Fetal-maternal crosstalk in holstein-friesian heifers experimentally infected with *neospora caninum*

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FETAL-MATERNAL CROSSTALK IN HOLSTEIN-FRIESIAN HEIFERS EXPERIMENTALLY INFECTED WITH *NEOSPORA CANINUM*

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SUMMARY

SUMMARY

The global aim of this thesis was to expand the knowledge about how *Neospora caninum* infection affects fetal-maternal crosstalk and uterine immunomodulation.

In the first study we evaluated the outcome of pregnancy in dairy heifers after an experimental infection with *N. caninum* in the second trimester of gestation and we examined the immune response in the fetus. Fetal death occurred in 3 of 6 experimentally infected dams, the parasite was found in all fetuses and IFN-γ production was detected only in fetal fluids of a dead fetus found upon euthanasia.

The aim of the second study was to examine PAG-1 and PAG-2 dynamics and trophoblast cell populations following an experimental infection with *N. caninum*. Plasma PAG-1 and PAG-2 concentrations in non-infected heifers increased until the day of euthanasia, non-aborting infected heifers showed a temporary fall in PAG-1 and PAG-2 concentrations from 7 to 14 days post infection (dpi) and aborting infected heifers showed dramatic PAG-1 and PAG-2 reductions from 21 dpi to euthanasia. A stereological study of placentomes revealed significantly higher relative proportions of mononucleate and binucleate trophoblast cells at 42 dpi in non-infected heifers than infected non-aborting heifers.

The third study was designed to determine the effect of an experimental infection of pregnant dairy heifers with *N. caninum* on PAG-1 and PAG-2 concentrations and pH in amniotic and allantoic fluids. Fetal fluids from infected heifers showed higher levels of PAG-1, PAG-2

and pH than non-infected heifers. Furthermore, fetal fluids presenting antibodies against *N. caninum* showed the highest levels.

In the fourth study we examined the expression patterns of the genes *SERPINA14, PAG1*, and *PAG2* at the fetal-maternal interface in dairy heifers experimentally infected with *N. caninum.* Expression of *SERPINA14* was higher in intercaruncular endometrium of control dams than in infected animals. There was also a negative association between *N. caninum* antibody titers with *SERPINA14* and *PAG* expression in infected animals.

In the fifth chapter we estimated the cost of *N. caninum* infection for the Spanish dairy industry to be $42 million per year. Research to mitigate *Neospora caninum* negative effects and efforts to eradicate *N. caninum* from dairy farms are economically justified.

In conclusion, *N. caninum* infection modifies the fetal-maternal crosstalk and the immunological balance of gestation and an increased Th1 immune response during gestation is needed to prevent *N. caninum* induced abortion.

RESUMEN

El objetivo global de esta tesis fue expandir el conocimiento sobre cómo afecta una infección con *Neospora caninum* a la comunicación materno-fetal y a la inmunomodulación uterina.

En el primer estudio se evaluó el desenlace de la gestación de novillas experimentalmente infectadas con *N. caninum* en el segundo trimestre de gestación y la respuesta inmune del feto tras la infección. La muerte fetal se produjo en 3 de las 6 novillas infectadas, el parasito se encontró en todos los fetos y tan solo se detecto IFN-γ en los fluidos fetales de un feto encontrado muerto durante la eutanasia.

El objetivo del segundo estudio fue examinar las dinámicas de PAG-1 y PAG-2 y las poblaciones de células trofoblasticas después de una infección con *N. caninum*. Las concentraciones de PAG-1 y PAG-2 aumentaron de manera constante en las novillas no infectadas hasta el momento de la eutanasia, las novillas infectadas que no abortaron mostraron un descenso temporal de PAG-1 y PAG-2 entre los días 7 y 14 post infección y la novillas infectadas que abortaron mostraron un gran descenso de PAG-1 y PAG-2 a partir del segundo día de gestación. El estudio estereológico de los placentomos reveló una mayor concentración de células mononucleadas y binucleadas a los 42 días post-infección en las novillas no infectadas respecto a las infectadas.

El tercer estudio se diseñó para determinar el efecto de una infección experimental con *N. caninum* en novillas lecheras en las concentraciones de PAG-1 y PAG-2 y los niveles de pH en los fluidos amniótico y alantoideo. Los fluidos fetales de las novillas infectadas mostraron

niveles más elevados de PAG-1, PAG-2 y pH. Ademas los niveles mas altos se encontraron en aquellos fluidos en los que también se detectaron anticuerpos contra *N. caninum*.

En el cuarto estudio se examinaron los patrones de expresión de los genes *SERPINA14*, *PAG1* y *PAG2* en la interfase materno-fetal de novillas lecheras experimentalmente infectadas con *N. caninum*. La expresión de *SERPINA14* fue mayor en el endometrio intercaruncular de las novillas control que en el de las novillas infectadas. También se halló una correlación negativa entre los títulos de anticuerpos contra *N. caninum* y la expresión de *SERPINA14* y *PAG1*.

En el quinto capitulo se calculó el coste de la neosporosis bovina para las granjas lecheras españolas en 42 millones de dólares al año. Las investigaciones destinadas a mitigar los efectos negativos de la neosporosis y los esfuerzos para erradicar *N. caninum* en las lecherías están justificados económicamente.

En conclusión, la infección por *N. caninum* modifica la comunicación materno-fetal y el equilibrio inmunológico de la gestación. Ademas, un aumento de la respuesta inmune de tipo Th1 es necesario para prevenir un aborto inducido por *N. caninum*.

RESUM

L'objectiu global d'aquesta tesi ha estat ampliar el coneixement sobre com afecta una infecció amb *Neospora caninum* a la comunicació materno-fetal i a la immunomodulació uterina.

En el primer estudi es va avaluar el desenllaç de la gestació de vedelles experimentalment infectades amb *N. caninum* al segon trimestre de la gestació i la resposta immune del fetus un cop iniciada la infecció. La mort fetal es va produïr en 3 de les 6 vedelles infectades, el paràsit es va detectar en tots els fetus i només es va detectar IFN- γ en els fluïds d'un fetus trobat mort durant l'eutanàsia.

L'objectiu del segon estudi va ser examinar les dinàmiques de la PAG-1 i la PAG-2 en les poblacions de cèl.lules trofoblàstiques després d'una infecció amb *N. caninum*. Les concentracions de PAG-1 i PAG-2 van créixer de manera constant en les vedelles no infectades fins al moment de l'eutanàsia, les vedelles infectades que no van abortar van mostrar una baixada temporal de PAG-1 i PAG-2 entre els dies 7 i 14 post infecció i les vedelles infectades abortades van mostrar una gran baixada de PAG-1 i PAG-2 a partir del segon dia de gestació. L'estudi estereològic dels placentomes va revelar una major concentració de cèl.lules mononucleades i binucleades als 42 dies post-infecció en les vedelles no infectades respecte a les infectades.

El tercer estudi es va dissenyar per determinar l'efecte d' una infecció experimental de *N. caninum* en vedellles lleteres sobre les concentracions de PAG-1 i PAG-2 i els nivells de pH

en els fluïds amniòtic i alantoideu. Els fluïds fetals de les vedelles infectades van mostrar nivells més elevats de PAG-1, PAG-2 i pH. A més, els nivells més alts es van trobar en aquells fluïds en els que també es van detectar anticossos contra *N. caninum*.

Al quart estudi es van examinar els patrons d'expressió dels gens *SERPINA14, PAG1* i *PAG2* en l'interfase materno-fetal de les vedelles lleteres experimentalment infectades amb *N. caninum*. L'expressió de *SERPINA14* fou major a l'endometri intercaruncular de les vedelles control que al de les vedellles infectades. També es va trovar correlació negativa entre els títols d'anticossos contra *N. caninum* i l'expressió de *SERPINA14* i *PAG1*.

Al cinquè capítol es detalla el cost de la neosporosi bovina per a les granges lleteres espanyoles en 42 milions de dòlars a l'any. Les investigacions destinades a mitigar els efectes negatius de la neosporosi i els esforços per erradicar *N. caninum* en les lleteries estàn justificats econòmicament.

En conclusió, la infecció per *N. caninum* modifica la comunicació materno-fetal i l'equilibri immunològic de la gestació i és necessari un augment de la resposta immune de tipus Th1 per a prevenir un abortament induït per *N. caninum*.

GENERAL INTRODUCTION

INTRODUCTION

In 2013 there were 271 million of dairy cows that produced 638 million tones of milk worldwide (FAOstat). In order to start milk production and take advantage of the most efficient part of the lactation curve, cows have to calf (Ferguson and Galligan, 1999). Individual milk production mainly depends on the cow's ability to have more calvings during its career (Sørensen and Østergard, 2001) and the profitability and the sustainability of dairy farms are strongly associated with reproductive performance (Giordano *et al*., 2012; Galvao *et al*., 2013). After cows get pregnant, the economic loss attributed to pregnancy loss increase with pregnancy age (Cabrera, 2014). If cows abort after month 4 of gestation can be expected to miss one lactation and usually are culled and replaced (Reichel *et al*., 2013) Thus, abortion in dairy cattle is cause of great economic losses and efforts should be done to study and prevent it.

Nowadays one of the major cause of abortion in cattle worldwide is an obligate intracellular protozoan parasite known as *Neospora caninum* (Almería and López-Gatius, 2015). Economic losses attributable to bovine neosporosis amount to over US$1 billion every year (Reichel *et al*., 2013) and are mainly the outcome of a lengthened calving interval, reduced milk production, falling stock value and an elevated culling rate (Trees *et al*., 1999). In our geographical area of study (North-East Spain), *Neospora*-seropositive cows carry a 12–19 times greater risk of abortion than seronegative cows, abortion affecting 30 to 44% of seropositive animals (López-Gatius *et al*., 2004a,b). As a global problem, *N. caninum* has been widely studied since its discovery in 1988 but, the main questions such as 'Why do some cows abort and others do not? or How *Neospora* induced abortion could be prevented?' still remain without clear answers (reviewed by Almería and López-Gatius, 2015; Almería

and López-Gatius, 2013; Goodswen *et al*., 2013; Dubey and Shares, 2011; Dubey *et al*., 2007).

Cows have cotyledonary synepitheliochorial placentation. In this type of placentation binucleated and mononucleated trophoblast cells transport products between the dam and the fetus due to fetal and maternal circulatory systems are not connected (Haeger *et al*., 2016). These cells produce Pregnancy Associated Glycoproteins (PAGs) which are implicated in fetal-maternal communication and immunomodulation (Wallace *et al*., 2015). PAGs interact with uterine products such as Serine Proteinase Inhibitor 14 (SERPINA14) that has been attributed with immunosuppressive properties (Padua and Hansen, 2010).

Neospora caninum

History of discovery*:* *Neospora caninum* is an obligate intracellular protozoan that was first described in dogs in Norway in 1984 (Bjerkas et al., 1984).It was misdiagnosed as *Toxoplasma gondii* until 1988, when the new genus and species, *Neospora caninum*, was described (Dubey *et al*., 1988). *N. caninum* is also extremely similar to *Hammondia heydorni* and the definitive differentiation among them was not achieved until 2002 when *N. caninum* was redescripted (Dubey *et al*., 2002). *N. caninum* was first associated with abortion in cows at a Holstein-Friesian dairy farm in north-central New Mexico in 1989 (Thilsted and Dubey, 1989). Since then, it has been found in cattle and dogs worldwide (Dubey and Shares, 2011).

Life cycle: In the life cycle of *N. caninum*, there are three known infectious stages: tachyzoites, bradizoites and oocyst. Unsporulated oocysts are shed in the faeces from definitive host where sexual replication occur (Reichel *et al*., 2007). Outside the host the oocysts sporulate to contain two sporocysts of which each contains four sporozoites (Dubey

et al., 2002). The intermediary host can ingest sporulated oocysts found on contaminated food or water and become infected. Sporozoites are liberated from oocysts in the gut of intermediary host and invade the gut wall transforming into tachyzoites. Tachyzoites reside in unique intracellular compartments called parasitophorous vacuoles and rapidly replicate asexually. Tachyzoites can invade different types of cells like neural, macrophage, fibroblast, vascular endothelial, muscle and liver cells (Barr *et al*., 1993; Dubey *et al*., 2002). The rapid replication of tachyzoites last about 3 weeks and then they differentiate into bradyzoites that form cysts in tissues (Dubey *et al*., 1990). The tissue cysts are typically found in neural or skeletal muscle cells and can persist for the life of the intermediate host without clinical manifestations (Dubey and Lindsay, 1996). The life cycle is completed when a tissue cysts present in contaminated meat are ingested by the definitive host where sexual replication occurs in the gut forming new oocysts (Dubey *et al*., 2007).

The parasite was found in wide range of warm-blooded animals worldwide, but only canids like the domestic dog, the Australian dingo (both *Canis domesticus*), the coyote (*Canis latrans*) and the grey wolf (*Canis lupus*) were proved as definitive hosts for *N. caninum* (Dubey and Shares, 2011; Dubey *et al*., 2011). Proved intermediary host which viable *Neospora* have been isolated are only cattle, sheep, water buffalo, dog, horse, bison and white-tailed deer (Dubey and Shares, 2011).

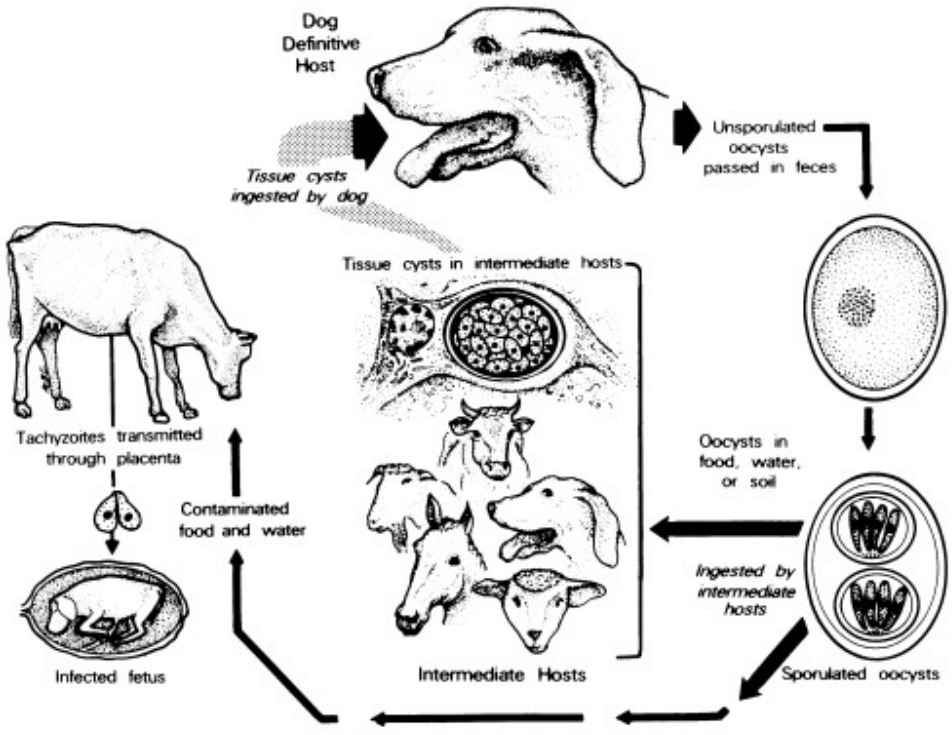

Figure 1. Life cycle of *Nespora caninum* (Dubey *et al*., 2007)

Bovine neosporosis

Clinical signs: For the most part, *Neospora*-infected adult cattle do not exhibit clinical signs of the disease (Goodswen *et al*., 2013), being the abortion the main clinical sign of bovine neosporosis (Almería and López-Gatius, 2013). Fetuses may die *in utero* or be reabsorbed, mummified, autolyzed, stillborn, born alive with clinical signs or born clinically normal but persistently infected (Dubey *et al*. 2007). In neonatal calves signs like poor suckling reflex, poor balance, improper positioning of rear fetlocks, inability to stand, and seizures ("dummy calves") can be attributed to congenital neurological defects produced by *N. caninum* (Mcallister, 2007).

Transmission: Vertical or transplacental is the major route of transmission of *N. caninum* in cattle (Barr *et al*., 1993; Schares *et al*., 1998; Hietala and Thurmond, 1999). The rate of vertical transmission may vary among herds, from 0% to 85.7% (Bergeron *et al*., 2000) and between cows with low and high antibody titers from 14,8% to 94,8%, respectively (Moré *et al*., 2009). Horizontal or postnatal transmission rarely occurs in nature (Hall *et al*., 2005). There is no cow to cow (Anderson *et al*., 1997), venereal (Osoro *et al*., 2009) or lactogenic (Dijkstra *et al*., 2001) transmission although the parasite was found in semen (Ferre *et al*., 2008), milk and calostrum (Moskwa *et al*., 2007). The ingestion of oocysts is the only demonstrated mode of horizontal transmission in cattle (Dubey *et al*., 2007), thus the rate of postnatal transmission is related to herd exposure to domestic and wild canids (Barling *et al*., 2000).

N. caninum is one of the most efficiently transplacentally transmitted parasites among all known microbes in cattle (Dubey *et al*., 2006). Transplacental transmission can occur in postnatally acquired infections by ingestion of oocysts (exogenous) or reactivation of infection in a chronically infected cow (endogenous) and the rate of transmission may differ in these two scenarios (Williams *et al*., 2009). In both exogenous and endogenous infections, the gestation age when taquizoites arrive to placenta and fetus seems to be a key factor. The immunocompetence of the foetus develops progressively over the approximately 280 days of cattle pregnancy. Therefore, a foetus is exceptionally vulnerable to *N. caninum* infection during early gestation (e.g. <100 days gestation) when the thymus, spleen and peripheral lymph nodes are immature (Swift and Kennedy, 1972). If recrudescence transpires within this gestation period an abortion is most likely to occur (Guy *et al*., 2001). During mid-gestation (e.g. 100–150 days) the tissues and organs of the foetus's immune system start to

rudimentarily recognise and respond to microorganisms but ineffectively for its survival because most abortions are reported to occur during this gestation period (Dubey *et al*., 2006). The increasingly immunocompetent foetus during third trimester can survive to develop into a clinically normal, but congenitally infected calf (Williams *et al*., 2000). Congenital infection is also more likely than abortion if recrudescence transpires during the third trimester (Guy *et al*., 2001).

Abortion: *Neospora*-infected cows may abort from 3 months of gestation to term, though most abortions take place in the second trimester of gestation (López-Gatius *et al*. 2004a). For *N. caninum*-associated abortion to occur, the fetus or placenta has to be damaged to the extent that it is no longer viable (Dubey *et al*. 2006; Gibney *et al*. 2008).

The precise causes of fetal or placental damage are not well-known although it has been proposed that the parasite or parasite-induced pro- inflammatory immune response, the only effective response against *N. caninum*, may cause fetal or placental damage (Quinn *et al*. 2002; Innes *et al*. 2005; Almería and López-Gatius 2015).

Nowadays still remain unclear why some *Neospora*-infected cows abort and others do not. Even so, a large number of factors have been proved as increasers or reducers of *Neospora*-induced abortion risk (reviewed by Dubey *et al*. 2007; Almería and López-Gatius, 2013). Between these factors highlight the *N. caninum* antibodies titers, the climate stress, the breed, the cross-breed pregnancies and strain of the parasite. Several studies have proved that abortion risk increases with increasing levels of *N. caninum*-specific antibodies in individual animals (López-Gatius *et al*. 2005a; Quintanilla-Gozalo *et al*. 2000; Stenlund *et al*. 2003; Wouda *et al*. 1999). Also, *N. caninum* abortion outbreaks have been related with high

rainfalls in dry environment in Spain (López-Gatius *et al*., 2005b), cold season in California (Thurmond *et al*., 1995) and with hot season in The Netherlands (Wouda *et al*., 1999). Besides, a lower risk of abortion has been noted in infected beef cows compared to dairy cows (De Meerschman *et al*., 2000), and insemination of Holstein-Friesian dairy cows with beef bull semen, especially Limousin breed, reduces dramatically the risk of abortion in seropositive cows (López-Gatius *et al*., 2005a; Almería *et al*. 2009; Yaniz *et al*. 2010). Finally, different strains of *N. caninum* show different virulence and abortion and transmission rate (Benavides *et al*., 2014).

