonin dose was used, C_{max} and $AUC_{0\text{-}49d}$ were either insufficiently increased, as in the case of the group receiving 249 µg/kg (162 mg/cow; 9 implants) or not increased at all, as in cows receiving 166 or 83 µg/kg (108 and 54 mg/cow; 6 and 3 implants, respectively). In both cases, no statistical difference was detected

according to the corresponding dose. In addition, a non-linear relationship was evident between dose and C_{max} and AUC_{0-49d}, whereby doubling the melatonin dose from 83 to 166 µg/kg failed to produce any corresponding increase, while after increasing the dose by 50% (166 to 249 µg/kg) C_{max} was tripled (from 10.20 to 31.24 ng/ml), and a further dose increase by 25% (249 to 332 µg/kg) further doubled the C_{max} (from 31.24 to 64.44 ng/ml). The AUC_{0-49d} response showed a similar pattern to that of C_{max}. This non-linearity in the melatonin dose relationship with C_{max} and AUC has also been observed in humans (Mulchahey et al., 2004). The clinical significance of such a non-linear relationship is unknown and it could be that melatonin receptors play a role in the kinetics of this hormone. The MRT_{0-49d} was relatively similar among the different dose groups, suggesting the dose-independence of this pharmacokinetic variable, in agreement with the findings of other studies (Berthelot et al., 1990; Eriksson et al., 1998).

Large inter-individual differences were observed in most PK variables. For example, the C_{max} observed after the administration of 249 µg/kg of melatonin ranged from 19.4 to 43.6 ng/ml. These large inter-individual differences could explain why in cows receiving 249 µg/kg (9 implants), although showing higher C_{max} and AUC_{0-49d} on average, the values of these variables were similar statistically to those recorded in cows receiving 166, 83 or 0 µg/kg doses of melatonin. These inter-individual differences could be due to differences in melatonin metabolism, and may indicate that a dose of 249 µg/kg is insufficient to overcome melatonin metabolism in some cows. Such inter-individual variability has also been observed in humans (Mulchahey et al., 2004).

In response to an intravenous injection of 270 µg/kg of melatonin in lactating cows (Eriksson et al., 1998), PK parameters differed from those of the present study, except for a

fairly similar clearance ($C_{l/F}$) value obtained for our dose of 249 µg/kg administered subcutaneously. The route of administration could be the reason for such differences as proposed in small ruminants (English et al., 1987). In addition, such differences should not be the difference in the type of PK analysis used, 3-compartment model (Eriksson et al., 1998) against linear trapezoid method (this study), because in the same study the 3-compartment analysis gave practically identical results to the trapezoid analysis.

Care should be taken to consider milk production when interpreting melatonin PK data in lactating animals. Thus, we would expect that cows producing more milk will show lower plasma melatonin levels, irrespective of the dose given. In pregnant cows, plasma levels of hormones such as progesterone and pregnancy-associated glycoproteins are negatively correlated with milk production (Bech-Sàbat et al., 2008; Lopez-Gatius et al., 2007). In effect, it has been possible to detect melatonin in milk as early as 15-45 minutes after its administration (Eriksson et al., 1998). Consequently, cows producing more milk would be expected to return a shorter half-life ($t_{1/2}$) and lower C_{max} of melatonin and this was the case. Furthermore, when data was extrapolated to infinity ($AUC_{0-\infty}$, $AUC_{0-48d}/AUC_{0-\infty}$, $AUMC_{0-\infty}$ and $MRT_{0-\infty}$) less values was also obtained in those cows. Milk production has also been reported to affect melatonin PK in goats (Eriksson et al., 1998).

Correcting for significant inter-individual variation in milk production as determined one week before the study (introduced as covariate in the mixed model), neither melatonin treatment nor its interaction with time had any effect on milk production in the cows examined. This finding is consistent, at least partly, with a previous study in which a daily dose of 22.5 mg melatonin given in the feed for 8 weeks did not affect lactation in younger cows (Dahl et al., 2000). The fact that subcutaneous melatonin doses of up to 332 µg/kg seem

to have no effect on milk production is of clinical importance, especially for the dairy industry.

5.5 Conclusions

According to the results of this study, exogenous subcutaneous melatonin doses of 83, 166 and 249 μg/kg failed to increase plasma melatonin concentrations (C_{max} and AUC) of the hormone and should therefore not be considered for studies designed to assess the effects of pharmacological doses of subcutaneous melatonin in lactating dairy cows. The only subcutaneous dose capable of inducing pharmacological levels of melatonin in lactating cows was the highest dose tested, 332 μg/kg, and was able to do so for at least 40 days after its administration. Milk production at the time of treatment was negatively correlated with the subsequent half-life of melatonin in the bloodstream.

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Chapter 6: Melatonin Treatment at Dry-off Improves Reproductive Performance Postpartum in High Producing Dairy Cows under Heat Stress Conditions

6. Chapter 6

Melatonin Treatment at Dry-off Improves Reproductive Performance Postpartum in High Producing Dairy Cows under Heat Stress Conditions

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Abstract

The aim of this study was to determine the effect of melatonin treatment during the early dry-off period on subsequent reproductive performance and milk production in high producing dairy cows under heat stress conditions. The study was performed during the warm season on 25 heifers and 114 high milk-producing Holstein-Friesian cows from a commercial dairy herd. Animals were randomly assigned to a Control group (C) or Melatonin group (M). Heifers or cows in the M group received 332 µg/kg body weight of melatonin on Days 220-226 of gestation. Four blood samples were collected before parturition (on gestation Days 220–226, 234–240, 248–254, and 262–268) and two samples after parturition (on Days 14–21, and 28–34 postpartum) to determine pregnancy associated glycoprotein-1 (PAG-1) and prolactin. Through binary logistic regression, we observed that the likelihood of repeat breeding syndrome and pregnancy loss were lower (OR 0.36 and 0.19) in treated than control animals, respectively. Through general linear model repeated measures ANOVA, significant effects were detected of treatment on prolactin levels throughout the study period (between subject effects, P=0.01). Plasma prolactin levels decreased after melatonin treatment and recovered during the postpartum compared to control cows. No significant effects were observed on normalized total milk production in the previous and subsequent lactations, somatic cell counts and plasma PAG-1 concentrations throughout the study period. Kaplan-Meier survival curves for the study period revealed significant differences in estimated days open between groups (means 123±71.9 and 103±43, respectively for C and M; Log Rank Mantel-Cox P=0.02). In conclusion, melatonin treatment in the early dry off period improves the reproductive performance of dairy cattle, reducing the number of days open, repeat breeding syndrome and pregnancy loss.

Key words: somatic cell counts, repeat breeding syndrome, reproductive failure, transition period

6.1 Introduction

The postpartum is the most critical period for the reproductive and productive success of the dairy cow. Since the 1980s, milk yield has been increasing at the expense of a decline in fertility (Lucy, 2001; López-Gatius, 2003). As a focused solution to this problem, many strategies have been targeted at both the postpartum and dry-off period. Apart from nutrition, such strategies have included synchronization protocols and antibiotic therapy to improve postpartum fertility, yet we are still far from the ideal solution at the herd level. Moreover, the problem of heat stress is on the rise worldwide, impairing fertility, and it is foreseeable that over the years to come, the negative effects of heat stress on reproductive function will continue to increase (Intergovernmental Panel of Climatic Change, 2007). Heat, besides reducing fertility and increasing pregnancy losses (Garcia-Ispierto et al, 2006, 2007), can also worsen postpartum recovery and impair the return to cyclicity (De Rensis and Scaramuzzi, 2003).

