Embryo Reduction:

An Open Window to Decreasing the Twinning Rate

in High-Producing Dairy Cattle

Cover picture: Greta and Audrey. Twin female calves born in the Ramaderia Anibal S.L. (2012)

Back cover picture: Probably the first representation of a twin pregnancy. Monograph reproducing the prehistoric rock painting "Phallic dance" from La Roca de los Moros in El Cogul (Lleida, Spain) by Breuil and Izquierdo (1908)

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An Open Window to Decreasing the Twinning Rate in High-Producing Dairy Cattle

DISSERTATION

to obtain the Degree of Doctor at the Autonomous University of Barcelona

by

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A mi madre y hermanas,
y al pedacito de papá que vive en cada una de nosotras

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0. Summaries

0.1 Summary

Twin pregnancy is an increasing and current topic of the high-producing dairy herds due to the economic losses caused by their great risk of pregnancy failure and the detrimental effects of twinning on the postpartum reproductive efficiency. Transforming a multiple pregnancy into a singleton pregnancy by inducing embryo reduction may circumvent those problems. Therefore, the main aim of this thesis was to develop and evaluate the potential risks and benefits of embryo reduction in dairy cattle as a therapeutic strategy addressed to prevent cows from delivering twins and to reduce the twinning rate in high-producing dairy herds. In order to offer a holistic perspective on the problem of twins and to determine its magnitude, we also studied the factors that affect the incidence of twin pregnancies and the effects of twinning on the productive lifespan of the cows. Research included in this thesis was divided in four studies published or submitted for publication in peer-reviewed journals.

The first study addressed potential management risk factors affecting the incidence of twin pregnancies in high-producing dairy cows. Special attention was paid to the estrus synchronization protocol used before the AI resulting in pregnancy. Twin pregnancy was recorded in the 17.9% of the 2015 pregnancy diagnoses, and the incidence was affected by cow factors (lactation number and previous twining), environmental factors (photoperiod and season) and management related to synchronization protocols.

The second study analyzed data from complete reproductive records for 4861 high milk-producing dairy cows including 12587 calving events to determine the effects of twinning on the subsequent reproductive performance and productive lifespan of the cows. The twinning rate was 5.6%, and 9.8% of the cows delivered twins at least once during their life. Cows delivering twins, besides being less likely to conceive and more likely to be culled in the subsequent lactation, also carry a higher risk of abortion on the subsequent lactation and show a reduced mean productive lifespan.

In the third study we evaluated the effect on pregnancy maintenance of embryo reduction by manual amnion rupture in unilateral and bilateral twin pregnant cows. Embryo reduction by manual amnion rupture did not carry an additional risk of pregnancy loss for unilateral twin pregnancies, whereas the treatment increased the risk of pregnancy failure in bilateral twin pregnancies.

The last study compared the effect on pregnancy maintenance of two embryo reduction techniques, Manual Rupture (MR) and Transvaginal Ultrasound Guided Aspiration (TUGA) of allanto-amniotic fluid in dairy cows with multiple pregnancies. No effect was detected on pregnancy maintenance of the technique used.

0.2 Resumen en castellano

Las gestaciones gemelares tienen un impacto negativo sobre la economía de las explotaciones de vacuno lechero por su elevado riesgo de aborto y el efecto del parto gemelar sobre la eficiencia reproductiva postparto de la vaca. La reducción embrionaria brinda la oportunidad de transformar una gestación múltiple en una simple y se presenta como una herramienta para disminuir la creciente tasa de partos gestaciones y sus efectos negativos. El principal objetivo de esta tesis fue desarrollar y evaluar los beneficios y riesgos potenciales de la reducción embrionaria en vacas de alta producción. Se estudiaron además los factores que afectan a la incidencia de gestaciones gemelares y los efectos de los partos de gemelos sobre la vida productiva de la vaca. Los cuatro estudios experimentales y epidemiológicos incluidos en esta tesis han sido publicados o están sometidos a revisión para su publicación en revistas científicas.

En el primer estudio se analizaron los factores de manejo y especialmente de los diferentes protocolos de sincronización de celo empleados sobre la incidencia de gestaciones gemelares. La tasa de gestaciones gemelares sobre un total de 2015 gestaciones fue del 17.9% y la incidencia se vio significativamente afectada por factores individuales (número de lactación y gemelos en el parto previo), factores ambientales (fotoperiodo y estación) y por el protocolo de sincronización aplicado antes de la inseminación.

En el secundo estudio se recogieron los historiales reproductivos completos de 4861 vacas lecheras de alta producción con un total de 12587 partos con el objetivo de determinar los efectos del parto gemelar sobre la eficiencia reproductiva postparto y la vida productiva de las vacas. La tasa de partos gemelares durante los 11 años de estudio fue del 5.6% y el 9.8% de las vacas parieron gemelos al menos en una ocasión a lo largo de sus vidas. Las vacas con partos gemelares presentaron, además de una menor tasa de concepción y un mayor riesgo de ser eliminadas durante la siguiente lactación, un mayor riesgo de aborto y una menor vida productiva que las vacas con un único ternero al parto.

El tercer estudio evaluó el efecto de la reducción embrionaria mediante la ruptura manual del amnios de uno de los embriones en gestaciones gemelares unilaterales y bilaterales sobre el mantenimiento de la gestación. El riesgo de pérdida de la gestación fue similar para el grupo de vacas con gestación unilateral tratadas y control mientras que la reducción embrionaria incrementó el riesgo de pierda de la gestación en las vacas con gestaciones bilaterales.

En el último estudio se compararon las tasas de mantenimiento de la gestación tras reducir gestaciones múltiples mediante la ruptura manual del amnios o mediante aspiración transvaginal del fluido fetal guiado por ecografía. El riesgo de pérdida de la gestación fue similar para ambos métodos de reducción.

0.3 Resum en català

Les gestacions de bessons tenen un impacte negatiu sobre l'economia de les explotacions lleteres per l'elevat risc d'avortament i els efectes negatius del part de bessons sobre l'eficiència reproductiva postpart de la vaca. La reducció embrionària ofereix l'oportunitat de transformar una gestació múltiple en una simple i es presenta con una eina per a disminuir la creixent taxa de bessons i els seus efectes negatius. El principal objectiu d'aquesta tesi ha estat desenvolupar i avaluar els beneficis i riscs potencials de la reducció embrionària en vaques d'alta producció. També es van estudiar els factors de maneig que poden afectar la incidència de gestacions de bessons i els efectes del part de bessons sobre la vida productiva de la vaca. Els quatre estudis que s'inclouen en aquesta tesi han estat publicats o estan sotmesos a revisió per a la seva publicació en revistes científiques.

En el primer estudi es van analitzar l'efecte dels factors de maneig i especialment els diferents protocols de sincronització de cel emprats sobre la incidència de gestacions de bessons. La taxa de gestació de bessons sobre un total de 2015 gestacions va ser del 17.9% i la incidència es va veure significativament afectada per factors individuals (lactació i bessons en el part anterior), factors ambientals (fotoperíode y estació) i pel protocol de sincronització aplicat abans de la inseminació.

El segon estudi recull els historials reproductius complets de 4861 vaques lleteres d'alta producció amb un total de 12587 parts amb l'objectiu de determinar els efectes del part de bessons sobre l'eficiència reproductiva postpart i la vida productiva de les vaques. La taxa de parts de bessons durant el 11 anys d'estudi va ser del 5.6% y el 9.8% de las vaques van tenir bessons al menys en una ocasió al llarg de la seva vida. Les vaques amb part de bessons van presentar una menor taxa de concepció i un major risc d'eliminació en la lactació següent, major risc d'avortament i menor la vida productiva que les vaques amb un únic vedell al part.

En el tercer estudi es va avaluar l'efecte de la reducció embrionària per la ruptura manual de l'amnios d'un dels embrions en gestacions de bessons unilaterals i bilaterals sobre el manteniment de la gestació. El risc de pèrdua de la gestació va ser similar en el grup de vaques amb gestacions de bessons unilaterals tractades i control. Tanmateix, la reducció embrionària va incrementar el risc de pèrdua en les vaques amb gestacions de bessons bilaterals.

A l'últim estudi es van comparar les taxes de manteniment de la gestació en gestacions múltiples reduïdes mitjançant la ruptura manual de l'amnios o mitjançant aspiració transvaginal del fluid fetal guiada per ecografia. El risc de pèrdua de la gestació va ser similar per ambdós mètodes de reducció.

1. General introduction

1.1 Introduction

Intensification procedures of the dairy production systems have culminated in an increase of milk production and the number of animals per herd over the past 50 years in the developed countries. In 1944, the dairy cattle population in USA exceeded 25 millions of animals and the mean number of cows per herd was as low as 6 (Capper *et al.* 2009). In 2007, North American herds' averaged size was 155 cows, with a total dairy cattle population in USA of approximately 9 million cows producing over 84 million tonnes of milk per year (Capper *et al.* 2009). Also in the European Union (EU-27), with 23 millions of dairy cows that produce annually almost 140 million tonnes of milk, which represents the 22% of the world production (EUROSTAT 2011), countries have followed the trend towards intensive systems on a smaller number of larger and more specialized production units. In Spain, from the early nineties onwards, the number of dairy cows and herds has descended from 1.5 million to 900000, and from 140000 to 24000, respectively, with no impact on the amount of milk produced, which approximates 6 million tonnes (MAGRAMA 2012).

The emergence of high-producing rentable herds, that actually displace more traditional dairy systems, is the result of an intense genetic selection for milk production, together with nutrition and general management improvements. However, genetic selection for a higher milk production has been related to the worldwide decline in reproductive performance reported over the last 30 years, with dramatically dropping fertility rates and longer calving to conception intervals (Lucy 2001; López-Gatius 2003). Cattle reproduction efficiency is extremely linked to the economic success in dairy herds (Britt 1985; Plaizier *et al.* 1997, Meadows *et al.* 2005), especially in high-producing systems, because it is just after parturition that lactation initiates. The premise of "a calf per cow per year" is a main goal for farmers and practitioners as it is known that milk production of individual cows is maximized when the mean calving interval approaches 12-13 months. However, the gestation period averages 282 days, and postpartum uterine involution and resumption of the ovarian cyclicity determine at least a 50 to 60 days voluntary waiting period until first breeding. Achieving satisfactory fertility rates in order to get the cow pregnant again in a short interval, during which cows are suffering a great metabolic stress due to the peak of milk production, has become a major challenge for researchers, veterinarians and farmers.

Once the cow becomes pregnant, however, it is equally important for further productivity that the pregnancy progresses safely to term. In fact, pregnancy failure has been described as the major single source of economic losses for dairy producers (Ball 1997). Most pregnancy losses take place during the first two weeks after conception (Ball 1997; Hanzen *et al.* 1999; Vanroose *et al.* 2000; Inskeep and Dailey 2005) and are, therefore, commonly unnoticed. Cyclicity is minimally disrupted and cows returning to estrus are submitted for re-insemination before pregnancy diagnosis. In contrast accurate early pregnancy diagnosis (conducted by ultrasonography on days 28-34 post insemination) and subsequent pregnancy controls, have allowed determining the actual rates of losses. The economic impact, the critical periods and the factors affecting pregnancy loss have been established. Pregnancy loss rates during the first trimester of gestation may exceed 20% in high-producing dairy herds (Bartolomé *et al.* 2005a-c; Grimard *et al.* 2006; López-Gatius *et al.* 2009). Risk factors for early fetal loss include not only viral, bacterial, protozoal and mycoplasmal infections which are rare in herds under strict health programs, but also non-infectious causes (Vanroose *et al.* 2000). Once infectious agents are discarded, determining the exact etiology of a pregnancy loss may be difficult, because non-infectious causes are often multifactorial. Some specific individual, environmental and management factors directly affecting pregnancy maintenance have been identified in our area of study (Labèrnia *et al.* 1996; López-Gatius *et al.* 2002, 2009) and elsewhere (Silke *et al.*, 2002; Santos *et al.* 2004; Grimard *et al.* 2006; Gabor *et al.* 2008). Among them, the presence of twins has been described as the main factor of a non-infectious nature related to pregnancy loss in high-producing dairy herds (López-Gatius *et al.* 2009). The likelihood of pregnancy loss during the first trimester of gestation for cows carrying twins, that may reach 60% during the warm period (López-Gatius *et al.* 2004), has been reported to be 3 to 7 times-higher than for cows carrying singletons (López-Gatius *et al.* 2002, 2009; López-Gatius and Garcia-Ispierto 2010).

Twin pregnancies are undesirable in dairy herds not only because of their greater risk of pregnancy loss but also because of a high incidence of dystocia, stillbirths and calf mortality registered for the cows delivering twins (Chapin *et al.* 1980; Nielen *et al.* 1989; Gregory *et al.* 1990; Eddy *et al.* 1991; Eckternkamp *et al.* 1999a, 1999b; Bicalho *et al.* 2007). Furthermore, twin calving births are responsible for most periparturient diseases such as, for example, placental retention, a disorder that negatively affects the subsequent fertility (reviewed by Laven and Peters 1996). Cows delivering twins require longer and more expensive treatments during the postpartum period and account wider calving to conception intervals (Nielen *et al.* 1989; Eddy *et al.* 1991; Bicalho *et al.* 2007). In addition, and closely related to their impaired postpartum reproductive performance, cows delivering twins suffer a higher risk of culling (Nielen *et al.* 1989; Eddy *et al.* 1991; Bicalho *et al.* 2007). Thus, twin births represent a serious management and economic problem and have been reported to reduce the profitability of the herd (Eddy *et al.* 1991; Beerepoot *et al.* 1992).

The cow, given its monovular condition, normally produces one offspring per pregnancy. However, the twinning has increased over the past two decades rate in dairy cattle (Kinsel *et al.* 1998; Silva del Rio *et al.* 2007) so that the actual estimates run at 9% and even exceed 12% in some herds (Silva del Rio *et al.* 2007). High milk production has been reported to be the single largest contributor to this recent increase in the twinning rate (Kinsel *et al.* 1998), and it has been linked to the incidence of double ovulation (Fricke and Wiltbank 1999; López *et al.* 2005). Genetic selection for high milk production seems to be to blame for the increase on the incidence of twin pregnancies (Johanson *et al.* 2001),whereas management practices related to high production should also reduce the risk of losses in twin pregnancies (López-Gatius and Garcia-Ispierto 2010). Therefore, it is foreseeable that the twinning rate will continue to increase along with milk production in the years to come. However, the contribution to the twinning rate of other factors such as the application of recently developed estrus synchronization protocols, combining several hormones, should be checked.

Under the current framework, twin pregnancies should be considered as an emerging disorder with negative impact on herds' economy. This thesis assesses the factors affecting the incidence of twin pregnancies in high-producing dairy cows and evaluates the effects of twin birth calvings on the subsequent reproductive performance and the productive lifespan of the cow. Furthermore, the thesis proposes a "therapeutic approach" to the problem of twinning based on two different embryo reduction techniques adapted from human obstetrics (Mansour *et al.* 1999; Ibérico *et al.* 2000; Lee *et al.* 2008) and equine reproduction (Bracher *et al.* 1993; Macpherson and Reimer 2000; Frazer *et al.* 2003; Mari *et al.* 2004). Special attention is given to the risk of pregnancy loss following induced twin embryo reduction. Thus, twin embryo reduction is proposed herein as a strategy to lowering the twinning rate in high-producing dairy herds. Applicability and profitability of the techniques by facing both the risks and benefits at a farm level are also focused.

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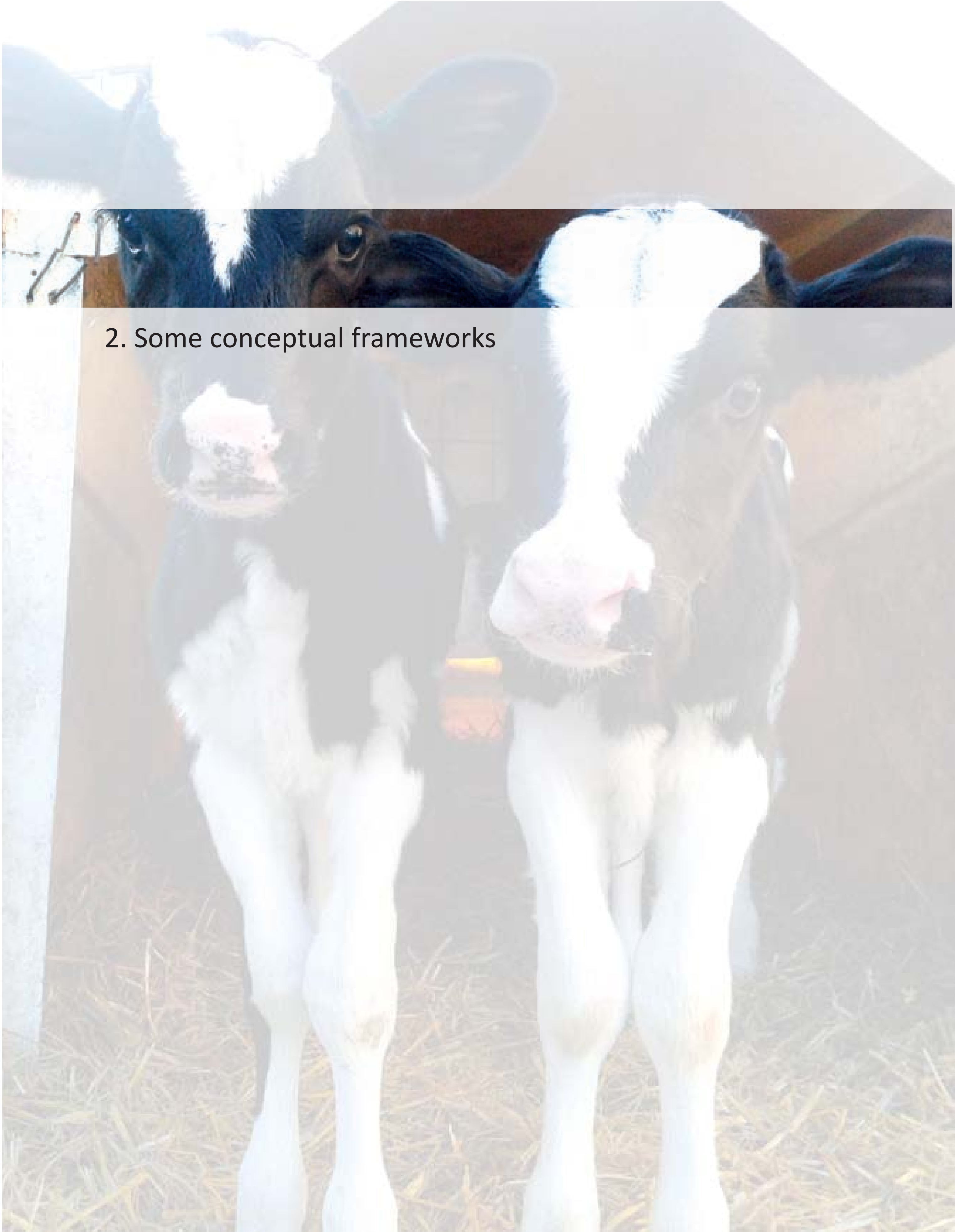