Pregnancy-associated glycoproteins (PAGs)

PAGs are expressed products of the placenta of species within the Cetartiodactyla order, including Suidae, Cetacea and Pecora. These proteins are specially expressed in Bovidae (Wallace *et al*., 2015) and were discovered attempting to develop an early pregnancy test in cattle (Butler *et al*., 1982; Zoli *et al*., 1991; Mialon *et al* 1993). Since then a great number of different PAGs have been discovered in cattle but can be segregated in two main groups defined as ancient (PAG-2) and modern (PAG-1) based on the time when each group arose (Hughes *et al*., 2000). PAG-2 molecules are exclusively expressed in mono-nucleate trophoblast cells, while PAG-1 molecules are expressed in binucleate cells of both the intercotyledonary and cotyledonary chorion (Touzard *et al*. 2013). PAG-2 molecules are accumulated at the microvillar junction between the trophoblast and uterine epithelium and PAG-1 are accumulated mainly in the maternal connective tissue of the caruncle and slightly at the microvillar junction (Wooding *et al*. 2005). Through radioimmunoassay (RIA), PAG-1 and PAG-2 levels can be measured in foetal blood plasma, foetal fluids and maternal blood plasma (Zoli *et al*. 1992; Bella *et al*. 2011). Although the functions of PAGs are not yet fully

understood, maternal plasma PAG-1 concentrations have been used for pregnancy diagnosis and as a marker of placental/fetal well-being (Zoli *et al*. 1992; López-Gatius *et al*. 2007) while, maternal plasma PAG-2 concentrations may serve as an indicator of abortion risk in *N. caninum*-infected cows (García-Ispierto *et al*. 2013). Several studies have shown that *N. caninum* infection (López-Gatius *et al*. 2007; García-Ispierto *et al*. 2010, 2013, 2015) and crossbreed pregnancies (Serrano *et al*., 2009) modifies PAG-1 and PAG-2 patterns in maternal blood plasma.

Uterine serpins (SERPINA14)

Uterine serpins (SERPINA14) are proteins belonging to the serine proteinase inhibitor (serpin) superfamily. These proteins are present in some mammals in the Laurasiatheria superorder (ruminants, horses, pigs, dolphins and some carnivores) (Padua and Hansen, 2010). In cattle SERPINA14 is expressed mainly in the uterus, but also can be found in the ovarian follicles, corpus luteum, cumulus oocyte complex and placental cotyledon (Ulbrich *et al*., 2009; Khatib *et al*., 2006). Secretion of bovine SERPINA14 can be induced by progesterone and is maximum during late pregnancy (Leslie and Hansen, 2001). It is not clear yet the role of uterine serpins but has been demonstrated that inhibits lymphocyte function in vitro (Padua and Hansen, 2008). Therefore, SERPINA14 may be responsible in part of the uterine immunosuppressive actions attributed to progesterone (Hansen *et al*., 1987). Uterine serpins could also be implicated in transplacental transport due to have been detected in fetal fluids and interact with other uterine proteins such as PAGs (Mathialagan and Hansen, 1996), IgM and IgA (Hansen and Newton, 1988) and activin A (McFarlane *et al*., 1999). The effects of *N. caninum* infection in *SERPINA14* expression have not been investigated yet.

Thus, the genaral target of this thesis is to contribute in the response of the main unresolved N.caninum questions, 'Why do some cows abort and others do not? and How *Neospora* induced abortion could be prevented?', through the study of maternal-fetal crosstalk and uterine immunomodulation in the presence of the *Neospora caninum*.

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Web pages

FAOstat, faostat3.fao.org.

MAIN OBJECTIVES

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The present work is composed of four studies, all of them directed to understand how *N. caninum* infection affects fetal-maternal crosstalk and uterine immunomodulation. To reach this global objective, the individual objectives of each work were:

- To evaluate the outcome of pregnancy in dairy heifers after an experimental infection with *N. caninum* in the second trimester of gestation and, to examine the immune response in the fetus.

- To determine maternal plasma PAG-1 and PAG-2 dynamics following experimental *N. caninum* infection in dairy heifers.

- To examine the possible effects of *N. caninum* infection in dairy heifers on mono- and binucleate trophoblast cell numbers in large placentomes 42 days after experimental infection.

- To determine the effect of the experimental infection of pregnant dairy heifers with *N. caninum* on PAG-1 and PAG-2 concentrations and pH in amniotic and allantoic fluids.

- To examine expression of *SERPINA14, PAG1*, and *PAG2* genes at the fetal-maternal interface in the second trimester of gestation after an experimental infection with *N. caninum.*

- To assess possible interrelations between expression patterns of *SERPINA14* and *PAG* with plasma *N. caninum* antibodies and plasma concentrations of PAG-1 and PAG-2 after an experimental infection with *N. caninum*.

- An additional study was included with the objective of evaluating the economical impact of *N. caninum* in dairy herds and the potential impact of inseminate seropositive cows with Limousin semen.

CHAPTER 1

FETAL DEATH IN NAIVE HEIFERS INOCULATED WITH *NEOSPORA CANINUM* ISOLATE NC-SPAIN7 AT 110 DAYS OF PREGNANCY

Abstract

Neospora caninum infection is a leading cause of abortion in cattle worldwide. The pathogenesis of bovine neosporosis, particularly during the second term of gestation, when most abortions occur in naturally infected dams, is poorly understood. In the present study fetal death was observed in 3 of 6 experimentally infected dams at 110 days of gestation after 6 weeks of experimental period. All the experimental heifers were febrile between 3 to 5 days post infection (dpi). Inoculated dams seroconverted by 3-4 weeks post infection with higher mean antibody titers in aborting dams compared to non-aborting heifers. *Neospora caninum* DNA was detected in all infected fetuses and placentas, and three infected fetuses also had *N. caninum* antibodies. The parasite burden was higher in the brain of dead/aborted fetuses than in live fetuses. Interestingly, high IFN-γ production was detected in fetal fluids of a dead fetus found upon euthanasia of its dam, while no IFN-γ was observed in amniotic, allantoic and /or fetal fluids in the three infected fetuses that were alive upon maternal euthanasia. The present study confirms that the infection of dams on gestation day 110 with 10^7 tachyzoites of the Nc-Spain7 isolate causes abortion. The fact that some infected dams aborted and some did not make this model of bovine neosporosis useful to try to understand *N. caninum* pathogenesis of abortion in the second trimester of gestation.

1. Introduction

Neospora caninum is considered as one of the main causes of bovine abortion worldwide (Almería and López-Gatius, 2013; Dubey *et al*., 2007; Dubey and Schares, 2011).

The pathogenesis of bovine neosporosis is complex and only partially understood. Abortion occurs in both naive and chronically infected cows, and abortion is not consistently induced in experimentally infected cows. Many factors including, breed, gestational age, immune status of the cow, route of inoculation, dose, stage of the parasite and the strain/isolate of the parasite inoculated affect the outcome of pregnancy, and these were very recently reviewed in detail by Benavides *et al*. (2014). It is generally agreed that gestational age is one of the important factors; disease is most severe in cows inoculated early in gestation versus late gestation. In most experimental infections established using different isolates in early stages of pregnancy (90 days of gestation or earlier) in naïve cattle, fetal death is the most common finding (e.g. Bacigalupe *et al*., 2013; Bartley *et al*., 2012; Caspe *et al*., 2012; Gibney *et al*., 2008; Macaldowie *et al*., 2004; Regidor-Cerrillo *et al*., 2014; Rosbottom *et al*., 2008; Williams *et al*., 2000) and such deaths have been widely attributed to a lack of fetal immunocompetence. Later in gestation, after 120 days of pregnancy or later (at 210 days), infections mostly give rise to the birth of full-term congenitally infected fetuses (Almería *et al*., 2003; Andrianarivo *et al*., 2001; Benavides *et al*., 2012; Gibney *et al*., 2008; Maley *et al*., 2003; Rosbottom *et al*., 2008; Williams et al., 2000).

The pathogenesis of infection during the second term of gestation, when most abortions occur in naturally infected dams, is poorly understood. Transitory immune-suppression of T lymphocytes, starting at around 18 weeks of gestation, has been observed in cattle experimentally infected with *N. caninum* (Innes *et al*., 2001) and could be the cause of the

increased susceptibility of these animals to parasitaemia at that time. A previous study in heifers experimentally infected at 110 days of gestation and an experimental period of 6 weeks after infection was the first report of fetopahty in dams experimentally infected in the second trimester of gestation (Almería *et al*., 2010). The objectives of the present study were to further evaluate the outcome of pregnancy in cows inoculated following the same experimental infection in the second trimester of gestation and, to examine the immune response in the fetus. A proven virulent isolate of *N. caninum*, Nc-Spain7 was used. This isolate has been recently shown to induce severe neonatal neosporosis in cows inoculated in early pregnancy (65-70 days of gestation) (Caspe *et al*., 2012; Regidor-Cerrillo *et al*., 2014).

2. Material and methods

2.1. Animals and infection

Ten Friesian heifers that were seronegative for *N. caninum* (CIVTEST, Spain) and free or vaccinated against the main abortifacient agents (*Brucella abortus*, bovine viral diarrhoea virus (BVDV) and infectious bovine rhinotracheitis (IBR) virus) were synchronized and artificially inseminated. Pregnancy was assessed by ultrasonography at 30, 45 and 90 days after insemination. At 110 days of pregnancy, 6 of the heifers were intravenously (i.v.) inoculated with 10^7 culture-derived tachyzoites of the *N. caninum* isolate Nc-Spain7 (passage 15), which was kindly donated by Dr. L. M. Ortega-Mora, SALUVET, University Complutense of Madrid, Spain. These 6 heifers were euthanized at 6 weeks post-infection (wpi) (Table 1). The four remaining heifers were kept as uninoculated controls and were euthanized at the same time as inoculated dams. In addition, three Friesian heifers from the same herd, that had tested seropositive for *N. caninum* prior to gestation were inseminated at

the same time and followed during the experimental period to compare the response in naturally infected dams versus the experimentally infected dams. These chronically infected seropositive dams had healthy calves at parturition and were not euthanized.

Table 1. Neosporosis in cows inoculated with 10^7 N. caninum Nc-Spain7 tachyzoites at 110 days of gestation.

Cow				**Fetus**		
No.	*N. caninum* inoculated	Pregnancy Outcome	Histopathology in placenta	Histopathology fetus	DNA detection (organs) (Nc5)	*N. caninum* antibodies
6683	Yes	Live fetus	Pl++	H+, Lu, CNS	CNS, Lu	No
203	Yes	Live fetus	Pl+	Li+, Lu+, CNS+	CNS, Lu, Sk	Yes
641	Yes	Live fetus	Pl++	Li+, CNS++	CNS, H, Lu	Yes
659	Yes	Aborted 3 wpi	Pl+	H+, Lu+, Sp**, CNS**	CNS, H, Lu, Sk	NA
649	Yes	No viable fetus	Pl+**	Lu++, Li**, Sp**, CNS**	CNS, H, Lu, Sk	Yes
635	Yes	Aborted 2 wpi	Pl+	NA	NA	NA
101	No	Live fetus	No lesion	No lesion	Negative	No
199	No	Live fetus	No lesion	No lesion	Negative	No
982	No	Live fetus	No lesion	No lesion	Negative	No
418	No	Live fetus	No lesion	No lesion	Negative	No

+, ++: arbitrary degree of lesions
** Severe autolysis
NA: Not available
H/E tissues positive to lesions in the study: H: heart, CNS (brain and Spinal cord), Li: liver, Lu: lung, PL: placenta, Sk: skeletal muscle.

2.2. Sample collection

Heifers were observed daily throughout the experimental period for possible abortion. Rectal temperatures were recorded daily for the first week after infection and at weekly intervals thereafter until euthanasia. Heifers with a temperature >39.5ºC were considered febrile.

Blood samples were collected from the dams by tail vein puncture on the day before infection and regularly at weekly intervals until culling 6 wpi. In the three chronically infected dams, blood was collected at the same time points. Plasma was obtained by centrifugation within 30 min of sampling and stored at -20ºC until analysis.

At 6 wpi, the six experimentally infected and the four controls uninfected heifers were sedated with xylazine hydrochloride (Rompun; Bayer) and immediately euthanized by an intravenous (i.v.) overdose of embutramide and mebezonio iodide (T61; Intervet). Immediately after death, heifers were necropsied. Amniotic and allantoic fluids were collected before the placenta was opened and fetuses separated from the placenta. Fetal blood samples were obtained by cardiac puncture or peritoneal fluids were collected. Fetuses were measured from crown to rump.Two dams aborted dead fetuses at 2-3 wpi (one could not be recovered and the second was autolytic) and one dam had a non-viable fetus upon euthanasia at 6 wpi (Table 1).

Samples of nine randomly selected placentomes (three cranial, three medial and three caudal) were removed. Fetal tissue specimens collected were: CNS (brain and spinal cord), heart, lung, liver, skeletal muscle, spleen, and thymus.

2.3. Ethics

All procedures were approved by the Ethics Committees on Animal Experimentation of the Autonomous University of Barcelona (license number CEEAH.1426-08/02/2012) and of the University of Lleida (license number CEEA.06-01/12). Heifers were handled in strict accordance with good animal practices and the conditions defined by the Animal Ethics Committee at the Autonomous University of Barcelona and CReSA, Spain. Every effort was made to minimize suffering.

2.4. Sample analysis

2.4.1. Specific anti-*N. caninum* antibodies in infected heifers and fetuses

Serum samples collected before infection and at weekly intervals for 6 weeks from the dams were tested for anti-*N. caninum* antibodies using a commercial ELISA kits based on the whole tachyzoite lysate of *Neospora* NC-1, according to the manufacturers' instructions (CIVTEST Spain). The cut-off used for a positive test result was an S/P ratio of 6.0, as established by López-Gatius *et al*. (2004). Sera and/or fetal fluids were analyzed using the same technique on undiluted samples.

2.4.2. Interferon-γ production in plasma and fetal fluids

In serum samples collected weekly from the experimental dams, and/or in fetal serum/fluids and ammniotic and allantoid fluids collected upon maternal euthanasia or when abortion

occurred, interferon-γ (IFN-γ) production was measured using the kit Bovigam IFN-γ (CSL Veterinary, Victoria, Australia). To quantify IFN-γ levels in the test samples, a standard curve was prepared using serial dilutions of a recombinant bovine IFN-γ standard (rboIFN- γ), as previously described by López-Gatius *et al*. (2007). Mean optical density (OD) values were plotted against units/ml of rboIFN- γ. A regression line was then calculated and the quantity of IFN-γ present in each test sample determined from the standard curve. Results are expressed as pg/ml.

2.4.3. Histopathology

Paraffin-embedded 5-µm sections of the tissues harvested from dams and fetuses were prepared and stained with haematoxylin–eosin (H–E) for histopathological examination.

2.4.4. Parasite detection by *N. caninum*-specific PCR (Nc-5)

Portions of placenta and fetal tissues were aseptically obtained and stored in liquid nitrogen at -80ºC until DNA extraction.

At least 0.5–1 g of each tissue was homogenized with a pestle and mortar in liquid nitrogen and DNA extracted as described by Almería *et al* (2002). Briefly, after lysis of red blood cells, tissue samples were incubated in proteinase K buffer (200 mg of proteinase K/ml) at 37ºC overnight, phenol extracted and precipitated. For PCR-based diagnosis of *N. caninum*, the specific genomic Nc-5 region (Genbank accession X84238) was selected as the target sequence for DNA amplification using the primers Np21+ and Np6+. The PCR reaction was performed as described by Almería *et al*. (2002) including 40 amplification cycles. Amplification products were analyzed by electrophoresis on a 2% agarose gel. DNA extracted from Nc-1 tachyzoites was used to prepare positive controls for *N. caninum*. As a

negative control, PCR was performed on samples lacking template DNA. The sensitivity of this reaction has been established as the detection of the DNA of 1 tachyzoite in a background of host DNA (Almería *et al*., 2002).

2.4.5 Real time PCR for the quantitation of Neospora caninum (Neospora-SP qPCR)

2.4.5.1. Set–up conditions

A real time fluorogenic 5'-nuclease PCR assay (TaqMan assay) was designed for the detection and quantification of *N. caninum*-specific DNA (*Neospora-SP* qPCR). Using published nucleotide sequences of the *N. caninum* Nc-5 repeat element (Genbank accession number X84238), a TaqMan probe and corresponding primers were designed with Primer Express software and custom synthesized by Applied Biosystems (ABI, Foster City, CA, USA). Optimized primer and probe concentrations were tested. The nucleotide sequences of the primers and probe and conditions selected for *Neospora-SP* qPCR were forward primer: 5'CTGTGCTCGCTGGGACTTC (30µM); reverse primer: 5'CGATTTACGACA TACGGTGTTCA (30µM); probe: 6FAM-CATCGGAGGACATCGCTCACTGACTG-TAMRA (20 µM). The *Neospora-SP* qPCR system designed was tested on serial 5-fold dilutions of parasite DNA, equivalent to 10^6 (approximately equal to 100 ng of genomic *Neospora* DNA) to 10^{-1} NC-1 strain tachyzoites Since this test was designed to amplify *N. caninum* DNA in a background of cow DNA, a second standard curve was prepared using the same NC-1 tachyzoite dilutions in the presence of 50 ng/µl of endogenous bovine DNA isolated from non-infected skeletal muscle. Cycle threshold (Ct) values increased linearly as the target DNA quantity decreased until a level of 10^{-1} tachyzoite equivalents was reached. This meant that as for the standard PCR assay, the detection limit of *Neospora-SP* qPCR was 1 tachyzoite in 50 ng of bovine DNA.

As a PCR amplification control in bovine tissues, a second TaqMan assay targeting the conserved region of the bovine β-actin gene was developed using primers and a probe based on those described by Moniwa *et al*. (2007) for the corresponding gene sequence. Optimized primer and probe concentrations were determined. The nucleotide sequences and conditions of the selected primers and probe for bovine β-actin-SP were: forward primer: 5'CCTGCGGCATTCACGAA (30 µM); reverse primer: 5'GCGGATGTCGACGTCACA (30 µM); probe: 6FAM-CTACCTTCAATTCCATCATG-MGBNFQ (20 µM). This procedure should also compensate for the presence of potential PCR-inhibiting compounds and exclude false-negative results due to poor-quality DNA. When reactions had Ct values higher than 30.0, indicating a non-optimal sample quality, DNA extraction was repeated.

2.4.5.2. Neospora-SP qPCR and bovine β-actin-SP TaqMan PCR in experimental samples

DNA from samples testing positive in the *N. caninum*-specific PCR (Nc5) were quantified by the nanodrop method and diluted to a final concentration of 50 ng of DNA/µl.

For the molecular analysis of experimental samples, 50 ng of DNA (1 µl) from each tissue sample was used in a 25-µl reaction in a 96-well reaction plate. Each 25-µl reaction mixture contained TaqMan Universal PCR Master Mix, noAmpliErase UNG (Applied biosystems, Foster City, CA, USA) (2.0x), 0.75 µl of 30 µM forward primer, 0.75 µl of 30 µM reverse primer, 0.3 µl of 20 µM TaqMan probe, 1 µl containing 50 ng of DNA, and 9.7 µl of nuclease-free water. Thermal cycling was carried out following a standard protocol recommended by the manufacturer (1× (50 ºC for 2 min), 1× (95 ºC for 10 min), and 40× (95 ºC for 15 s, 60 ºC for 1 min). All samples were run in triplicate. An ABI 7500 Prism Sequence Detector (Applied Biosystems, Foster City, CA, USA) was used for amplification, data acquisition

and data analysis. A linear *Neospora-SP* qPCR standard curve was routinely generated in each real-time PCR run from serial 10-fold dilutions of parasite DNA equivalent to 10^{-1} tachyzoite to 10^4 tachyzoites. The number of parasites in the samples was calculated from a standard curve of Ct values plotted against the log of known concentrations of the parasite. Reactions for the *Neospora-SP* qPCR sequence and bovine β-actin-SP qPCR were performed in separate tubes. Parasite burdens are expressed as parasite numbers/50 ng bovine tissue.

2.5. Statistical analysis

Data were compared among groups (uninfected controls, seropositive chronically infected dams, infected dams with aborted fetuses, and infected dams with live fetuses) by one-way ANOVA. When significant differences were detected, the Bonferroni or Tukey's Multiple Range test was used to examine all possible pairwise comparisons at each sampling time. All statistical tests were performed using the software package SPSS v17 (Statistical Package for Social Sciences Inc., Chicago, IL, USA). Significance was set at a $P < 0.05$. Data are provided as the mean ± standard deviation.