Melatonin, a hormone synthesized mainly by the pineal gland, has a multifactorial effect on animals. It modulates circadian rhythm (Gillette and Tischkau, 1999), reproduction (Reiter, 1991), the neuroendocrine (Cardinali and Pevet, 1998), immunological and cardiovascular systems (Reiter, 1991; Cardinali and Pevet, 1998; Srinivasan, 2005; Reiter et al, 2009), pregnancy and parturition times (Takayama et al, 2003; Tamura et al, 2008a,b), and corpus luteum function (Tamura et al, 1998), among others. Then, there is some evidence that melatonin could enhance ovarian function and embryo survival in vitro (Shi et al, 2009). Thus, during the dry off period melatonin could be a potential tool to boost the cow's immune system and help reduce postpartum disorders. Against this hypothesis, at least in dairy cattle, it has been reported that melatonin supplementation during late lactation reduces

prolactin levels and milk yields in grazing cattle (Auldist et al, 2007). This study was therefore designed to determine the effect of melatonin administered during the early dry-off period on subsequent reproductive performance and milk production in high producing dairy cows under heat stress conditions.

6.2 Materials and methods

6.2.1 Study population and management

The study was performed on 25 heifers and 114 high milk-producing Holstein-Friesian cows from a commercial dairy herd in northeast Spain, which included a mean of 562 lactating cows during the study period. Mean annual milk production was 11,020 kg per cow. The animals were reared within the herd and milked three times per day before drying-off and fed complete rations according to NRC recommendations (2001). The mean annual culling rate for the study period was 28%. All animals were tuberculosis- and brucellosis free, as shown by yearly tests from 1985 to 2012. The reproductive management of this herd has been described by López-Gatius et al. (2008).

6.2.2 Experimental design

Pregnancy was diagnosed by trans-rectal ultrasound between Days 28–34 postinsemination and confirmed on Day 60. Thereafter, pregnancy was followed by trans-rectal palpation on Days 90, 120, 150, and 180 and at dry-off on Day 210 of gestation. Only confirmed pregnant animals on Day 210 were included in the study. Animals were randomly assigned to a Control (C) or Melatonin (M) group. Heifers or cows in M received 249 or 332

µg/kg of the hormone on Days 220-226 of gestation, respectively (Abdelfatah-Hassan, submitted). All animals in C and M were treated during the warm season (May-August). During the study period, six blood samples were collected from each animal. Four samples were collected before parturition (on gestation Days 220–226, 234–240, 248–254, and 262–268) and two samples after parturition (on Days 14–21, and 28–34 postpartum). Blood samples could not be obtained at parturition because of farm management policy. The blood samples (10 ml) were collected from the coccygeal vein of each animal into EDTA vacuum tubes (BD Vacutainer, Becton, Dickinson, and Company, Plymouth, UK). The remaining blood was rapidly centrifuged (within 30 min of collection) at 2,500 rpm for 10 min, and the plasma stored at -26 °C until serological analysis.

6.2.3 PAG radioimmunoassay

Plasma PAG concentrations in the samples were determined by double antibody radioimmunoassay (RIA-706) (López-Gatius et al., 2007). Rabbit polyclonal antiserum AS# 706 was raised against goat $PAG_{55kDa+62kDa}$ (accession numbers P80935 and P80933) and prepared using the method described by Vaitukaitis and others (1971). Briefly, plasma (10 µl of each sample) was incubated for 16 h (at room temperature) with 100 µl of tracer (28,000 cpm) and 100 µl of primary antiserum (AS#706 diluted to 1:240,000). The total assay volume was adjusted to 0.5 ml using Tris-BSA buffer (pH 7.6). The rest of the procedure was as previously described (López-Gatius et al, 2007). Samples showing high PAG concentrations (>50 ng/ml) were diluted (1/50, 1/100 and 1/200) and re-assayed. The minimum detection limit (MDL) for this assay is 1.5 ng/ml. Intra-assay and interassay coefficients of variance (CV) are 7.8 (4.90±0.38 ng/ml) and 16.0% (4.32±0.69 ng/ml), respectively.

6.2.4 Prolactin

Plasma prolactin concentrations were determined using a double antibody radioimmunoassay procedure (Ayad et al, 2007) with some modifications. Bovine prolactin (NIH-B5 bPRL, NIH, Bethesda, MD, USA) diluted in assay buffer (Tris–BSA) was used as standard (0.8 to 200 ng/mL) and tracer. Iodination (Na–I125, PerkinElmer, Belgium) was conducted according to the iodogen method (Greenwood et al, 1963). Briefly, 50 µl of each plasma sample in duplicate and/or 0.1 mL of standard preparation were diluted in Tris–BSA buffer. Next, 0.1 mL of radiolabeled prolactin (26,000 cpm) and 0.1 mL of the diluted antiserum (R#144; 1:100,000) were added to all tubes followed by an overnight incubation at room temperature (20–23 ◦C). Bound- and free prolactin were separated after addition of the double antibody precipitation system, as previously described (Ayad et al, 2007).

Estimated doses (ED) at 20%, 50% and 80% B/B0 (bound tracer/unbound tracer in the zero standard) (mean±S.D.) were 51.51±5.53, 9.62±0.92 and 1.82±0.34 ng/mL, respectively. The minimum detection limit (MDL) is 0.30 ng/mL. Intra- and inter-assay coefficients of variation for the prolactin RIA are 5.23% (14.03±0.73 ng/mL) and 5.97% (12.43±0.74 ng/mL), respectively.

6.2.5 Data collection and statistical analysis

The data obtained for each animal were: treatment group (C versus M), parturition date, lactation number, stillbirth, placenta retention, days open, repeat breeding syndrome (<3 versus ≥ 4 AIs per cow) and pregnancy losses (first trimester) in the subsequent gestation, normalized total milk production of the previous and subsequent lactations, somatic cell

counts (in 2 milk samples before the dry-off period and 3 samples after parturition), and plasma prolactin and PAG-1 concentrations (in 4 blood samples before and 2 samples after parturition).

Three binary logistic regression analyses were performed. The dependent variables entered in these three analyses were placenta retention, repeat breeding syndrome or pregnancy losses. Regression analyses were conducted according to the method of Hosmer and Lemeshow (1989).

The effects of the above variables on normalized total milk production of the previous and subsequent lactations, somatic cell counts, and plasma prolactin and PAG-1 concentrations through the study period were assessed by GLM repeated measures analysis of variance.

Finally, Kaplan-Meier survival analysis was used to detect possible differences in days open between the treatment and control groups. All statistical analyses were performed using the SPSS computer package, version 17.0 (SPSS Inc., Chicago, IL, USA).

6.3 Results

The study included 67 control and 72 melatonin-treated animals. After parturition, nineteen and fifteen of these animals suffered retention of placenta and pregnancy loss, respectively. A total of 28 animals were inseminated 4 times or more in the next lactation (repeat breeding syndrome). The mean lactation number for the study period was 2.4 ± 1.2

(mean ± SD) for mature cows (cows calving at least once before treatment). Normalized total milk production for the subsequent lactation was 14097 ± 3452 kg per cow.