2. Some conceptual frameworks

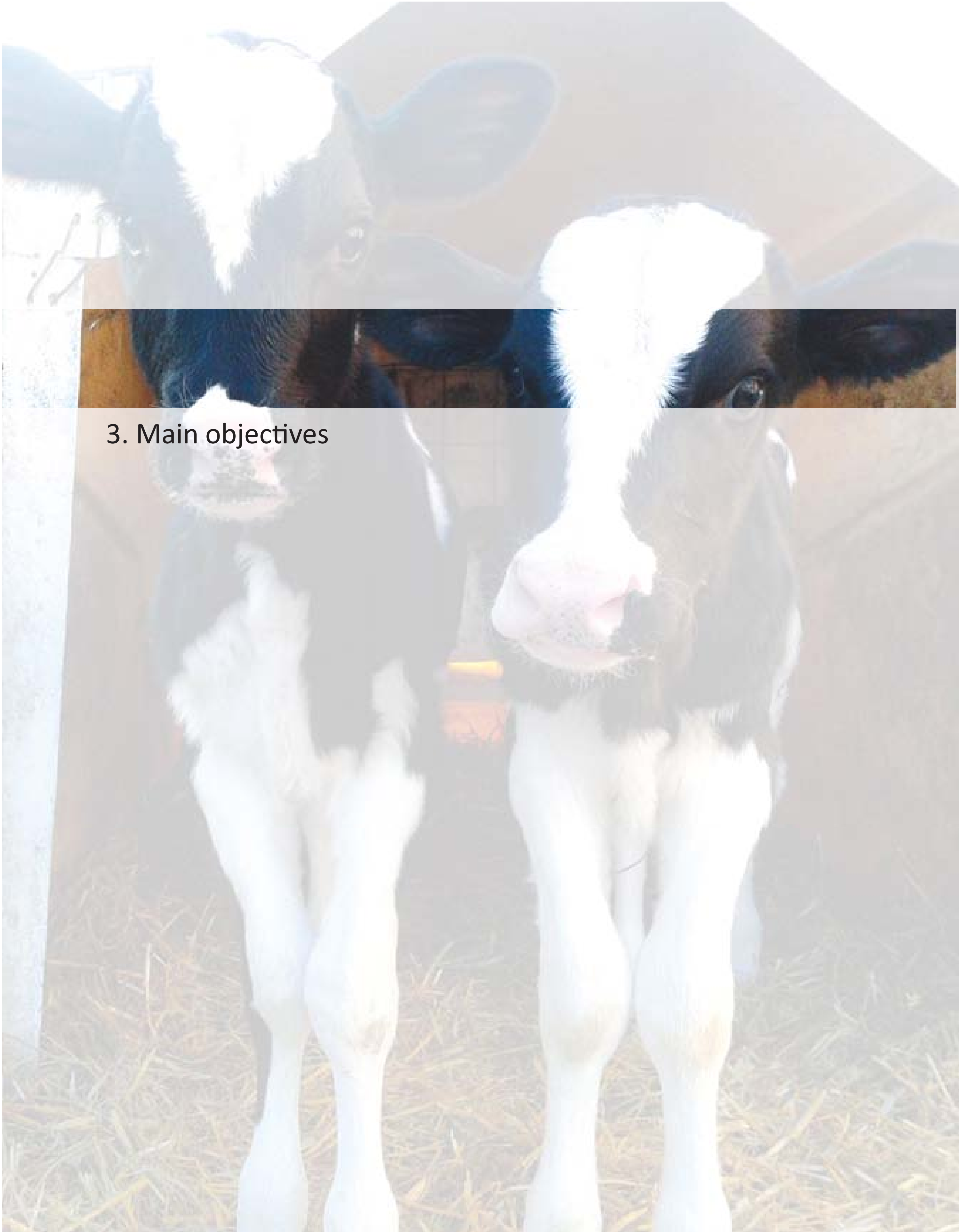
2.1 Epidemiological-observational *versus* experimental studies

Conceptually, two kinds of studies were included in this thesis: epidemiological-observational and experimental studies. Whereas the goal of epidemiological-observational studies, often concerning a large population, is to assess the incidence and the factors related to a certain disease, experimental studies focus in a small number of individuals assigned to specific groups with the purpose of evaluating a particular preventive or therapeutic agent.

According to these premises, the two first studies included in this thesis could be considered as retrospective epidemiological-observational studies as they aim to establish the incidence and to identify the factors affecting twin pregnancy, and to evaluate the effects of a twin birth calving on the subsequent reproductive performance and productive lifespan of the cow. The last two studies constituted experimental studies as they proposed and evaluated the success of two embryo reduction techniques conducted in a limited number of cows bearing multiple pregnancies and compared them in terms of pregnancy maintenance. Aspects on data collection and statistic analyses are provided in appendix 1.

2.2 Outline of the thesis

Globally, and independently of their nature, the four studies included in this thesis aim to offer a holistic perspective on the problem of twinning. Knowing the real incidence of twin pregnancies in the herd, as well as the management, environmental, and cow-related factors that may affect the incidence of twin pregnancies, overall could be used to modify management and herd health practices (first study). Not least important information could be drawn by a retrospective exhaustive analysis and comparison of the productivity of cows that give birth to singletons or twin calves (second study). Results from these first two studies are essential to assess the real magnitude of the twinning problem in the dairies and, therefore, may respond to whether it could be reasonable to implement embryo reduction treatments. Finally, evaluating the risk of the embryo reduction techniques and analyzing the factors that affect pregnancy maintenance following embryo reduction could provide answers to further questions such as in which cows should we conduct embryo reduction, which is the best method and moment to perform it and which additional therapies should be administered following embryo reduction in order to maximize its success (third and fourth studies).



3. Main objectives

The main aim of this thesis was to develop and evaluate the applicability of inducing embryo reduction in high-producing dairy cows as a therapeutic strategy to transform multiple pregnancies into singleton pregnancies. The therapeutic approach to the problem of twinning in dairy herds was constructed under the hypothesis that embryo reduction methods developed in human obstetrics and equine medicine might be adapted and applied to cattle in order to prevent the negative effects of delivering twins and to achieve a lower twinning rate.

The analysis of the potential management risk factors affecting the incidence of twin pregnancies in high-producing dairy cows, and the assessment of the detrimental effects of twinning on cows' subsequent reproductive and productive performance were additional objectives.

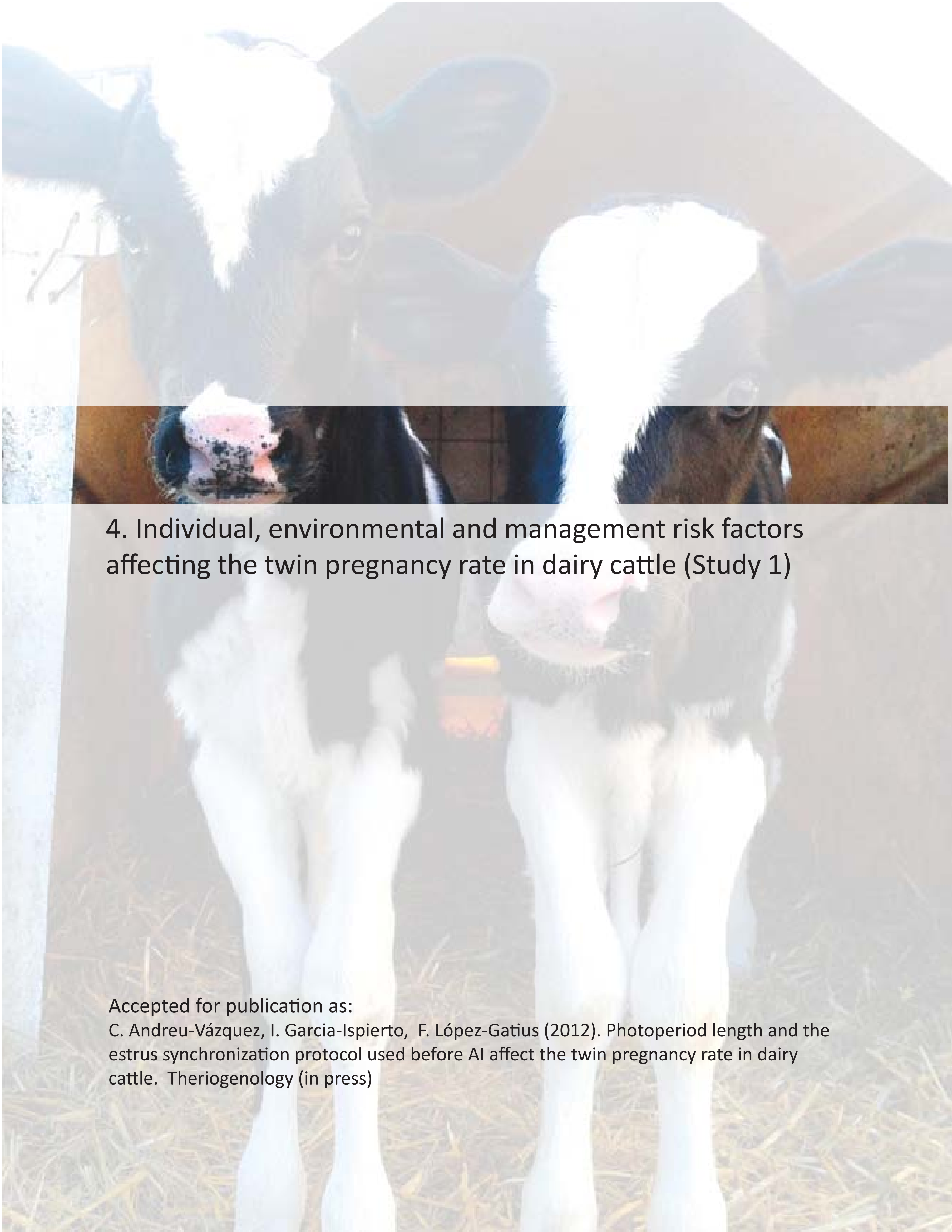
These general objectives were split in four specific objectives, as follows:

1) To assess potential cow, environmental and management risk factors affecting the incidence of twin pregnancies in high-producing dairy cows with special emphasis placed on the estrus synchronization protocols applied before insemination.

2) To assess the effects of twinning on the subsequent reproductive performance and the productive lifespan of high-producing dairy cows.

3) To evaluate in a large population of unilateral and bilateral twin pregnancies the effect of inducing embryo reduction by MR (manual rupture of the amnion) on pregnancy maintenance, and to assess the dynamics of the endocrine factors progesterone, pregnancy associated glycoprotein (PAG-1) and prolactin following treatment.

4) To compare the effect on pregnancy maintenance of inducing embryo reduction by MR (manual rupture of the amnion) or TUGA (transvaginal ultrasound guided aspiration of the fetal fluid) in dairy cows with multiple pregnancies.



4. Individual, environmental and management risk factors affecting the twin pregnancy rate in dairy cattle (Study 1)

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Abstract

This study addresses potential management risk factors affecting the incidence of twin pregnancies in high-producing dairy cows. Special attention was paid to the estrus synchronization protocol used before the AI resulting in pregnancy. Possible factors affecting the twin pregnancy rate were analyzed through binary logistic regression procedures on 2015 pregnant cows from July 2010 to July 2011. Twin pregnancy was recorded in 361 of the 2015 pregnancy diagnoses made (17.9%). Twin pregnancy rates differed among herds ($P<0.001$) and ranged from 12.4% to 23.9%. Based on the odds ratios, the risk of twin pregnancy was reduced by factors of 0.65 or 0.71 when AI was performed during the warm season or an increasing photoperiod, respectively and increased by a factor of 1.11 for each unit increase in lactation number; by factors of 4.57 or 6.33 in cows that received a progesterone-releasing intravaginal device (PRID) plus 500 IU or 750 IU of equine chorionic gonadotropin (eCG) 28 days before the pregnancy AI, respectively; by a factor of 2.39 in cows with an ovarian cyst diagnosed in the 14 d prior to AI and treated with prostaglandins (PG); by factors of 1.94 or 3.91 in cows that received two PG doses during the 14 days prior to AI or cows that following failed PRID treatment had received PG started over the 28 days prior to AI, respectively; and by a factor of 2.58 in cows that had previously delivered twins compared to cows delivering singletons. Our results indicate that cow factors such as lactation number and previous twining, as well as environmental factors such as photoperiod and season and management related to synchronization protocols affect significantly the incidence of twin pregnancies.

Keywords

Multiple pregnancies; Environmental factors; Hormones; Pregnancy diagnosis; Cattle

4.1 Introduction

Interest in controlling the twinning rate in dairy herds has varied over the past decades. Early studies sought to increase the twinning rate by genetic selection and hormone administration to improve milk production and progeny per cow (Rudledge 1975; Echternkamp *et al.* 1990; Echternkamp 1992; Van Vleck *et al.* 1991). However, most authors today agree that twin pregnancies are undesirable in a dairy herd, and any anticipated benefits of twin calving are insufficient to outweigh such undesirable effects as a higher risk of pregnancy loss (López-Gatius *et al.* 2002; López-Gatius *et al.* 2009; López-Gatius and Garcia-Ispierto 2010), abortion, retained placenta, dystocia, stillbirths and freemartins (Echternkamp and Gregory 1999 a, 1999 b), or a longer calving to conception interval and higher culling rate for cows delivering twins (Chapin and Van Vleck 1980; Gregory *et al.* 1990, Bilcalho *et al.* 2007). In effect, the few studies assessing the economic consequences of twinning have described that twin pregnancies reduce herd profitability, with an average loss of income attributed to cows delivering twins estimated at 74$ to 108$ (Eddy *et al.* 1991; Beerepoot *et al.* 1992). In addition, the real economic impacts of twinning are probably on the rise since the twinning rate has considerable increased over the past 20 years and estimates run at 9%, and even exceed 12% in some herds (Silva del Rio *et al.* 2007).

Prior work has addressed the management factors affecting the prevalence of twinning and double ovulation (Cady and Van Vleck 1978; Nielen *et al.* 1989; Kinsel *et al.* 1998; Fricke and Wiltbank 1999; López *et al.* 2005; López-Gatius *et al.* 2005a). However, it should be noted that neither the incidence of twins at calving nor double ovulation are a true reflection of the rate of twin pregnancy. Thus, a vast difference exists between the incidence of double ovulation and the twinning rate (Kidder *et al.* 1952). Recording twins on pregnancy diagnosis (PD) is a more appropriate endpoint to consider when examining possible factors related to the incidence of twin pregnancies. Given the high risk of pregnancy loss and abortion, the actual occurrence of twin pregnancies may have been underestimated in studies designed to examine twin births. In addition, while the twinning rate (i.e., incidence of twin birth calvings) has been steadily rising, fertility has drastically declined in high-producing cows (Lucy 2001; López-Gatius 2003), and this decline has prompted the development of estrus synchronization protocols based on several combinations of different hormones. However, as far as we are aware no study has yet explored how these protocols could affect the incidence of twin pregnancies. The present study was designed to assess potential cow, environmental and management risk factors affecting the incidence of twin pregnancies in high-producing dairy cows with special emphasis placed on the estrus synchronization protocols applied before insemination.

4.2 Material and methods

4.2.1 Animals

The data analyzed were derived from 2015 cows that became pregnant over a one-year period (July 2010- July 2011) belonging to three commercial dairy herds in northeastern Spain. Mean annual milk production for the three herds, comprising 1600, 640 and 1091 lactating cows, were 11797 kg, 11350 kg and 11150 kg, respectively. Cows were housed in free stalls with concrete slatted floors and cubicles, milked three times daily and fed complete rations. Feeds consisted of cotton-seed hulls, barley, corn, soybean and bran, and roughage, primarily corn, barley and alfalfa silages and alfalfa hay. Rations were in line with NRC recommendations (National Research Council 2001). The culling rates for the three herds during the study period were 27%, 27% and 34%, respectively.

4.2.2 Reproductive management

Herds were maintained on a weekly reproductive health program. Daily checks were performed during the first two weeks after calving to diagnose and treat any puerperal disease. Cows were examined at least once within 35–50 d postpartum to check for ovarian and uterine structures.

Reproductive disorders such as incomplete uterine involution, endometritis, pyometra, and ovarian cysts, diagnosed either in a gynecological postpartum examination or at any subsequent time, were treated until resolved. The voluntary waiting period for the herds was 50 d. Only cows free of detectable reproductive disorders were inseminated. Cows more than 60 d in milk showing no estrus signs within the previous 21 d were examined weekly until specific estrus synchronization or until AI during a natural estrus (López-Gatius *et al.* 2008). At the weekly visit, ovarian structures were recorded. If a cow had a corpus luteum (CL) estimated to be at least 15 mm (mean of the maximum and minimum diameters), the animal was synchronized for estrus with a single prostaglandin dose (PG; 25 mg dinoprost i.m.; Enzaprost, CEVA Salud Animal, Barcelona, Spain). Cows with persistent follicles (a follicular structure of at least 8–15 mm detected in two consecutive exams 7 d apart in the absence of a corpus luteum and no estrous signs, López-Gatius *et al.* 2001) were fitted with a progesterone releasing intravaginal device (PRID, containing 1.55 g of progesterone; CEVA Salud Animal, Barcelona, Spain) for 9 d and given the PG dose 24 h before PRID removal (López-Gatius *et al.* 2008; Garcia-Ispierto *et al.* 2010). A subset of cows with CL also received a PRID for 9 d. At the time of PRID removal, some cows received randomly a dose of 500 IU or 750 IU of equine chorionic gonadotropin (eCG; i.m.; Syncrostim, CEVA Salud Animal, Barcelona, Spain). In one of the herds, PRIDs were removed on day 5 followed by two doses of PG given 24 h apart. Ovarian cysts (a follicular structure larger than 15 mm detected in either one or both ovaries in the absence of CL and uterine tone, Hanzen *et al.* 2008) were ruptured and treated either with PG (López-Gatius *et al.* 2008; Hanzen *et al.* 2008) or PRID (Hanzen *et al.* 2008) following rupture. Under a reproductive control on a weekly basis, cyst treatment with manual rupture favors reproductive efficiency of the cow and does not impair her productive life [29]. All cows not showing estrus signs during the 14 days after a treatment based on PG or PRID (considered as a failed synchronization protocol) were returned to the reproductive control program. Cows were inseminated after a time-fixed schedule or at estrus detection based on walking activity (López-Gatius *et al.* 2005b, pedometer system: Afifarm; SAE Afikim) using the frozen sperm of bulls of proven fertility. A dose of gonadotropin releasing hormone (GnRH; 100 µg i.m.; Cystoreline, CEVA Salud Animal, Barcelona, Spain) was given 12 h or 24 h before AI to all cows under a time-fixed protocol or at the time of AI to cows showing natural estrus.