3. Results

3.1. Clinical observations

Two of the 6 inoculated dams aborted, one at 2 wpi (#635) and another at 3 wpi (#659) (Table 1). The first fetus could not be recovered and the second was very autolytic. Another dam

had a non-viable fetus upon euthanasia at 6 wpi (#649); based on fetal length, this fetus was estimated to have died 2 weeks before euthanasia, at approximately 28 days post infection (dpi). The other 3 infected dams had live fetuses at 6 wpi (Table 1).

All 6 infected dams had fever (>39.5ºC) at 3-5 dpi (Figure 1). Maximum average temperatures were 40.2 ºC, 40.1 ºC and 39.5 ºC at 3 dpi, 4 dpi and 5 dpi, respectively. At these time points, 6, 5 and 3 cows were febrile, respectively. By 6 dpi, temperatures had returned to normal in most heifers, though one dam was still febrile at 6 dpi and another at 7 dpi. Rectal temperatures in uninfected controls and chronically infected seropositive dams were <39°C throughout the experiment (Figure 1).

Significant differences in temperature among groups were observed at 3 dpi, 4 dpi and 5 dpi (< 0.01, P<0.001 and P=0.012, respectively). Significantly higher temperatures were observed in infected heifers (with aborted or live fetuses) compared to uninfected control and naturally infected dams at the three time points (Figure 1).

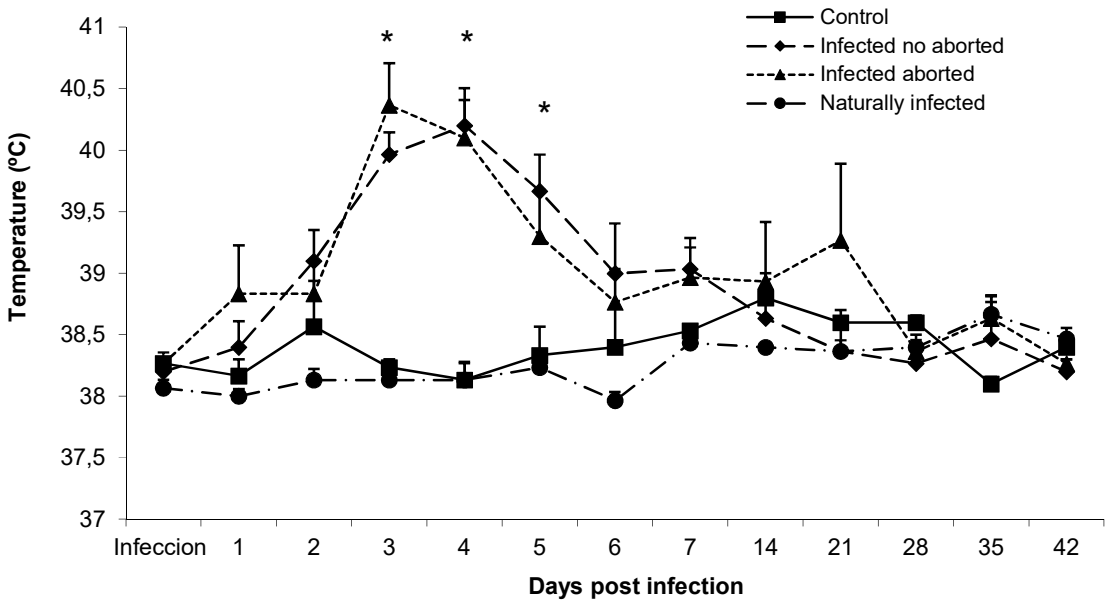

Figure 1. Dynamics of rectal temperatures recorded in the *N. caninum* experimentally infected dams with aborted/non viable fetuses and dams with live fetuses, in control uninfected animals, and in chronically infected seropositive dams throughout the experimental period (baseline to 42 days post infection (dpi)).

3.2. Distribution of parasites and lesions

N. caninum DNA was amplified from at least one tissue type in all 5 infected fetuses examined (one fetus could not be recovered). Parasite DNA was more frequently detected in fetal CNS (brain and spinal cord) and lung tissue (both tissue types in all 5 fetuses tested positive) (Table 1). Three of the 5 infected fetuses analyzed had parasite DNA in skeletal muscle and/or heart tissue. No parasite DNA was observed in the liver of any infected fetus. Aborted/dead fetuses had *N. caninum* DNA in the brain, heart, lung and skeletal muscle (Table 1). *Neospora* DNA was detected in the placenta (caruncles and/or cotyledons) of all infected dams. All DNA samples from control uninfected fetuses were negative (Table 1).

Microscopic lesions were observed in the placenta of all 6 inoculated dams. The placenta of the dam with a non-viable fetus was severely autolysed. Placentomes were not found in the cows that had aborted at 2-3 wpi. In the 3 cows with live fetuses, the fetal and maternal placentas had not separated and contained focal areas of placentitis at the materno-fetal junction. The placentitis was characterized by focal necrosis and infiltrates of neutrophils and mononuclear cells.

Lesions were also seen in at least one tissue section of all fetuses (Table 1). The 3 live fetuses had neural lesions. Severe autolysis in the CNS tissues of the aborted and non-viable fetuses prevented their histological examination. In the recovered aborted fetus and in the fetus that was non-viable upon euthanasia of the mother severe autolysis was also observed in the spleen (both fetuses) and liver (1 fetus). Inflammatory lesions were also identified in the lung of 4 fetuses (including the non-viable fetus), and in the heart and/or liver of 2 fetuses (Table 1). CNS lesions in live fetuses were mainly haemorrhage, mild necrosis, and mononuclear cells infiltrates. Lesions in other organs were predominantly mononuclear cell infiltrates.
Tissue samples testing positive for the presence of *N. caninum* DNA by Nc-5 PCR in fetal tissues were quantified for parasite burdens by real-time PCR (*Neospora-SP* qPCR). All samples scoring positive by Nc-5 PCR (Table 2) also proved positive by real-time PCR. Parasite burdens determined as tachyzoites per 50 ng of bovine DNA in the Nc-5 PCR-positive samples are provided in Table 2.

Table 2. Quantification of the number of tachyzoites/50 ng of bovine DNA tissue by real time PCR (*Neospora*-SP qPCR) in samples previously testing positive by Nc-5 qualitative PCR in fetal tissues and the CNS of their dams. Results provided as the mean± standard deviation.

Fetus	Fetal tissues¶				Dam
	CNS	HEART	LUNG	MUSCLE	CNS
F6683	19.5±11.0	Neg.	32.5±15.6	Neg.	5.2±5.0
F203	24.8±.12.9	Neg.	15.3±7.9	25.5±13.8	Neg.
F641	275.1±6.4	36.9±20.5	15,34±9.2	Neg.	13.2±6.7
F659*	853.7±63.7	8.7±8.0	19.5±19.0	19.8±12.3	Neg.
F649**	425.0±27.8	11.3±6.6	11.0±10.0	30.6±13.1	Neg.
F635*	NA	NA	NA	NA	Neg.
Average burden in live fetuses	106.5±146.5	12.3±21.3	21.0±9.9	8.5±14.7	6.1±6.6
Average burden in aborted/nonviable fetuses	639.3±426.8	10.0±5.9	15.2±9.8	25.2±15.5	0±0

¶All lung tissue samples were negative by Nc-5PCR
Neg.: Negative by Nc-5PCR and undertermined by *Neospora-SP* qPCR
NA: sample not available
CNS: Brain and spinal cord
* Aborted fetus
** Fetus dead upon maternal euthanasia

The highest parasite burdens in the infected fetuses were observed in the CNS, particularly in aborted/dead fetuses which had higher burdens than the CNS tissues of live fetuses (mean

burdens were 639.3 ± 426.8 in aborted/dead fetuses versus 106.5 ± 146.5 in live fetuses) but only a trend towards statistical significance was observed (P=0.06) (Table 2).

3.3. *Neospora caninum*-specific antibodies in dams and fetuses

In five of the six infected cows, anti-*N.caninum* antibodies were detected by 21 days post inoculation (dpi); the remaining infected animal was seropositive 28 dpi (Figure 2). In the 3 experimentally infected heifers with dead/aborted fetuses, antibody titres rose over the study course up to 35 dpi. Moreover, two of the 3 experimentally infected dams with live fetuses showed low antibody levels throughout the study; of note, dam #203 was seropositive by 28 dpi and showed S/P ratios <12.0 over the study period. In contrast, dam #641 with a live fetus showed elevated and increasing antibody levels throughout the study and, by the end of the study period, this dam had the highest levels of all (Figure 2).

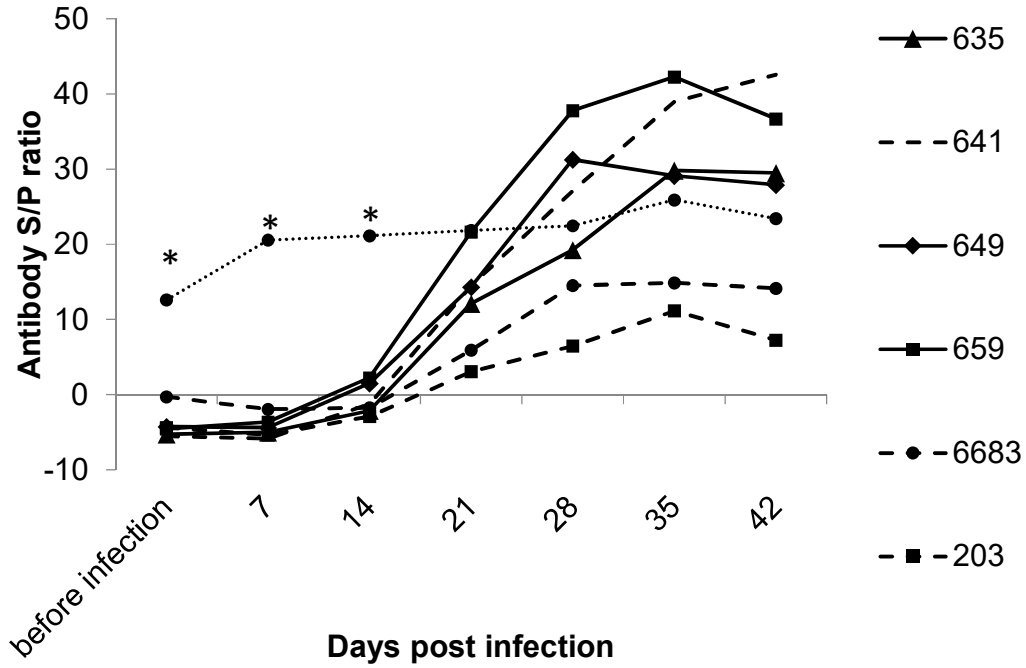

Figure 2. Dynamics of total IgG antibodies against *Neospora caninum* in the individual experimentally infected dams throughout the experimental period (baseline to 42 days post infection (dpi)). Solid line: dams with aborted/non viable fetuses. Dashed line: dams with live fetuses, and avarege levels in naturally infected dams throughout the experimental period.

Dams with aborted/dead fetuses had higher average levels of total antibodies compared to non-aborting dams throughout the study (Figure 2) but differences between the two groups were non-significant at all time points owing to the individual variations indicated. Antibodies were not detected in control uninoculated cows during the study period. The chronically naturally infected dams remained seropositive during the entire course of the study. In naturally infected dams, antibody levels were similar from 14 dpi until the end of the study period (the mean S/P ratio was 21.2) (Figure 2).

Significantly higher antibody levels were observed in naturally infected dams versus experimentally infected heifers at the time points before infection, 7 dpi and 14 dpi (P=0.006, P=0.015 and P<0.001, respectively). From 21 dpi onwards, antibody levels rose in the experimentally infected dams such that differences between the two groups of dams disappeared (Figure 2).

N. caninum antibodies were detected by ELISA in serum (2 fetuses) and in allantoic fluid (3 fetuses) or amniotic fluid (2 fetuses) (Table 3). Unfortunately, fluids in the other aborted fetuses (F635 and F659) were not available or in poor condition. No antibodies were detected in any of the analyzed fluids in control fetuses.

3.4. IFN-γ in plasma from dams and in fetal fluids

No IFN-γ was observed in any plasma sample collected weekly from the experimental dams, with the exception of the sample from dam #659 (which aborted) collected at 42 dpi (536 pg/ml).

The fetus found dead upon maternal euthanasia (F-649) showed high IFN- γ concentration in peritoneal fluids (1466 pg/ml) and very high production in the amniotic fluid (22,886 pg/ml) (Table 3). All 4 control uninfected fetuses showed no IFN-γ in all analyzed samples, and neither was evidence of IFN-γ production detected in amniotic, allantoic and /or fetal fluids in live fetuses upon maternal euthanasia (Table 3).

Table 3 *Neospora caninum* antibodies and IFN-γ levels detected in fetal serum and fetal fluids after experimental infection with *Neospora caninum* at 110 days of gestation

Fetus	Antibodies (cut-off S/P ratio 6.0)			IFN-γ production (pg/ml)	
	Fetalserum	Amniotic Fluid	Allantoic fluid	Fetalserum	Amniotic fluid
F6683	0	0	0	0	0
F203	0	0	7.3	0	0
F641	17.0	15.8	13.5	0	0
F649**	82.4	94.7	71.9	1466	22886

** fetus dead on maternal euthanasia

4. Discussion

The present study confirmed that fetal death occurs when heifers are experimentally infected with virulent isolates of *N. caninum* at 110 days of gestation after an experimental period of 6 wpi. In the present study 50% (3 of 6) of infected dams lost their fetuses during the duration of the study. In contrast, in dams infected in early pregnancy (gestation day 70) using the same strain and dose, Regidor-Cerrrillo *et al*., (2014) reported 100% fetal mortality in seven dams inoculated. These observations confirm the hypothesis that gestational age at the time of infection is an important determinant with respect to fetal loss.

Besides, fetal loss, pyrexia was the main clinical sign. All infected dams became febrile by 3-5 dpi. Transient rises in body temperature are consistent with other reports and are likely the consequence of the first cycles of parasite replication in host tissues (Caspe *et al*., 2012; Regidor-Cerrillo *et al*., 2014). In general, the rectal temperature increase observed here occurred a little sooner and lasted longer than that reported in pregnant cattle infected with the same strain (Nc-Spain7) at 70 days of gestation (Regidor-Cerrillo *et al*., 2014) in which fever was observed between 5 and 7 dpi. No differences in temperatures were observed among infected dams which will suggest homogeneity in the administrated dose of viable tachyzoites.

Specific *N. caninum* humoral immune responses were observed in all *N. caninum* inoculated dams, the threshold for seropositivity being attained by 3-4 wpi. Higher mean antibody levels were found in dams with dead/aborted fetuses versus those with live fetuses though differences were not significant, probably because of high individual variation within groups. While no antibodies were detected in control uninfected heifers at any time, levels of specific antibodies in the chronically infected seropositive dams practically remained constant throughout the study, in agreement with the results of previous studies in our area indicating that *Neospora* seropositivity can be very stable over time (Pabón *et al*., 2007). Antibody levels were significantly higher in the naturally infected dams than the experimentally infected dams until 21 dpi when antibody levels were comparable in the two groups owing to levels increasing in the inoculated heifers. This suggests similar antigen exposure in the heifers from 21 dpi onwards.

Importantly, 3 of the infected fetuses developed *N. caninum* antibodies by 6 wpi. In a prior study, we did not find antibodies in fetuses of dams inoculated with Illinois strain of *N. caninum* at 110 days of gestation and euthanized at 3 wpi, though antibodies were detected in some fetuses at 6 wpi and 9 wpi (Almería *et al*., 2010). Similarly, antibodies were not detected in fetuses at 2 wpi (Regidor-Cerrillo *et al*., 2014) but were present at 6 wpi in cows infected with the Nc-Spain 7 isolate (Caspe *et al*., 2012). These observations seems to indicate that, irrespective of the isolate, around 6 wpi are needed for antibody detection in infected fetuses, which is important since the presence of antibody in fetal fluid or serum indicates transplacental infection because maternal antibodies do not cross placental barrier in ruminants.

Higher parasite burdens, and thus greater parasite multiplication, were observed in CNS tissues compared to other organs examined in the fetuses after 6 weeks of infection, and the parasite loads were higher in the CNS of aborted/non viable fetuses than the CNS of infected fetuses that were alive upon maternal euthanasia. Unfortunately, severe autolysis prevented histological examination of CNS tissue samples in aborted/non viable fetuses. The highest antibody level was observed in the dead fetus found upon euthanasia. We could thus speculate that the greater parasitaemia observed in the dams with aborted/non viable fetuses and in the non-viable fetus itself could have induced such an elevated humoral immune response. The immune response detected in the experimental heifers indicates early passage of tachyzoites after infection. Maley *et al*. (2003) observed tachyzoites in fetal tissues as early as 14 days postinfection and Barr *et al*. (1994) suggested that tachyzoites cross the placenta and reach the fetus around 10 days after maternal infection.

In fetuses, *N. caninum* DNA was more frequently found in CNS (brain and spinal cord) and lung tissues. The central nervous system has been described as the target tissue in *N. caninum* infected fetuses harvested after 150 days of gestation (Almería *et al*., 2010; Pereira *et al*., 2014). A preference of *N. caninum* for lung tissue has been also suggested (Pescador *et al*., 2007; Rojo-Montejo *et al*., 2011). In our study, parasite DNA was also observed in the CNS of two infected dams, consistent with observations in natural infections in which the parasite persists in the host as replicating bradyzoites in tissue cysts mainly in the central nervous system (Collantes-Fernandez *et al*., 2006; Dubey *et al*., 2006). On the other hand, no parasite DNA was observed here in the liver of fetuses at 6 wpi, in agreement with Almería *et al*., (2010) which observed DNA in the liver of fetuses euthanized at 3 wpi but not at later times. Hepatic lesions are more prominent in fetuses from epidemic outbreaks than in fetuses from endemic cases (Collantes-Fernandez *et al*., 2006; Wouda *et al*., 1997), probably related to ingestion of large numbers of oocysts by dams. Using the same *N. caninum* strain as used here for experimental infection early in gestation, Regidor-Cerrillo *et al*. (2014) observed the presence of the parasite in the liver of all infected fetuses and higher burdens were observed in the heart and liver than in the CNS at the time of fetal death between 24 and 49 dpi (Regidor-Cerrillo *et al*., 2014). Taken together, these results clearly indicate that the parasite follows a dynamic pattern of infection in different organs in the fetuses.

Although the presence and replication of the parasite at the materno-fetal interface is a key determinant of fetal mortality, immunological mechanisms in both dams and fetuses also play an important role in fetal death. Several mechanisms could lead to abortion or fetal damage. Direct tissue damage can be caused by the multiplication of parasites in the placenta or in fetal tissues (Buxton *et al*., 2002; Innes *et al*., 2007) or by insufficient oxygen/nutrition as a

consequence of placental damage (Dubey *et al*., 2006). Tissue damage may also occur through maternal immune system activation, which elicits the production of pro-inflammatory cytokines, chemokines, nitric oxide or prostaglandins in the placenta (Buxton *et al*., 2002; Innes *et al*., 2007). In the present study, a dead fetus found when the mother was euthanized was seropositive for the parasite and showed high IFN-γ production in fetal fluids. In contrast, no IFN-γ was observed in amniotic, allantoic and /or fetal fluids in any of the infected fetuses that were alive upon maternal euthanasia. Practically identical results were observed in the dead fetus examined in our previous study, the first report of foetopathy in response to experimental infection at 110 days of gestation (Almería *et al*., 2010). Recently, Cantón *et al*. (2014) suggested that an observed high pro-inflammatory response, especially early in gestation, plays a significant role in disease pathogenesis by inducing placental deterioration with the consequence of a reduced fetal vascular supply of nutrients, leading ultimately to abortion. Therefore, a threshold IFN-γ response seems to be needed to be beneficial against *Neospora*-infection, as we previously suggested (Almería et al., 2014; Almería and López Gatius, 2015).

Interestingly, in this study we were able to describe some parasitological and immunological differences in experimentally infected dams carrying live or dead fetuses and in their fetuses. Live infected fetuses showed lower levels of total antibodies, no IFN-γ production, and lower burdens in their CNS than those observed in infected dead/non viable fetuses. In the dams, both groups of infected dams showed a similar rectal temperature response yet biphasic temperature increases were observed in 2 dams with dead/aborted fetuses which could suggest increased replication of the parasite in these dams; lower antibody levels were observed in dams with live fetuses than with dead/aborted fetuses (with the exception of one

dam that may have aborted later) and, one dam with an aborted fetus was the only animal to show plasma IFN-γ production.