No significant effects were found of any of the variables on placenta retention. Based on the odds ratios, the likelihoods of repeat breeding syndrome and pregnancy loss were lower in treated (OR 0.36 and 0.19, respectively) compared to control animals (Tables 6.1 and 6.2), respectively.

Table 6.1. Odds ratios of the variables included in the final logistic regression model for factors affecting repeat breeding syndrome

Factor	Class	n	% Repeat breeding syndrome	Odds ratio	95% Confidence Interval	P
Treatment	Control	19/67	28.4	Reference		
	Melatonin	9/72	12.5	0.36	0.15-0.86	0.002

P=0.0001. R^2 Nagelkerke = 0.21

Table 6.2. Odds ratios of the variables included in the final logistic regression model for factors affecting pregnancy loss (before 90 days of gestation).

Factor	Class	n	% Pregnancy loss	Odds ratio	95% Confidence Interval	P
Treatment	Control	12/67	17.9	Reference		
	Melatonin	3/72	4.1	0.19	0.05-0.7	0.001

P=0.0001. R^2 Nagelkerke = 0.15

Through GLM repeated measures ANOVA, significant effects were observed of treatment on prolactin levels throughout the study period (between subject effects, P=0.01). Thus, plasma prolactin levels decreased after melatonin treatment and recovered during the postpartum compared to control cows (Figure 6.1). No significant effects were found of

normalized total milk production of the previous and the subsequent lactation, somatic cell counts and plasma PAG-1 concentrations throughout the study period.

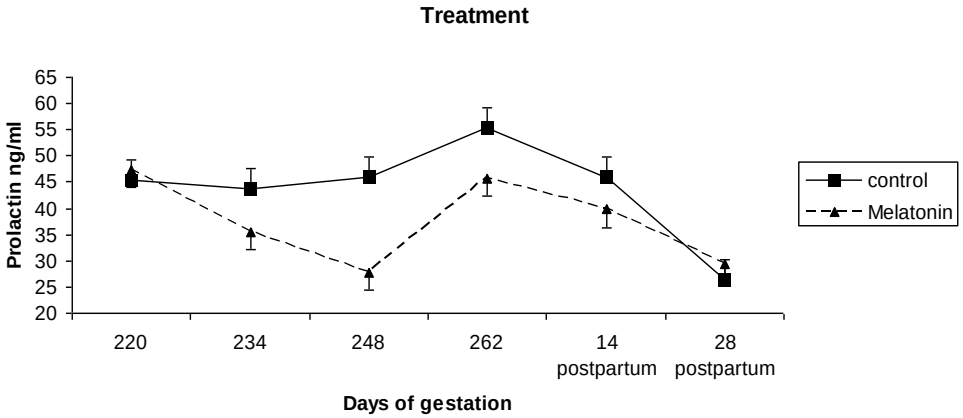

Figure 6.1. Mean plasma prolactin concentrations recorded during the study period in control (n=67) or melatonin treated animals (n=72) (mean ± S.E.M)

Kaplan-Meier survival analysis revealed significant differences between estimated days open in the control and treatment groups (means of 123±71.9 in C and 103±43 in M; Log Rank Mantel-Cox P=0.02) (Figure 6.2).

6.4 Discussion

Melatonin treatment at the beginning of the dry-off period improved the reproductive performance of dairy cattle during the warm season. Treatment not only reduced the incidence of repeat breeding syndrome but also decreased pregnancy losses during the first trimester of gestation. Moreover, although plasma prolactin concentrations decreased during the dry off period in treated animals, normal levels were recovered during the early postpartum, and no negative effect of melatonin treatment was found on the subsequent lactation.

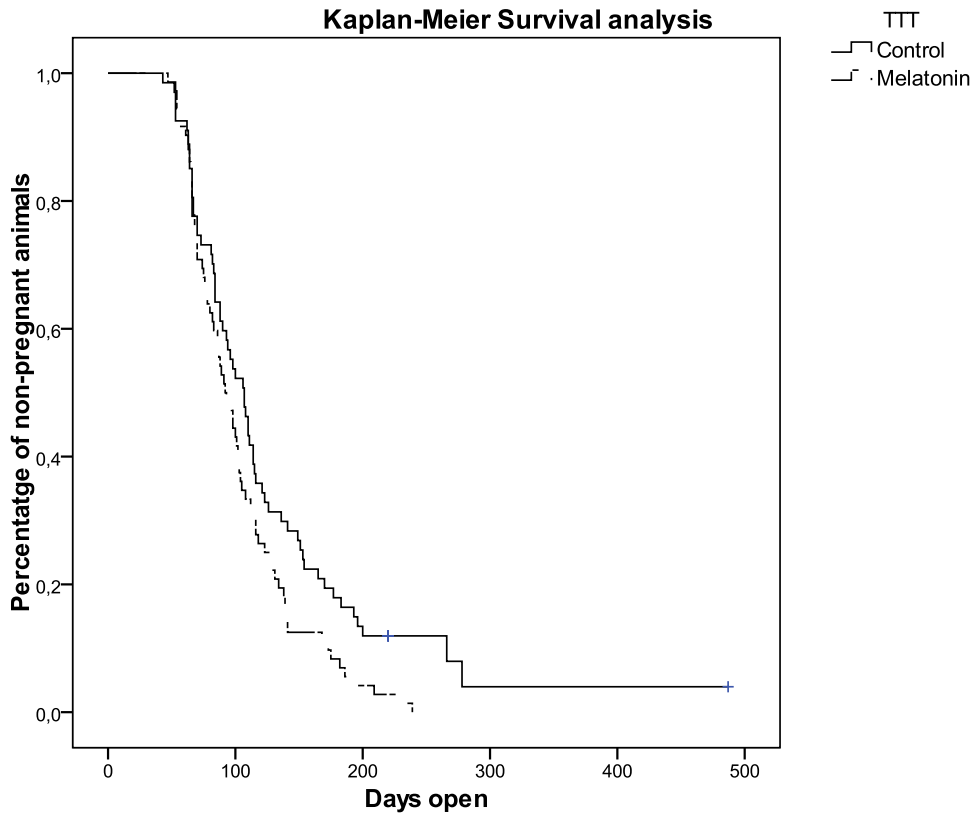

Figure 6.2. Kaplan-Meier survival curve of days open in control (n=67) or melatonin treated animals (n=72).

The photoperiod has a great impact on reproduction in many mammals. Photoperiod changes in cattle include low concentrations of melatonin observed under light conditions and their marked increase under conditions of darkness (Dahl et al., 2012). The effects of these changes on the reproductive performance of cattle seem limited (Hansen et al., 1983; Rius and Dahl, 2006), but the same does not occur for milk yield. Thus, a decreasing day length during the dry off period increases mammary cell proliferation and reduces cell apoptosis, increasing milk production (Wall et al., 2005). In response to this, plasma prolactin concentrations decline but prolactin receptor expression increases in many tissues, including the liver, mammary gland, and lymphocytes (Auchtung et al., 2003, 2005). Because of this, melatonin treatment should be given during the dry-off period and will probably be more

effective during the longer days of the positive photoperiod. More studies are needed to examine differences in treatment during the negative or positive photoperiods in dairy cows.