Pregnancy diagnosis (PD) was performed by ultrasonography 28 to 34 days post AI. Scanning was performed along the dorso/lateral surface of each uterine horn. The presence of twins was recorded after the observation of two embryos in different positions within one uterine horn on two screen scans, two embryos simultaneously present on the screen or one embryo in each uterine horn. Cows diagnosed as not pregnant were either returned to the reproductive program or registered for culling. All gynecological exams and pregnancy diagnoses were performed by the same operator.

4.2.3 Data collection and analysis

The following data were recorded for each cow: number of embryos observed on PD (twins/triplets or singletons), cow factors: lactation number, milk production and milk protein and fat contents (average for the month of AI), days in milk at AI, insemination number and date, ovarian structures recorded in the 14 d prior to AI, walking activity on the day of AI, twinning and occurrence of stillbirth at previous calving, pregnancy loss (before or after day 90 of gestation) over the current lactation and prior to the analyzed positive PD, and number of embryos/fetuses aborted, and management factors: herd, inseminating bull, estrus synchronization protocol in the 28 d prior to AI, type of AI (time-fixed or at estrus detection) and GnRH administration and time before AI (0, 12 or 24 h).

In our geographical area of study there are two clearly differentiated weather periods: warm (May to September) and cold (October to April, Labèrnia *et al.* 1998). Reproductive variables are impaired during the warm season (López-Gatius 2003; Garcia-Ispierto *et al.* 2007). For this reason, insemination dates were used to analyze the effect of AI season (environmental factor, warm versus cold period) on the occurrence of a twin PD. Additionally, since endocrine patterns of gestation are affected by photoperiod length (Garcia-Ispierto *et al.* 2009), insemination dates were also used to analyze the effect of the photoperiod (environmental factor, increasing day length, 21st December: sunrise at 8:20 h and sunset at 17:29 h, to 20th June, versus decreasing day length, 21st June: sunrise at 5:23 h and sunset at 20:35 h, to 20th December) on the occurrence of twin pregnancies. For the analyses, data on ovarian structures, the estrus synchronization protocol and type of AI were grouped and coded (Table 4.1).

A binary logistic regression analysis was performed to determine the relative contribution of each variable to the probability of a twin pregnancy. Triplet pregnancies (n=43) were considered as twin pregnancies. The dependent variable was twin pregnancy; the categorical and continuous variables possibly affecting twin PD are listed in Table 5.2. Regression analysis (PASW Statistics 18, SPSS Inc., Chicago, IL, USA) was performed according to the method of Hosmer and Lemeshow (1989). Basically, this method consists of six steps as follows: preliminary screening of all variables for univariate associations; construction of a full model using all the significant variables resulting from the univariate analysis; stepwise removal of non-significant variables from the full model; comparison of the reduced model with the previous model for model fit and confounding; evaluation of interactions among variables; and assessment of model fit using Hosmer-Lemeshow statistics. Variables with univariate associations showing P-values <0.25 were included in the initial model. Modeling was continued until all the main effects or interaction terms were significant according to the Wald statistic at $P < 0.05$.

Regression coefficients from the logistic regression were exponentiated to obtain the odds ratio and corresponding 95% confidence interval associated with each factor. An odds ratio significantly higher (or lower) than 1 indicates an increased (or reduced) risk of twin PD if the factor is present and is a dichotomous variable. For continuous variables, an odds ratio significantly higher (or

Table 4.1. Hormonal treatments.

Hormonal treatment code	Ovarian structure recorded over the 14d prior to AI	Estrus synchonization protocol applied over the 28d prior to AI	Type of AI	n of cows enrolled
0 Natural estrus	Unknown or follicle	Absence of treatment	estrus detection	1241
1 PRID_FOL	Persistent follicle	PRID[ab] during 9d	estrus detection	171
2 PRID_FOL5d	Persistent follicle	PRID[ac] during 5d	time-fixed	97
3 PRID_FOL500eCG	Persistent follicle	PRID[ab] during 9d and 500 UI of eCG[d] at the time of PRID removal	estrus detection or time-fixed	23
4 PRID_FOL750eCG	Persistent follicle	PRID[ab] during 9d and 750 UI of eCG[d] at the time of PRID removal	estrus detection or time-fixed	15
5 PRID_CL	Corpus luteum	PRID[ab] during 9d	estrus detection or time-fixed	29
6 PRID_CL500eCG	Corpus luteum	PRID[ab] during 9d and 500 UI of eCG[d] at the time of PRID removal	estrus detection or time-fixed	20
7 PRID_CL750eCG	Corpus luteum	PRID[ab] during 9d and 750 UI of eCG[d] at the time of PRID removal	estrus detection or time-fixed	21
8 PG	Corpus luteum	PG[e] (with no previous protocol over the 28d prior to AI)	estrus detection	304
9 PRID_Cyst	Ovarian cyst	PRID[a] during 9d	estrus detection	55
10 PG_Cyst	Ovarian cyst	PG[e]	estrus detection	28
11 Failed PG then PG	Corpus luteum	PG[e] (with a failed[f] PG given over the 14d prior to AI)	estrus detection	116
12 Failed PRID then PG	Corpus luteum	PG[e] (with a failed[f] PRID[ab] given over the 28d prior to AI)	estrus detection	10

[a] Progesterone releasing intravaginal device (containing 1.55 g of progesterone; CEVA Salud Animal,

lower) than 1 implies an increased (or reduced) risk of twin PD with each 1 unit increase in the value of this factor. For class variables, one class of each variable was considered as the reference, and an odds ratio significantly higher (or lower) than 1 for any other class of this variable was taken to indicate an increased (or reduced) risk of twin PD when compared to the reference class. Mean values are expressed as the mean ± standard deviation (SD).

4.3 Results

Twin PD was recorded in 361 of the 2015 pregnancies analyzed (17.9%). In 208 of the 361 pregnancies (57.6%) the twin embryos were located in the same uterine horn (152 and 56 in the right and the left uterine horns, respectively) and 153 were bilateral twin pregnancies. The incidence of twin pregnancies ranged from 12.4% to 23.9% among the three herds included in the study. Twin PD was recorded in 132 out of 885 (14.9%), 90 out of 483 (18.7%), 71 out of 348 (20.4%), 41 out of 187 (21.9%) and 27 out of 112 (24.1%) cows in their first, second, third, fourth and fifth or higher lactation, respectively. The odds ratios for the factors found to significantly affect twin pregnancies are shown in Table 4.2. No significant effects were observed of days in milk at AI, milk production, milk fat and protein contents, insemination number, inseminating bull, walking activity recorded on the day of AI, GnRH administration before AI, the occurrence of stillbirth in previous calvings and previous single or twin pregnancy losses during the current lactation. Plausible interactions such as season-photoperiod and lactation number-synchronized/natural estrus were not detected.

4.4 Discussion

The overall incidence of twins was 17.9%, a figure closer to the lower end of the range of 15 to 37% reported for double ovulation in high-producing dairy cows (Santos et el. 2000; Sartori *et al.* 2004; López-Gatius *et al.* 2005a) than the 9% currently reported for twin births (Silva del Rio *et al.* 2007). This expected result reinforces the idea that neither the incidence of twins at calving nor double ovulation following insemination are a true reflection of the twin pregnancy rate. The high incidence of pregnancy loss in twin pregnancies and, no less important, the fact that natural twin reduction usually occurs following a positive pregnancy diagnosis (López-Gatius and Hunter 2005; López-Gatius *et al.* 2010; Andreu-Vázquez *et al.* 2011), will logically reduce the number of twins recorded at parturition. On the contrary, since double ovulation may occur in cows conceiving a single embryo (López-Gatius *et al.* 2009; López-Gatius and Garcia-Ispierto 2010), the rate of twin pregnancy will be lower than that of double ovulation.

The twin pregnancy rate differed significantly among the three herds. This could reflect differences in management practices not considered in this study. However, irrespective of the herd, our results still reveal how management factors can intensely contribute to the incidence of twin pregnancies.

The estrus synchronization protocol used before AI affected the twin pregnancy rate. Among the 12 estrus synchronization protocols investigated here, six were found to significantly increase the incidence of twins compared to cows conceiving at natural estrus. The risk of twin pregnancy was not significantly greater in the cows subjected to a progesterone-based protocol for 5 or 9 d (PRID). However, although calculated on a small study population, the odds ratio reached figures of 4.6 or 6.3 for the subset of anestrous cows that were administered 500 IU or 750 IU of eCG at the time of PRID removal. Because of its LH- and FSH like activity and its long half-life (Bevers *et al.* 1989), eCG has been widely used to induce multiple ovulation in small ruminants (Rahman *et al.*

Table 4.2 Cow, environmental and management factors assessed and odds ratios for the factors included in the final binary logistic regression model for twin pregnancy (TP).

Factor	Mean ± S.D. (ranges)	Class description	N of pregnancies	TP (%)	Odds Ratio	95%CI	P- value
Environmental factors							
Season		Cold	1560	19.3	Reference	-	-
		Warm	455	13.2	0.646	0.47 - 0.90	0.009
Photoperiod		Decreasing	1058	17.7	Reference	-	-
		Increasing	957	18.2	0.705	0.54 - 092	0.010
Management factors							
Herd		1	925	12.4	Reference	-	-
		2	429	20.5	-	-	NS
		3	661	23.9	2.413	1.78-3.26	<0.001
Inseminating bull[a]		30	2015	17.9	-	-	NS
Hormonal treatment[b]		0 Natural estrus	1241	16.1	Reference	-	-
		1 PRID_FOL	131	20.6	-	-	NS
		2 PRID_FOL5d	22	13.6	-	-	NS
		3 PRID_FOL500eCG	23	43.5	4.572	1. 9 - 11.08	0.001
		4 PRID_FOL750eCG	15	53.3	6.329	2.17 - 18.5	0.001
		5 PRID_CL	29	20.7	-	-	NS
		6 PRID_CL500eCG	20	15.0	-	-	NS
		7 PRID_CL750eCG	21	38.1	3.045	1.17 - 7.94	0.023
		8 PG	304	14.1	-	-	NS
		9 PRID_Cyst	55	14.5	-	-	NS
		10 PG_Cyst	28	35.7	2.390	1.06 - 5.38	0.035
		11 Failed PG then PG	116	26.7	1.937	1.23 - 3.06	0.005
		12 Failed PRID then PG	10	40.6	3.913	1.06-14.51	0.041

GnRH[c]		Absence	656	23.8	Reference	-	-
		At AI	1311	15.1	-	-	NS
		12 h prior to AI	22	13.6	-	-	NS
		24 h prior to AI	26	23.1	-	-	NS
Cow factors							
Previous calving		Singleton	1941	17.4	Reference	-	-
		Twins	74	32.4	2.572	1.52 - 4.36	<0.001
Stillbirth/s previous calving		Absence	1831	17.7	Reference	-	-
		Presence	184	19.6	-	-	NS
Previous pregnancy loss		Absence	1867	17.7	Reference	-	-
		Single pregnancy loss	103	17.5	-	-	NS
		Twin pregnancy loss	45	26.7	-	-	NS
Lactation number	2.12 ± 1.33 (1-10)	Continuous	2015	17.9	1.106	1.01 - 1.21	0.028
Days in milk at conception	125.16 ± 71.11 (34-635)	Continuous	2015	17.9	-	-	NS
Insemination number	2.40 ± 1.84 (1-14)	Continuous	2015	17.9	-	-	NS
Milk production (Kg)[d]	41.41 ± 9.18 (15.0-78.2)	Continuous	2015	17.9	-	-	NS
Protein content (%)[d]	3.34 ± 0.28 (1.94-4.67)	Continuous	2015	17.9	-	-	NS
Fat content (%)[d]	3.32 ± 0.75 (1.20-6.68)	Continuous	2015	17.9	-	-	NS
Walking activity at estrus	562.27 ± 221 (43-1498)	Continuous	2015	17.9	-	-	NS

Likelihood ratio test= 102.711; 18 d.f.; $P<0.001$. Hosmer Lemeshow goodness-of-fit test= 5.695; 7 d.f.; $P=0.576$ (the model fits). Nagelkerke R^2= 0.084.

NS=Not significant

[a] Bulls with less than 10 AI recorded (n=38) were group together.

[b] List of abbreviations for the hormonal treatments: PRID= Progesterone releasing intravaginal device (containing 1.55 g of progesterone; CEVA Salud Animal, Barcelona, Spain); eCG= Equine chorionic gonadotropin (i.m.; Syncrostim, CEVA Salud Animal, Barcelona, Spain); PG= Prostaglandin G2α (25 mg dinoprost i.m.; Enzaprost, CEVA Salud Animal, Barcelona, Spain).

[c] Cystoreline (CEVA Salud Animal, Barcelona, Spain) 100 µg i.m.

[d] Mean at the month of pregnant AI.

2008), beef and dairy cattle (Mapletoft *et al.* 2002). Notwithstanding, the mechanisms whereby eCG exerts its effects remain to be determined. Thus, if eCG promotes double ovulation, it could be that in cows producing two oocytes, short exposure to exogenous elevated progesterone concentrations immediately prior to insemination will enhance embryo survival [43].

The condition of ovarian cysts has been linked to subsequent double ovulation and twinning (Kinsel *et al.* 1998; Labhsetwar *et al.* 1963; Kesler and Garverick 1982). With regard to twinning, Kinsel *et al.* (1998) stated that the probability of delivering twins was increased by a factor of 2.52 in cows with an untreated ovarian cyst, but was lowered when the cows had been treated with PG or GnRH. The authors of this study failed to mention the time interval between ovarian cyst diagnosis and pregnancy AI. In our study, all cows diagnosed with an ovarian cyst over the 14 d prior to AI received treatment. The risk of twins on PD was 2.39 times higher in cows treated with PG whereas PRID treatment did not increase the twin pregnancy rate. Differences between treatments could be attributed to the time from treatment to AI. Thus, while cows receiving a PRID were not inseminated during the following 9 d, cows treated with PG that showed signs of estrus within the following 24-48 h were inseminated. In the latter cows, the presence of the ovarian cyst could be related to double ovulation (Labhsetwar *et al.* 1963). Similarly, cows that received 2 doses of PG over the 14 d prior to AI (indicating a possible persistent CL) and cows that received a PG dose following a failed PRID protocol over the 28 d prior to AI had a 1.94 and 3.91 times higher risk of twin PD, respectively, than cows conceiving after a natural estrus. In these cows ovulating after a failed estrus synchronization protocol, a certain degree of ovarian dysfunction might be assumed and this could be responsible for the higher risk of double ovulation. The scenario might be similar to that of anovular cows spontaneously recovering, in which the multiple ovulation rate at first ovulation reaches 46.3% (López *et al.* 2005). On the other hand, the incidence of twin pregnancy was unaffected by synchronization with a single dose of PG or application of GnRH immediately before or at AI, in agreement with previous studies in which PG, human chorionic gonadotropin and estradiol were included in the synchronization protocol

(López-Gatius *et al.* 2005a), nor by the administration of GnRH at AI (López-Gatius *et al.* 2006). In contrast, some authors noted an increased risk of twinning when cows had received PG alone (Kinsel *et al.* 1998) or in combination with FSH/LH or GnRH (Nielen *et al.* 1989). The authors of these studies also reported a significant effect of the administration of GnRH prior to AI (Nielen *et al.* 1989; Kinsel *et al.* 18). The timing of these hormones was not mentioned in the latter studies and this might be crucial for the outcome of the subsequent AI.

The duration of estrus has been reported to be shorter in cows experiencing multiple ovulations (López *et al.* 2005), and walking activity has been strongly correlated to fertility (López-Gatius *et al.* 2005b). Thus, we should expect some link between walking activity during the day of AI and the number of embryos at pregnancy diagnosis. However, differences were not found in walking activity in cows with single or twin pregnancies. This could be explained by the inclusion of cows conceiving after a time-fixed insemination protocol in which an increase in activity prior to AI might have not occurred. Further, eCG treatment on PRID removal seems to reduce estrous behavior (Garcia-Ispierto *et al.* 2012).

As anticipated, the likelihood of twin pregnancy increased with parity (a unit increase in lactation number led to a 1.11-fold increased risk of twin pregnancy). Likewise, older cows have been described to be more likely to deliver twins (Cady and Van Vleck 1978; Nielen *et al.* 1989; Eddy *et al.* 1991; Kinsel *et al.* 1998) and to experience double ovulation (Fricke and Wiltbank 1999; López *et al.* 2005; López-Gatius *et al.* 2005a). Further, cows that had delivered twins in the previous calving showed an increased risk of twin PD. This is in agreement with the findings of previous studies (Nielen *et al.* 1989), and, besides increased parity, probably reflects a maternal trait.

Cows inseminated during the cold season were more likely to conceive twins. This is in agreement with studies describing a higher rate of double ovulation during the cold season (López-Gatius *et al.* 200a) and the fact that most twins are born during the summer (Cady and Van Vleck 1978; Nielen *et al.* 1989). Some authors attribute seasonality of twinning to feed supplementation during the fall (Cady and Van Vleck 1978). However, in the current intensive-production setting, disturbed ovarian activity due to heat stress (López-Gatius *et al.* 2005a) seems to be a more plausible explanation. Regardless of season, decreasing day length at the time of conception was found to increase the likelihood of twin pregnancy. Whether to do with seasonality (Garcia-Ispierto *et al.* 2007) or with what remains of an ancient strategy in cows and other mammals increasing the chances of parturition when feed avalilabilty is higher, warrants further investigation.

Although high milk production has been traditionally linked to the incidence of double ovulation (Fricke and Wiltbank 1999; López *et al.* 2005) and is thought to be the single largest contributor to the recent increase in the twinning rate (Kinsel *et al.* 1998), we observed no effect of milk production, milk fat and protein contents or days in milk at AI on the risk of twin pregnancy. Expedited liver metabolism and hormonal clearance in high-producing dairy cows have been linked to impaired follicular development favoring follicular codominance and double ovulation (Fricke and Wiltbank 1999). Our results however do not support this hypothesis. In fact, our univariate analyses performed prior to logistic regression (data not shown) indicated that milk production was lower for cows carrying twins than cows carrying singletons (40.5 ± 9.3 kg vs. 41.7 ± 9.1 kg, twins vs. single PD respectively, P=0.032). However, this effect was not detectable after adjusting for other risk factors in the logistic regression analysis. This finding reinforces the results of a study conducted in our geographical area in which a 1 kg increase in milk yield led to a 0.97-fold reduced risk of double ovulation (López-Gatius *et al.* 2005a).

Collectively, our results indicate that cow factors such as lactation number and previous twining, as well as environmental factors such as photoperiod and season and management related to synchronization protocols affect significantly the incidence of twin pregnancies. Attention should be focused on implementing palliative practices, such as embryo reduction in twin pregnant cows (López-Gatius 2005; Andreu-Vázquez *et al.* 2011), in combination to reduce estrous synchronization protocols to prevent increasing twinning rates in the herds.