In conclusion, our findings confirm the occurrence of abortion in response to the experimental infection of naïve cows with 10^7 tachyzoites of the highly pathogenic *N. caninum* strain Nc-Spain7 at 110 days of pregnancy within an experimental period of 6 wpi. The fact that some dams aborted and some did not makes this model of bovine neosporosis useful to try to understand *N. caninum* pathogenesis of abortion in the second trimester of gestation, when most abortions occurs in field conditions. In future studies conducted on larger numbers of animals using this cow model of experimental infection, we hope to clarify the causes of *N. caninum*-associated abortions.

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CHAPTER 2

EXPERIMENTAL *NEOSPORA CANINUM* INFECTION MODIFIES TROPHOBLAST CELL POPULATIONS AND PLASMA PREGNANCY-ASSOCIATED GLYCOPROTEIN 1 AND 2 DYNAMICS IN PREGNANT DAIRY HEIFERS

Abstract

Neospora caninum is an obligate intracellular protozoan that causes abortion in cattle worldwide. Plasma concentrations of pregnancy-associated glycoprotein 1 (PAG-1), produced by binucleate trophoblast cells, are used for pregnancy diagnosis and as a marker of foetal-placental well-being, while PAG-2, produced by both mono- and binucleate trophoblast cells, may serve as an indicator of abortion risk. In prior work, natural *N. caninum* infection was found to modify plasma PAG-1 and PAG-2 patterns. The present study examines PAG-1 and PAG-2 dynamics and trophoblast cell populations following experimental infection with *N. caninum.* The study population was comprised of 17 *N. caninum* seronegative Holstein-Friesian heifers. On Day 110 of gestation, 6 heifers were inoculated intravenously with 10^7 taquyzoites of *N. canimum.* Plasma samples for PAG-1 and PAG-2 determinations were collected on Days 0, 7, 14, 21 and 42 post-infection. During the study course, pregnancy was normally expressed in all controls while three infected heifers lost their foetuses. All heifers were euthanized on Day 42 post infection and placentome samples from the 14 non-aborting heifers were collected to examine trophoblast cell populations. Plasma PAG-1 and PAG-2 concentrations in non-infected heifers increased until the day of euthanasia while non-aborting infected heifers showed a temporary fall in PAG-1 ($P<0.004$) and PAG-2 ($P<0.002$) concentrations from 7 to 14 days post infection (dpi). The two dams aborting at 14 and 21 dpi and a third dam with a mummified foetus upon euthanasia showed dramatic PAG-1 and PAG-2 reductions from 14 dpi to undetectable levels upon euthanasia. A stereological study of placentomes revealed significantly higher relative proportions of mono- ($P=0.035$) and binucleate ($P=0.029$) trophoblast cells at 42 dpi in non-infected heifers than infected non-aborting heifers. According to our findings, following experimental *N. caninum* infection on Day 110 of gestation, non-aborting heifers showed a

brief reversible drop in plasma PAG-1 and -2 concentrations two weeks later and reduced proportions of bi- and mononucleate trophoblast cells 42 days after infection. In aborting dams, dramatically reduced PAG levels were related to severe placental damage and a non-viable pregnancy.

1. Introduction

Neospora caninum is an obligate intracellular protozoan that causes abortion in cattle with severe economic impacts worldwide (Dubey and Schares, 2011; Reichel *et al*., 2013). *Neospora*-infected cows may abort from 3 months of gestation to term, though most abortions occur at 5-6 months of gestation (Dubey *et al*., 2007). However, the precise causes of abortion are not well-known. Some authors propose that the parasite or parasite-induced pro-inflammatory immune response, as the only effective response against *N. caninum*, causes foetal or placental damage (Innes *et al*., 2005; Almería and López-Gatius, 2015).

The pregnancy-associated glycoproteins 1 (PAG-1) and 2 (PAG-2) belong to the aspartic proteinase family and are synthesised in ruminant trophectoderm (Wallace *et al*., 2015). PAG-2 molecules are exclusively expressed in mono-nucleate trophoblast cells, whereas PAG-1 molecules are expressed in binucleate cells of both the intercotiledonary and the cotyledonary chorion (Touzard *et al*., 2013). Although the functions of these glycoproteins are not yet fully understood, PAG-1 concentrations are useful for pregnancy diagnosis and as a marker of placental/foetal well-being (Wallace *et al*., 2015), whilst some PAG-2 concentrations may be a useful indicator of abortion risk in *N. caninum*-infected cows (García-Ispierto *et al*., 2013). Both PAG-1 and PAG-2 patterns are modified by *N. caninum* infection (López-Gatius *et al*., 2007b; García-Ispierto *et al*., 2013). This study was designed to: 1) determine plasma PAG-1 and PAG-2 dynamics following experimental *N. caninum* infection in dairy heifers, and 2) examine the possible effects of *N. caninum* infection on mono- and binucleate trophoblast cell numbers in large placentomes 42 days after infection.

2. Materials and methods

2.1. Animals and infection

Seventeen 14-16 month old Holstein-Friesian heifers that were seronegative against *N. caninum* (CIVTEST, Spain) were synchronized and artificially inseminated. Seronegativity against the parasite was assessed before AI and on Days 60 and 90 of gestation. Heifers were previously vaccinated (6-8 months of age) against bovine viral diarrhoea virus (BVDV) and infectious bovine rhinotracheitis virus (IBR). Pregnancy was assessed by ultrasonography 30, 45, 90 and 110 days after insemination. On Day 110 of gestation, 6 of the heifers were intravenously (i.v.) inoculated with 10^7 culture-derived tachyzoites of the *N. caninum* isolate Nc-Spain7, kindly donated by Dr. L. M. Ortega-Mora (SALUVET, Universidad Complutense, Madrid, Spain). These 6 animals were euthanized on Day 152 of gestation. The 11 remaining heifers were kept as un-inoculated controls and were euthanized at the same time as inoculated dams. After Day 110, heifers were visually inspected daily for possible abortion until euthanasia.

2.2. Sample collection

Blood samples for antibody and placental protein determinations were collected by tail vein puncture from each heifer into heparinized vacuum tubes (BD Vacutainer, Becton-Dickinson and Company, Plymouth, UK) on Days 110, 117, 124, 131 and 152 of gestation. On Day 110, samples were collected immediately before infection. Plasma was obtained by centrifugation within 30 min of sampling and stored at -20ºC until analysis.

On Day 152 of gestation (6 weeks after infection) all animals were sedated with xylazine hydrochloride (Rompun; Bayer) and immediately euthanized by an intravenous (i.v.) overdose of embutramide and mebezonio iodide (T61; Intervet). Immediately after death, heifers were necropsied. Amniotic and allantoic fluids were collected before the placenta was opened and foetuses separated from the placenta. Foetal blood samples were obtained by cardiac puncture. Samples of nine a convenience selected placentomes (three cranial, three medial and three caudal) were removed from each dam. The foetal tissue specimens collected were: CNS (brain and spinal cord), heart, lung, liver, skeletal muscle, spleen, and thymus. A full description of inocula and the 6 experimentally infected heifers is provided in Almería *et al*. (2016).

2.3. Ethics

All procedures were approved by the Ethics Committees on Animal Experimentation of the Autonomous University of Barcelona (license number CEEAH.1426-08/02/2012) and of the University of Lleida (license number CEEA.06-01/12). Animals were handled in strict accordance with good animal practices and the conditions defined by the Animal Ethics Committee at the Autonomous University of Barcelona and CReSA, Spain. Every effort was made to minimize suffering.

2.4. Sample analyses

2.4.1. Plasma antibodies against *Neospora caninum*

Plasma samples collected from the dams were tested for anti-*Neospora caninum* antibodies using a commercial ELISA kit based on the whole tachyzoite lysate of *Neospora* NC-1, according to the manufacturers' instructions (CIVTEST® anti-*Neospora*; Hipra, Girona,

Spain). The cut-off used for a positive test result was an S/P ratio of 6.0, as established by López-Gatius *et al*. (2004). Sera and/or foetal fluids were analysed using the same technique on undiluted samples.

2.4.2. PCR-based diagnosis of Neospora caninum

Portions of placenta and foetal tissues were aseptically obtained and stored in liquid nitrogen at -196ºC until DNA extraction. At least 0.5–1 g of each tissue was homogenised with a pestle and mortar in liquid nitrogen and DNA extracted as described by Almería *et al*. (2002).

2.4.3. PAG-1 and PAG-2 determinations

PAG-1 concentrations were determined in plasma using a double antibody radioimmunoassay procedure (RIA-706) (López-Gatius *et al*., 2007a). Rabbit polyclonal antiserum AS#706 raised against caprine $PAG_{55\ kDa+62\ kDa}$ (accession numbers P80935 and P80933) according to the method of Vaitukaitis *et al*. (1971) was used as the primary antibody. The minimum detection limit for the RIA procedure was 1.2 ng/mL. Intra- and inter-assay coefficients of variation were 5.3% and 6%, respectively.

The bovine PAG-2 radioimmunoassay used has been recently described (García-Ispierto *et al*., 2013). Briefly, PAG-2 was purified according to the method detailed by Beckers *et al*. (1988). The primary antibody was rabbit polyclonal antiserum against boPAG-2 (AS#438) raised according to the method of Vaitukaitis (1971). Owing to the instability of the boPAG-2 molecule, boPAG-1 (67 kDa) was used as a standard (dilutions ranging from 100 to 0.2 ng/mL) and for iodination with the 125-I isotope (Thorell and Johansson 1971). The initial

dilution for primary AS#438 was 1:2500. The minimal detection limit calculated for RIA-438 was 2.3 ng/mL. Intra- and inter-assay coefficients were 4.6% and 4.8%, respectively.

2.4.4. Histology and stereological examination of placentomes

Placentome samples were embedded in paraffin and 5 μm sections of each placentome prepared for histology and stereological observations. For observation, sections were deparaffinized, dehydrated, and stained with haematoxylin-eosin. Stereological observations were performed in a similar way to that previously described by Kannekens *et al*. (2006). Ten pictures including the foetal-maternal area were taken of each placentome sample and visualised at a magnification of 400x using a projection microscope. A uniformly spaced 8x10 point grid was projected for each picture (Figure 1) and images analysed to estimate relative cell densities using the software package ImageJ 1.47 (http://imagej.nih.gov/ij). In total, 800 points per heifer were assigned to the following categories of cells or tissues: maternal tissues (Mt), maternal epithelium (Me), maternal-foetal interface (If), mono-nucleate trophoblast cells (MNC), binucleate trophoblast cells (BNC) and foetal tissues (foetal cells other than MNC and BNC: Ft) (Figure 1). Since most mono- and binucleate cells occur in the trophoectoderm, only points in the foetal tissue area were counted.

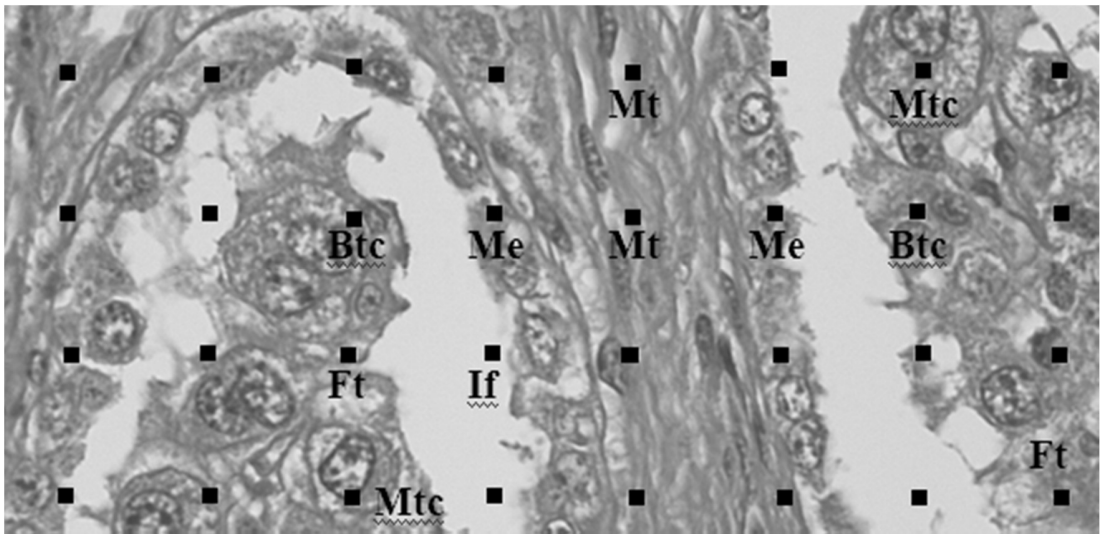

Figure 1. Micrograph (magnification 400X) of a bovine placentome at 152 days of gestation with a point grid projected onto the image. Each point was assigned a specific cell or tissue type: maternal tissues (Mt), maternal epithelium (Me), maternal-foetal interface (If), mono-nucleate trophoblast cells (Mtc), binucleate trophoblast cells (Btc) or foetal tissues (Ft).

2.5. Statistical analysis

The following data were recorded for each animal: experimental infection with *N. caninum* (presence or absence), plasma levels of PAG-1 and PAG-2 on Days 110, 117, 124, 131 and 152 of gestation, slope of PAG-1 and PAG-2 concentration changes (PAG-1S, PAG-2S) and the following stereological ratios: BNC/MNC, BNC/(BNC+MNC), MNC/(BNC+MNC), BNC/(BNC+MNC+Ft) and MNC/(BNC+MNC+Ft).

The slope of PAG-1 and PAG-2 concentration changes was defined by the expression:

$PAG\text{-}S_{Y\text{-}X} = (PAG_X - PAG_Y) / (X - Y)$

Where PAG_Y and PAG_X were two consecutive plasma levels of PAGs measured on Days Y and X after insemination, respectively.

The possible effects of variables on PAG-1 and PAG-2 concentrations, PAG-1S and PAG-2S were evaluated in non-aborting heifers by GLM repeated measures analysis of variance. *Neospora caninum* infection was included in the model as fixed effect. Stereological ratios were compared by ANOVA. Correlations between stereological ratios and PAG-1 and PAG-2 concentrations before euthanasia were assessed through Spearman correlation. All analyses were performed by using the SPSS software package, version 17.0 (SPSS Inc., Chicago, IL).

3. Results

From the time of experimental infection to euthanasia, of the six infected heifers, two underwent abortion on Days 14 and 21 post infection, respectively, and in one a mummified foetus was detected upon euthanasia. Pathological findings in these three foetuses are described in Almería *et al*. (2016).

At 21 days post inoculation, anti-*Neospora caninum* antibodies were detected in the plasma of all six infected dams, while control heifers (n=11) remained seronegative for the parasite. *N. caninum* antibodies were also detected in serum (2 foetuses) and in allantoic fluid (3 foetuses) or amniotic fluid (2 foetuses) collected from the infected dams. *N. caninum* DNA was observed in at least one tissue type and in the placenta (caruncles and/or cotyledons) of all recovered infected foetuses. Further data on the experimental protocol are provided in

Almería *et al*. (2016, submitted). All DNA samples from control, uninfected foetuses were negative.

PAG-1 and PAG-2 were detected in all plasma samples obtained from non-aborting heifers. Figure 2 shows how plasma PAG-1 and PAG-2 concentrations increased significantly (within-subject effect; $P<0.016$; $P<0.021$, respectively) during the study in both non-aborting infected and non-infected heifers. In the three infected dams with dead/aborted foetuses, PAG-1 and PAG-2 concentrations fell drastically from 14 dpi onwards and levels were undetectable by the end of the study.

Figure 3 shows the changes produced in levels of both glycoproteins referred to as the slopes of the concentration curves PAG-1S and PAG-2S. Changes in PAG-1 and PAG-2 concentrations were significantly higher ($P<0.004$ and $P<0.013$, respectively) from 0 to 7 dpi, and lower ($P<0.004$ and $P<0.002$, respectively) from 7 to 14 dpi in non-aborting infected than in non-infected heifers. From 14 to 21 dpi, PAG-1S and PAG-2S increased in both infected and non-infected heifers (within-subject effect; $P<0.004$ and $P<0.002$, respectively).

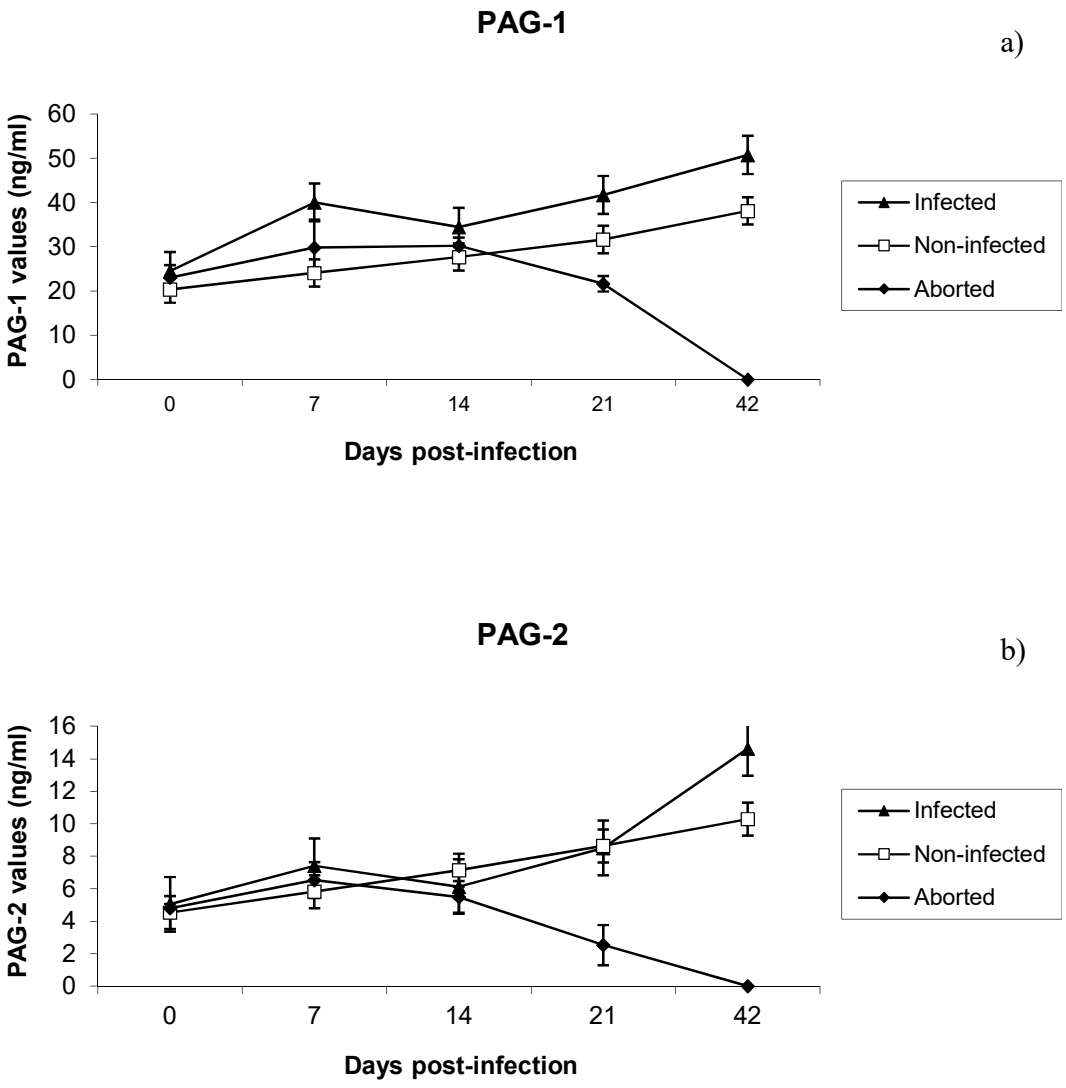

Figure 2. Plasma PAG-1 (a) and PAG-2 (b) concentrations (Mean ± S.E.M.) recorded from infection to euthanasia in heifers infected and non-aborted with *N. caninum* on day 110 of gestation (n=3), infected and aborted during the experiment (n=3) or non-infected (n=11). Within subject effect was significant in both cases: $P<0.016$ and $P<0.021$, respectively.