In this study, cows in the M group showed fewer days open and were less likely to be repeat breeders compared to control animals. Melatonin is directly involved in ovarian function in mammals (Tamura et al., 2008). Recently, el-Raey et al. (2011) demonstrated that melatonin supplementation during an *in vitro* maturation process had a positive effect on bovine oocyte quality. Repeat breeding is a multifactorial syndrome, but probably ovarian function and oocyte quality at AI may be a determining factor of the fertility of a cow. Thus, it is possible that exogenous melatonin enhances oocyte viability.

Surprisingly, melatonin treated animals showed a 5.2 (1/0.19) times lower risk of pregnancy loss than control animals. Pregnancy loss of non-infectious cause during the early foetal period has been extensively described in our geographical area and elsewhere in more than 12% of pregnant cows (López-Gatius et al., 2009; López-Gatius, 2012). It is known that melatonin is a potent antioxidant. In humans, melatonin treatment has been described to reduce preeclampsia improving placental well-being (Milczarek et al., 2010) and possibly other clinical states involving excessive free radical production, such as intrauterine foetal growth retardation and foetal hypoxia. Moreover, antioxidant defence impairment in women has been associated with recurrent pregnancy loss (Simsek et al., 1998). In other species, such as rats, pinealectomy increases the frequency of spontaneous abortions. Because of these actions and the inhibition of prostaglandin synthesis (Tamura et al., 2008), melatonin seems to be the perfect candidate to enhance embryo/foetal well-being. The question that arises now concerns the mechanism by which melatonin improves reproductive performance nearly 6 months after treatment.

The photoperiod and circulating prolactin show a consistent, established relationship in the cow (Dahl et al., 2000; Garcia-Ispierto et al., 2009). Thus, when the animals in this study were treated with melatonin, plasma prolactin levels were expectedly low. Thus, it is likely that the optimal time to treat lactating animals is when this hormonal treatment does not affect its milk production (i.e. the dry-off period).

Finally, somatic cell counts seem not to be related to treatment. It seems reasonable that a potent free radical scavenger and antioxidant improves mammary gland health. Somatic cell counts showed a large standard deviation, especially at the beginning of the postpartum period. More animals are needed to confirm whether melatonin has an effect or not on somatic cell counts.

In conclusion, melatonin treatment in the early dry off period improves reproductive performance in dairy cattle, reducing the number of days open, repeat breeding syndrome and pregnancy losses.

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Chapter 7: General discussion

7. Chapter 7

General discussion

This thesis represent a follow-up study of factors with potential influence on the kinetics of blood leukocytes, considered an indicator to immune soundness (and health in general), from Day 90 post-insemination till 30 days postpartum and considering possible improvement of cows' health using melatonin. Factors such as *Neospora caninum*-seropositivity interaction with parity, season, age, twin-pregnancy and milk production were related to important variations in peripheral blood leukocytes between Days 90-210 of gestation (Chapter 2). Whereas, factors such as, artificial inseminating bull, plasma pregnancy associated glycoproteins (PAGs), *N. caninum-Coxiella burnetii* interaction, season, age and twin pregnancy affected the kinetics of blood leukocytes studied from Day 220 of gestation till 30 days postpartum (Chapters 3,4). In order to evaluate melatonin importance in dairy cows, first pharmacokinetics of long-term melatonin administration were evaluated in order to determine the best dose to be used in dairy cows (Chapter 5), followed by an assessment of possible melatonin effects on dairy cows (Chapter 6). *N. caninum* and *C. burnetii* evaluated in the present thesis provide two examples of chronic diseases affecting cows' immune system during gestation.

Why in this thesis leukocyte counts were used as immune indicators? Most laboratories in developing countries or at field conditions do not have the sophisticated and expensive equipment used to detect immune system changes in response to surrounding factors (Such as Flow cytometry, PCR, real-time PCR, immunoassays, … etc.). However, blood cell counts represent a much easier and much cheaper method, plus they provide a

global image of the state of immune system responses. In fact, blood cell counts have been efficiently used to evaluate immune system changes in response to different conditions in human (Ohlsson and Vearncombe 1987, Hansen *et al.*, 1990, Facchini *et al.*, 1992, Arber *et al.*, 1996, Litos *et al.*, 2007, Lee *et al.*, 2009, Brüske *et al.*, 2010, Lee *et al.*, 2010), in cows (Bednarek *et al.*, 1998, Meglia *et al.*, 2001, Kulberg *et al.*, 2002, Zadnik 2003, Nazifi *et al.*, 2008, Wathes *et al.*, 2009) and in other species [For example, but not limited to these species, sheep (Onah *et al.*, 1996, Peña *et al.*, 2004, Raadsma *et al.*, 2007, Yates *et al.*, 2011), goats (Van Miert *et al.*, 1984, Takeuchi *et al.*, 1997, May *et al.*, 2002, Youssif *et al.*, 2007), Pigs (Stegeman *et al.*, 2000) and dogs (Farabaugh *et al.*, 2004, Choi *et al.*, 2008, Mori *et al.*, 2009, Willesen *et al.*, 2009)].

Data for the entire thesis articles were obtained from two commercial high-producing dairy farms in Lleida province (located in north-east Spain). Effects of nutrition and reproductive management (except, artificial inseminating bull) on the kinetics of blood leukocytes were not evaluated in the present thesis. This is due to the fact that both farms followed NRC recommendations for feeding dairy cows (NRC, 2001), and both farms had a rigorous reproductive control performed by the same veterinarian. Additionally, the distance in between the two farms was less than 10 km, which indicate that the availability of feed is practically the same.

In lights of this thesis the immune system, using leukocyte counts as an indicator, of pregnant dairy cows (and probably pregnant cattle in general) is subjected to various stimuli which could render the pregnant-cow immune repressed and/or seriously jeopardise the gestation. However, pregnant-cows' immune system seemed to be able to interact with each of the studied factors in a particular way that is not detrimental either to the gestation or

cow´s health. For example, maternal immune system responded in a different way towards infection with *N. caninum* or *C. burnetii* or both (Chapter 4), and different leukocyte counts were obtained in cow-groups inseminated with different bulls (Chapter 3).

N. caninum and *C. burnetii* represent two important infections in our geographical region. As abortion and subsequent drawbacks due to *N. caninum* has been observed (Pabón *et al.,* 2007). Meanwhile, *C. burnetii* represents a new challenge in our cows; with seroprevalence rates exceeding 50% (López-Gatius *et al.,* 2012, Nogareda *et al.,* 2012) and detected reproductive problems (López-Gatius *et al.,* 2012). Abortion due to *N. caninum* is mainly observed in the second trimester of gestation; however cows may abort any time between 3 months and full term (Anderson *et al.,* 2000, Jenkins *et al.,* 2000, López-Gatius *et al.,* 2004, López-Gatius *et al.,* 2004). On the other hand, *C. burnetii* was associated with sporadic abortion cases; some noted in late gestation (Cabassi *et al.,* 2006, Jensen *et al.,* 2007). Abortion due to *N. caninum* has been noted in this thesis; in Chapter 2 but not in Chapter 4. Which causes a question to urge, why some *N. caninum*-infected cows in this thesis did abort while others did not?