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5. Assessing short, mid, and long term detrimental effects of twinning (Study 2)

Submitted for publication as:
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Effects of twinning on the subsequent reproductive performance and productive lifespan of high -producing dairy cows.

Abstract

This study was designed to determine the effects of twinning on the subsequent reproductive performance and productive lifespan of high milk-producing dairy cows. The data analyzed were taken from complete reproductive records for 4861 Holstein Friesian cows comprising a commercial herd, including data for 12587 calving events from 1st April 2001 to 31st March 2012. The twinning rate was 5.6%, and 9.8% of the cows delivered twins at least once during their life. Conception rates before Days 90, 120 and 300 postpartum were 20.8%, 36.9% and 74.5%, and 34.2%, 51.8% and 85.0% for cows delivering twins and singletons, respectively. Cox regression analysis revealed that twinning reduced the chances of conception before Day 90 by a factor of 0.76. Also, the median calving to conception interval was significantly longer for cows calving twins (134 ± 4.5 d) than for cows delivering singletons (108 ± 0.8 d; P<0.001). Moreover, in cows in which conception was successful, the risk of abortion was higher for cows that calved twins than for those calving singletons (13.7% vs. 10.3%, P=0.01, respectively). Culling rates before Days 90, 120 and 300 postpartum were 15.6%, 16.1% and 28.6%, and 7.6%, 8.7% and 15.9% for cows calving twins and singletons, respectively. Cox regression analysis of the factors affecting the culling rate before Day 300 postpartum revealed a 1.41 times greater hazards ratio of culling for cows calving twins than cows calving singletons. Further, mean productive lifespan was almost 300 days shorter for primiparous twinners (n=48, 602 ± 493 days) than for non-twinners (n=2592; 899 ± 581 days; P<0.01), and 200 days shorter for secundiparous twinners (n=126, 914 ± 429 days) than for non-twinners undergoing at least two lactations (n=1936, 1101 ± 522 days; P<0.01). Kaplan-Meier survival curves for productive lifespan differed between primiparous twinners and non-twinners (P<0.001), and between secundiparous twinners and non-twinners having at least two lactations (P=0.017). Differences in culling-patterns for twinners and non-twinners were not restricted to the subsequent lactation but continued as long as 800 days after first calving, strongly suggesting long-term negative effects of twinning. As an economic implication of our findings, we suggest that twin embryo reduction at the moment of pregnancy diagnosis could be a profitable

strategy to cut twinning rates and abolish their detrimental effects on subsequent reproductive performance and productive lifespan.

Keywords

Culling rate, Fertility, Pregnancy loss, Bovine

5.1 Introduction

Over the years, the occurrence of twinning in dairy herds has been viewed from very different standpoints. While studies in the 1980s sought to increase the twinning rate to improve milk production and progeny per cow (Rutledge 1975; Echternkamp *et al.* 1990; Van Vleck *et al.* 1991; Echternkamp 1992), most authors today would agree that twin pregnancies are undesirable in a dairy herd (Chapin and Van Vleck 1980; Gregory *et al.* 1990; Echternkamp and Gregory 1999a, 1999b; López-Gatius *et al.* 2002, 2009; Bicalho *et al.* 2007; López-Gatius and Garcia-Ispierto 2010). The presence of twins has been described as the main negative factor of a non-infectious nature affecting pregnancy maintenance (López-Gatius *et al.* 2002, 2009; López-Gatius and Garcia-Ispierto 2010). For example, the risk of pregnancy loss during the first trimester of gestation for cows carrying twins is 3 to 9 times-higher than for cows carrying singletons [5-7]. Higher milk production related to twinning is controversial (Chapin and Van Vleck 1980; Nielen *et al.* 1989; Eddy *et al.* 1991; Beerepot *et al.* 1992; Bicalho *et al.* 2007), and this possible benefit will never outweigh the higher incidence of dystocia, stillbirths and retained placenta (Eddy *et al.* 1991; Beerepot *et al.* 1992; Echternkamp and Gregory 1999a). In effect, longer calving to conception intervals and higher culling rates have been reported for cows delivering twins compared to cows delivering singletons (Nielen *et al.* 1989; Eddy *et al.* 1991; Bicalho *et al.* 2007). It is now clear that twin pregnancies reduce herd profitability, and the average loss of income attributed to cows delivering twins has been estimated at 74$ to 108$ (Eddy *et al.* 1991; Beerepot *et al.* 1992). The real economic impacts of twinning are probably on the rise since twinning rates have increased considerable over the past 20 years and estimates currently run at 9%, or even 12% in some herds (Silva del Rio *et al.* 2007).

Recent studies have proposed the idea of inducing embryo reduction to lower the twinning rate in dairy herds. This is usually conducted through manual rupture of the amnios (López-Gatius 2005; Andreu-Vázquez *et al.* 2011) or transvaginal ultrasound-guided aspiration of allanto-amniotic fluid (Andreu-Vázquez *et al.* 2012a) at pregnancy diagnosis. However, transforming a multiple pregnancy into a singleton pregnancy is not a risk-free procedure (López-Gatius 2005; Andreu-Vázquez *et al.* 2011, 2012a) and there is a need to quantify the benefits of this practice and validate its cost-effectiveness. A report including data for over 33,000 calvings in 20 herds in New York and Indiana, has provided current figures on the effects of twinning on herd profitability in terms of reproductive performance and survival of lactating cows in the 300 days following calving (Bicalho *et al.* 2007). However, since only a single parturition per cow was examined, no information was gained on the subsequent productive lifespan of cows delivering twins. In the present study, we assessed the effects of twinning on the subsequent reproductive performance and productive lifespan of high-producing dairy cows.

5.2 Material and methods

5.2.1 Animals

The data analyzed were derived from the records of 12839 calvings over eleven years (1st April 2001- 31st March 2012) for 4896 Holstein Friesian cows comprising to a commercial dairy herd in northeastern Spain. Table 5.1 provides production data for the herd over the study period. Cows

Table 5.1. Productive data for the herd recorded over the 11-year study period.

Period (1st April-31st March)	No. of lactating cows	Annual milk production (Kg/cow)	Annual culling rate (%)
2001-2002	911	11607	33.9
2002-2003	944	11797	31.0
2003-2004	976	11790	31.0
2004-2005	1020	11826	32.0
2005-2006	1052	12739	36.0
2006-2007	1005	12848	33.0
2007-2008	1084	12498	32.0
2008-2009	1161	12381	31.6
2009-2010	1270	11954	33.0
2010-2011	1343	12187	33.0
2011-2012	1596	12651	25.0

were housed in free stalls with concrete slatted floors and cubicles, milked three times daily and fed complete rations in line with NRC recommendations (National Research Council 2001).

5.2.2 Reproductive management

The herd was maintained on a weekly reproductive health program. Daily checks were performed during the first two weeks after calving to diagnose and treat any puerperal disease. Cows were then examined within 35-50 d postpartum to check for ovarian and uterine structures. Reproductive disorders such as incomplete uterine involution, endometritis, pyometra, and ovarian cysts, diagnosed either in a gynecological postpartum examination or at any subsequent time, were treated until resolved or until culling. The voluntary waiting period for the herd was 50 d. Cows more than 60 d in milk showing no estrus signs within the previous 21 d were examined weekly until specific estrus synchronization or until AI during a natural estrus (López-Gatius *et al.* 2008). Only cows free of detectable reproductive disorders were artificially inseminated using semen from sires of proven fertility. Estrus was confirmed by rectal palpation at AI in cows returning to estrus before pregnancy diagnosis. Pregnancy diagnosis was performed by ultrasonography 28 to 34 days post AI. Cows diagnosed as not pregnant were either returned to the reproductive program or registered for culling. All gynecological exams and pregnancy diagnoses were performed by the same operator.

5.2.3 Data collection and analysis

The following data were collected for each cow: lactation number, date of calving, calving assistance required, number of dead/live calves at birth or immediately after, occurrence of retained placenta (retention of the placental membranes for a period longer than 12 h after calving) and metritis (purulent uterine content/ vaginal discharge and fever over two weeks postpartum), date of pregnancy AI, occurrence of pregnancy loss or abortion over the subsequent lactation, and date of culling. Premature calvings (gestation period lower than 265 days, n=64), abortions (n=169) and calvings requiring cesarean section (n=30) where excluded from the study. The final data set corresponded to 12587 calvings in 4861 cows.

All statistics procedures were performed using the PASW Statistics 18 (SPSS Inc., Chicago, IL, USA) with 0.05 as the level of significance.

5.2.3.1 Incidence and immediate effects of twinning

The incidence of twin birth calvings during the 11-year period and the lactation number were compared by Student Newman-Keuls post-hoc tests. Differences between percentages of calvings requiring assistance, stillbirths, retained placenta and metritis for singleton and twin calvings were determined using a Chi squared test.

5.2.3.2 Effects of twinning on subsequent reproductive performance

Kaplan-Meier survival curves were constructed to detect possible differences in calving to conception intervals on Days 90, 120 and 300 postpartum between singleton and twin calvings. Cows that were culled or not pregnant on Days 90, 120 or 300 postpartum or at the end of data collection were censored. Differences in the median calving to conception interval between singleton and twin calvings were determined by Log-Rank tests.

Three consecutive Cox regression analyses were performed to determine the relative contributions of each variable to the probability of conception before 90, 120 and 300 days postpartum. In each analysis, conception before Day 90, 120 or 300 was considered as the dependent variable. Categorical independent variables for each model were: season of calving (cold: October-April vs. warm: May-September), calving assistance (unassisted calving vs. easy assistance/slight traction, and position correction/hard traction), number of calves at parturition (singleton calving vs. twin calving), stillbirths (absence vs. presence of at least one dead calf), placenta retention (absence vs. presence) and metritis (absence vs. presence). Year and lactation number were considered continuous independent variables.

The hazard ratios associated with each factor significantly affecting conception ($P<0.05$) were obtained from the Cox regression coefficients after modeling, which basically consisted of stepwise removal of non-significant variables from a full model. For categorical variables, one class of each variable was considered as the reference, and a hazard ratio significantly higher (or lower) than 1 for any other class of this variable was taken to indicate an increased (or reduced) risk of conception before Day 90, 120 or 300 postpartum when compared to the reference class. For continuous variables, a hazard ratio significantly higher (or lower) than 1 determines an increased (or reduced) risk of conception before Day 90, 120 or 300 postpartum with each 1 unit increase in the value of this factor.

Differences in the rate of pregnancy loss/abortions during the lactation that followed singleton versus twin calvings were determined using the Chi squared test.

5.2.3.3 Effects of twinning on the culling rate during the subsequent lactation

Kaplan-Meier survival curves were constructed to determine the possible differences in the calving to culling interval on 90, 120 and 300 days postpartum between singleton and twin calvings. Cows alive on Days 90, 120 or 300 postpartum or at the end of the data collection period were considered censored. Differences in the median calving to culling interval between singleton and twin calvings were determined by Log-Rank tests.

Three consecutive Cox regression analyses were performed to determine the relative contributions of each variable to the likelihood of culling before 90, 120 and 300 days postpartum. In each analysis, culling before Day 90, 120 or 300 was considered as the dependent variable. Categorical and continuous independent variables for each model and modeling to determine the hazards ratio associated with each factor significantly affecting culling were similar to those described for the effects of twinning on conception.

5.2.3.4 Effect of twinning on productive lifespan

The effect of twinning on productive lifespan was examined in complete reproductive records (cows calving for the first time after 1st April 2001, n=4298 of the original 4861 cows enrolled in the study). Cows were grouped as twinners (cows delivering twins at least once during their lifetime) or non-twinners. Cows delivering twins in their first or second lactation were recorded as primiparous or secundiparous twinners, respectively. Cows having born twins for the first time in their third or higher lactation were excluded from the analyses.

Through Kaplan-Meier survival analysis, differences were detected in productive lifespan (time from first calving to culling) between primiparous twinners and non-twinners. Cows still alive 1200 days after their first calving or at the end of the data collection period were considered censored. Differences in the median productive lifespan between primiparous twinners and non-twinners were determined by Log-Rank tests.

A second Kaplan-Meier survival analysis was performed to determine differences in productive lifespan between secundiparous twinners and non-twinners. Non-twinners culled before their second lactation were excluded from the analysis. Cows that were still alive 1500 days after their first calving or at the end of the data collection period were considered censored. Differences in median productive lifespan between secundiparous twinners and non-twinners having been through at least two lactations were determined by Log-Rank tests.

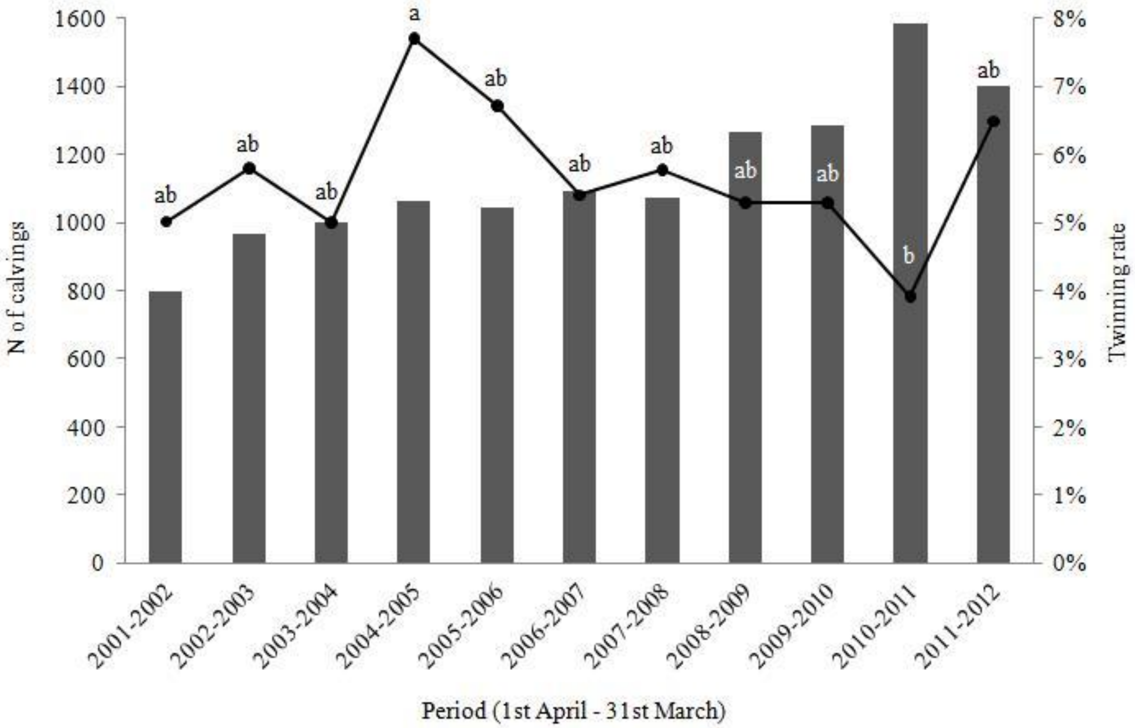
For the subset of cows with known first calving and culling dates (calving for the first time after 1st April 2001 and culled before 31st March 2012, n=2892 from the original 4861 cows enrolled in the study) differences in the mean productive lifespan length between primiparous twinners and non-twinners and between secundiparous twinners and non-twinners with at least two lactations were determined by unpaired Student t-tests.

5. 3 Results

The recorded twinning rate was 5.6% and ranged from 3.9% to 7.7% across the 11-year study period (Figure 5.1) and from 1.5% to 8.9% across lactations (Figure 5.2). Table 5.2 provides the percentages of calvings requiring assistance, stillbirths, retained placenta and metritis for singleton and twin calvings.

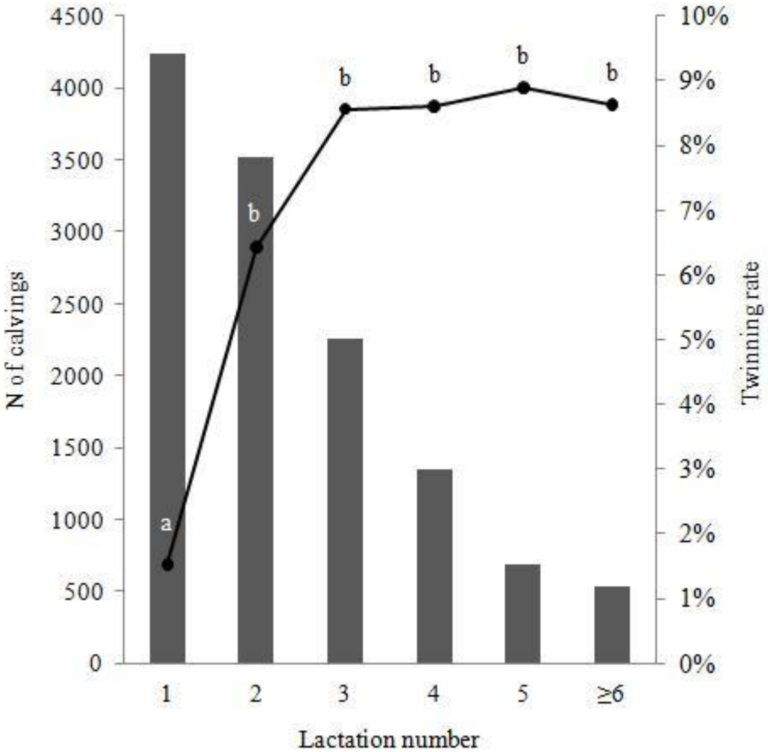
Conception rates before Days 90, 120 and 300 postpartum were 20.8%, 36.9% and 74.5%, and 34.2%, 51.8% and 85.0% for twin and singleton calvings, respectively. Kaplan-Meier survival curves for the interval calving to conception on 90, 120 and 300 days postpartum were different

Figure 5.1. Number of calvings (bars) recorded from 1st April 2001 to 31st March 2012 by period. The twinning rate (line) was similar for all periods except for 2004 -2005 and 2010-2011 (a,b; Student Newman-Keuls post-hoc test P<0.001).



(P<0.001) for twin and singleton calvings (Figure 5.3a). Thus, the median calving to conception interval (days after parturition at which 50% of the cows become pregnant, ± S.D.) was significantly longer for cows delivering twins (134 ± 4.5 d) than for cows delivering singletons (108 ± 0.8 d; P<0.001). Hazard ratios for the variables significantly affecting conception before Days 90, 120 and 300 postpartum are provided in Table 5.3. When we compared the 10401 and 546 cows that become pregnant after calving singletons and twins, respectively, the pregnancy loss or abortion rate during the subsequent lactation was higher following a twin calving than a singleton calving (13.7% and 10.3%, P=0.01, respectively).