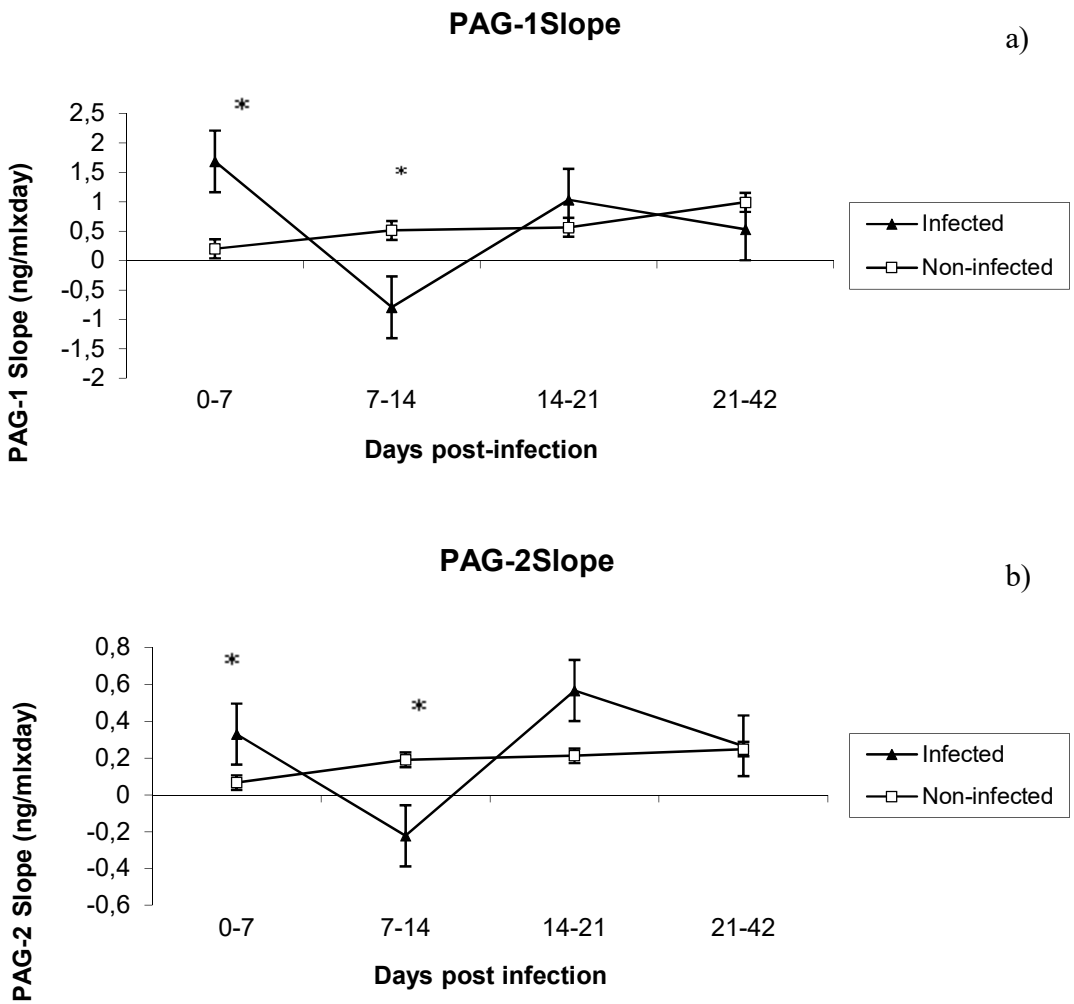

Figure 3. Slopes of plasma PAG-1 (a) and PAG-2 (b) concentrations (Mean ± S.E.M.) recorded from infection to euthanasia in heifers infected with *N. caninum* on day 110 of gestation (n=3) or non-infected (n=11). Slopes of PAG-1 and PAG-2 concentration changes were significantly higher ($P<0.004$ and $P<0.013$, respectively) from 0 to 7 days post infection (dpi), and lower ($P<0.004$ and $P<0.002$, respectively) from 7 to 14 dpi in infected than in non-infected heifers. From 14 to 21 dpi PAG-1S and PAG-2S increased in both, infected and non-infected heifers (Within subject effect; $P<0,004$ and $P<0.002$, respectively).

Stereological examinations revealed significantly higher relative proportions of mono-nucleate and binucleate trophoblast cells in foetal tissues from non-infected heifers than from non-aborting infected heifers upon euthanasia (Table 1). No correlations were found between PAG levels upon euthanasia and stereological ratios. Unfortunately, severe autolysis prevented histological examination of placental tissue samples in aborted/non-viable foetuses.

Table 1. Stereological ratios recorded upon euthanasia between numbers of points in foetal tissue of binucleate trophoblast cells (BNC), mono-nucleate trophoblast cells (MNC) and other foetal cells (Ft).

Stereological ratio	Group	Mean value	P
BNC/MNC	Non-infected	1.24	0.921
	Infected	1.26	
BNC/(BNC+MNC)	Non-infected	0.53	0.669
	Infected	0.56	
MNC/(BNC+MNC)	Non-infected	0.47	0.669
	Infected	0.44	
BNC/(BNC+MNC+Ft)	Non-infected	0.32	0.029*
	Infected	0.19	
MNC/(BNC+MNC+Ft)	Non-infected	0.29	0.035*
	Infected	0.15	

*: Difference significant according to ANOVA tests.

4. Discussion

To the best of our knowledge, no prior study has examined trophoblast cell populations and plasma PAG 1 and 2 dynamics in response to experimental infection with *N. caninum*. Non-

aborting *Neospora*-infected dairy heifers showed a brief transient reduction in PAG-1 and PAG-2 concentrations from 7 to 14 dpi, and lower relative proportions of bi- and mono-nucleate trophoblast cells upon euthanasia. The PAG-2 concentration patterns registered herein indicate a good treatment of plasma samples overcoming the low stability of PAG-2 molecules. These results reinforce previous findings in naturally infected cows in which lower plasma PAG-1 concentrations throughout gestation were detected in *Neospora*-seropositive cows than in their seronegative peers (Garcia-Ispierto *et al*., 2010), while PAG-1 concentrations dramatically fell in cows suffering *Neospora*-associated abortion (López-Gatius *et al*., 2007b). Similarly, in a recent study, low plasma PAG-2 concentrations on day 120 of gestation were identified as a marker of abortion in *Neospora*-seropositive cows (García-Ispierto *et al*, 2013). Thus, it seems that after infection by, or the recrudescence of, *N. caninum* a significant decline is produced in both PAG-1 and PAG-2. The mechanism for this decline seems to be linked to a reduction in active (or secreting) bi- and mono-nucleate trophoblast cells.

Following experimental infection with *N. caninum* at different point times during gestation, peaks in Th1 immune-response players such as CD4+ and CD8+ T cells and IFN-γ and IL-4 cytokines have been observed between one and three weeks post infection. This response has been detected in dams in peripheral blood mononuclear cells (Rosbottom *et al*., 2007; Regidor-Cerrillo *et al*., 2014), spleen, peripheral lymph nodes and placenta (Bartley *et al*., 2013; Almería *et al*., 2014), at the foetal-maternal interface (Maley *et al*., 2006) and also in foetal tissues (Almería *et al*., 2014). The Th1 immune-response is characterized, among other features, by phagocytic activity against genetically different cells. Thus, Th1 activity inside the placenta could promote the destruction of foetal-genotyped cells such as bi- and mono-

nucleate cells and this would explain the lower relative densities of these cells observed here at 42 dpi. Accordingly, the reduction noted in PAG-1 and PAG-2 concentrations in non-aborting infected dams could have been a consequence of the Th1 response. However, this decline was transient and could be perhaps related to the recovery of lesions in the placenta, as indicated by Maley *et al*. (2006). It also suggests that the presence of a parasite-induced, maternal immune response in the placenta may not be detrimental to foetal survival, and, rather, could help control the placental parasitosis (Rosbottom *et al*., 2011). Another explanation could be that increased PAG levels are the consequence of PAGs secreted by other binucleate cells in small cotyledons. These small cotyledons develop during pregnancy in both uterine horns. In contrast, in aborting heifers, the decline in PAGs was constant and levels were undetectable upon euthanasia. These findings support the hypothesis that a strong Th1 immune response following the recrudescence (multiplication or cross over) of *N. caninum* provokes severe lesions in the placenta causing the death of the foetus, as observed in some studies (Almería and López-Gatius, 2015).

Probably because of the small population size of the present study, no correlations between bi- or mono-nucleate trophoblast cell proportions and plasma PAG concentrations were detected.

In conclusion, following experimental infection with *N. caninum* on Day 110 of gestation, non-aborting heifers showed a short-lived transient decline in plasma PAG-1 and 2 concentrations within 14 dpi and reduced populations of bi- and mono-nucleate trophoblast cells at 42 dpi. In contrast, infected aborting dams showed a dramatic decline in PAGs until undetectable levels were recorded upon euthanasia. The clinical implication of these findings

is that following infection with *N. caninum* or recrudescence of the pathogen, most cows will suffer placental damage, though, in some animals, this damage will be overcome and pregnancy will successfully continue to term.

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CHAPTER 3

EXPERIMENTAL *NEOSPORA CANINUM* INFECTION IN PREGNANT DAIRY HEIFERS INCREASES CONCENTRATIONS OF PREGNANCY-ASSOCIATED GLYCOPROTEIN 1 AND 2 IN FOETAL FLUIDS

Abstract

Plasma concentrations of PAG-1 are used for pregnancy diagnosis and as a marker of placental/foetal well-being, while those of PAG-2 may be an indicator of abortion risk in *N. caninum*-infected cows. Studies have shown that *N. caninum* infection modifies PAG-1 and PAG-2 patterns in maternal blood plasma. However, no prior work has examined the effects of *N. caninum* infection on concentrations of PAGs in foetal fluids.In the present study, PAG-1, PAG-2 and pH levels were determined in the amniotic allantoic fluids of foetuses collected at 152 days of gestation from control uninfected dams and from dams experimentally infected with *N. caninum* on Day 110 of gestation. Foetal fluids from infected foetuses had significantly higher PAG-2 concentrations (P=0.026) and pH values (P=0.02) than fluids from non-infected foetuses. In infected foetuses, significantly higher concentrations of PAG-1 (P<0.001) and PAG-2 (P<0.001) were detected in fluid samples showing antibodies against *N. caninum* than those without antibodies. Moreover, pH values were significantly higher (P=0.011) in foetal fluid samples with antibodies than in samples from non-infected foetuses. In conclusion, this is the first report on the effect of *N. caninum* infection on PAG levels in foetal fluids. Our results indicate that following the experimental infection of dams with *N. caninum* on Day 110 of gestation, foetal fluids collected from the infected foetuses of these dams featured higher PAG-1 and PAG-2 levels and pH values than fluids from non-infected controls, provided the samples tested showed the presence of antibodies. The clinical implications of these findings are that following infection with *N. caninum*, most cows will experience some level of placental damage and that this injury correlates with foetal fluid PAG levels and pH.

1. Introduction

Neospora caninum is an obligate intracellular protozoan parasite that is considered a major cause of abortion in cattle worldwide (Dubey and Schares 2011; Almería and López-Gatius 2013). Neosporosis involves more than US$1 billion of economic losses every year worldwide (Reichel *et al*. 2013), attributed mainly to a lengthened calving interval, reduced milk production, falling stock value and an elevated culling rate (Trees *et al*. 1999). More specifically, in our geographical area of study in Northeasten of Spain, *Neospora*-seropositive cows show a 12–19 times greater risk of abortion than seronegative cows, ranging abortion from 30 to 44% of seropositive animals (López-Gatius *et al*. 2004a,b).

Neospora-infected cows may abort from 3 months of gestation to term, with most abortions occurring at second trimester of gestation (Dubey *et al*. 2007; Almería and López-Gatius 2013). For *N. caninum*-associated abortion to occur, the foetus or placenta has to be damaged to the extent that they are no longer viable (Dubey *et al*. 2006; Gibney *et al*. 2008). The precise causes of foetal or placental damage are not well-known. It is hypothetized that the parasite or the parasite-induced pro-inflammatory immune response, the only effective against *N.caninum*, can cause foetal or placental damage (Quinn *et al*. 2002; Innes *et al*. 2005; Almería and López-Gatius 2015).

The pregnancy-associated glycoproteins 1 (PAG-1) and 2 (PAG-2) belong to the aspartic proteinase family (Beckers *et al*. 1994; Xie *et al*. 1994) and coexist in ruminant trophectoderm (Zoli *et al*. 1991; Garbayo *et al*. 2008; Wallace *et al*. 2015). PAG-2 molecules are exclusively expressed in mono-nucleate trophoblast cells, whereas PAG-1 molecules are

expressed in binucleate cells of both the intercotiledonary and the cotyledonary chorion (Touzard *et al*. 2013) and accumulated at the microvillar junction of the placenta (Wooding *et al*. 2005). These proteins can be also detected through radioimmunoassay (RIA) in foetal blood plasma, foetal fluids and maternal blood plasma (Zoli *et al*. 1992; Bella *et al*. 2011). Although the functions of PAGs are not completely understood, plasma PAG-1 concentrations have been used for pregnancy diagnosis and as a marker of placental/foetal well-being (Zoli *et al*. 1992; López-Gatius *et al*. 2007a), whereas low plasma concentrations of PAG-2 molecules may serve as an indicator of abortion risk in *N. caninum*-infected cows (García-Ispierto *et al*. 2013). Previous studies have demonstrated that *N. caninum* infection modifies PAG-1 and PAG-2 patterns in maternal blood plasma (López-Gatius *et al*. 2007a; García-Ispierto *et al*. 2010, 2013, 2015; Mur-Novales *et al*. 2015). However, to the best of our knowledge there are no studies analysing the possible influence of *N. caninum* infection on concentrations of PAGs in foetal plasma and fluids. Therefore, the aim of this study was to determine the possible effects of experimental infection with *N. caninum* of pregnant dairy heifers on PAG-1 and PAG-2 concentrations in foetal plasma and in amniotic and allantoic fluids. Since PAG-2 molecules can act as proteolytic depending on pH (Telugu *et al*. 2010), the possible effects of the infection with the parasite on the pH of the foetal fluids were also evaluated.

2. Materials and methods

2.1. Animals and infection

Six 14-16 months old Holstein-Friesian heifers *N. caninum* seronegative (CIVTEST, Spain) were synchronized and artificially inseminated. Seronegativity was confirmed before insemination and on Days 60, 90 and 110 of gestation. All animals included in the present study were healthy and vaccinated (6-8 months old) against bovine viral diarrhoea virus (BVDV) and infectious bovine rhinotracheitis virus (IBR). Pregnancy was assessed by ultrasonography 30, 45, 90 and 110 days after insemination. On Day 110 of gestation, three of the heifers were intravenously (i.v.) inoculated with 10^7 culture-derived tachyzoites of the *N. caninum* isolate Nc-Spain7, kindly donated by Dr. L. M. Ortega-Mora (SALUVET, Universidad Complutense, Madrid, Spain). These three animals were euthanized on Day 152 of gestation. The three remaining heifers were kept as un-inoculated controls and were euthanized at the same time as inoculated dams.

2.2. Sample collection

On Day 152 of gestation (Day 42 after infection), blood samples for maternal antibodies determination were collected by tail vein puncture into heparinized vacuum tubes (BD Vacutainer, Becton-Dickinson and Company, Plymouth, UK). Plasma was obtained by centrifugation within 30 min after sampling. After blood collection all animals were sedated with xylazine hydrochloride (Rompun; Bayer) and immediately euthanized by an intravenous (i.v.) overdose of embutramide and mebezonio iodide (T61; Intervet). Immediately after death, heifers were necropsied. Information on lesions of heifers and foetuses is provided in a previous study (Almería *et al*. 2016). The uterus was removed and foetal fluids were

collected by puncture. Amniotic and allantoic fluids were collected before the placenta was opened. The needle was inserted in the contralateral uterine horn to gestation for allantoic fluid, and in the ipsilateral horn with the foetus for amniotic fluid. Following sampling of foetal fluids, the uterus was opened and foetal central nervous system (CNS) tissue samples were collected. Blood samples were collected by cardiac puncture and plasma was obtained by centrifugation. Plasma and foetal fluid samples were stored at -20ºC and CNS tissues at -80ºC until analysis. Portions of CNS tissues were aseptically obtained and stored in liquid nitrogen at -196ºC until DNA extraction.

2.3. Ethics

All procedures were approved by the Ethics Committees on Animal Experimentation of the Autonomous University of Barcelona (license number CEEAH.1426-08/02/2012) and of the University of Lleida (license number CEEA.06-01/12). Animals were handled in strict accordance with good animal practices and the conditions defined by the Animal Ethics Committee at the Autonomous University of Barcelona and CReSA, Spain. Every effort was made to minimize suffering.

2.4. Sample analyses

2.4.1. Plasma antibodies determinations and PCR-based diagnosis of *Neospora caninum*

Plasma samples collected from the dams were tested for anti-*Neospora caninum* antibodies using a commercial ELISA kit based on the whole tachyzoite lysate of *Neospora* NC-1, according to the manufacturers' instructions (CIVTEST® anti-*Neospora*; Hipra, Girona, Spain). The cut-off used for a positive test result was an S/P ratio of 6.0, as established by

López-Gatius *et al*. (2004a). Foetal plasma and fluids were analysed using the same technique on undiluted samples.

At least 0.5–1 g of each CNS sample was homogenised with a pestle and mortar in liquid nitrogen and DNA extracted as described by Almería *et al*. (2002).

2.4.2. PAG-1 and PAG-2 determinations

PAG-1 concentrations were determined in plasma using a double antibody radioimmunoassay procedure (RIA-706) (López-Gatius *et al*., 2007b). Rabbit polyclonal antiserum AS#706 raised against caprine PAG55 kDa+62 kDa (accession numbers P80935 and P80933) according to the method of Vaitukaitis *et al*. (1971) was used as the primary antibody. The minimum detection limit for the RIA procedure was 1.2 ng/mL. Intra- and inter-assay coefficients of variation were 5.3% and 6%, respectively.

The bovine PAG-2 radioimmunoassay used has been recently described (García-Ispierto *et al*., 2013; Serrano *et al*., 2014). Briefly, PAG-2 was purified according to the method detailed by Beckers *et al*. (1988). The primary antibody was rabbit polyclonal antiserum against boPAG-2 (AS#438) raised according to the method of Vaitukaitis (1971). Owing to the instability of the boPAG-2 molecule, boPAG-1 (67 kDa) was used as a standard (dilutions ranging from 100 to 0.2 ng/mL) and for iodination with the 125-I isotope (Thorell and Johansson 1971). The initial dilution for primary AS#438 was 1:2500. The minimal detection limit calculated for RIA-438 was 2.3 ng/mL. Intra- and inter-assay coefficients were 4.6% and 4.8%, respectively.

2.4.3. pH measuring

In foetal fluids pH was measured using a Crison micro-pH 2000® pH-meter. The pH- meter was calibrated every 5 samples. Three measures were taken from each sample and the mean value was used for statistical analysis.

2.5. Statistical analyses

The following data were recorded for each foetus: *N. caninum* antibodies (presence or absence) in the plasma and foetal fluids, presence of the parasite in the CNS (presence or absence), PAG-1 and PAG-2 concentrations and pH value (continuous) in the foetal fluid. A foetus was considered to be infected when *N. caninum* DNA was found in its CNS.

The differences of PAG-1 and PAG-2 levels and pH measurements from foetal fluid samples between infected and non-infected dams and between amniotic and allantoic fluids were tested by paired samples T-Student tests. Since differences between amniotic and allantoic fluid measurements were not detected, samples derived from both amnion and allantois were combined and classified with respect to presence or not of *N. caninum* antibodies. Thus, foetal fluids were tested through ANOVA and a Tuckey HSD tests to compare PAGs concentrations and pH values among amnion or allantoic samples from non-infected foetuses, from infected foetuses without antibodies in their corresponding foetal fluids and from infected foetuses with antibodies in the foetal fluids. All analyses were performed by using the SPSS computer package, version 17.0 (SPSS Inc., Chicago, IL)

3. Results

All heifers carried out viable foetuses at euthanasia. Presence of antibodies against *N. caninum* in plasma of dams and in plasma of their foetuses and in foetal fluids, PAG-1 and PAG-2 concentrations and pH measurements in foetal fluids, and presence of *N. caninum* DNA in foetal central nervous system (CNS) on Day 152 of gestation, 42 days after experimental infection, are showed in Table 1. The three experimentally infected heifers were seropositive to *N. caninum* at euthanasia and vertical transmission occurred in all the experimentally foetuses since the parasite was detected by PCR in all foetuses from infected dams. In the infected foetuses, one foetus showed antibodies in plasma and in amniotic and allantoic fluids, whereas a further foetus had antibodies in the allantoic fluid (Table 1).