Some reports concluded that *C. burnetii* abortion could be due to placentitis (Bildfell *et al.,* 2000). Whilst other reports, hypothesised that *C. burnetii* lives inside the placental trophoblasts; which serve as a replication niche for the bacteria (Ben Amara *et al.,* 2010). However the absence of thorough description of the sampling strategy and the lack of sensitivity of testing procedures implied in many studies of *C. burnetii* in domestic ruminants are hiding information necessary for accurate judgment (Guatteo *et al.,* 2011). In addition, variable genotypes of *C. burnetii* has been found in Spain (Jado *et al.,* 2012), which also could be responsible for variable findings related to bovine abortion. *C. burnetii* infected

cows in this thesis (with or without *N. caninum* infection) did not abort. Contradictory data exists on *C. burnetii*-related bovine abortion, this unquestionably needs further investigation.

N. caninum-related abortion, on the other hand, is very frequent. It can be due to excessive maternal cellular immune-response (related to T_{h1} or pro-inflammatory cytokines) and/or due to extensive placental and foetal (mainly in the brain and heart) damage (Dubey and Schares, 2006). Nevertheless, weaker maternal cellular immune responses lead also to abortion, for example, in cows with high *N. caninum* titre, abortion likelihood increased 14.3 times in exogenous-progesterone treated than untreated animals; this was probably due to further weakening of the already down-regulated T_{h1} immunity (Bech-Sabat *et al.,* 2007).

The reason why *N. caninum* seropositive cows (with *C. burnetii* infection or not) in chapter 4 did not abort is not totally understood; although abortion was observed in late gestation in Chapter 2 and in other *Neospora*-seroporsitive cows (Wouda *et al.,* 1997, Anderson *et al.,* 2000, Collantes-Fernández *et al.,* 2006). Innes and others (2002) reviewed possible host-parasite relationships in case of *N. caninum* infection in cattle. They concluded that *N. caninum* infection or reactivation of infection in the first trimester of gestation is usually related to excessive maternal cellular immune response (mediated through T_{h1} cytokines) which ends up with abortion, however if this occurs during mid-gestation it is more probable that abortion occurs due to extensive placental and foetal damage (Innes *et al.,* 2002). While, during the late stage of gestation abortion is less frequent (but can still happen), due to the fact that the maternal immune system is recovering from gestational immune-modulation and the foetal immune system has already matured enough to fight against *N. caninum* infection (Innes *et al.,* 2002). The study cows in Chapter 4 were in the late gestation period and did not abort which is in accordance with the hypothesis of Innes and others

(2002). Then, why other cows do abort in late-gestation (as in Chapter 2)? The answer to this question is still missing. However, individual variation in the speed of recovery of the maternal immune system during late-gestation could be a reason. Also, individual variations in the immune responses being mounted against *N. caninum* infection (during late gestation) might be another possible reason. In effect, variable individual immune response against *N. caninum* has been previously observed during mid and late gestation (Innes *et al.,* 2002, Innes *et al.,* 2005, Rosbottom *et al.,* 2011). Further investigation is necessary to clarify this point.

Chapter 3 demonstrated interesting variations in peripheral blood leukocytes due to different artificial inseminating bulls and different pregnancy associated glycoproteins (PAGs) levels. Inseminating bulls are usually given less importance in most studies evaluating maternal immune response during gestation. While it was not evaluated in Chapter 2 (although it would have been interesting), this effect was detected in both Chapters 3 and 4. The clinical relevance of such effect is yet to be evaluated. However, on an individual farm level, detection of certain bulls with stimulant capacity on maternal immunity (increasing their total and differential leukocytes) could be of great benefit to inseminators' decision at artificial insemination. Especially, when the inseminators know from farm records that some cows have weaker immunity during gestation.

Of interest was the finding that higher levels (≥900 ng/ml, on Day 262-268 of gestation) of bovine pregnancy associated glycoproteins (PAGs) were associated with elevated total leukocytes and neutrophils throughout the peripartum period (Chapter 3). In addition, on the 2nd week postpartum total leukocytes and neutrophils in that chapter suffered sudden decline in almost all cows, even those in the low and medium PAGs group. If previous observations that higher PAGs are related to lower immunity, in particular lower

oxidative burst and numbers of neutrophils just after parturition (Dosogne *et al.*, 1999, Hoeben *et al.,* 2000), then in Chapter 3 a decreased leukocytes and neutrophils should have been observed only in cows with high PAGs, which was not the case. So far, no direct immune inhibitory effect of bovine PAGs has been reported. Hormonal changes around parturition seem the best candidate to be blamed for the postpartum immune dysfunctions in cows. In fact, levels of sex steroids (oestradiol and progesterone) and glucocorticoids change remarkably around parturition, and these hormones in particular have been shown to directly affect neutrophil functions around parturition (Da Silva *et al.*, 1998, Preisler *et al.,* 2000, Burton *et al.,* 2005); even during normal oestrus cycle (Chaveiro and Moreira Da Silva, 2009). So, together with results obtained in Chapter 3 of the present thesis, this should indicate that higher PAGs could not be responsible for the postpartum immune dysfunctions.

Melatonin is a pleotrophic orchestrating molecule, with multiple target organs and multiple mechanisms of actions with multiple functions (Reiter 2003, Hardeland *et al.*, 2011). From results of Chapter 6, it could be added (to the multiple functions that melatonin already has in different species) that in dairy cows melatonin implants (administered at dry-off) were able to improve post-partum reproductive performance, by decreasing the incidence of repeat breeding and reducing embryonic mortalities. In small ruminants, seasonal reproduction and its relation to melatonin is well documented (Thiéry *et al.*, 2002). Recently, in addition, it was found that polymorphisms of melatonin receptor genes were related to the seasonality of reproduction in sheep and buffalos (Carcangiu *et al.*, 2011, Carcangiu *et al.*, 2011, Luridiana *et al.*, 2012). In the latter studies, effects of melatonin have been found on, fertility after artificial insemination and lambing throughout the year in sheep and seasonal reproduction and possibility of calving throughout the year in buffalos. Hence, more studies are necessary

to assess whether certain gene polymorphisms do exist in cows or not, and to test if they are related to enhancing the reproductive functions reported in Chapter 6 of this thesis.

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Chapter 8: Conclusiones

8. Chapter 8

Conclusions

From the results of the present thesis, the following conclusions can be stated,

1- During gestation (between Days 90-210 of gestation), cows sampled in warm season had lower but rising concentrations of total leukocytes, neutrophils, lymphocytes and monocytes than those sampled during the cool season. Whilst during the peripartum period, cows calving in the cold season had higher total leukocytes, neutrophils, monocytes and eosinophils, while they had lower lymphocytes than those sampled during the warm season.

2- Twin carrying cows between Days 90-210 of gestation had lower total leukocytes and a tendency for lower lymphocytes (around Day 180 of gestation) than cows carrying singltons. While during the peripartum they had lower TLC, neutrophils and eosinophils with higher lymphocyte percentages.

3- Milk production was negatively correlated with total leukocyte counts.

4- Primiparous *Neospora*-seropositive cows showed different patterns of total leukocytes, neutrophils, and monocytes (particularly around Day 180 of gestation), compared to primiparous *Neospora*-seronegative and multiparous *Neospora*-seronegative or seropositive cows, indicating a parity-dependent effect of chronic *N. caninum* infection on peripheral blood leukocytes.