Figure 5.2. Number of calvings (bars) recorded from 1st April 2001 to 31st March 2012 by lactation number. A greater twining rate (line) existed for cows in their second and higher lactation than for primiparous cows (a,b; Student Newman-Keuls post-hoc test P<0.001).



Culling rates before Day 90, 120 and 300 postpartum were 15.6%, 16.1% and 28.6%, and 7.6%, 8.7% and 15.9% for cows calving twins and singletons, respectively. Kaplan-Meier survival curves for the calving to culling interval at 90, 120 and 300 days postpartum differed (P<0.001) for twin and singleton deliveries (Figure 5.3b). The median calving to culling interval could not be determined for cows calving twins or singletons by Day 300 postpartum on 50% of the cows that were still alive. Hazard ratios for the variables significantly affecting culling before Days 90, 120 and 300 postpartum are provided in Table 5.4.

Table 5.2. Frequency of calvings requiring assistance, stillbirths and early postpartum disorders following singleton and twin calvings (n=12576).

	Singleton calving (n=11869)		Twin calving (n=707)	
Calvings requiring assistance n (%)	4518	(38.1)[a]	379	(53.6)[b]
Easy assistance/Slight traction n (%)	3695	(31.1)[a]	337	(47.7)[b]
Position correction/Hard traction n (%)	823	(6.9)	42	(5.9)
Calvings with at least 1 stillborn calf n (%)	716	(6.0)[a]	188	(26.6)[b]
1 dead singleton/twin calf n (%)	716	(6.0)[a]	120	(17.0)[b]
2 dead twin calves n (%)	-		68	(9.6)
Placental retention[c] n (%)	1333	(11.2)[a]	334	(47.2)[b]
Metritis[d] n (%)	1346	(11.3)[a]	142	(20.1)[b]

[a,b] Different letters indicate significant differences between values in columns (Chi-square, P<0.01)
[c] Retention of placental membranes for a period longer than 12 h after calving
[d] Purulent uterine content/vaginal discharge and fever over two weeks postpartum

Out of 4298 cows, 423 (9.8%) calved twins at least once during their productive life and 3875 were non-twinners. Sixty-five, 204 and 154 of the cows gave birth to twins the first time in their first, second and third or higher lactations, respectively. Kaplan-Meier survival curves for productive lifespan differed between primiparous twinners and non-twinners (P<0.001, figure 5.4a), and between secundiparous twinners and non-twinners having undergone at least two lactations (P=0.017, figure 5.4b). Mean productive lifespan (± S.D.) was significantly shorter for primiparous twinners (n=48, 602 ± 493 days) than non-twinners (n=2592; 899 ± 581 days; P<0.01), and shorter for secundiparous twinners (n=126, 914 ± 429 days) than non-twinners having had at least two lactations (n=1936, 1101 ± 522 days; P<0.01).

Figure 5.3. Kaplan-Meier survival curves for conception (a) and culling (b) before Days 90 (1), 120 (2) and 300 (3) after singleton (continuous line, n=11869) or twin birth calving (discontinuous line; n=707).

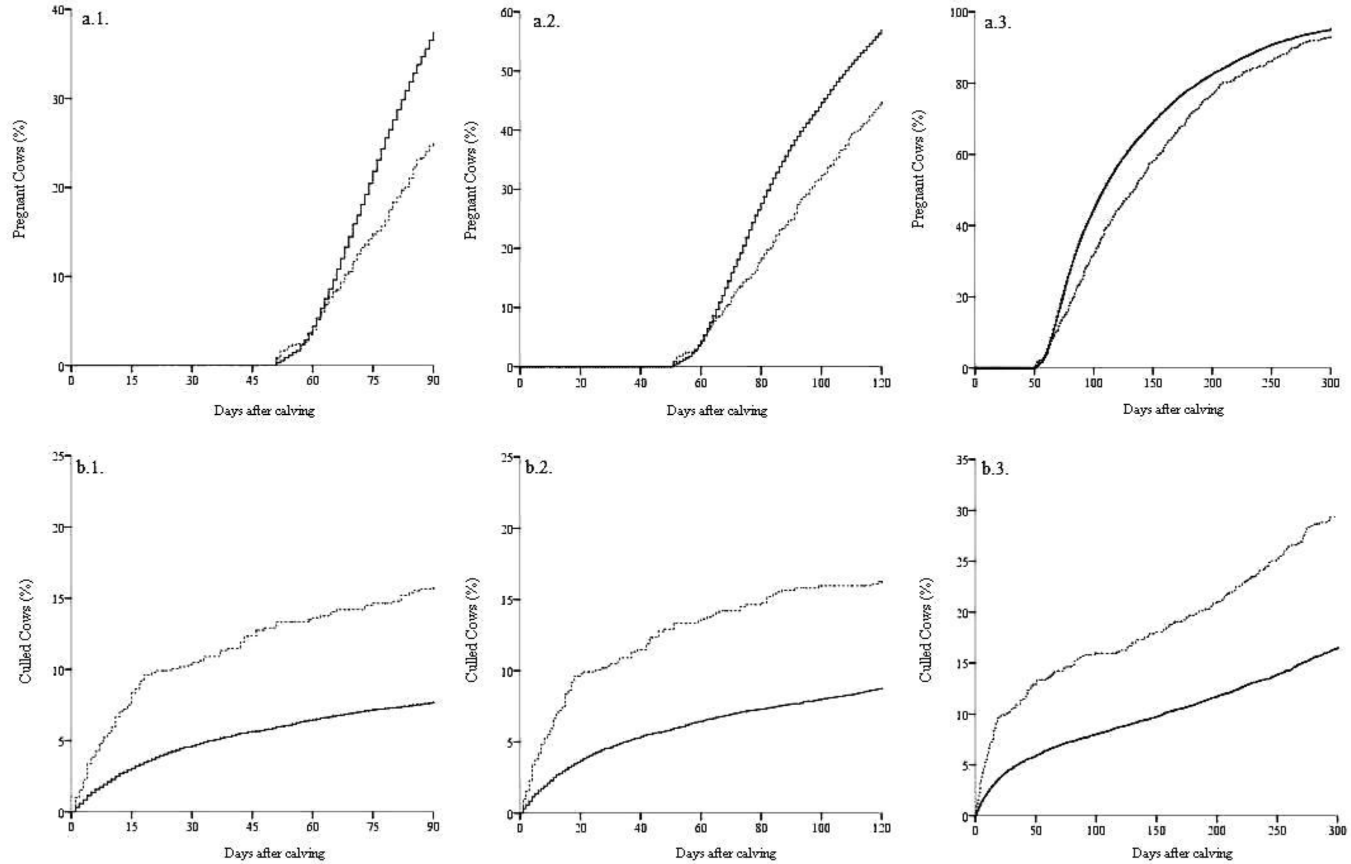


Table 5.3. Conception rates before Days 90, 120 and 300 postpartum and hazard ratios (HR) for the factors included in the COX regression analyses for conception before Days 90, 120, and 300 postpartum (n=12576)[*].

		Conception before					
		Day 90 postpartum[a]		Day 120 postpartum[b]		Day 300 postpartum[c]	
Pregnant cows n (%)		4215 (33.5%)		6414 (51.0%)		10619 (84.4%)	
Factor and classes	n	HR	p-value	HR	p-value	HR	p-value
Season of calving							
Cold (October-April)	7518	reference	-	reference	-	reference	-
Warm (May-September)	5058	0.863	<0.001	0.904	<0.001	-	NS
Calving Assistance							
Unassisted calving	7679	reference	-	reference	-	reference	-
Easy assistance/Slight traction	4032	0.902	0.004	0.905	0.001	0.891	<0.001
Position correction/Hard traction	865	0.753	<0.001	0.785	<0.001	0.831	<0.001
Singleton /Twin birth calving							
Singleton birth calving	11869	reference	-	reference	-	reference	-
Twin birth calving	707	0.764	0.002	0.846	0.011	-	NS
Stillbirths							
Absence	11672	reference	-	reference	-	reference	-
Presence (1 or more)	904	-	NS	-	NS	-	NS
Placenta retention							
Absence	10909	reference	-	reference	-	reference	-
Presence	1667	0.746	<0.001	0.748	<0.001	0.821	<0.001
Metritis							
Absence	11088	reference	-	reference	-	reference	-
Presence	1488	0.764	<0.001	0.794	<0.001	0.83	<0.001
Lactation number (continuous)	12576	0.924	<0.001	0.919	<0.001	0.926	<0.001
Year (continuous)	12576	1.068	<0.001	1.056	<0.001	1.047	<0.001

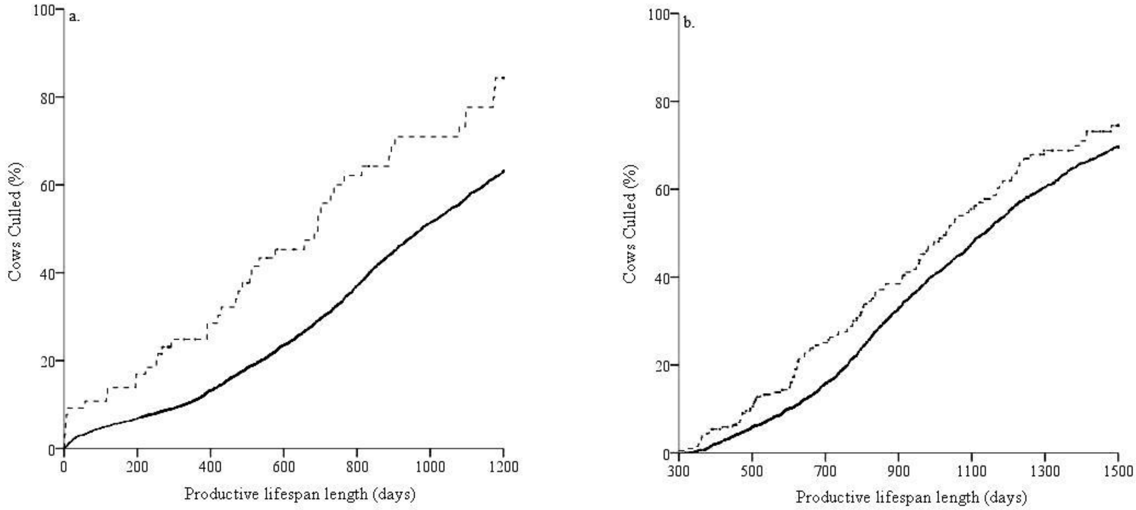
[*] For categorical variables, one class of each variable was considered as the reference, and a hazard ratio significantly higher (or lower) than 1 for any other class of this variable was taken to indicate an increased (or reduced) risk of conception before Day 90, 120 or 300 postpartum when compared to the reference class. For continuous variables, a hazard ratio significantly higher (or lower) than 1 indicates an increased (or reduced) risk of conception before Day 90, 120 or 300 postpartum with each 1 unit increase in the value of this factor.
NS=Not significant
[a] Likelihood ratio test= 321.65; 8 d.f.; P<0.001
[b] Likelihood ratio test= 377.87; 8 d.f.; P<0.001
[c] Likelihood ratio test= 394.42; 6 d.f.; P<0.001

Table 5.4. Culling rates before Day 90, 120 and 300 postpartum and hazard ratios (HR) for the factors included in the COX regression analyses for culling before Days 90, 120, and 300 postpartum (n=12576)*.

		Culling before					
		Day 90 postpartum[a]		Day 120 postpartum[b]		Day 300 postpartum[c]	
Culled cows n (%)		1014 (8.1%)		1142 (9.1%)		2090 (16.6%)	
Factor and classes	n	HR	p-value	HR	p-value	HR	p-value
Season of calving							
Cold (October-March)	7518	reference	-	reference	-	reference	-
Warm (March-September)	5058	-	NS	1.156	0.015	-	NS
Calving Assistance							
Unassisted calving	7679	reference	-	reference	-	reference	-
Easy assistance/Slight traction	4032	1.171	0.021	1.163	0.019	1.1	0.048
Position correction/Hard traction	865	-	NS	-	NS	1.331	0.001
Singleton /Twin calving							
Singleton calving	11869	reference	-	reference	-	reference	-
Twin calving	707	1.321	0.012	1.261	0.031	1.41	0.001
Stillbirth							
Absence	11672	reference	-	reference	-	reference	-
Presence (1 or more)	904	1.557	<0.001	1.435	0.001	1.204	0.025
Placenta retention							
Absence	10909	reference	-	reference	-	reference	-
Presence	1667	1.396	<0.001	1.346	<0.001	1.179	0.006
Metritis							
Absence	11088	reference	-	reference	-	reference	-
Presence	1488	-	NS	-	NS	-	NS
Lactation number (continuous)	12576	1.425	<0.001	1.407	<0.001	1.414	<0.001
Year (continuous)	12576	-	NS	-	NS	-	NS

* For categorical variables, one class of each variable was considered as the reference, and a hazard ratio significantly higher (or lower) than 1 for any other class of this variable was taken to indicate an increased (or reduced) risk of culling before Days 90, 120 or 300 postpartum when compared to the reference class. For continuous variables, a hazard ratio significantly higher (or lower) than 1 indicates an increased (or reduced) risk of culling before Day 90, 120 or 300 postpartum with each 1 unit increase in the value of this factor.
NS=Not significant
[a] Likelihood ratio test= 475.40; 6 d.f.; P<0.001
[b] Likelihood ratio test= 471.88; 7 d.f.; P<0.001
[c] Likelihood ratio test= 820.85; 6 d.f.; P<0.001

Figure 5.4. Kaplan-Meier survival curves for productive lifespan length (time from first calving to culling) for cows calving twins (discontinuous line) or singletons (continuous line) at first (a; n=65 primiparous twinners and 3875 non-twinner cows) and second lactation (b; n=204 secundiparous twinners and 2948 non-twinner cows).



5.4 Discussion

The mean twinning rate for the 12587 calving events in the herd's reproductive records was 5.6%, slightly higher than the rates of 4.6% and 4.2% reported recently in North-American dairy herds (Bicalho *et al.* 2007; Silva del Rio *et al.* 2007). Over the 11 year-period examined here (1st April 2001- 31st March 2012), the twinning rate remained stable, contrary to previous reports that have described increases of 1.4% to 2.4% from 1983 to 1993 (Kinsel *et al.* 1998), and of 3.4% to 4.8% from 1996 to 2004 (Kinsel *et al.* 1998). A change in the steadily rising-tendency in the incidence of twin pregnancies in dairy cattle over the last decades is, however, unlikely. In effect, the actual percentage of cows carrying twins on the day of pregnancy diagnosis (28-34 days postAI) for the herd examined here was as high as 12.6% in 2011 (Andreu-Vázquez *et al.* 2012b). The difference between the incidence of twin pregnancies and the twinning rate can be attributed to an increased risk of pregnancy loss in cows carrying twins, reported to be 3- to 9 times greater than for singleton pregnancies (López-Gatius *et al.* 2009; López-Gatius and Garcia-Ispierto 2010). Also, culling policies aimed at eliminating twin pregnant cows (Day *et al.* 1995) would explain the substantially lower number of twin calvings and the stable twinning rates over time. More importantly, complete reproductive records for 4298 cows for the 11 year period revealed that 9.8% of the cows in the herd gave birth to twins at least once during their life. This figure, considerably higher than the mean twinning rate for all calving events, provides a different perspective to the problem of twinning in dairy herds, which might have been underestimated because of a high frequency of calvings in primiparous cows (which carry a lower risk of twinning) and animals eliminated before their second calving.

Our results besides confirming the higher incidence of extensively reported postpartum disorders, impaired fertility and a higher risk of culling over the subsequent lactation for cows calving twins (Chapin and Van Vleck 1980; Nielen *et al.* 1989; Gregory *et al.* 1990; Eddy *et al.* 1991; Echternkamp and Gregory 1999a, 1999b; Bicalho *et al.* 2007), also stress that the detrimental effects of a twin calving extend beyond into the following lactations and reduce the mean productive lifespan of the cow. Twin birthing has a dramatic impact on the profitability of primiparous cows although its incidence is low (1.5% this study, 1.1% to 1.3 previous studies, Bicalho *et al.* 2007; Silva del Rio *et al.* 2007; Ghavi Hossein-Zadeh *et al.* 2008). The mean productive lifespan of the primiparous twinners was almost 300 days shorter than the non-twinners. Impaired reproductive performance (i.e. a high number of unobserved heats, and many services needed to achieve pregnancy) at first lactation has been described as a factor that negatively affects the length of a cow's productive life(Pasman *et al.* 1995). Interestingly, over 80% of the primiparous twinners were culled before 1200 days after their first parturition, in contrast to a culling rate of 60% for non-twinners. Kaplan-Meier curves revealed significant differences between culling-patterns over time for the two groups and these differences were not merely a consequence of a higher elimination rate during the subsequent lactation. In fact, a pronounced slope in the curve for the twinners may be noted at around Days 400-550, and Days 650-800 after first calving, strongly suggesting long-term negative effects of twinning. Similarly, the mean productive lifespan of the secundiparous twinners (with an overall incidence of 6.4% herein, and ranging from 4.2% to 5.5% in previous studies, Silva del Rio *et al.* 2007; Ghavi Hossein-Zadeh *et al.* 2008) was almost 200 days shorter than for non-twinner cows having undergone at least two lactations. The highest incidence of twinning corresponded to multiparous cows (from 8.6 to 8.9 for cows in their third or higher lactation) in agreement with previous studies, Bicalho *et al.* 2007; Silva del Rio *et al.* 2007; Ghavi Hossein-Zadeh *et al.* 2008). Delivering twins in a third or higher parturition would have scarce long-term impacts, as in current dairy systems the mean lactation number is as low as 2.4 for lactating cows (Garcia-Ispierto *et al.* 2007) and 3.4 at culling (López-Gatius *et al.* 2006). Cows giving birth to twins have been reported to reduce herd incomes (Eddy *et al.* 1991; Beerepoot *et al.* 1992), and to be 3 times more often classified as “loser cows” (Thompsen *et al.* 2007), accompanied by a higher risk of being culled (Thompsen *et al.* 2007b). To the best of our knowledge, this is the first study that determines the impact of twinning on productive lifespan. A reduced productive lifespan, meaning lower total milk production per cow and increased culling rates and costs associated with breeding heifers for replacement, may seriously affect the profitability of a herd in today’s intensive dairy production systems. In this context, transforming a twin pregnancy into a singleton pregnancy thought embryo reduction techniques at the moment of pregnancy diagnosis (López-Gatius 2005; Andreu-Vázquez *et al.* 2011, 2012a) could be a profitable strategy to decrease the twinning rate and its detrimental effects.