The concentrations (ng/ml) of PAG-2 were higher than those PAG-1 in all foetal fluids ($P<0.001$); whereas foetal fluids from infected foetuses had significantly higher PAG-2 concentrations ($P=0.026$) and pH values ($P=0.02$) than those foetal fluids from non-infected foetuses (Table 2). Foetal fluid samples with *N. caninum* antibodies showed significant higher concentrations of PAG-1($P<0.001$) and PAG-2 ($P<0.001$) than those foetal fluids without antibodies, whereas pH values from foetal fluids with antibodies were significantly higher than those foetal fluids from non-infected foetuses.

Table 1. Presence of antibodies against *Neospora caninum* in plasma of pregnant dairy heifers and their foetuses and in foetal fluids, PAG-1 and PAG-2 concentrations (ng/ml) and pH measurements in foetal fluids, and presence of *N. caninum* DNA in central nervous system (CNS) of the foetus on Day 152 of gestation, 42 days after experimental infection of the dams with *N. caninum*.

Heifer	101	199	982	203	641	6683
Dams						
Neospora-infection	-	-	-	+	+	+
Neospora-seropositivity	-	-	-	+	+	+
Foetuses						
Neospora-seropositivity	-	-	-	-	+	-
N. caninum antibodies in amnion	-	-	-	-	+	-
N. caninum antibodies in allantois	-	-	-	+	+	-
N. caninum DNA in CNS	-	-	-	+	+	+
Amnion PAG-1 concentrations	6.78	8.56	0.31	14.74	44.40	19.49
Amnion PAG-2 concentrations	24.96	26.66	12.86	49.94	103.02	67.02
Allantois PAG-1 concentrations	9.93	8.10	5.31	96.19	155.84	8.35
Allantois PAG-2 concentrations	23.15	22.73	17.13	183.99	168.84	33.40
Amnion pH	6.96	7.06	6.81	7	7.35	7.01
Allantois pH	6.65	7.12	6.74	7.29	7.37	7.17

Table 2. PAG-1 and PAG-2 concentrations (ng/ml) and pH values in amniotic and allantoic fluid samples on Day 152 of gestation from infected and non-infected foetuses. 2

Foetuses	Non-Infected	Infected	P
N samples	6	6	
	(Mean ± SD)	(Mean ± SD)	
PAG-1	6.1 ± 3.3	56.5 ± 58.3	0.088
PAG-2	20.4 ± 5.3	101 ± 63.0	0.026*
pH	6.92 ± 0.19	7.19 ± 0.16	0.02*

*Differences were significant by T-student test
Samples from non-infected foetuses: 3 amnion + 3 allantois samples. Samples from infected foetuses: 3 amnion + 3 allantois samples.

4. Discussion

To the best of our knowledge, there are not previous studies analysing the possible influence of *N. caninum* infection on concentrations of PAGs in amnion and allantois fluids. Dairy heifers were experimentally infected with the parasite at Day 110 of gestation and their foetuses showed higher levels of PAG-1, PAG-2 and pH values in foetal fluids on Day 152 of gestation than in non-infected controls. In infected foetuses, antibodies against *N. caninum* were found in some foetal fluid samples so that samples with antibodies showed higher levels of PAGs than those samples without antibodies.

Since the bovine placenta presents amniochorion and allantochorion in contact with the caruncles (Reviewed by Favaron *et al*. 2015), antibodies found in amniotic and allantoic fluids may derive from both the foetal immune-system and arriving from maternal blood

through placental lesions. Although is well-known that the cotyledonary synepitheliochorial placentation characteristic of cows do not allow maternal antibodies to cross the placental barrier to foetal fluids (Kruse 1983; Latshaw 1987), some studies performed in cattle (Gabriël *et al*. 2005) and sheep (Poitras *et al*. 1986) have shown that antibody transfer is observed in association with placental lesions. Placental lesions have been related to *N. caninum* infection (Maley *et al*. 2006; Almería *et al*. 2010).

Owing to the proximity of both PAG-1 and PAG-2 to the placenta it is reasonable to think that after a placental damage episode, PAGs can cross the placenta easier and arrive to the foetal fluids in a similar manner than antibodies. This idea is supported by the higher levels of PAG-2 compared with PAG-1 found in all foetal fluids. PAG-2 molecules are accumulated at the placenta–uterine interface in a more direct contact with possible placental lesions than those PAG-1 molecules which are released by binucleated trophoblast cells towards the maternal uterine stroma (Wallace *et al*. 2015). Therefore, it is also reasonable to hypothesize that levels of PAGs in the foetal fluids can be proportional to placental damage. This could explain why PAGs levels were higher when *N. caninum* antibodies were detected in foetal fluids and why the low plasma concentrations of PAG-2 were related to a higher risk of abortion in *Neospora*-infected dams (Garcia-Ispierto *et al*. 2013). *Neospora*-induced placental damage can increase the migration of PAG-2 from the microvilli junction to foetal fluids reducing its concentration in the maternal blood.

In the present study the presence of *N. caninum* antibodies and high levels of PAG-1 and PAG-2 in foetal fluids was related with higher levels of pH values in foetal fluids. This change in pH values could be explained by the activity of PAGs, of antibodies or other

maternal proteins which crossed the damaged placenta altering the chemical balance of the foetal fluids. This change in pH may compromise the foetal and placental development because some pH values could favour the proteolytic activity of PAG-2 (Telugu *et al*. 2010). Some data indicate that the foetal fluid environment may be slightly acidic (pH 6.5) (Punturieri *et al*., 2000), very similar than pH values observed in the present study in foetal fluid samples from non-infected foetuses.

In conclusion, following an experimental infection with *N. caninum* on Day 110 of gestation, the infected foetuses showed higher levels of PAG-1, PAG-2 and pH values in foetal fluids than non-infected controls. Foetal fluids from infected foetuses with *N. caninum* antibodies had higher levels of PAG-1 and PAG-2 than the foetal fluids from infected foetuses without antibodies in their corresponding fluids. The clinical implication of these findings is that following an infection with *N. caninum*, most cows suffer different levels of damage in their placentas.

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CHAPTER 4

CROSSTALK BETWEEN UTERINE SERPIN (SERPINA14) AND PREGNANCY-ASSOCIATED GLYCOPROTEINS AT THE FETAL-MATERNAL INTERFACE IN PREGNANT DAIRY HEIFERS EXPERIMENTALLY INFECTED WITH *NEOSPORA CANINUM*

Abstract

Infection with *Neospora caninum* is the leading cause of abortion in cattle. In cows naturally infected with *N. caninum*, plasma concentrations of pregnancy-associated glycoproteins (PAG) 1 and 2 indicate fetal-placental well-being, while an excess of progesterone (P4) in the second trimester of gestation has been related to high abortion rate. The immunosuppressive action of P4 on the uterus during gestation has been attributed in part to the uterine serpins (SERPINA14). This study examines expression patterns of the genes *SERPINA14, PAG1*, and *PAG2* at the fetal-maternal interface in dairy heifers experimentally infected with *N. caninum* during the second trimester of pregnancy, when most abortions takes place in natural conditions. Irrespective of infection, expression of *SERPINA14* was higher, and expression of *PAG1* and *PAG2* lower, for intercaruncular endometrium than for caruncles or cotyledons. Cotyledonary tissues showed the highest expression of both *PAG* genes but lowest expression of *SERPINA14*. Expression of *SERPINA14* was significantly higher in intercaruncular endometrium of control dams than for infected animals, pointing to potential disruption of modulation of maternal immune function during infection. Dramatically reduced *SERPINA14* was particularly apparent in infected dams with aborted fetuses. There was also a negative association between *N. caninum* antibody titers with *SERPINA14* and *PAG* expression in infected animals, further suggesting that *N. caninum* infection down-regulates the uterine immunosuppressive function of SERPINA14.

1. Introduction

Neospora caninum is considered a main cause of abortion in cattle worldwide (Dubey and Lindsay, 1996; Dubey *et al*., 2007; Dubey and Schares, 2011; Almería and López-Gatius, 2013). *Neospora*-seropositive cows carry a 12–19 times greater risk of abortion than seronegative cows; the incidence of abortion ranges from 30% to 44% in seropositive animals (López-Gatius *et al.,* 2004a; López-Gatius *et al.,* 2004b). Further, the risk of repeat abortion persists in seropositive cows (Mazuz *et al.,* 2014). The major route of *N. caninum* infection in dairy herds is transplacental, meaning that the parasite passes from dams to their fetuses during pregnancy (Schares *et al.,* 1998; Davison *et al*., 1999). Maternal immunity, host susceptibility, parasite strain diversity, and the stage of fetal development at which infection is acquired have all been related to transplacental infection and abortion (Williams *et al.*, 2000; Gibney *et al.*, 2008; Almería and López-Gatius, 2015). Parasites may provoke lesions in the placenta that are severe enough to cause fetal death and pregnancy termination (Dubey and Schares, 2011; Almería and López-Gatius, 2013). Among several cell-mediated immunity mechanisms, those induced by T helper 1 cells (Th1) have been described as the most important for reducing parasite multiplication in the host (Dubey *et al.*, 2007; Almería and López-Gatius, 2015). However, Th1 activity, although effective in non-pregnant animals, could play a role in the pathogenesis of fetal rejection during gestation (Hansen, 2013).

Pregnancy-associated glycoproteins (PAGs) are a multigene family related to aspartic proteinases that are expressed in the placenta of artiodactyls. Ruminant PAGs are classified into two main groups: one of ancient origin (the PAG-2 subgroup, including PAG-2, PAG-

8, PAG-10, PAG-11, PAG-12, PAG-12, and PAG-22), largely occurring at the placental fetal-maternal interface, and one produced by a more recent series of gene duplications (the PAG-1 subgroup, including PAG-1, PAG-3, PAG-15, PAG-17, and PAG-21), expressed primarily in trophoblast binucleate cells (Wooding *et al.*, 2005; Garbayo *et al.*, 2008). Plasma PAG-1 concentrations are unaffected by chronic *N. caninum*-infection in dams although PAG-1 and PAG-2 concentrations in aborting animals are useful indicators of fetal-placental distress (López-Gatius *et al.*, 2007a; García-Ispierto *et al.*, 2013; García-Ispierto *et al.*, 2015).

In high-producing dairy cows, *Neospora* infection affects endocrine patterns during gestation such that *Neospora*-seropositivity has been associated with higher plasma progesterone (P4) concentrations (García-Ispierto *et al.*, 2010). Progesterone (P4), a key pregnancy hormone, participates in the natural immunomodulation of gestation, reducing the Th1 response to induce maternal immunologic tolerance to the fetus (Druckmann and Druckmann, 2005; Szekeres-Bartho *et al.*, 2001). However, excess progesterone in the second trimester of gestation leads to a higher abortion rate in cows chronically infected with *N. caninum* (Bech-Sabat *et al.*, 2007). Thus, a threshold Th1 immune response such as gamma interferon production seems necessary to confer protection against abortion (López-Gatius *et al.*, 2007b). The maternal immune system can therefore tolerate the presence of paternal alloantigens without affecting anti-infection mechanisms during gestation. The uterine immunosuppressive actions of P4 have been attributed in part to uterine serpins (Hansen *et al.*, 1987). These basic glycoproteins are members of the serpin superfamily of serine peptidase inhibitors. One such member, SERPINA 14 (Ing and Roberts, 1989), is expressed in response to progesterone in the endometrium. According to its uterine expression and loss of proteinase inhibitory activity, a new function in establishing and maintaining pregnancy

in ruminants has been suggested (Padua *et al.*, 2010). SERPINA14 inhibits lymphocyte function in vitro (Padua *et al.*, 2008) and selectively interacts with other uterine proteins, such as PAGs (Mathialagan and Hansen, 1996), uteroferrin (Baumbach *et al.*, 1986), IgM and IgA (Hansen and Newton, 1988), and activin (McFarlane *et al.*, 1999). SERPINA14 is not only secreted by the endometrium of the pregnant ruminant, it is also present in fetal fluids (allantoic and amniotic fluids) (Newton *et al.*, 1989), and ovarian luteal and follicular structures (Ulbrich *et al.*, 2009).

The present study is one of a series of investigations performed in pregnant dairy heifers experimentally infected with *N. caninum*. The objective was to examine expression of *SERPINA14*, *PAG1* and *PAG2* genes at the fetal-maternal interface in the second trimester of gestation of infected animals. This stage of pregnancy was selected as the time when most abortions occur in field conditions. Also assessed were possible interrelations between expression patterns of *SERPINA14* and *PAG* with plasma *N. caninum* antibodies and plasma concentrations of PAG-1 and PAG-2 in the infected dams.

2. Materials and methods

2.1. Animals and experimental design

A full description of the parasite inocula used and the characteristics of the experimentally infected heifers is provided by Almería *et al*. (2016). Briefly, ten 14-16 month old Holstein-Friesian heifers that were seronegative against *N. caninum* (CIVTEST, Spain) were synchronized and artificially inseminated (AI). Seronegativity against the parasite was assessed before AI and on Days 60 and 90 of gestation. Heifers were previously vaccinated

(6-8 months of age) against bovine viral diarrhea virus (BVDV) and infectious bovine rhinotracheitis virus (IBR). Pregnancy was assessed by ultrasound at 30, 45, 90 and 110 days after insemination. On Day 110 of gestation, 6 of the heifers were intravenously inoculated with 10^7 culture-derived tachyzoites of the *N. caninum* isolate Nc-Spain7, kindly donated by Dr. L. M. Ortega-Mora (SALUVET, Universidad Complutense, Madrid, Spain). These 6 animals were euthanized on Day 152 of gestation. The 4 remaining heifers remained as uninoculated controls and euthanized at the same time as inoculated dams. After Day 110, heifers were visually inspected daily for possible abortion until their sacrifice.

2.2. Sample collection

Blood samples for antibody and placental protein determinations were collected from each heifer by tail vein puncture into heparinized vacuum tubes (BD Vacutainer, Becton-Dickinson and Company, Plymouth, UK) on day 152 of gestation. Plasma obtained by centrifugation within 30 min of sampling was stored at -20ºC until analysis.

On Day 152 of gestation (6 weeks after infection) all animals were sedated with xylazine hydrochloride (Rompun; Bayer) and euthanized by an intravenous overdose of embutramide and mebezonio iodide (T61; Intervet). Immediately after death, heifers were necropsied. Amniotic and allantoic fluids were collected before the placenta was opened, and fetuses separated from the placenta. Fetal blood samples were obtained by cardiac puncture. Samples of nine selected placentomes (three cranial, three medial and three caudal) were removed from each dam. Both the maternal side of the placenta (caruncle) and its corresponding fetal side (cotyledon) were carefully separated manually from each placentome. Intercaruncular

tissue was also collected. Tissues collected from fetuses were: CNS (brain and spinal cord), heart, lung, liver, skeletal muscle, spleen, and thymus.

2.3. Ethics

All procedures were approved by the Ethics Committees on Animal Experimentation of the Autonomous University of Barcelona (license number CEEAH.1426-08/02/2012) and University of Lleida (license number CEEA.06-01/12). Animals were handled in strict accordance with good animal practices and the conditions defined by the Animal Ethics Committee of the Autonomous University of Barcelona and CReSA, Spain. Every effort was made to minimize suffering.

2.4. Sample analyses

2.4.1. Plasma antibodies against *Neospora caninum*

Plasma samples collected from the dams and fetuses were tested for anti-*N. caninum* antibodies using a commercial ELISA kit based on the whole tachyzoite lysate of *Neospora* NC-1 according to the manufacturer's instructions (CIVTEST® anti-*Neospora*; Hipra, Girona, Spain). The cut-off used for a positive test result was an S/P ratio of 6.0, as previously established (López-Gatius *et al.*, 2004b) . Fetal fluids were analyzed using the same technique on undiluted samples.

2.4.2. PCR-based diagnosis of *N. caninum*

Portions of placenta and fetal tissues were aseptically obtained and stored in liquid nitrogen at -196ºC until DNA extraction. At least 0.5–1 g of each tissue were homogenized with a

pestle and mortar in liquid nitrogen and DNA extracted using Nc5 PCR (Almería *et al.*, 2002).

2.4.3 PAG-1 and PAG-2

PAG-1 concentrations were determined in plasma using a double antibody radioimmunoassay procedure (RIA-706) (López-Gatius *et al.*, 2007c). As the primary antibody, rabbit polyclonal antiserum AS#706 raised against caprine $PAG_{55\ kDa+62\ kDa}$ (accession numbers P80935 and P80933) was used (Vaitukaitis *et al.*, 1971). The minimum detection limit for the RIA procedure was 1.2 ng/mL. Intra- and inter-assay coefficients of variation were 5.3% and 6%, respectively.

The bovine PAG-2 radioimmunoassay procedure used has been recently described (López-Gatius *et al.*, 2007; Serrano-Perez *et al.*, 2014) . Briefly, PAG-2 was purified according to the method detailed by Beckers *et al*. (Beckers *et al.*, 1988). The primary antibody was rabbit polyclonal antiserum raised against boPAG2 (AS#438) according to the method of Vaitukaitis *et al.* (1971). Owing to the instability of the boPAG-2 molecule, boPAG-1 (67 kDa) was used as a standard (dilutions of 100 to 0.2 ng/mL) and for iodination with the ^{125}I isotope (Thorell and Johansson, 1971). The initial dilution of the primary AS#438 was 1:2500. The minimal detection limit was 2.3 ng/mL. Intra- and inter-assay coefficients were 4.6% and 4.8%, respectively.

2.4.4. RNA extraction and gene expression

Intercaruncular and placental tissue samples were kept frozen in liquid nitrogen, homogenized in a mortar in the presence of additional liquid nitrogen and maintained in trizol

(Invitrogen Corp., Carlsbad, CA, USA) at -80ºC. For caruncle or cotyledon tissue gene expression analysis, a mixed sample of RNA from the three different sections (cranial, medial and caudal) of each tissue was used as template.

Total RNA was extracted according to the method of Chomczynski and Sacchi (1987). Samples were treated with DNAse in the presence of RNAse inhibitors to eliminate contaminating genomic DNA. Concentrations of RNA were determined spectrophotometrically, and RNA integrity was checked by denaturing agarose gel electrophoresis. Complementary DNA was synthesized from 4 µg of total RNA in the presence of random primers using the High Capacity cDNA Reverse Transcription kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer's recommendations.

Messenger RNA expression was determined by real time RT-PCR for three target genes: serpin peptidase inhibitor, clade A member 14 (*SERPINA14*), *PAG1*, and *PAG2*. The genes β-actin (*ACTB*) and ribosomal protein L19 (*RPL19*) were used as housekeeping genes. Sequences of primers for *PAG1* and *PAG2* were those described by Touzard *et al*. (2013). The primers used for ACTB have been described elsewhere (Ribeiro *et al.*, 2014). Primers for *RPL19* (forward primer: 5'-GATCCGGAAGCTGATCAAAG-3, reverse primer: 5-ATTCGAGCATTGGCAGTACC-3') and S*ERPINA14* (Accession number NM_174797; forward primer: 5'- TTTGGAGGCCCTACATCAAG-3, reverse primer: 5-GACCTCCTTTGCCTTCATTG-3') were designed with the Primer3Plus tool (www.bioinformatics.nl/primer3plus) and synthesized by Isogen-Life Sciences (Isogen-Life Science B.V., De Meern, The Netherlands). To avoid genomic contamination, all primers were designed to span an intron. For each gene, we generated a standard curve by amplifying

serial dilutions of a control cDNA to check for linearity between initial template concentration and cycle threshold (Ct) values. Amplifications were conducted using the SYBRgreen method and ABI PRISM^{TM} 7500 sequence detector (Applied Biosystem, Foster City, CA, USA) under the conditions specified by the manufacturer: an initial activation and denaturation step of 10 min at 95 °C followed by 40 cycles consisting of 10 s at 95 °C and 1 min at 60 °C. PCR reactions were run using 3 μL of 30-fold diluted cDNA as template in a total volume of 8 μL containing 1× Maxima SYBR Green/ROX qPCR Master Mix (Fermentas Inc., MD, USA), and 200 nM forward and reverse primers. Each measurement was carried out in triplicate and the average used to calculate the relative amount of gene. The $2^{-\Delta\Delta Ct}$ method was used for data normalization and analysis employing as calibrator a pool of RNA from the tissues used in this study (Livak and Schmittgen, 2001).

2.5. Statistical analysis

One-way ANOVA was used to compare relative *SERPINA14*, *PAG1* and *PAG2* expression for maternal (caruncle and intercaruncle) and fetal (cotyledon) sides of the placenta and among groups (uninfected controls, infected dams with aborted fetuses, and infected dams with live fetuses). When significant differences were detected, the Bonferroni test was used to examine all possible pairwise comparisons. Spearman's rho (sr) test was used to identify possible relationships between gene expression levels of *SERPINA14*, *PAG1* and *PAG2* and *Neospora*-seropositivity or plasma PAG-1 and PAG-2 concentrations before euthanasia in controls and infected non-aborting animals. All tests were performed using the computer package SPSS version 17.0 (SPSS Inc., Chicago, IL). Significance was set at $P \leq 0.05$.