5- The inseminating bull proved to be an important factor affecting maternal total and differential leukocyte counts during the peripartum period.

6- Cows with levels of 900 ng/mL or higher of bovine pregnancy associated glycoproteins (PAGs) on Days 260-268 of gestation, were found to have higher total leukocyte and neutrophil counts during the peripartum period compared to cows with PAGs less than 900 ng/mL.

7- Different maternal peripheral leukocyte subpopulations (indicator to maternal immune responses) were obtained during the peripartum period in response to infection with *N. caninum*, *C. burnetii* or both *N. caninum* and *C. burnetii* compared to control cows. Indicating that maternal immune system was able to respond to *N. caninum* and/or *C. burnetii* infections during the last trimester of gestation.

8- Although the cows' immune system responded differently to the infection with *N. caninum*, *C. burnetii* or both, the cows maintained the gestation and did not abort. Indicating that, even if reactivation of *N. caninum* and/or *C. burnetii* infection could have taken place in the last trimester of gestation, this did not jeopardise the gestation.

9- Subcutaneous melatonin dose of 332 µg/kg was able to increase plasma melatonin concentrations (C_{max} and AUC) for at least 40 days after its administration, consequently this dose is recommended for studying melatonin effects on dairy cows. In addition, such dose seems to have no effect on milk production.

10- Melatonin half-life in the blood stream is negatively correlated with milk production at the time of administration.

11- Melatonin administration at dry-off improved reproductive performance of dairy cattle as a consequence to reducing the number of days open and decreasing the rates of repeat breeding syndrome and pregnancy losses during the postpartum.

Conclusiones

De los resultados obtenidos en la presente tesis, se puede concluir que

1- Durante la gestación (entre los días 90 y 210), las vacas en las que se tomaron muestras durante la estación cálida tenían menos, pero a su vez incrementando, niveles de leucocitos totales, neutrófilos, linfocitos y monocitos comparados con las vacas muestreadas durante la estación fría. Mientras que durante el periodo periparto, las vacas que parieron durante la estación fría tenían niveles más altos los recuentos de leucocitos totales, neutrófilos, monocitos e eosinófilos, pero tenían menos linfocitos comparadas con las vacas que parieron durante la estación cálida.

2- Las vacas con gestación gemelar entre los días 90-210 de gestación tenían menos leucocitos totales y una tendencia para tener menos linfocitos (alrededor del Día 180 de gestación) en comparación con vacas con gestación simple. Mientras durante el periodo periparto tenían menos leucocitos totales, neutrófilos e eosinófilos pero con altos porcentajes de linfocitos.

3- La producción de leche fue negativamente correlacionada con los niveles de leucocitos totales.

4- Las vacas primíparas y *Neospora*-seropositivas mostraron diferentes patrones de leucocitos totales, neutrófilos y monocitos (especialmente alrededor del Día 180 de gestación), en comparación con los de vacas primíparas y *Neospora*-seronegativas y vacas multíparas y *Neospora*-seropositivas o -seronegativas.

5- El toro usado en la inseminación artificial resultó ser un factor importante alterando los leucocitos totales y diferenciales maternos durante el periodo periparto.

6- Las vacas con niveles de 900 ng/mL o más de las Proteinas Associadas a la Gestación (PAGs) entre los Días 260-268 de gestación tenían recuentos de leucocitos totales y neutrófilos más altos durante todo el periodo periparto que los de las vacas con niveles de PAGs menos de 900 ng/mL durante los mismos días de gestación.

7- Diferentes subpoblaciones de leucocitos periféricos maternos (indicador de la respuesta inmune materna) durante el periodo periparto se observaron en respuesta a la infección con *N. caninum*, *C. burnetii* o ambas comparados con vacas seronegativas a ambas enfermedades. Esto indica que el sistema inmune materno fue capaz en responder a la infección con *N. caninum* y/o *C. burnetii* durante el último trimestre de gestación.

8- Aunque el sistema inmune materno de las vacas respondía de una forma diferente frente a la infección con *N. caninum*, *C. burnetii* o ambos, estas vacas no abortaron. Indicando que, a pesar de una posible reactivación de la infección con *N. caninum* y/o *C. burnetii* ocurrida durante el último trimestre de gestación, ésta no afectaba negativamente a la gestación.

9- Una dosis subcutánea de 332 µg/kg de melatonina fue capaz a incrementar los niveles plasmáticos de melatonina (C_{max} y AUC) para un mínimo de 40 días después de su administración, y por lo tanto esta fue la dosis recomendada para estudiar los efectos

de la melatonina en las vacas lecheras. Dicha dosis no afectó negativamente a la producción lechera.

10- La vida media de la melatonina en sangre se correlacionó negativamente con la producción de leche en el momento del tratamiento.

11- La administración de melatonina en el momento del secado mejoró el rendimiento reproductivo de las vacas lecheras, como consecuencia de la reducción del número de días abiertos, la disminución del número de inseminaciones para que quede una vaca gestante y las pérdidas de gestación durante el posparto.

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Professional category and starting date: Investigador en formación MEC-FPI - 01/12/2008 (Full-time contract)

ACADEMIC TRAINING

Bachelor's Degree	**Centre**	**Date**
Doctor of Veterinary Medicine (DVM)	Zagazig University	06/08/2006
Master Degree	**Centre**	**Date**
Official master with European mention	ETSEA, Universidad de Lleida	24/02/2010

PREVIOUS SCIENTIFIC AND/OR TEACHING ACTIVITIES

Activity	**Center/Institution**	**Dates**
Lecturer of Anatomy and Embryology, for first and second year veterinary students	Faculty of Veterinary Medicine, Zagazig Univ.	24/03/2007 - 30/11/2008
Member of the Quality Assurance Unit	Faculty of Veterinary Medicine, Zagazig Univ.	2006 - 2008

Languages of scientific interest *(Regular, Sufficient, Well)*

Language	**Speak**	**Read**	**Write**
**Arabic	Well	Well	Well
**English	Well	Well	Well
**Spanish	Well	Well	Well
**Catalan	Regular	Sufficient	Regular

Participation in funded R+D+I projects from public calls

(national and/or international)

Projects

Title of the project / contract: Grupo consolidado sobre factores que afectan a la fertilidad y al mantenimiento de lagestación en vacuno lechero
Kind of contract/Program: Projectes de recerca per potenciar els grups de recerca consolidats
Financing Firm/administration: Departament d'Innovació, Universitats i Empresa de la Generalitat de Catalunya (DIUE)
Number of the project / contract: 2009 SGR 816 **Amount:** 42.640,00 **Duration, since:** 2009 **Until:** 2012
Main researcher: Fernando Roman Lopez Gatius
Number of researchers participating: 6
Keywords: Fertilidad / Vacuno lechero / estrés térmico calórico / aborto / Terapia Hormonal
Code of the project / contract: 006970 **Order:** 001