When we screened for early fertility (i.e. conception before Day 90 postpartum, López-Gatius *et al.* 2006), with a mean incidence of 33.5% and increasing yearly by 6.8% over the study period, lactation number emerged as a negative factor in agreement with previous studies (Grönh and Rajala 2000; López-Gatius *et al.* 2006; Garcia-Ispierto *et al.* 2007). More importantly, twinning reduces the chances of conception before Day 90 by a factor of 0.76 (i.e. a 24% lower risk of pregnancy in cows calving twins compared to cows calving singletons). Similar conception rates before Day 90 postpartum for cows calving twins and singletons were reported in a previous study conducted in our geographical area (López-Gatius *et al.* 2006). Differences between the results of these studies can be explained by the different statistical approaches used to determine the factors affecting early fertility. Whereas, in the present study, animals culled before Day 90 were censored, logistic regression procedures excluded culled animals from the analysis. Accordingly, in our earlier study (López-Gatius *et al.* 2006), conception rates could have been overestimated, especially for the twinner cows. The negative effect of twinning on conception was still significant, yet less pronounced 120 days after calving but, surprisingly, was no longer detectable when assessing factors affecting fertility on Day 300 postpartum. This contrasts with a reported 22% decreased hazard ratio of conception before Day 300 for cows calving twins (Bicalho *et al.* 2007). In the latter study, median calving to conception intervals were 175 days and 220 days for cows calving singletons and live twins, respectively, whereas herein both median calving to conception intervals were considerably narrower (108 and 134 days for singleton and twin calvings, respectively). The hazards ratios of culling before Day 300 for cows calving twins compared to cows calving singletons were very similar in both studies (1.41 herein and 1.42 in the study by Bicalho *et al*., 2007). Differences between studies in the number of days open could consequently be attributable to the particular herd management policies. An intensive postpartum health program and the use of synchronization protocols could have improved reproductive performance and reduced the number of days open in both twinner and non-twinner cows. Other factors found to negatively affect early fertility were season of calving (with lower conception rates for cows calving during the warm season), calving assistance required, placenta retention and metritis, reinforcing previous studies (López-Gatius *et al.* 2006; Bicalho *et al.* 2007; Garcia-Ispierto *et al.* 2007). On the other hand, we found no difference in conception rates for cows delivering still or live calves, in contrast to previous studies describing longer calving to conception intervals for cows delivering a dead singleton or at least one dead twin compared to cows delivering live singletons (Bicalho *et al.* 2008) or twins (Bicalho *et al.* 2007). Nonetheless, the presence of at least one dead calf was found to dramatically affect the risk of culling at 90, 120 and 300 days postpartum, with hazards ratios of culling 56%, 44% and 20% greater than for cows delivering live calves, respectively. This higher elimination pressure towards cows delivering still calves would have reduced the number of animals inseminated with a potential risk of failing to conceive.

Culling rates did not vary throughout the 11-year study period, but increased with lactation number. More importantly, cows calving twins as well as cows requiring calving assistance or delivering stillborn calves were at a higher risk of being culled not only in the first 90 days after parturition but also within 120 and 300 days postpartum. As discussed above, subsequent impaired fertility for these groups of cows could explain the higher culling rate. Although the elimination of the cows is mainly based on milk production and heavily influenced by the farmer, it is extensively assumed that pregnant cows are more likely to remain in the herd. Interestingly, the effect of twinning on culling seems to be attenuated from Day 90 to Day 120 postpartum, as the hazard ratio for this factor decreased from 1.32 to 1.26, but dramatically increased later when the risk of culling was 1.41 times higher before day 300 postpartum for cows calving twins than for cows calving singletons. This latter peak may be related to the finding that cows delivering twins suffered pregnancy loss or abortion more often compared to cows calving singletons. No previous report has described a cause-effect relationship between previous twinning and abortion. However, a retained placenta, whose incidence among cows calving twins was 47.2% here and ranged from 45% to 63.5% in previous studies (López-Gatius *et al.* 2006; Murugavel *et al.* 2007), has been described to increase the risk of early pregnancy loss by a factor of 1.8 (López-Gatius *et al.* 1996). Moreover, abortion was the main reason for elimination in this herd where one out of six cows is culled due to abortion (personal communication). Thus, it can be hypothesized that some of the cows calving twins that managed to conceive before Day 120 might have lost their pregnancy later on and, therefore, might have been sold or sent to slaughter when diagnosed as empty in a pregnancy check or later in lactation.

In conclusion, cows delivering twins, besides being less likely to conceive and more likely to be culled in the subsequent lactation, also carry a higher risk of abortion and show a reduced mean productive lifespan. From an economic perspective, the practice of embryo reduction on twin pregnancy diagnosis (López-Gatius 2005; Andreu-Vázquez *et al.* 2011, 2012a) might be profitable to prevent cows from delivering twins and thus improve their productive lifespan length.

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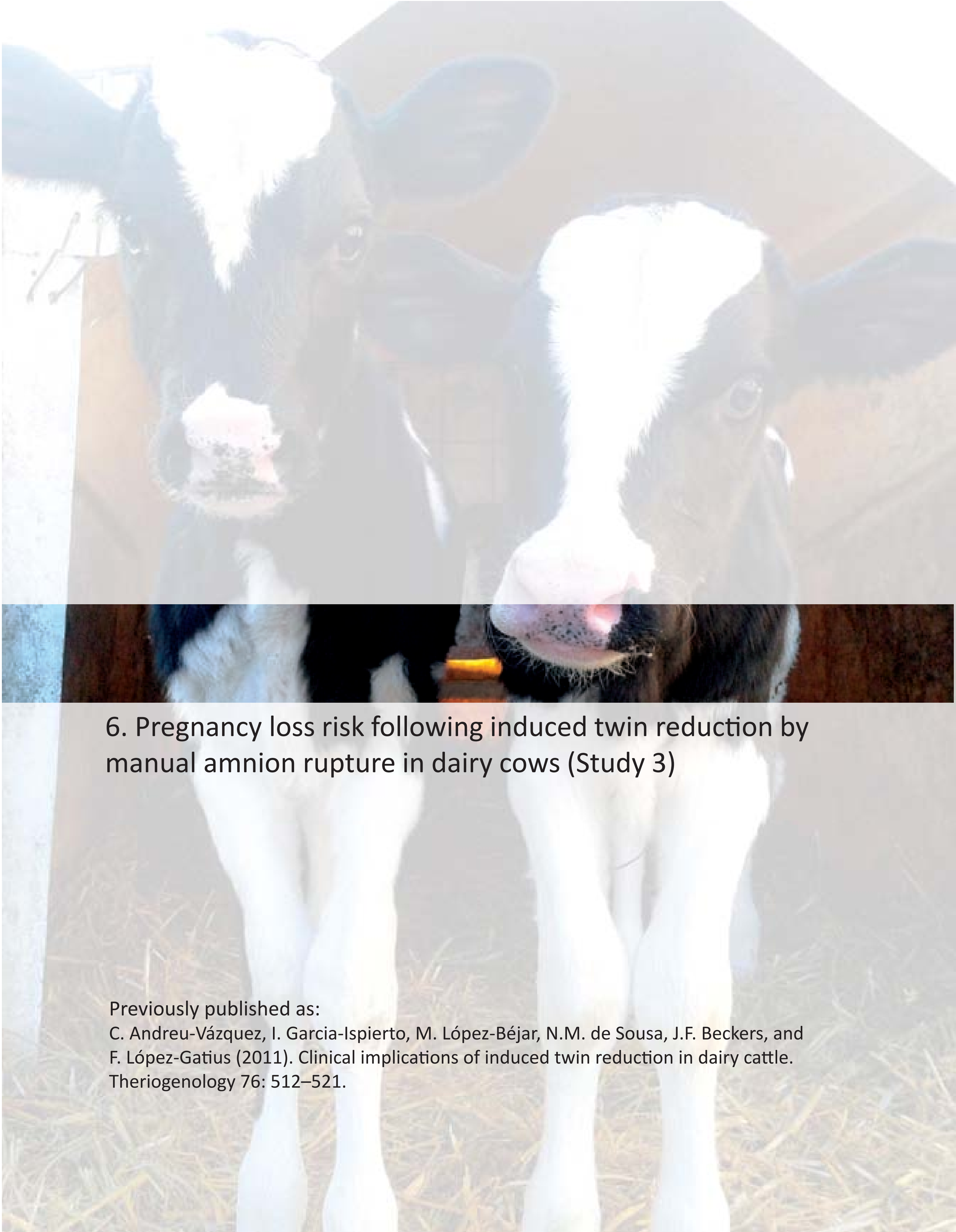

6. Pregnancy loss risk following induced twin reduction by manual amnion rupture in dairy cows (Study 3)

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Abstract

Embryo reduction may prevent the negative effects of twinning in dairy cattle; however, the technique may carry an additional risk of pregnancy loss. The aim of this study was to evaluate the effect on pregnancy maintenance of embryo reduction by manual amnion rupture in unilateral and bilateral twin pregnant cows. A secondary objective was to examine the dynamics of endocrine factors following the treatment. On Day 35–41 of gestation 55 cows bearing two live twin embryos (28 bilateral, 27 unilateral) were randomly assigned to a twin reduction group (n = 27; cows fitted with a progesterone releasing intra-vaginal device for 21 days after manual amnion rupture) or control group (n = 28; untreated cows). Pregnancy loss before Day 90 was recorded in nine control and eleven twin reduction cows (32.1% vs. 40.7%, respectively, p = 0.508). Logistic regression models indicated that laterality was the only variable significantly affecting pregnancy loss. The pregnancy loss risk was 8.7 times higher for unilateral than for bilateral twin pregnancies (59.3% vs. 14.3%, respectively, P = 0.001) yet was similar in the unilateral control and unilateral twin reduction cows (62.3% vs. 53.8%, respectively, P = 0.581). In contrast, four of 14 cows with bilateral twin pregnancies undergoing twin reduction lost their pregnancies while no losses were recorded in control cows with bilateral pregnancies (P = 0.049). A rise in plasma progesterone concentration was detected on the day following treatment in the twin reduction group and concentrations remained high within the first week of treatment. Plasma pregnancy-associated glycoprotein-1 (PAG-1) concentrations fell between Day 35–41 and Day 42–48, regardless of treatment. Our findings indicate that embryo reduction by manual amnion rupture did not carry an additional risk of pregnancy loss for unilateral twin pregnancies, whereas it increased the risk of pregnancy failure in bilateral twin pregnancies. However, benefits of preventing cows from delivering twins might also be considered when assessing the success of embryo reduction in bilateral twin pregnancies.

Keywords

Twin reduction; Amnion rupture; Progesterone; Pregnancy-associated glycoprotein; Dairy cattle

6.1 Introduction

The cow is a monovular species usually producing one offspring per pregnancy. However, over the past two decades the twinning rate in dairy cattle has increased alongside milk production (Nielen *et al.* 1989; Kinsel *et al.* 1998; Bicalho *et al.* 2007) and estimates run at 9%, with rates of 0.3% to 12% reported among herds (Silva del Rio *et al.* 2007). In an epidemiological study of 52,362 lactations, the rising twinning rate was linked to increased milk production (Kinsel *et al.* 1998). In high producers, the rate of double ovulation may reach 20% (Fricke and Wiltbank 1999) and even exceed 25% in cows in their third or higher lactation period (López-Gatius *et al.* 2005). In a further analysis of data related to about 1.3 million births of 37,174 sires of cows, it was shown that sires born after 1990 had a higher incidence of twins than sires born before 1980 (Johanson *et al.* 2001). Besides genetic progress, improvements in nutrition and management practices related to high milk production have probably diminished the risk of twin pregnancy loss and thus raised the twinning rate. It is therefore foreseeable that in the years to come, the twinning rate will continue to increase along with milk production.

Twin pregnancies in dairy cattle are undesirable as they pose serious management problems and reduce the profitability of the herd (Eddy *et al.* 1991; Beerepoot *et al.* 1992). Cows carrying twins have a risk of pregnancy loss 3.1– 6.9 times greater than those carrying singletons (López-Gatius *et al.* 2002, 2009). Insufficient space in the uterus for both growing fetuses seems to be the major cause of pregnancy failure (Vanroose *et al.* 2000), which may occur throughout the gestation period (Day *et al.* 1995). A higher risk of abortion, dystocia, stillbirth, calf mortality and freemartinism are further negative aspects of twin pregnancies (Nielen *et al.* 1989; Gregory *et al.*

1990, 1996). Further, twin births are responsible for most periparturient diseases, for example, retention of the placenta. This disorder has been related to longer and more expensive postpartum management and to a longer calving to conception interval (Nielen *et al.* 1989; Gregory *et al.* 1996). In fact, in our geographical area of study, 45% (Murugavel *et al.* 2007) to 63.5% (López-Gatius *et al.* 2006) of cows carrying twins suffer placental retention and the likelihood of early fetal loss was found to be 1.8 times greater in cows suffering this disorder compared to cows without a retained placenta in a study on 3,022 gestations (López-Gatius el al. 1996). In addition, cows with twin births were reported to produce less milk and were 1.42 times more likely to die or be culled than cows delivering singletons (Bicalho *et al.* 2007).

Transforming a multiple pregnancy into a singleton pregnancy by inducing embryo reduction should prevent the negative effects of twining. Embryo reduction methods have been successfully used to treat potentially dangerous multiple pregnancies in women (Ibérico *et al.* 2000) and mares (Macpherson and Reimer 2000). While sophisticated techniques are used in human medicine, manual crushing of one embryo vesicle has become a widespread strategy among equine medicine practitioners to avoid twin pregnancies in the mare, with acceptable results (Frazer 2003). As far as we are aware, only one study (López-Gatius 2005) has assessed the practice of embryo reduction to address the problem of twinning in high-producing dairy cows. The results of this study indicated that manual rupture of the amniotic vesicle of a twin embryo without further treatment resulted in pregnancy loss in 100% of cases (11/11), whereas the addition of progesterone supplementation to this procedure decreased the changes of pregnancy loss; four of 11 animals maintained their pregnancies. Only cows carrying unilateral twin pregnancies were included in the above mentioned study. The present study was designed to evaluate in a larger population of unilateral and bilateral twin pregnancies the effect of embryo reduction on pregnancy maintenance. An additional objective was to assess the dynamics of the endocrine factors progesterone, pregnancy associated glycoprotein (PAG-1) and prolactin following treatment.

6.2 Materials and methods

6.2.1 Animal management

This study was performed on a commercial dairy herd of 1,074 mature cows in northeastern Spain from April 2009 to April 2010. This herd was selected because of its high twinning rate (17.1%) recorded over the preceding 12 mo period. Mean annual milk production was 10,749 kg per cow. Fat and protein content of the milk were 3.38% and 3.32%, respectively. The cows were milked three times daily, kept in open stalls and fed complete rations. Feeds consisted of cotton-seed hulls, barley, corn, soybean and bran, and roughage, primarily corn, barley and alfalfa silages and alfalfa hay. Rations were in line with NRC recommendations (National Research Council 2001). All cows were tested free of tuberculosis and brucellosis and artificially inseminated using semen from sires of proven fertility. The mean annual culling rate for the study period was 34%.

The herd was maintained on a weekly reproductive program. Normal uterine involution and the morphology of ovarian structures were checked by palpation per rectum 30–36 d postpartum. Any postpartum reproductive disorders diagnosed were treated until resolved or until culling. The voluntary waiting period from calving to first insemination was 60 d. Only cows devoid of detectable reproductive disorders were inseminated.

6.2.2 Pregnancy diagnosis number and viability of embryos and number of corpora lutea

Pregnancy was diagnosed by transrectal ultrasonography from Days 28 to 34 post-insemination using a portable B-mode ultrasound scanner (Scanner SonoSite 180 PLUS Vet equipped with a 5–10 MHz transducer, SonoSite, Bothell, WA, USA). Scanning was performed along the dorso/lateral surface of each uterine horn. The presence of twins was established through the observation of two embryos in different positions within one uterine horn on two screen scans or two embryos simultaneously present on the screen (unilateral twin pregnancy), or one embryo in each uterine horn (bilateral twin pregnancy). The viability of the embryos was determined by detecting their heartbeat. Only cows carrying two live embryos were included in the study and given a GnRH dose (100 µg i.m.; Cystoreline, CEVA Salud Animal, Barcelona, Spain). Administration of GnRH on the day of pregnancy diagnosis is a routine treatment in our reproductive management protocol as it reduces the risk of early pregnancy loss in cows with twins (Bech-Sàbat *et al.* 2009). Each ovary was scanned in several planes by moving the transducer along its surface to identify luteal structures, and the number and location of corpora lutea recorded.

6.2.3 Twin reduction treatment, pregnancy controls and blood sample collection

Cows maintaining both embryos live in the following week (Day 35–41 post-insemination) were randomly assigned to the control or twin reduction group. In the control group no manipulations were done. In the twin reduction group, the amniotic vesicle of a twin embryo was pressed with the thumb to cause its rupture. Amnion rupture was always conducted by the same operator. The time lapsed between vesicle manipulation and the rupture was not longer than five seconds. Embryo death was assessed by the disappearance of the heartbeat detected by ultrasonography. Immediately after amnion rupture, cows were fitted with a progesterone releasing intra-vaginal device (PRID, containing 1.55 g of progesterone; CEVA Salud Animal) for 21 d. Cows showed no signs of discomfort throughout the procedure. Control cows did not receive PRID.

Subsequent weekly pregnancy follow-ups were performed by ultrasound until Day 56–62 post-insemination or until fetal loss. We recorded the number and viability of embryos and the number of corpora lutea. Pregnancy was further confirmed by palpation per rectum on Days 90–96 and 150–156 post-insemination. Pregnancy loss was recorded when a subsequent examination to the first pregnancy check proved negative. The date of the negative diagnosis was registered as the day of pregnancy loss. The day of calving and the number of calves delivered were recorded in cows not suffering pregnancy loss.

Blood samples were withdrawn from the coccygeal vein of all animals on the day of twin reduction (Day 0), on Days 1 and 3 after treatment, and at each weekly pregnancy follow-up session on Days 7, 14 and 21 after treatment. The samples were collected into heparinized vacuum tubes (BD VacutainerTM, Becton, Dickenson and Company, Plymouth, UK), centrifuged (10 min at 1 600 g) within 30 min of collection, and plasma stored at -20 °C until assayed.

6.2.4 Hormone assays

Prolactin concentrations were determined in plasma using a double antibody radioimmunoassay procedure (Ayad *et al.* 2007) with some modifications. Bovine prolactin (NIHB5bPRL, NIH, Bethesda, MD, USA) was diluted in assay buffer (Tris–BSA, pH 8) and was used as standard (0.8–200 ng/mL) and tracer. Iodination (Na-I125, Perkin Elmer, Belgium) was carried out according to the lactoperoxidase method (Greenwood *et al.* 1963). Briefly, 50 µL of each plasma sample and/or 0.1 mL of standard preparation were diluted in Tris-BSA buffer. Next, 0.1 mL of radiolabeled prolactin (26,000 cpm) and 0.1 mL of the diluted antiserum (R#144; 1:100,000) were added to the tubes followed by overnight incubation at room temperature (20–23 °C). Bound and free prolactin were separated after addition of the double antibody precipitation system, as previously described (Ayad *et al.* 2007). The minimum detection limit (MDL) of the method was 0.63 ng/mL. Intra- and inter-assay coefficients of variation were 9.8% (9.48 ± 0.93 ng/mL) and 10.6% (8.52 ± 0.9 ng/mL), respectively.