3. Results

3.1. Fetal viability

Fetal death was recorded in 3 of 6 infected dams: two dams aborted 14 and 21 days after infection and a third dam showed a mummified fetus upon euthanasia. Pathological findings in these three fetuses are described in Almería *et al*. (2015). For this study, placentomes were not available or were in too poor a condition for analysis in the aborted fetuses.

3.2. Plasma antibody response

Anti-*N. caninum* antibodies were detected in plasma from all infected dams whereas control heifers (n=4) remained seronegative. *N. caninum* antibodies were also detected in serum (2 fetuses), allantoic fluid (3 fetuses), and amniotic fluid (2 fetuses) collected from the infected dams.

3.3. Detection of N. caninum DNA

N. caninum DNA was observed in at least one tissue type and in the placentas (caruncles and/or cotyledons) from all recovered infected fetuses (Almería *et al.*, 2015). All DNA samples from control, uninfected fetuses were negative.

3.4. Gene expression for *SERPINA14*, *PAG1* and *PAG2*.

Significantly higher (P=0.006) expression of *SERPINA14* and significantly lower (P<0.001) expression of *PAG1* and *PAG2* were observed in intercaruncular tissues when compared with cotyledon and caruncle samples (Figure 1A, 1B, 1C). Highest expression levels of *PAG1* and *PAG2* were observed in the cotyledons compared with caruncular or intercaruncular tissues

(P<0.01) (Figure 1B, 1C). In maternal tissues (caruncle vs. intercaruncle), significant differences were observed for expression of *PAG1* (P=0.013) (Figure 1B) but not *PAG2* (Figure 1C).

Expression of *SERPINA14* in intercaruncular tissues was higher for control (uninfected) dams as compared with infected animals (P=0.001) (Figure 2A). Infected dams carrying a live fetus showed higher *PAG1* expression in intercaruncular tissues compared with that for control or aborting infected dams (P=0.017). Expression of *PAG2* was higher for infected dams carrying a live fetus compared with that for aborting infected dams (P=0.027) but not for control dams (Figure 2B). No differences were detected in *SERPINA14*, *PAG1* or *PAG2* expression between the caruncles and cotyledons of non-aborting infected dams versus control dams.

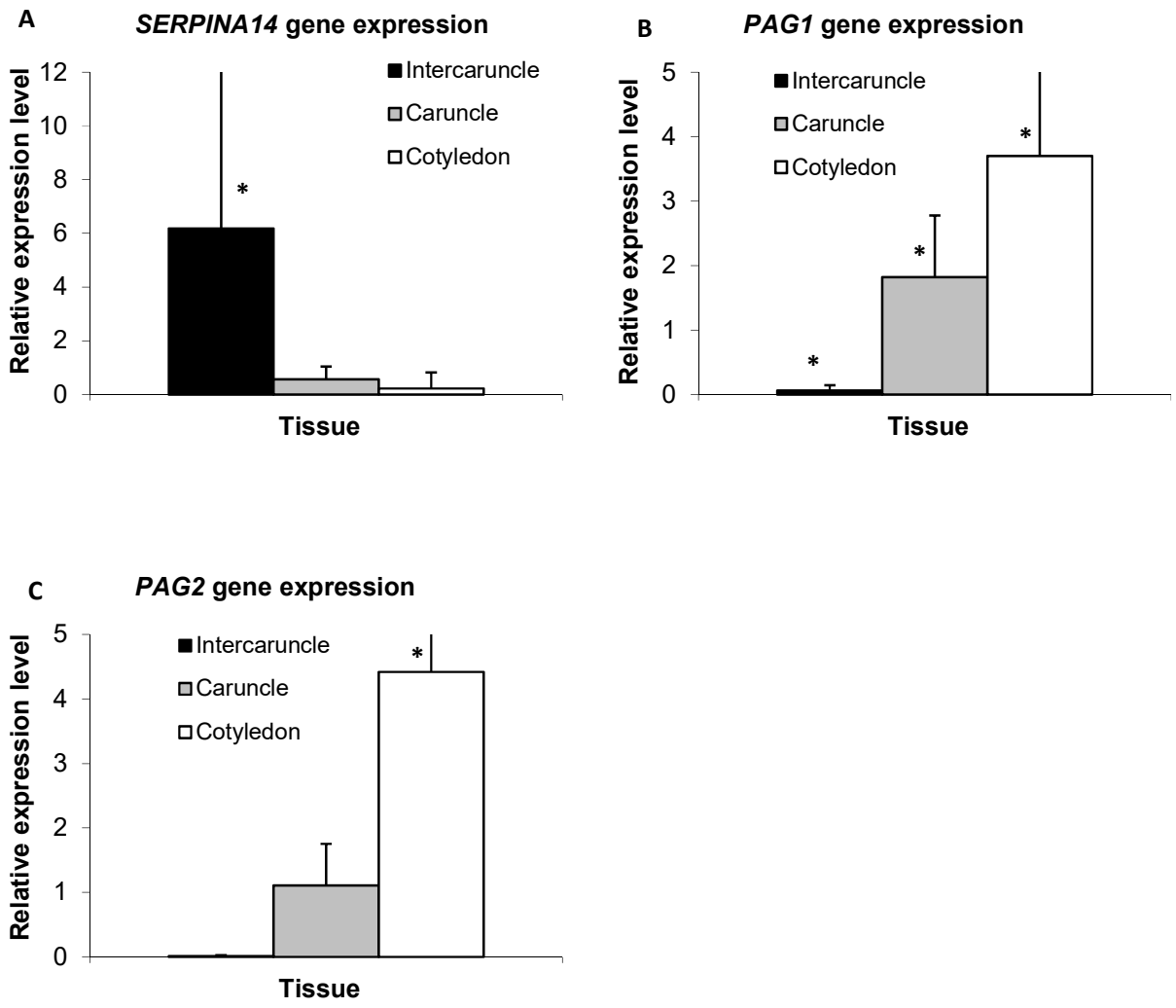

Figure 1. Relative expression of *SERPINA14* (A), *PAG1* (B) and *PAG2* (C) in the cotyledons (n=7), caruncle (n=8) and intercaruncular tissues (n=10) of control and experimental dairy heifers infected with *N. caninum* on Day 152 of gestation. Bars represent the mean ± SEM. *Values for each tissue differing according to the Bonferroni test ($P<0.05$).

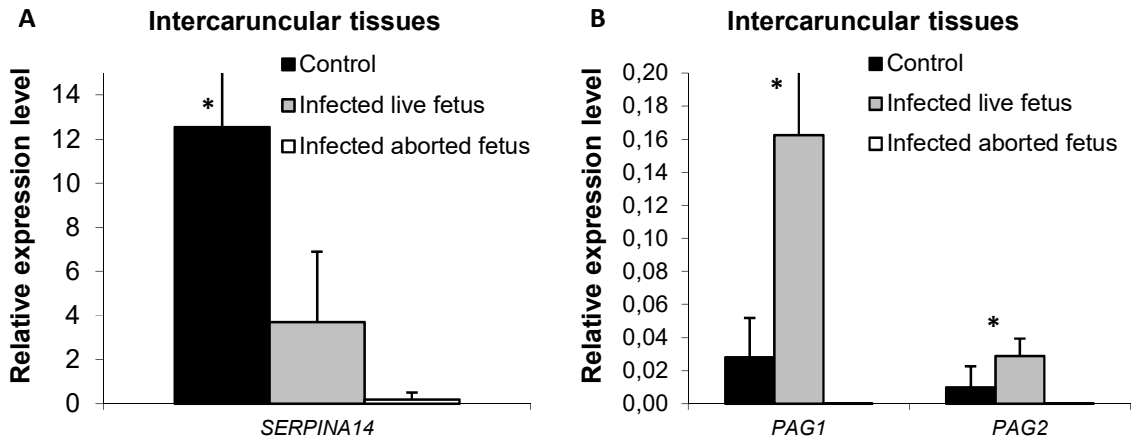

Figure 2. Relative gene expression levels of *SERPINA14* (A), *PAG1* and *PAG2* (B) in intercaruncular tissue samples from: heifers experimentally infected with *N. caninum* on Day 152 of gestation; infected heifers carrying aborted/non-viable fetuses on Day 42 post infection (n=3); live fetuses (n=3); and control uninfected animals (n=4). Bars represent the mean ± SEM. *Values for each group differing according to the Bonferroni test (P<0.05).

Neospora-seropositivity was negatively correlated with *SERPINA14* expression in intercaruncular (r: -0.786, P= 0.036) and cotyledon samples (r: -0.786, P= 0.036) and positively correlated with *PAG1* expression in intercaruncular tissues (r: 0.821, P= 0.023).

3.5. Plasma PAG concentrations

Significantly higher plasma PAG-1 and PAG-2 concentrations were observed in both control and non-aborting infected dams versus aborting infected animals upon euthanasia (P=0.001 and P=0.004, respectively) (Figure 3).

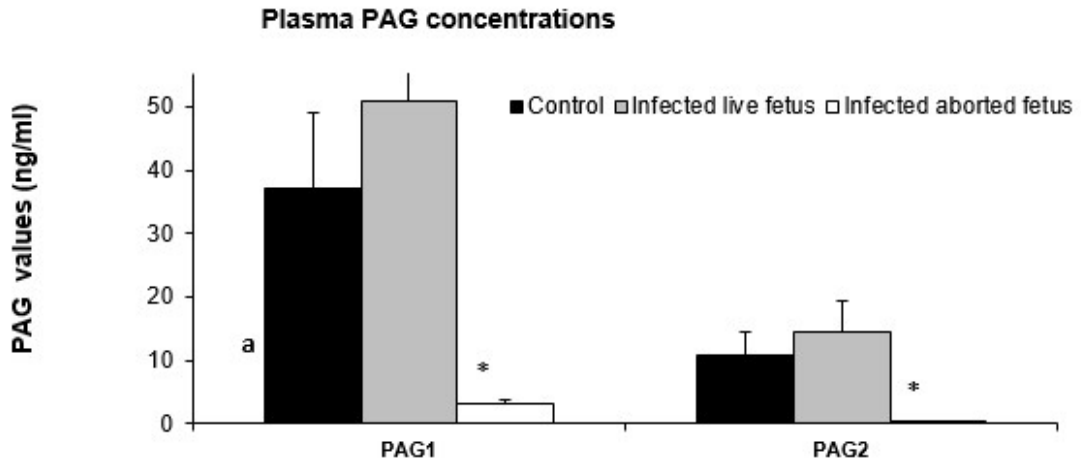

Figure 3. Plasma PAG-1 and PAG-2 concentrations recorded in: heifers infected with *N. caninum* on Day 42 post infection, infected heifers carrying aborted/non viable fetuses (n=3); infected heifers carrying live fetuses (n=3), and control uninfected animals (n=4). Bars represent the mean ± SEM. * Values of each variable differing according to the Bonferroni test ($P<0.05$).

In controls and non-aborting infected dams, *SERPINA14* expression in the cotyledons was negatively associated with plasma PAG-2 concentrations (sr: -0.929, P= 0.003). Expression of *PAG1* in caruncles was positively associated with plasma PAG-1 concentrations (sr: 0.857, P=0.014), whereas *PAG2* expression in cotyledons was positively correlated with plasma PAG-1 and PAG-2 concentrations (sr: 0.857, P=0.014 and sr: 0.750; P=0.052, respectively).

4. Discussion

In this report, we characterize immune-endocrine responses in bovine neosporosis by examining expression of *SERPINA14*, *PAG1* and *PAG2* expression at the fetal-maternal interface following experimental *N. caninum* infection in pregnant dairy heifers. Our main findings were that: 1) irrespective of infection, intercaruncular, caruncular and cotyledonary tissue samples showed different degrees of expression for *SERPINA14*, *PAG1* and *PAG2*; 2) *N. caninum* infection affected expression of all three genes in intercaruncular endometrium; 3) *SERPINA14* expression in intercaruncular endometrium was negatively correlated with *PAG1* and *PAG2* expression levels and negatively correlated with antibody production against *N. caninum* and; 4) *PAG1* and *PAG2* expression were positively correlated with plasma concentrations of both PAGs and antibody production against *N. caninum*.

Greater *SERPINA14* expression was observed in intercaruncular tissue than placentome tissue, in agreement with the findings of other authors (Stephenson *et al.*, 1989). On the contrary, the lowest expression levels of *PAG*s were detected in the intercaruncular area, while placentomes showed the highest *PAG* expression levels, especially in cotyledonary tissues. The segregation of modern PAGs in cotyledons and of ancient PAGs in the intercotyledonary chorion suggests their distinct biological functions within placental tissues (Touzard *et al.*, 2013). The differences observed between caruncular and cotyledonary tissues also indicate good tissue separation in our study.

Experimental infection with *N. caninum* on Day 110 of gestation clearly modified the expression of *SERPINA14*, *PAG1* and *PAG2* in intercaruncular tissues. Unfortunately, we

could not obtain intercotyledonary tissue samples in aborted/mummified fetuses. Uterine serpins can inhibit a wide variety of lymphocyte functions such as mitogen-induced proliferation and natural killer cytotoxicity (Tekin *et al.*, 2005). The significantly higher expression of *SERPINA14* observed here in the intercaruncular tissues of control dams point to a role of this molecule in maternal immune modulation of gestation. However, the dramatically reduced *SERPINA14* expression detected in infected animals, especially in aborting dams, and the negative correlation observed between *SERPINA14* and *PAG* expression along with antibody production suggest that *N. caninum* infection is able to down-regulate *SERPINA14* expression. One possible consequence would be to prevent possible antiproliferative actions of the serpin on *N. caninum* growth. It may also be, however, that reduced *SERPINA14* expression during infection represents a maternal immunological adjustment to enhance maternal immune responses against *N. caninum* by reducing local immunosuppression. It may also be be that disruption of pregnancy leads to a reduction in expression of many pregnancy-associated uterine proteins. This could be tested by examining expression of some other progesterone-induced uterine proteins. The interaction of this molecule with cellular and humoral immune responses against *N. caninum* infection remains to be clarified.

As we anticipated, *PAG* expression in placentome tissues showed positive correlation with plasma PAG concentrations in control and non-aborting infected dams. Despite the low *PAG* expression observed in intercaruncular tissues compared with the levels detected in the placentomes, *N. caninum* infection significantly up-regulated *PAG* expression in this endometrial area in dams with live fetuses compared to uninfected controls. As noted for plasma PAG levels, *PAG* expression in the intercaruncular tissues of aborting infected dams

was practically undetectable, probably due to uterine tissue damage caused by parasite multiplication. We hypothesize that a strong Th1 immune response following multiplication of *N. caninum* and reduction in ummunosuppression caused by reduced *SERPINA14* expression provokes severe lesions in the placenta and endometrium causing the death of the fetus.

As argued by Wallace *et al*. (2015), the accumulation of binucleated trophoblast cells [cells related to the secretion of the modern (type I) PAGs] in the maternal uterine stroma would position them to potentially influence lymphocyte or PMN leukocyte migration and/or activation. Thus, the positive correlation observed between *PAG1* expression and *N. caninum* antibody production could reflect parasite replication in the endometrium of the non-aborting infected heifers.

In summary, our findings indicate differential expression of *SERPINA14*, *PAG1* and *PAG2* in intercaruncular and placental tissues. The dramatic decrease in *SERPINA14* expression detected in infected dams, particularly in those with aborted fetuses, and the negative correlation observed between expression of *SERPINA14*, *PAG1*, and *PAG2* expression with *N. caninum* antibody production in infected animals seem to indicate that *N. caninum* infection can directly or indirectly regulate expression of *SERPINA14* so that uterine immunosuppression is reduced.

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CHAPTER 5

ECONOMIC IMPACT OF *NEOSPORA CANINUM* IN DAIRY FARMS

1. Bio-economical model

1.1. Overview of the Model

A model was built in Microsoft EXCEL® to evaluate the economical impact of *Neospora caninum* in dairy herds and the potential impact of inseminate seropositive cows with Limousin semen. A probabilistic tree considering the main effect of *Neospora*-seropositivity, abortion as well as other reproductive events such as early pregnancy loss, metritis and retained of placenta was developed (Figure 1). Probabilities were derived from research literature and expert knowledge (Table 1). A value was given to every branch of the tree through simulating a cow state (CS) in a farm during 1,400 d that it is the period that a cow needs to complete 4 305-d lactations considering a 60-d dry period. The gross value of every management option GV_i was calculated as follows:

$$GV_i = \sum p_i \times V_i$$

where p_i = probability of every branch and V_i = value of every branch. Finally, the net cost of different management strategies was the result of subtracting the value of a pregnant seronegative cow from the value of a seropositive cow inseminated with Holstein-Friesian and of a pregnant seropositive cow inseminated with Limousin.

1.2. Calculation of branch value

The incomes considered in the simulation were milk income over feed cost (IOFC), income from calves born, and slaughter value when culling occurred. The extra expenses taken into account depending on each branch were additional inseminations and synchronization protocols, embryo reduction, induction of abortion, replacement heifers, and costs due to metritis and retained placenta.

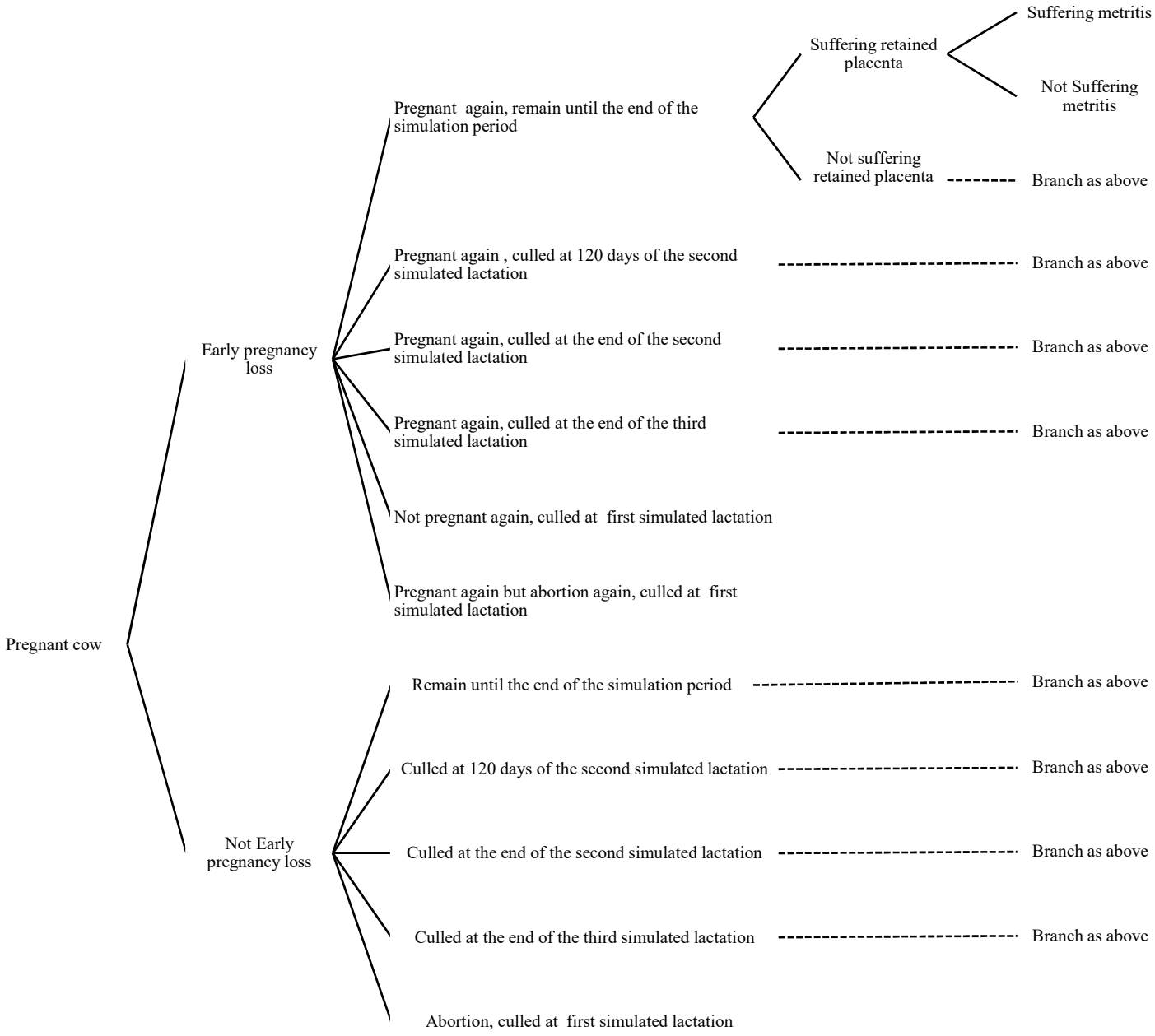

Figure 1. Probabilistic tree showing all the different outcomes of the bio-economical mode1.