Title of the project / contract: Aspectos endocrinos e inmunológicos asociados con la infección y el aborto en gando vacuno y estudio del papel de los carnívoros silvestres en el ciclo silvático del parásito
Kind of contract/Program: Programa Nacional de Recursos y Tecnologías Agroalimentarias (AGL)
Financing Firm/administration: Ministerio de Educación y Ciencia
Number of the project / contract: AGL2007-65521-C02-01 **Amount:** 181.500,00 **Duration, since:** 2007 **Until:** 2010
Main researcher: Fernando Roman Lopez Gatius
Number of researchers participating: 6
Keywords: Glicoproteína / Neospora caninum / Carnivoros silvestres
Code of the project / contract: 005509 **Order:** 002

Title of the project / contract: Effects of exogenous melatonin during the dry period during the warm period of the year on gestation health and subsequent postpartum health, milk production and reproductive performance in high producing dairy cows.
Kind of contract/Program: Contracte de recerca
Financing Firm/administration: CEVA Sante Animale
Number of the project / contract: C08023 **Amount:** 64.000,00 **Duration, since:** 2008 **Until:** 2010
Main researcher: Fernando Roman Lopez Gatius
Number of researchers participating: 5

Title of the project / contract: Interacciones inmunoendocrinas materno-fetal y con Coxiella burnetii en vacas infectadas con Neospora caninum. Efecto del tratamiento con melatonina en ambas enfermedades
Kind of contract/Program: Programa Nacional de Recursos y Tecnologías Agroalimentarias (AGL)
Financing Firm/administration: Ministerio de Ciencia e Innovación
Number of the project / contract: AGL2010-21273-C03/GAN **Amount:** --- **Duration, since:** 2010 **Until:** 2011
Main researcher: Fernando Roman Lopez Gatius
Number of researchers participating: 4

Title of the project / contract: Neosporosis bovina: interaciones materno-fetal y mecanismos asociados con la proteccion frente al aborto en gestaciones de razas cruzadas en condiciones de campo

Kind of contract/Program: Programa Nacional de Recursos y Tecnologías Agroalimentarias (AGL)

Financing Firm/administration: Ministerio de Ciencia e Innovación

Number of the project / contract: AGL2012-39830-C02-01 **Amount:** --- **Duration, since:** 2012 **Until:** 2013

Main researcher: Fernando Roman Lopez Gatius

Number of researchers participating: 4

Participation in R+D+I Contracts of special importance with companies and/or public services

(national and/or international)

Title of the project / contract: Effects of exogenous melatonin during the dry period during the warm period of the year on gestation health and subsequent postpartum health, milk production and reproductive performance in high producing dairy cows.

Kind of contract/Program: CORE - Contracte de recerca

Financing Firm/administration: CEVF - CEVA Sante Animale

Institutions participating: ---

Number of the project / contract: C08023 **Amount:** 64.000,00 **Duration, since:** 2008 **Until:** 2010

Main researcher: Fernando Roman Lopez Gatius

Number of researchers participating: 5

Keywords: 000312 - Fertilidad / 203101 - Vacuno lechero / 204456 - estrés térmico calórico / 204457 - aborto / 204458 - Terapia Hormonal

Code of the project / contract: 004777 **Order:** 003

Publications or Scientific-technical documents

(Journal publications)

Authors (signature): Serrano B, Almería S, García-Ispierto I, Yániz JL, Abdelfattah-Hassan A, López-Gatius F.

Title: Peripheral white blood cell counts throughout pregnancy in non-aborting Neospora caninum-seronegative and seropositive high-producing dairy cows in a Holstein Friesian herd

Journal: Research in Veterinary Science

Volume: 90 **Number:** 3 **Pages, Initial:** 457 **final:** 462 **Year:** 2011 **Place of publication:** ENGLAND **ISSN:** 0034-5288

Link: http://www.sciencedirect.com/science/article/pii/S0034528810002559

Authors (signature): Abdelfatah-Hassan A, Almería S, Serrano B, de Sousa NM, Beckers JF, López-Gatius F

Title: The Inseminating Bull and Plasma Pregnancy-Associated Glycoprotein (PAG) Levels Affect Peripheral Leukocyte Counts during the Late Pregnancy/Early Postpartum Period in High-Producing Dairy Cows

Journal: Theriogenology

Volume: 77 **Number:** 7 **Pages, Initial:** 1390 **final:** 1397 **Year:** 2012 **Place of publication:** UNITED STATES **ISSN:** 0093-691X

Link: http://www.sciencedirect.com/science/article/pii/S0093691X11005620

Authors (signature): García-Ispierto I, Abdelfattah-Hassan A, López-Gatius F.

Title: Melatonin Treatment at Dry-off Improves Reproductive Performance Postpartum in High-producing Dairy Cows under Heat Stress Conditions.

Journal: Reproduction in Domestic Animals. doi: 10.1111/rda.12128

Volume: Number: Pages, Initial: final: Year: 2012 **Place of publication: ISSN:** 1439-0531 (Online)

Link: http://onlinelibrary.wiley.com/doi/10.1111/rda.12128/abstract

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Research stays abroad
(longer than 30 days)

Center: Friedrich-Loeffler-Institut (FLI). Bundesforschungsinstitut für Tiergesundheit [Instituto Federal de Investigación en Sanidad Animal]. Institut für Epidemiologie (IfE) [Instituto de Epidemiología]
Place: Berlin-Brandenburg **Country:** GERMANY **Year:** 2010 **Duration:** 4 Months & 5 Days

Center: Department of Animal Sciences, University of Florida, USA.
Place: Florida **Country:** UNITED STATES **Year:** 2012 **Duration:** 4 Months

Conference contributions

Authors: Abdelfatah-Hassan A, Almería S, Tutusaus J, López-Gatius F
Title: Total Lymphocyte Counts are Affected by *Neospora Caninum* during the Peripartum Period in Dairy Cows
Kind of participation: Poster
Conference: 15th Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR 2011)
Publication: Reproduction in domestic animals, vol. 46, (suppl. s3), p. 78 (2011) Online ISSN: 0936-6768.
Number or authors: 4
Place of celebration: Antalya (TURKEY) **Year:** 2011

Authors: Serrano B, Abdelfatah-Hassan A, Andreu-Vázquez C, López-Gatius F
Title: Relationships between Placenta Retention and the Peripartum Leukocytic Counts in High-Producing Dairy Cows
Kind of participation: Poster
Conference: 15th Annual Conference of the European Society for Domestic Animal Reproduction (ESDAR 2011)
Publication: Reproduction in domestic animals, vol. 46, (suppl. s3), p. 149 (2011) Online ISSN: 0936-6768.
Number or authors: 4
Place of celebration: Antalya (TURKEY) **Year:** 2011

Authors: Abdelfatah-Hassan A; Serrano B; Almería S; López-Gatius F
Title: El recuento de Leucocitos Totales y Linfocitos durante el periodo Periparto es diferente entre vacas lecheras de alta y baja producción
Kind of participation: Oral presentation (en Spanish)
Conference: XIV Jornadas sobre Producción Animal de la Asociación Interprofesional para el Desarrollo Agrario (AIDA)
Publication: XIV Jornadas sobre Producción Animal. Vol. I. ISBN: 978-84-615-0064-02, pp. 356-358.
Number or authors: 4
Place of celebration: Zaragoza (SPAIN) **Year:** 2011