Progesterone concentrations were determined in plasma using a direct method (without extraction) as previously described in detail (López-Gatius *et al.* 2007). The MDL of the P4-RIA technique used was 0.15 ng/mL and intra-assay and inter-assay coefficients of variation were 13% (2.6 ± 0.4 ng/mL) and 19% (2.7 ± 0.5 ng/mL), respectively.

Pregnancy-associated glycoprotein-1 (PAG-1) concentrations were determined in plasma using a double antibody radioimmunoassay procedure (RIA-706, López-Gatius *et al.* 2007). Rabbit polyclonal antiserum AS#706 was raised against caprine PAG $_{55\ kDa\ +\ 62\ kDa}$ and prepared according to the method of Vaitukaitis *et al.* (1971). The MDL for the RIA procedure was 0.26 ng/mL. Intra-assay and inter-assay coefficients of variation were 8.8% (4.99 ± 0.44 ng/mL) and 12.8% (2.47 ± 0.31 ng/mL), respectively.

6.2.5 Data collection and statistical analysis

The following data were recorded for each animal: treatment (control vs. twin reduction); lactation number; date of conception; interval from calving to conception; semen-providing bull (4 bulls); milk production at pregnancy diagnosis; uterine laterality of twin embryos (unilateral vs. bilateral); conceptus age and number of corpora lutea on the day of treatment; plasma concentrations of progesterone, PAG-1 and prolactin on the day of treatment (Day 0) and on Days 1, 3, 7, 14 and 21 or until pregnancy loss; and the date of pregnancy loss or date of calving and its outcome.

All statistics procedures were performed using the SPSS package version 15.0 (SPSS Inc., Chicago, IL, USA) with 0.05 as the level of significance.

6.2.5.1 Pregnancy loss

The time during which pregnancy failure was deemed to have resulted from inducing embryo reduction was 55 days (Day 90 of gestation). Differences between pregnancy loss rate before Day 90 for control and twin reduction cows were determined by Chi square test. Logistic regression procedures were used to evaluate the relative contribution of each potentially affecting variable to the probability of pregnancy loss before day 90. Binary logistic regression was performed according to the method of Hosmer and Lemeshow (1989) considering pregnancy loss before Day 90 as the dependent variable, and treatment, lactation number, calving to conception interval, semen-providing bull, milk production, uterine laterality of twin embryos, conceptus age, number of corpora lutea on the day of treatment and concentrations of progesterone, PAG-1 and prolactin on the day of treatment as independent variables.

For unilateral and bilateral pregnancies, differences on the rates of pregnancy loss between control and twin reduction groups were compared by Chi square or Fisher's Exact test. Kaplan-Meier survival analysis was used to compare the mean time of pregnancy loss for each group of study. Cows that were culled before Day 90 of pregnancy were considered as censored cases.

6.2.5.2 Endocrine parameters

General lineal models (GLM) for repeated measures were built to detect the effect of each variable treatment, laterality of gestation, number of CL, age of conceptus, calving to conception interval and milk production) on PAG-1, progesterone and prolactin concentrations during the first week and four weeks after treatment.

6.3 Results

During the study period a total of 2,756 inseminations were performed in the herd. The conception rate and the twinning rate for this period were 33% and 6.6%, respectively. Seventeen of the 72 twin pregnancies diagnosed at the start of the experiment (Days 28–34) were excluded from the study because of spontaneous embryo reduction (10 pregnancies) or pregnancy loss (7 pregnancies). The remaining 55 twin pregnancies were assigned randomly into a control group (n = 28) and a twin reduction group (n = 27).

6.3.1 Pregnancy loss

Pregnancy loss before Day 90 was recorded in nine control and eleven twin reduction cows (32.1% vs. 40.7%, respectively, p = 0.508). Logistic regression analysis indicated no significant effects on pregnancy loss before Day 90 of treatment, parity, calving to conception interval, semen-providing bull, progesterone, prolactin and pregnancy-associated glycoprotein-1 plasma concentrations, conceptus age and the number of corpora lutea on the day of treatment. Laterality was the only factor significantly affecting pregnancy loss (Table 6.1). Based on the odds ratio, cows with unilateral twin gestation were 8.7 times more likely to suffer pregnancy loss than cows with bilateral twin gestation.

Figure 6.1 shows Kaplan-Meier survival curves until Day 90 of gestation for bilateral and unilateral twin pregnancies stratified by treatment. The pregnancy loss until Day 90 curves for the control and twin reduction treatments were similar for the unilateral twin pregnancies, while survival curves differed between treatments for the bilateral twin gestations.

Table 6.1. Odds ratios for the factor included in the final binary logistic regression model for pregnancy loss before Day 90.

Factor	Class	Fetal loss rate before Day 90 (%)		Odds Ratio*	95% CI	*P*
Uterine laterality of gestation	Bilateral	4/28	(14.3)	reference		
	Unilateral	16/27	(59.3)	8.73	2.63 - 32.27	0.001

Likelihood ratio test; 12.638; 1 d.f.; p<0.001. Nagelkerke R^2 =0.281. Overall success ratio; 72.7%.

*For class variables, one class is considered as the reference, and an odds ratio signifficantly higher (or lower) than 1 for any other class of the variable is indicative of an increased (or reduced) risk of pregnancy loss when compared to the reference class.

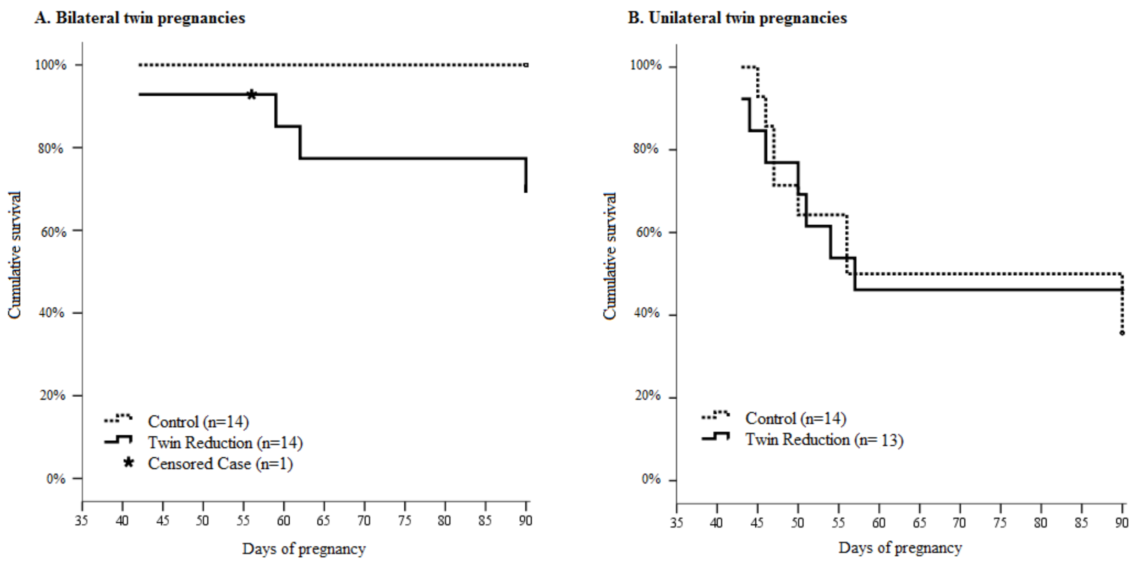

Figure 6.1. Kaplan-Meier survival curves for bilateral (A; n=28) and unilateral (B; n=27) twin pregnancies showing the percentages of control and twin reduction cows maintaining pregnancy (Y-axis) along time until day 90 of gestation (X-axis).

Pregnancy loss rates and mean estimated times of pregnancy loss for each treatment are shown in Table 6.2. None of the 14 control bilateral twin pregnancies suffered pregnancy loss before Day 90 whereas nine of the 14 control unilateral pregnancies resulted in pregnancy loss (0% vs. 64.3%, respectively, P = 0.001). Although not significant, the pregnancy loss rate for the unilateral pregnancies was higher in the control than twin reduction group (64.3% vs. 53.8%, respectively, P = 0.581).

6.3.2 Endocrine parameters during the first week of treatment (from Day 35–41 to Day 42–48).

GLM repeated measures revealed no effect on progesterone concentrations of uterine laterality of twin embryos, milk production on pregnancy diagnosis and number of corpora lutea on the day of treatment. Cows in the twin reduction group showed a significantly different pattern of progesterone concentration than control cows (between-effect) (Fig. 6.2). A significant increase in progesterone concentrations on the day following treatment was detected in the twin reduction group (within effect) and levels remained high during the study period.

GLM repeated measures revealed no effect on PAG-1 concentrations of treatment, uterine laterality of twin embryos, milk production on pregnancy diagnosis and conceptus age on the day of treatment. However, we did observe significant effects of the day after treatment and the interaction day by treatment (within effects). PAG-1 concentrations significantly decreased during the study period in both the control and twin reduction groups (Fig. 6.3A). Notably, on the day after manual amnion rupture, cows in the twin reduction group experienced a significantly more abrupt drop in PAG-1 concentrations than cows in the control group. Both treatments were related to similar PAG-1 levels on the following days.

Table 6.2. Rates of pregnancy loss before Day 90 and estimated time for pregnancy loss in the control and twin reduction groups for unilateral and bilateral twin pregnancies.

	n	Fetal loss rate before Day 90 (%)[c]		Estimated time of pregnancy loss (days)[d]	
				Mean	95% CI
Unilateral twin pregnancies					
Control[a]	14	9/14	(64.3)	69.8	58.4 - 81.1
Twin reduction[ab]	13	7/13	(53.8)	68.1	56.9 - 79.3
Bilateral twin pregnancies					
Control[a]	14	0/14	(0)[e]	_[g]	_
Twin reduction[ab]	14	4/14	(28.6)[f]	82.1[h]	72.5 - 91.5

[a] Cows treated with 100µg GnRH on pregnancy diagnosis (Day 28-34)
[b] Cows fitted with a progesterone releasing intra-vaginal device (PRID, containing 1.55 g of progesterone) for 21 days after manual amnion rupture (Day 35-41).
[c] Significant differences detected by Fisher's Exact test ([e,f] p=0.049).
[d] Significant differences detected by the LogRank Matel-Cox test ([g, h] p =0.028)

The cows in the study showed prolactin concentrations ranging from a minimum of 7.09 ng/ml to a maximum of 159.36 ng/ml. Prolactin concentrations (mean ± SD) on the day of the treatment and on the days 1, 3 and 7 after the treatment were 54.75 ± 26.4, 58.43 ± 33.6, 60.69 ± 34.8 and 55.41 ± 25.5 for control cows, and 54.03 ± 29.4, 45.97 ± 31.8, 60.62 ± 39.2 and 54.89 ± 32 for twin reduction cows, respectively. GLM repeated measures indicated no significant effects of any of the variables examined on plasma prolactin concentrations.

6.3.3 Endocrine parameters from Day 35–41 to Day 56–62 of gestation in cows not suffering pregnancy loss

GLM repeated measures analysis revealed that none of the factors examined were significantly affected by treatment (between effect). While progesterone and prolactin concentrations did not significantly vary during the four weeks of study, GLM repeated measures determined that PAG-1 concentrations suffered significant changes during this period. Significant effects of the day after treatment and the interaction day by treatment (within effects) were detected. PAG-1 significantly decreased in the first week of study in both treatment groups and the cows undergoing twin reduction showed a greater significant decrease in PAG-1 immediately after treatment compared to the control cows (Fig. 6.3B).

6.4 Discussion

The findings of our study we would highlight are: a similar incidence of pregnancy loss detected in both the control and twin reduction groups among the unilateral twin gestations, and a significant decline produced in plasma PAG-1 concentrations from Day 35–41 to Day 42–48 regardless of the treatment received.

The management strategy of embryo reduction with progesterone supplementation could be a satisfactory approach to the problem of twinning in dairy cattle. The main purpose of embryo reduction is the prevention of twins at calving in order to avoid peripartum and postpartum problems. Twelve of the 27 cows (44%) undergoing manual amnion rupture delivered a single calf after the procedure whereas 15 twin calvings were registered in the 28 control cows. However, the risk of pregnancy loss after the procedure of embryo reduction should be considered. The results indicate that embryo reduction did not carry an additional risk of pregnancy loss when conducted in unilateral twin pregnancies, whereas pregnancy loss is higher when conducted in bilateral twin pregnancies. A common figure for pregnancy loss in twin cattle pregnancies is around 20–25% (López-Gatius *et al.* 2002, 2009; López-Gatius and Hunter 2005; Silva del Rio *et al.* 2009), with losses occurring mostly before Day 90 of gestation (López-Gatius *et al.* 2004a; López-Gatius and Garcia-Ispierto 2010). The overall rate of pregnancy loss before Day 90 combined for the two groups was 36.4%. This relatively high figure may be due to heat stress. All pregnancies included in this study were diagnosed in the warm season (April–September). Previous studies in this area (López-Gatius *et al.* 2004a; López-Gatius and Hunter 2005) have reported similar pregnancy loss rates for twin pregnancies during the warm season to the rate observed in the present control animals.

The uterine laterality of the twin gestation was an important factor affecting pregnancy loss, in agreement with previous studies (López-Gatius and Hunter 2005; Echternkamp *et al.* 2007; López-Gatius and Garcia-Ispierto 2010). The pregnancy loss risk for the unilateral twin gestations was 8.7 times higher than for the bilateral twin pregnancies. Interestingly, the pregnancy loss rate for unilateral twin pregnancies was similar in the control and twin reduction groups. Thus, the survival curves of the twin reduction and control group unilateral pregnancies almost overlapped and suggest common mechanisms of pregnancy wastage for unilateral twin gestations regardless of treatment.

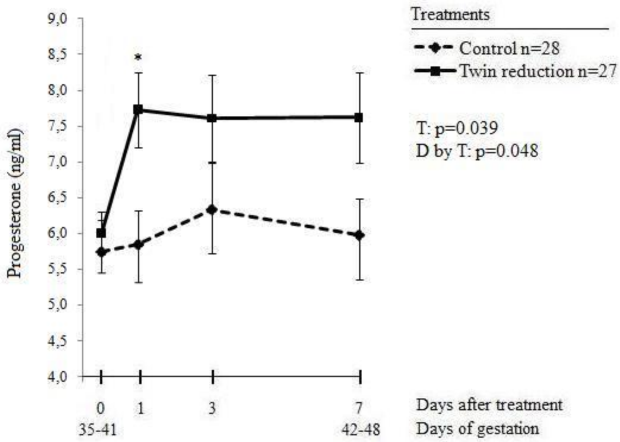

Figure 6.2. Plasma progesterone concentrations (ng/ml) recorded from Day 35-41 to Day 42-48 stratified by treatment (mean ± S.E.M). Probabilities for main effects of treatment (T) and day (D) and the interaction (D by T) that were significant are shown. An asterisk indicates a difference ($P<0.05$) between treatments within a day.

In contrast, four of the 14 cows with bilateral gestations undergoing embryo reduction treatment suffered pregnancy loss, a figure clearly lower than that observed for the unilateral pregnancies, but higher than the rate recorded for the control bilateral pregnancies (0%). Therefore, in order to prevent cows from twinning, our results suggest amnion rupture in unilateral twin pregnancies can be conducted without additional risk for pregnancy loss, but embryo reduction in bilateral twin gestations should be reconsidered. However, the results of this technique are rather poor. Twin reduction by transvaginal ecoguided amnion puncture and aspiration could offer more satisfactory rates of pregnancy maintenance in both unilateral and bilateral twin pregnancies. These techniques have been widely developed in equine reproductive medicine and are designed to reduce twin pregnancies following the implant of embryonic vesicles now that manual crushing is no longer considered safe (Macpherson and Reimer 2000; Mari *et al.* 2004).

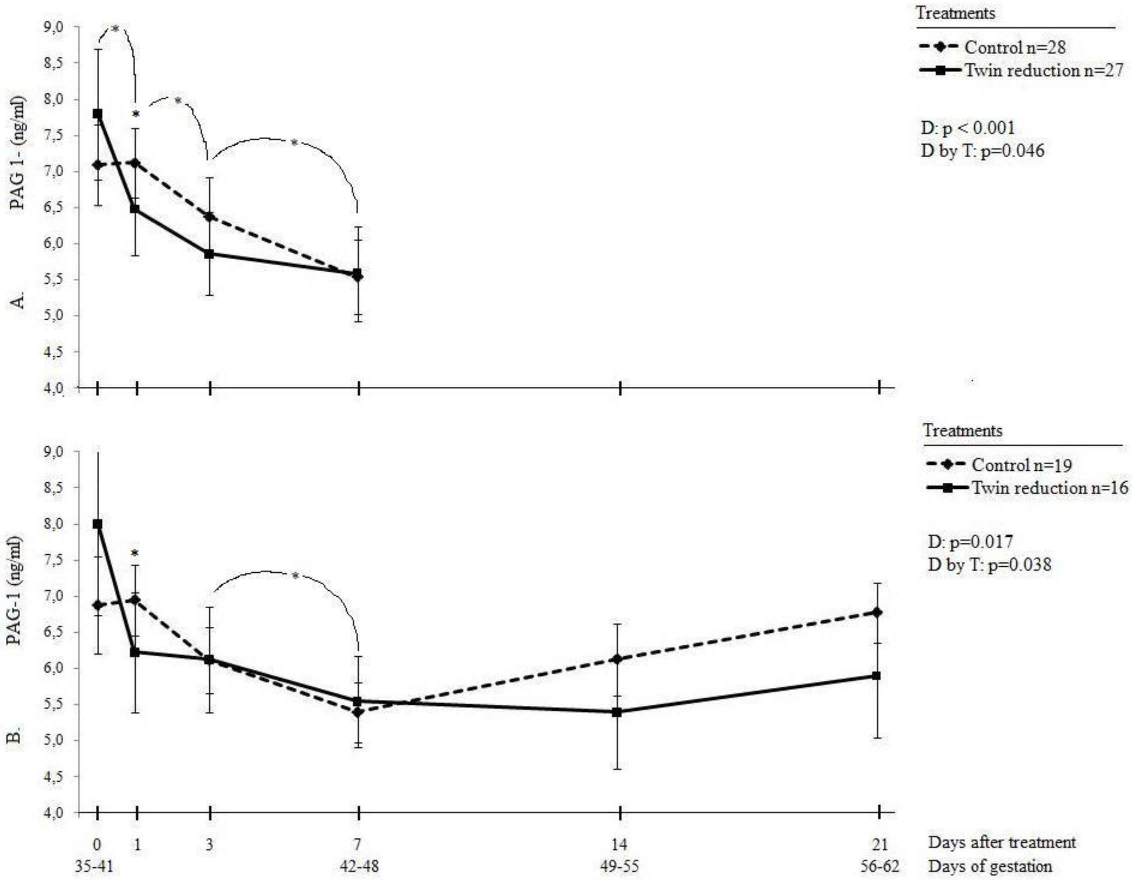

Figure 6.3. Plasma pregnancy-associated glycoproteins-1 (PAG-1) concentrations (ng/ml) recorded from Day 35-41 to Day 42-48 for all the cows included in the study (A) and from Day 35-41 to Day 56-62 for cows that did not suffer pregnancy loss (B) stratified by treatment (mean ± S.E.M). Probabilities for main effects of treatment (T) and day (D) and the interaction (D by T) that were significant are shown. An asterisk indicates a difference (P<0.05) between days combined for treatments and between treatments within a day.