Table 1. Probabilities applied in the probabilístic trees.

	Seronegative	Seropositive Holstein	Seropositive Limousin	Reference
Early pregnancy loss (%)	7.2a	5.5 a	5.5 a	a: López-Gatius et al., 2004
Abortion rate 2nd trimester (%)	5.5 a	25.3 a	10 b	b: Almería et al., 2009
Abortion rate 3nd trimester (%)	0.6 a	1.1 a	0.8b	
Retained placenta (%)	11.2	11.2	11.2	c: Andreu-Vazquez et al, 2012
Metritis (%)	11.3	11.3	11.3	c: Andreu-Vazquez et al, 2012
Accumulated culling rate at 120 d of the second simulated lactation (Primaparous cows) (%)	7.6 c	7.6 c	7.6 c	c: Andreu-Vazquez et al, 2012
Accumulated culling rate at the end of the second simulated lactation (Primiparous cows) (%)	15.9 c	34 c,b	23 c,b	c: Andreu-Vazquez et al, 2012
Accumulated culling rate at the end of the third simulated lactation (Primiparous cows) (%)	28 c	51 c,b	38 c,b	c: Andreu-Vazquez et al, 2012
Accumulated culling rate at 120 d of the second simulated lactation (Second lactation cows) (%)	2.4 c	2.4 c	9.6 c	c: Andreu-Vazquez et al, 2012
Accumulated culling rate at the end of the second simulated lactation (Second lactation cows) (%)	9.6 c	26 c,b	17 c,b	c: Andreu-Vazquez et al, 2012
Accumulated culling rate at the end of the third simulated lactation (Second lactation cows) (%)	35.2 c	56 c,b	44 c,b	c: Andreu-Vazquez et al, 2012
Accumulated culling rate at 120 d of the second simulated lactation (Third lactation cows) (%)	12.1	12.1	12.1 c,b	Data from commercial farm
Accumulated culling rate at the end of the second simulated lactation (Third lactation cows) (%)	40	62	54 c,b	Data from commercial farm
Accumulated culling rate at the end of the third simulated lactation (Third lactation cows) (%)	58	80	74 c,b	Data from commercial farm

*Accumulated culling rates due to not reproductive reasons for Seronegative cows and combination of not reproductive reasons with Neospora incuced abortions for Seropositive Holstein and Seropositive Limousine cows.

1.3. Income Over Feed Cost (IOFC) The IOFC for each case was calculated by subtracting the feed costs from the milk value produced during the 1,400-d simulation period.

Lactations length. The first step to calculate the IOFC was to determine the length of all lactations included in the simulation. Because the aim of this study was to evaluate the effects of *Neospora caninum*, the *Neospora*-associated abortion always occurred in the first simulated lactation, and its consequences were followed for 1,400 d since the beginning of the lactation when the first *Neospora*-associated abortion occurs. Thus, the length of the first lactation simulated (DIM_1) varied in every case and was calculated using the following formula:

$DIM_1 = CCI_S + DPL + DPA + GL - DRY$

where CCI_S = simulated calving to conception interval, DPL = days of gestation when early pregnancy loss or abortion occurs, DPA = days required to re-establish pregnancy, GL= gestation length, and DRY= dry period duration.

We assumed that after a pregnancy loss all cows were enrolled in a fixed time short progesterone synchronization protocol (Santos *et al*., 2015) and inseminated 8 d later. Cows that did not become pregnant after this synchronization had the chance to become pregnant every 21 d thereafter. Replacement occurred when cows failed to become pregnant by the time of a predefined DIM cutoff for AI services (DIM_{cutoff}) and they reached a minimum milk threshold. Therefore, if $CCI_S + DPL + DPA > DIM_{cutoff}$ the length of the first lactation (DIM_1) was the day when milk production was below a milk threshold ($DIM_{milk\ threshold}$) or CCI_S + DPL if. $CCI_S + DPL > DIM_{milk\ threshold}$.

As shown below, the length of the remaining lactations and the replacement lactations DIM_i were considered as farm average lactations until reaching the end of the simulation period at 1400 d:

$DIM_2 = CCI_{farm} + GL - DRY$

The values considered in each case for DPL, DPA, DIM_{cutoff}, CCI_{farm}, and the milk threshold are shown in Tables 2 and 3. The values applied for GL and DRY were 280 and 60 respectively (Nielen *et al*., 1989).

Table 2. Values used in the simulations for calculating diferent gestation lengths.

	Days	Reference
Moment of early pregnancy loss (DPL) (Days post AI)	52	López-Gatius *et al*., 2004
Moment of abortion 2nd trimester (DPL) (Days post AI)	135	Data from commercial farm
Moment of abortion 3rd trimester (DPL) (Days post AI)	225	Data from commercial farm
Farms average calving to conception interval (CCI_{farm})	120	Data from commercial farm

Table 3. Values used in the simulations for calculating the days that a cow requires to get pregnant again after a pregnancy loss (DPA) and the moment of culling when a not pregnant cow reaches a milk threshold ($DIM_{milk\ threshold}$).

Parameter	Value
Conception rate for first resynchronization after pregnancy loss (%)	35
21d pregnancy rate after resynchronization after pregnancy loss (%)	25
DIM cutoff for AI services (DIM_{cutoff}) (d)	250
Minimum milk threshold (kg)	25

Milk Production. The MilkBot model (Ehrlich, 2011) was used to predict the milk production for each lactation. The MilkBot model predicts milk production (MP) based on 4 parameters: scale (a), ramp (b), offset (c), and decay (d) (for more details, please refer to http://milkbot.com and Ehrlich, 2011).

$$MP_{DIM} = a \times \left(1 - \frac{e^{\left(\frac{c-DIM}{b}\right)}}{2} \right) \times e^{(-d \times DIM)}$$

The total amount of milk produced in each simulated lactation was calculated by integrating the previous function. The parameters used in the MilkBot model (Table 4) were obtained by fitting Lactation Benchmark Curves for Wisconsin dairy farms provided by AgSource Cooperative Services (AgSource.crinet.com) using the Milk Curve Fitter tool from the University of Wisconsin-Madison Dairy Management website (DairyMGT.info: Tools). We assumed that lactation curve parameters were similar for cows in their third and later lactations.

Table 4 Parameters used in the MilkBot[1] model for calculate milk production.

Average Farm Milk production (kg of milk per cow per year)	Lactation	Scale	Ramp	Offset	Decay
10,433	1	37.29	25.57	-1.53	0.0010
	2	47.50	16.86	-1.17	0.0019
	>2	51.03	18.60	0.45	0.0021

[1]Elhrich (2011)

DMI. The dry matter intake (DMI) (kg/cow per day) was a function of maintenance and milk production according to BW (kg) and 4% FCM being produced, according to NRC (2001):

$$DMI_{DIM} = \left(0.372 \times FCM_{DIM} + 0.0968 \times BW^{0.75}\right) \times \left(1 - e^{0.192 \times \left(\frac{DIM}{7}\right) + 3.67}\right)$$

BW. The BW was calculated for each cow using the Korver function as described by van Arendonk (1985) as a cumulative function of the age of a cow (according to parity), DIM, and pregnancy status using a similar approach as Kalantari *et al*. (2016). Simulated cows were assumed to be Holstein and entered the simulation at 0 DIM with 540 kg of BW for primiparous cows and 620 kg of BW for multiparous cows.

1.4. Calf Value Calculations

Calf value calculations were based on Silva del Río *et al*. (2007) in which a single calving produced 0.93 live calves (53.3% males and 46.7% females). The value assigned to female

calves born from a seropositive cow was set at 70% of a male calf value, to account for females expected to be useless as replacements and have reduced ability to produce beef compared with male calves.

1.5. Culling Value Calculations

The culling value was calculated by multiplying the body weight at culling by the market price of a kg of a live cow.

1.6. Additional Reproductive Cost Calculation

Synchronization Protocols. In all cases when early pregnancy loss or abortion occurred before DIM_{cutoff}, the cost of a fixed-time short progesterone protocol was added. The calculation included labor for administration of hormonal treatments and the cost of the hormones.

Artificial Insemination. Only in the branches where pregnancy loss occurred before DIM_{cutoff}, extra inseminations were counted. After a pregnancy loss, the number of extra inseminations was calculated by dividing by 21 the number of d since the end of the protocol until a new conception or until DIM_{cutoff}. The fixed time insemination from the synchronization protocol then was added. The cost of an insemination included semen and labor to perform the insemination.

Metritis and Retained Placenta. The cost of metritis and retained placenta included veterinary and treatment costs, milk discarded as consequence of antibiotic treatment, and a reduction in milk production. The economic losses associated with decreased reproductive performance and increased culling rate as a result of metritis and retained placenta were not included because these costs are accounted for in other parts of the model. When a cow

suffered both pathologies, the cost included the whole cost of metritis and the veterinary and treatment costs of retained placenta. This calculation and the values applied to it were adapted from Liang *et al*. (2017) (Table 5).

Table 5. Values used in the simulations for calculate the cost of metritis and retained placenta (Adapted from Liang et al., 2017).

Disease	Veterinary labor and treatment cost ($)	Decreased milk production (kg)	Milk discarded (kg)
Metritis	100	24	110
Retained Placenta	84	240	NA[1]

NA[1]: Not applied

1.7. Replacement Cost

The replacement cost included only the market value of a heifer. We assumed that the replacement heifer started her first lactation the day after the cow was culled.

1.8. Costs Used in the Simulations

All costs not specified above that were used in the simulations are shown in Table 6. Costs used in the simulations were collected from scientific journal articles published within the last 10 years as well as actual costs incurred by commercial dairy farms.

Table 6. Costs used in the simulations.

	Unit	Price	Reference
Milk	$/kg	0.36	Giordano *et al*., 2012
Lactation Food	$/kg	0.17	Giordano *et al*., 2012
Dry food	$/kg	0.13	Giordano *et al*., 2012
Male calf	$/head	50	Giordano *et al*., 2012
Female calf	$/head	136	Giordano *et al*., 2012
Culling	$/kg of alive cow	1.16	Giordano *et al*., 2012
Heifer	$/head	1302	Giordano *et al*., 2012
Dose of $PGF_{2\alpha}$..	$/dose	2.3	Giordano *et al*., 2012
Dose of GnRH	$/dose	2.6	Giordano *et al*., 2012
Intravaginal progesterone	$/dose	12	Data from commercial farm
Artificial insemination	$/AI	10	Giordano *et al*., 2012

2. Results and Discussion

As showed in table 7 the cost associated to a *Neospora* seropositive cow ranged between $ -81 and $ -116 and the benefit of inseminate seropositive cows with Limousin semen varied between $51 and $73.

Table 7. Net cost for a Neospora seropositive cow compared with a seronegative cow suffering the first abortion at different lactations.

Lactation of 1st abortion	1st	2nd	3rd
Cost *Neospor*a-seropositive cows using Holstein semen ($)	99	116	81
Cost *Neospor*a-seropositive cows using Limousin semen ($)	32	43	30
Benefit inseminate seropositive animals with Limousin ($)	67	73	51

Extrapolating our average cost for a *Neospora*-seropositive cow $98 along with the 35.9% of seroprevalence reported in Spain (Quintanilla-Gozaloa *et al*., 1999) and the current Spanish inventory of dairy cows (1.2 million), we can estimate the cost of *Neospora* for Spanish dairy industry to be $42 millions per year. Using a similar procedure, the estimated benefit of inseminate all this cows with Limousine semen was $26.7 million. Research to mitigate *Neospora* negative effects and efforts to eradicate *Neospora* from dairy farms are economically justified.

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GENERAL DISCUSSION

GENERAL DISCUSSION

This thesis focused mainly in how *Neospora caninum* infection affects fetal-maternal crosstalk and uterine immunomodulation. To carry out this research an experimental challenge with *N. caninum* in Holstein-Friesian heifers was performed. The heifers were inoculated on Day 110 of gestation and were monitored during 6 weeks until slaughter on Day 152 of gestation.

One of the main findings of this thesis was that a sudden drop of PAG-1 and PAG-2 levels during the second week post-infection (2nd wpi) prevented *N. caninum*-associated abortion. We found that infected non-aborting heifers showed a sudden drop of PAGs levels at 2nd wpi while infected aborting heifers showed a dramatic decrease of PAGs at 3rd wpi. Furthermore, aborting heifers presented higher levels of antibodies at 3rd wpi than non-aborting heifers. Previous studies showed that Th1 immune response is the only effective against *N.caninum* (Innes *et al*., 1995; Khan *et al*., 1997) and reaches its maximum activity during the 2nd wpi (Innes *et al*., 2002). After the Th1 immune response peak, antibodies against *N.caninum* appear in blood at 3rd wpi (Regidor-Cerrillo *et al*., 2014; Almería *et al*., 2010). Linking our observations with the above described immmune response after a *N. caninum* infection, we may hypothesize that non aborting heifers experienced a strong Th1 immune response during 2nd wpi capable to control the parasite and impair placental function while aborting heifers experienced a weak Th1 immune response that did not damage the placenta and failed to control the parasite. As consequence of the weak Th1 immune response during 2nd wpi, the parasite was able to induce a greater production of antibodies and fetal death during 3rd wpi in aborting heifers. This hypothesis would agree with previous studies

which argued that a moderate Th1 response is needed to prevent *N. caninum* induced abortion (López-Gatius *et al*., 2007; Williams *et al*., 2007).

After slaughter on Day 42, higher levels of PAGs were found in fetal fluids from infected dams as well as lesions in placentomes. These lesions could be the cause of the higher levels of PAGs found in fetal fluids from infected heifers. Moreover, the highest levels of PAGs in fetal fluids were associated with the presence of antibodies against *N. caninum*. Although it is accepted that the cotyledonary synepitheliochorial placentation characteristics of cows do not allow for maternal antibodies to cross (Kruse 1983; Latshaw 1987), antibodies can cross through placental lesions (Gabriël *et al*.2005; Poitras *et al*., 1986). Thus, PAGs which normally accumulate at the microvillar junction (Wooding *et al*. 2005) could cross along with antibodies through placental lesions to foetal fluids.

In the stereological study of the placentomes it was observed a lower concentration of Binucleated Trophoblast Cells (BNC) and Mononucleated Trophoblast Cells (MNC) in the infected heifers. The reduced concentration of these cells could be also produced by the Th1 immune response against the parasite. Both, BNC and MNC are responsible for PAGs synthesis and transport (Touzard *et al*., 2013). Despite the lower concentration of BNC and MNC in infected heifers, maternal blood plasmatic levels of PAGs were equal for infected and control heifers. This fact could be produced by a different PAG glycosylation pattern (Constant *et al*., 2011) or due to PAGs are produce in other places. The second idea is supported by findings of this thesis where a higher expression of *PAG1* and *PAG2* mRNA was found at the intercaruncle of infected non-aborting heifers than in control heifers. This

over expression of *PAGs* genes in intercaruncle of infected heifers could be a response to lesions and lower concentrations of BNC and MNC at placentomes.

Other finding of this thesis was that intercaruncles from infected non-aborting heifers showed a lower expression of *SERPINA14* than uninfected controls. Downregulation of *SERPINA14* could be the key for a successful pregnancy when *N. caninum* infection occurs, due to *SERPINA14* possess immune suppressive activity (Hansen *et al*., 1987). Thus, a downregulated expression of *SERPINA14* may allow a moderate immune response at uterine level which could be necessary to control the parasite as previously suggested (Rosbottom *et al*., 2011). In addition, negative correlation between *N. caninum* antibodies titres and *SERPINA14* mRNA expression and negative correlation between *PAG1* expression and *SERPINA14* expression were found. These correlations are a clear example of the delicate balance of gestation when *N. caninum* infection occurs. In one hand, the maternal immune system represented by antibody titres and the fetus represented by *PAGs* demanded less *SERPINA14* to favour a stronger immune response at placental level while in the other hand, PAGs production is relocated in the intercaruncular tissues due to the damages occasioned by the immune response in the placentomes.

The general conclusions of this thesis were that *N. caninum* infection modifies the fetal maternal cross-talk and the immunological balance of gestation and that an increased Th1 immune response during gestation is needed to prevent *N. caninum* induced abortion. In future studies could be interesting to slaughter some cows two weeks after infection and study the immune response at that time. The likelihood of abortion for the slaughtered animals

could be predicted through PAGs concenatration evolution during the second week post infection.

Finally, indicate that with the actual knowledge about bovine neosporosis the best tools to prevent *N. caninum* induced abortion in commercial dairy farms are to inseminate with Limousin sires and cull the seropositive possible reposition calves and cows whom present abortion background. The calculations carried out in this thesis showed that both measures might have a great economic impact. Only in Spain, the insemination of all seropositive cows with Limousin semen would increase the yearly profit of the dairy industry in $26.7 milllions and the erradication of *N. caninum* through selective culling would save $42 millions each year.

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CONCLUSIONS

CONCLUSIONS

The following conclusions were obtained for the objectives of this thesis:

Objective 1:

- To evaluate the outcome of pregnancy in dairy heifers after an experimental infection with *N. caninum* in the second trimester of gestation and, to examine the immune response in the fetus.

Conclusion 1:

- Experimental infections of naïve heifers with 10^7 tachyzoites of the highly pathogenic *N. caninum* strain Nc- Spain7 at 110 days of pregnancy within an experimental period of 6 wpi may cause abortion. The parasite was found in all fetuses from infected heifers and IFN-γ production was detected only in fetal fluids of a dead fetus found upon euthanasia.

Objective 2:

- To determine maternal plasma PAG-1 and PAG-2 dynamics following experimental *N. caninum* infection in dairy heifers.

Conclusion 2:

- Following experimental infection with *N. caninum* on day 110 of gestation, non-aborting heifers showed a short-lived transient decline in plasma PAG-1 and 2 concentrations within 14 dpi. In contrast, infected aborting dams showed a dramatic decline in PAGs until undetectable levels were recorded upon euthanasia.

Objective 3:

- To examine the possible effects of *N. caninum* infection in dairy heifers on mono- and binucleate trophoblast cell numbers in large placentomes 42 days after experimental infection.

Conclusion 3:

- Infected not aborted heifers presented reduced populations of bi- and mononucleate trophoblast cells at 42 dpi.

Objective 4:

- To determine the effect of the experimental infection of pregnant dairy heifers with *N. caninum* on PAG-1 and PAG-2 concentrations and pH in amniotic and allantoic fluids.

Conclusion 4:

- Infected foetuses showed higher PAG-1, PAG-2 and pH values in foetal fluids than non-infected controls. Furthermore, foetal fluids with *N. caninum* antibodies showed higher PAG-1 and PAG-2 levels than foetal fluids from infected foetuses lacking antibodies.

Objective 5:

- To examine expression of *SERPINA14, PAG1*, and *PAG2* genes at the fetal-maternal interface in the second trimester of gestation after an experimental infection with *N. caninum.*

Conclusion 5:

- *N.caninum* infection increased *PAG1* and *PAG2* expression and decreased *SERPINA14* expression in the intercaruncular tissues.

Objective 6:

- To assess possible interrelations between expression patterns of *SERPINA14* and *PAGs* with plasma *N. caninum* antibodies and plasma concentrations of PAG-1 and PAG-2 after an experimental infection with *N. caninum*.

Conclusion 6:

- Plasma *N. caninum* antibodies concentration was found negatively correlated with *SERPINA14* expression and positively correlated with intercaruncular *PAG1* expression.

Blood levels of PAG-2 were negatively correlated with *SERPINA14* expression and positively correlated with *PAG1* and *PAG2* expressions. Plasma PAG-1 concentration showed a positive correlation with *PAG1* expression.

Objective 7:

- To evaluate the economical impact of *Neospora caninum* in dairy herds and the potential impact of inseminate seropositive cows with Limousin semen.

Conclusion 7:

- The estimated cost associated to a *Neospora* seropositive cow ranged between $ -81 and $ -116 and the estimated benefit of inseminate seropositive cows with Limousin semen varied between $51 and $73.

APPENDIX

APPENDIX 1

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