Authors: Serrano B; Abdelfatah-Hassan A; Almería S; Uriarte J; López-Gatius F; García-Ispierto I;
Title: El estado de Inmunosupresión Materna durante el periodo Periparto aumenta la incidencia de desórdenes reproductivos postparto
Kind of participation: Oral presentation
Conference: XIV Jornadas sobre Producción Animal de la Asociación Interprofesional para el Desarrollo Agrario (AIDA)
Publication: XIV Jornadas sobre Producción Animal. Vol. I. ISBN: 978-84-615-0064-02, pp. 353-355.
Number or authors: 6
Place of celebration: Zaragoza (SPAIN) **Year:** 2011

Authors: Abdelfatah-Hassan A, Serrano B, García-Ispierto I, López-Gatius F
Title: Factors affecting Leukocytic Counts during the peripartum period in high producing dairy cattle
Kind of participation: Oral presentation
Conference: 10º Congreso de la Asociación Española de Reproducción Animal (AERA)
Publication: Reproduction in domestic animals, vol. 45, (suppl. s2), p. 81 (2010) Online ISSN: 1439-0531.
Number or authors: 4
Place of celebration: Cáceres (SPAIN) **Year:** 2010

Authors: Serrano B, Abdelfatah-Hassan A, García-Ispierto I, Nogareda C, Yániz JL, Almería S, López-Gatius F
Title: Relationships between *Neospora caninum* antibody responses and peripheral white blood cell counts during pregnancy in naturally *Neospora*-infected cattle
Kind of participation: Poster
Conference: 10º Congreso de la Asociación Española de Reproducción Animal (AERA)
Publication: Reproduction in Domestic Animals, vol. 45, (suppl. s2), p. 85 (2010) Online ISSN: 1439-0531.
Number or authors: 7
Place of celebration: Cáceres (SPAIN) **Year:** 2010

Authors: López-Helguera I, Garcia-Ispierto I, Abdelfatah-Hassan A, López-Gatius F
Title: Prepartum Lymphocytic counts are positively correlated to Somatic Cell count in the early lactation
Kind of participation: Poster
Conference: 10º Congreso Internacional de la Asociación Española de Reproducción Animal
Publication: Reproduction in Domestic Animals, vol. 45, (Suppl. s2), p. 84 (2010) Online ISSN: 1439-0531.
Number or authors: 4
Place of celebration: Cáceres (SPAIN) **Year:** 2010

Authors: Ahmed Abd-Elfatah Hassan
Kind of participation: Organization committee member
Conference: 'Adapting Animal Production to changes for Growing Human Population'
Place of celebration: Lleida (SPAIN) **Year:** 2010

Authors: Ahmed Abd-Elfatah Hassan
Kind of participation: Assistance
Conference: 1st conference of African Association of Veterinary Anatomists (AAVA)
Place of celebration: Cairo (EGYPT) **Year:** 2008

Authors: Ahmed Abd-Elfatah Hassan
Kind of participation: Assistance
Conference: '31st conference of Egyptian Anatomical Society (EAS)'
Place of celebration: Cairo (EGYPT) **Year:** 2007

Authors: Ahmed Abd-Elfatah Hassan
Title: 'Equine Stay Apparatus'
Kind of participation: Poster
Conference: '5th student symposium of Zagazig Veterinary Student Association (ZVSA)'
Number or authors: 1
Place of celebration: El Zagazig (EGYPT) **Year:** 2003

Techniques or Specialties

Technique: Transrectal ultrasound examination in cows.

Technique: Leukocyte counts (Both traditionally and using HEMAVET® HV-950FS Multispecies Haematology System).

Technique: Parasite In-vitro cultivation (*Neospora caninum*, *Toxoplasma gondii*, *Besnoitia besnoiti*) in tissue culture (vero cells and other cell lines).

Technique: Total antigen preparation (from parasites). Antigen purification by means of affinity chromatography.

Technique: ELISA

Technique: Immune Blotting 'Western Blotting'

Technique: Indirect Fluorescent Antibody Technique (IFAT)

Technique: PCR & Real-Time PCR

Technique: Loop-mediated Isotheral Amplification (LAMP)

Technique: Isolation and purification of different white blood cells subpopulations (Neutrophils or PBMCs).

Technique: Flow cytometry of different white blood cells subpopulations.

Technique: Neutrophil functions assays (phagocytosis and chemotaxis evaluation).

Specialized Equipments and Systems used

Equipment: Scanning Electron Microscopy (SEM)
Dates: 14/05/2010 - 26/05/2010

Other important activities (Teaching and Courses)

Activity: Neospora caninum, control and diagnosis. **Hours:** 2
Dates: 10/12/2012
Broad field: **Local (Grado Biotecnologia, ETSEA, Universidad de Lleida)
Classification: Teaching in grade

Activity: Assignatura: Histiofisiología de los Tejidos Animales. **Hours:** 5
Dates: 20/01/2011 - 20/01/2011
Broad field: **Local (Grado Ciencia y Salud Animal, ETSEA, Universidad de Lleida)
Classification: Teaching in grade

Activity: Assignatura: Histiofisiología de los Tejidos Animales **Hours:** 4
Dates: 2010
Broad field: **Local (Grado Ciencia y Salud Animal, ETSEA, Universidad de Lleida)
Classification: Teaching in grade

Activity: Teaching Animal Anatomy I & II **Hours:** 240
Dates: 24/03/2007 - 30/11/2008
Broad field: **Local (Faculty of Veterinary Medicine, Zagazig University)
Classification: Teaching in grade

Activity: Teaching Animal Embryology **Hours:** 60
Dates: 24/03/2007 - 30/11/2008
Broad field: **Local (Faculty of Veterinary Medicine, Zagazig University)
Classification: Teaching in grade

Activity: UNIVERSIDAD Y COEDUCACIÓN (U0539). LAS TÉCNICAS DE GRUPO COMO PROCEDIMIENTO PARA INCREMENTAR LOS COMPORTAMIENTOS ASERTIVOS DE LAS ESTUDIANTES EN LAS CLASES.
Dates: 21/02/2011 - 23/02/2011
Broad field: **Nacional (Universidad de Lleida)
Classification: **Courses

Activity: ESTRATEGIAS PARA ANALIZAR/EVALUAR DEBATES VIRTUALES (U0544).
Dates: 05/04/2011 - 05/04/2011
Broad field: **Nacional (Universidad de Lleida)
Classification: ** Courses

Activity: TALLER ABP: APRENDIZAJE BASADO EN PROBLEMAS Y EN PROYECTOS (U0548).
Dates: 06/04/2011 - 27/04/2011
Broad field: **Nacional (Universidad de Lleida)
Classification: ** Courses

Activity: TALLER DE APRENDIZAJE COOPERATIVO (U0549).
Dates: 28/04/2011 - 29/04/2011
Broad field: **Nacional (Universidad de Lleida)
Classification: ** Courses

Activity: NUEVAS METODOLOGÍAS DIDÁCTICAS, HACIA LA CLASE INTERACTIVA Y MÁS ALLÁ. DESDE EL JUST IN TIME TEACHING HASTA EL TEAM BASED LEARNING (U0552).
Dates: 26/05/2011 - 27/05/2011
Broad field: **Nacional (Universidad de Lleida)
Classification: ** Courses

“Opportunity is missed by most people because it is dressed in overalls and looks like work”

... Thomas A. Edison