Spontaneous embryo reduction of a twin embryo was recorded in ten cows out of the 72 cows forming the study population before Day 35–41. Six of the twin gestations were unilateral and four were bilateral twin pregnancies. Later on in gestation, four cows assigned to the control treatment underwent spontaneous fetal reduction. Of these, two suffered subsequent pregnancy loss while the two remaining cows maintained their pregnancies and delivered singletons. In two studies including 211 (López-Gatius and Hunter 2005) and 464 (López-Gatius *et al.* 2010) twin pregnancies, the incidences of spontaneous embryo reduction were 16.6% and 28.4% respectively. The probability of undergoing spontaneous embryo reduction was 3.5 times higher in the unilateral than bilateral twin pregnancies (López-Gatius *et al.* 2010). Moreover, the risk of pregnancy loss after spontaneous embryo reduction was significantly higher for unilateral than for bilateral pregnancies (López-Gatius and Hunter 2005). Close contact between unilateral twin conceptuses and interchorionic vascular anastomosis (Williams *et al.* 1963; Echternkamp 1992) are likely to be the cause of pregnancy failure after spontaneous embryo reduction, and could also affect manual embryo reduction.

In a prior study in which plasma hormone levels were not assessed, amnion rupture resulted in a gestation maintenance rate of 36% (four out of 11) when cows were fitted with a progesterone releasing intravaginal device, while the procedure caused pregnancy loss in all animals not receiving progesterone treatment (López-Gatius 2005). Herein, a significant increase in plasma progesterone concentrations was detected following twin reduction treatment on the day following insertion of the intra-vaginal device. Thereafter, progesterone levels remained elevated in the twin reduction group during the first week of treatment. A rise in plasma progesterone concentration was also detected (Bech-Sàbat el al. 2009) seven days after fitting cows with a progesterone releasing intra-vaginal device. In our circumstances, intra-vaginal progesterone supplementation has been effectively used to reduce the incidence of pregnancy loss during the late embryonic/early fetal period in single pregnancies (López-Gatius *et al.* 2004b; Bech-Sàbat *et al.* 2009, 2010). However, GnRH seems to offer more benefits than progesterone supplementation in cows bearing twins since it improves pregnancy maintenance in cows that undergo spontaneous embryo reduction (Bech-Sàbat *et al.* 2009).

Manual amnion rupture requires manipulation of the gravid uterus. This could promote prostaglandin release from the uterus and affect the developing placental membranes. Although the role of PAG-1 remains unknown, plasma levels of PAG-1 have been widely used as a marker of placental/fetal wellbeing (Szenci *et al.* 1998) and changes in PAG-1 concentrations may be predictive of pregnancy loss (López-Gatius *et al.* 2007; Whitlock and Maxwell 2008). Our results reveal a sudden drop in PAG-1 concentrations on the day after embryo reduction treatment. This could indicate placental membrane injury as a consequence of manipulation. However, no significant differences could be detected between treatments on the following days. Hence, seven days after treatment both the control and twin reduction group cows showed similar PAG-1 levels. This suggests the placenta may be able to rapidly recover from any possible damage incurred during amnion rupture. More importantly, a significant decline in PAG-1 levels was detected between Day 35–41 and Day 42–48 of gestation across both treatment groups in cows not suffering pregnancy loss. This finding is consistent with the results of a previous study including 80 cows with maintained pregnancies (López-Gatius *et al.* 2007) in which lower levels of plasma PAG-1 were observed on Day 42 and Day 49 of pregnancy compared to Day 35. Further investigation is need to clarify why levels of PAG-1 decrease during the late embryo/early fetal period.

Besides placental damage, manual amnion rupture would also be expected to cause inflammation of the endometrium. Moreover, the presence and re-absorption of dead embryo debris could trigger an inflammatory response that would potentially compromise the viability of the remaining co-twin. In effect, most pregnancy losses in our cows subjected to twin reduction occurred within two weeks of treatment. In some cases (data not shown), the remaining co-twin showed signs of degeneration membrane detachment and/or amniotic fluid turbidity) and the presence of debris from the dead embryo was also observed. Prolactin is a cytokine-like hormone (Kelly *et al.* 1991) involved in pro-inflammatory immune responses (Brand *et al.* 2004). However, no significant changes in plasma prolactin concentrations were detected in the week after embryo reduction treatment or until Day 56–62 of gestation. The immunomodulating effects of progesterone supplementation might mitigate the inflammatory response to amnion rupture and minimize the detrimental effects of uterine inflammation on conceptus viability. Progesterone plays a key role in modulating the maternal immune system by recruiting a wide range of pregnancy protective mechanisms (Druckmann and Druckmann 2005). However, it is puzzling why in some cases progesterone supplementation fails to maintain gestation after twin reduction treatment. Assessing other indicators of acute inflammation and stress such as haptoglobin (Skinner *et al.* 1991; Petersen el al. 2004), as well as monitoring uterine prostaglandin release could provide further insight into the physiology of the gravid uterus after embryo reduction on which to base future therapies.

By way of overall conclusion, embryo reduction by manual amnion rupture did not carry an additional risk of pregnancy loss for unilateral twin pregnancies, whereas it increases the risk of pregnancy failure in bilateral twin pregnancies. However, not only risk of pregnancy loss but also benefits of preventing cows from delivering twins might be also taken into count when assessing the success of embryo reduction in bilateral twin pregnancies. Despite the fact that spontaneous embryo reduction can reach levels of 28.4% (López-Gatius *et al.* 2010), further studies are needed to evaluate possible benefits of inducing embryo reduction in twin pregnant cows on their subsequent reproductive performance.

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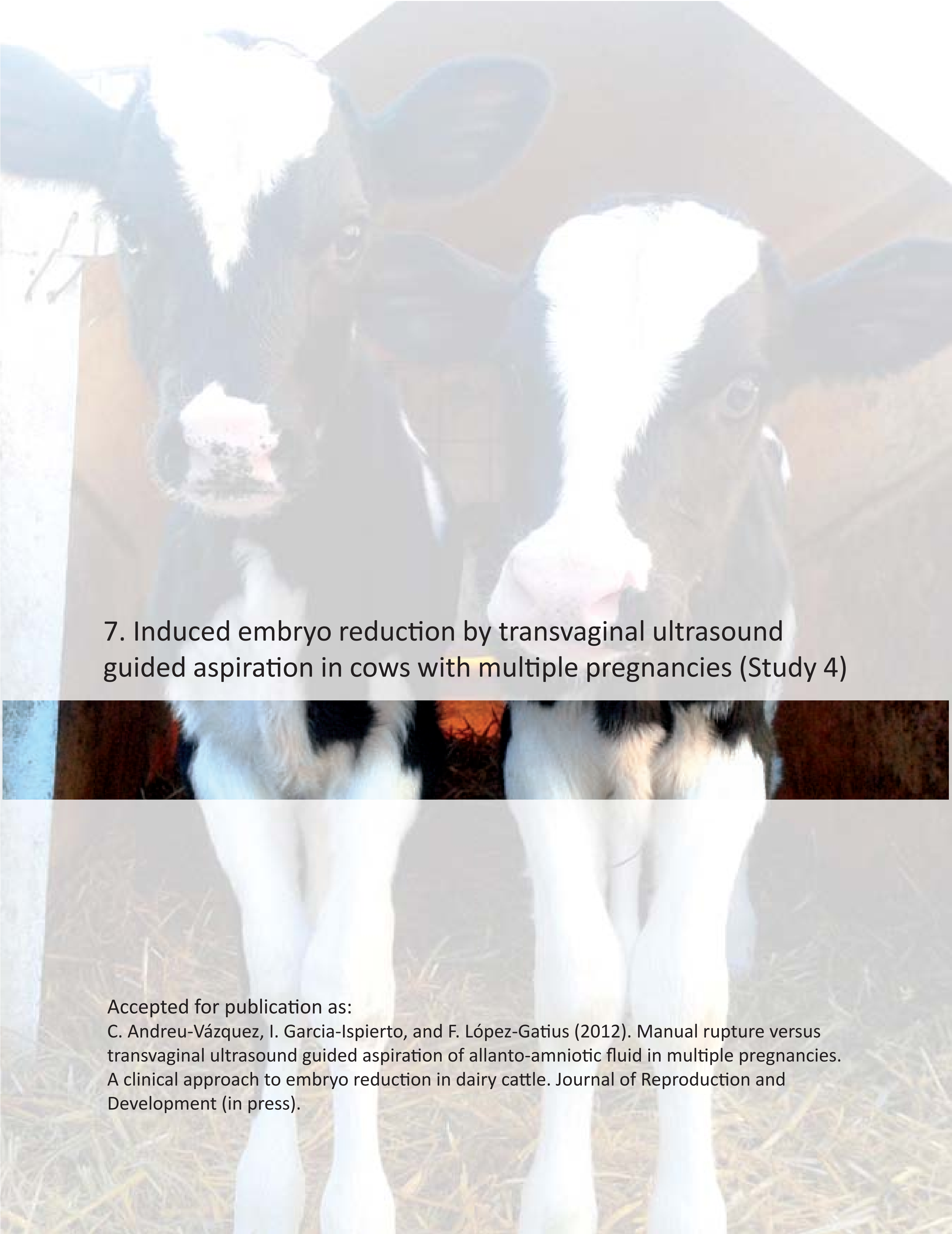

7. Induced embryo reduction by transvaginal ultrasound guided aspiration in cows with multiple pregnancies (Study 4)

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C. Andreu-Vázquez, I. Garcia-Ispierto, and F. López-Gatius (2012). Manual rupture versus transvaginal ultrasound guided aspiration of allanto-amniotic fluid in multiple pregnancies. A clinical approach to embryo reduction in dairy cattle. Journal of Reproduction and Development (in press).

Abstract

To avoid the problems associated with twinning in dairy cattle, one of the embryos may be eliminated. This study compares the effect on pregnancy maintenance of two embryo reduction techniques, Manual Rupture (MR) and Transvaginal Ultrasound Guided Aspiration (TUGA) of allanto-amniotic fluid in Holstein-Friesian cows with multiple pregnancies. In a first experiment, 61 lactating cows bearing unilateral twins (n=27), bilateral twins (n=30) or triplets/quadruplets (n=4) were subjected to MR (n=45) or TUGA using a 17G-neddle (n=16) on D28-34 of gestation. In 21 and 10 cows undergoing MR and TUGA embryo reduction, respectively, pregnancy loss occurred before D90 (46.7% vs. 62.5%, P= 0.28). Through binary logistic regression, the type of pregnancy was identified as the only variable significantly affecting pregnancy maintenance (P=0.03). Based on the odds ratio, the risk of pregnancy loss was 4.1 times higher for unilateral than for bilateral twins (70.4% vs. 36.7%, respectively, P=0.01). No effect was detected on pregnancy maintenance of the technique used (P=0.17) or of the interaction technique by type of pregnancy (P=0.22). In a second experiment, a 22G-needle was used to perform TUGA on 22 lactating cows. The pregnancy loss rates were 44.4% (4/9), 18.2% (2/11) and 50% (1/2) for cows bearing unilateral twins, bilateral twins and triplets, respectively. Total pregnancy loss rate following TUGA using the 22G-needle tended to be lower than using the 17G-needle (31.8% vs. 62.5%; P=0.06). Our results suggest that TUGA using a 22G-needle could be the method of choice to perform embryo reduction in cows carrying multiple pregnancies.

Keywords

Dairy cattle**,** embryo reduction, quadruplets, triplets, twin pregnancy.

7.1 Introduction

Multiple pregnancies (twins or higher-order pregnancies) are undesirable in dairy herds. Whilst cows carrying triplets and quadruplets are unlikely to maintain their pregnancies to term (Echternkamp 1992), cows delivering twins are more likely to suffer peripartum diseases and to be culled (Bicalho *et al.* 2007). Twinning increases not only the risk of pregnancy loss (López-Gatius *et al.* 2009; López-Gatius and Garcia-Ispierto 2010) but also those of dystocia, stillbirth and calf mortality (Nielen *et al.* 1989; Gregory *et al.* 1990; Eddy *et al.* 1991; Beerepoot *et al.* 1992). Over the past 20 years, twinning rates in dairy cattle have increased alongside milk production (Nielen *et al.* 1989; Kinsel *et al.* 1998; Bicalho *et al.* 2007). Estimates currently run at 9%, with rates of 0.3% to 12% reported among herds (Silva del Rio 2007) and it is foreseeable that this twinning rate will continue to increase.

Transforming a multiple pregnancy into a singleton pregnancy by inducing embryo reduction should in theory avoid the negative effects of multiple pregnancies in dairy cattle. However, the embryo reduction technique itself may carry an additional risk of pregnancy loss. Embryo reduction by manual rupture (MR) of the amniotic vesicle during the late embryonic/early foetal period has been assessed in unilateral (López-Gatius 2005; Andreu-Vázquez *et al.* 2011) and bilateral twin pregnancies (Andreu-Vázquez *et al.* 2011). In these studies, pregnancy loss rates of 28.6 to 100% were reported. Transvaginal Ultrasound Guided Aspiration (TUGA) is an alternative method to MR for embryo reduction in twin pregnant mares (Bracher *et al.* 1993; Macpherson and Reimer 2000; Mari *et al.* 2004) although as far as we know TUGA has not been used to address the problem of multiple pregnancies in high-producing dairy cows. The aim of this study was to compare the effect on pregnancy maintenance of inducing embryo reduction by MR or TUGA in dairy cows with multiple pregnancies.

7.2 Materials and Methods

7.2.1 Animal management

This study was performed on a commercial dairy herd comprised of 1128 mature Holstein-Friesian cows in northeastern Spain selected because of its high twinning rate (17.1%), and high pregnancy loss rate for cows carrying twins (32.1%) recorded over the preceding 6-month period (Andreu-Vázquez *et al.* 2011). Mean annual milk production for the herd was 10360 kg per cow. The cows were milked three times daily, kept in open stalls and fed complete rations in line with NRC recommendations (National Research Council 2001). All cows were tested free of tuberculosis and brucellosis and artificially inseminated using semen from sires of proven fertility. The mean annual culling rate for the study period was 33%.

The herd was maintained on a weekly reproductive programme. Normal uterine involution and the morphology of ovarian structures were checked by palpation per rectum 30-36 days post partum. Any postpartum reproductive disorders diagnosed were treated until resolved or until culling. The voluntary waiting period from calving to first insemination was 60 days. Only cows devoid of detectable reproductive disorders were inseminated.

7.2.2 Pregnancy diagnosis, number and viability of embryos

Pregnancy was diagnosed by transrectal ultrasonography on Day 28-34 post-insemination using a portable B-mode ultrasound scanner (Scanner SonoSite 180 PLUS Vet equipped with a 5-10 MHz transducer; SonoSite, Bothell, WA, USA). Scanning was performed along the dorso/lateral surface of each uterine horn. Twins were recorded on observation of two embryos in different positions within one uterine horn on two scans, two embryos simultaneously present on the screen (unilateral twin pregnancy), or one embryo in each uterine horn (bilateral twin pregnancy). Higher-order pregnancies were recorded when three or more embryos were observed in one or both uterine horns. The viability of the embryos was determined by detecting their heartbeat. Cows carrying one or more dead embryos were excluded from the study.

7.2.3 Embryo reduction by MR or TUGA and pregnancy follow-up examinations

All procedures were approved by the Ethics Committee on Animal Experimentation of the University of Lleida (license numbers CEEA.09-01/11 and CEEA.10-01/11).

Embryo reduction was always conducted on the day of pregnancy diagnosis (28-34d of gestation). For the first experiment 61 lactating cows bearing unilateral twins (n=27), bilateral twins (n=30) or with higher-order pregnancies (n=4) were subjected to embryo reduction. Because MR proved to be relatively successful in a previous study (Andreu-Vázquez *et al.* 2011), for the first experiment TUGA was carefully introduced as an alternative embryo reduction method, such that, in chronological order of pregnancy diagnosis, for every 3 cows undergoing MR (n=45), TUGA was conducted in a further cow (n=16). Thirty minutes prior to embryo reduction, all cows received 1250 mg flunixin meglumine i.m. (Flunex Industrial Veterinaria S.A., Barcelona, Spain) to counteract prostaglandin release due to manipulation of the uterus. For MR, the amniotic vesicle of a twin embryo was pressed with the thumb to cause its rupture (Andreu-Vázquez *et al.* 2011). For TUGA, we used a portable B-mode ultrasound scanner (HS-1500 V; Honda Electronics CO., Ltd, Toyohashi, Japan) equipped with a convex 5-10 MHz (HCV-3710MV; Honda Electronics CO., Ltd, Toyohashi, Japan) transducer for transvaginal use. A sterile 17G 50-cm long needle with an echogenic tip (COVA needle type-A; Misawa Medical Industry CO., Ltd, Tokyo, Japan) was used for puncture and aspiration. TUGA was performed on standing animals under epidural anesthesia by infusing 0.04 mg/kg of 2% xylacin (Rompun; Bayer, Barcelona, Spain). The transducer was inserted into the anterior vagina and the position of the uterus corrected per rectum until the amniotic vesicle and embryo could be visualized on the ultrasound screen. A puncture guide on the screen was used to draw the correct path for needle placement in the amniotic vesicle. An assistant passed the needle through the needle guide-tube located on the handle of the transducer and pushed it against the fornix and uterine wall until the echogenic tip of the needle could be seen inside the amniotic vesicle. A 20-ml syringe attached to the needle was used to aspirate allanto-amniotic fluid.

For a second experiment, 22 lactating cows bearing unilateral twins (n=9), bilateral twins (n=11) or triplets (n=2) were subjected to TUGA using a modified needle with a 22G 9-cm long tip attached to a 17G 40-cm long body (Figure 7.1). Drug administration and embryo reduction were conducted as described above. Additionally, cows were treated with non-teratogenic antibiotics, 750 mg of ceftiofur s.c. (Cevaxel; CEVA Salud Animal, Barcelona, Spain) immediately after TUGA, and 24h and 48h later.

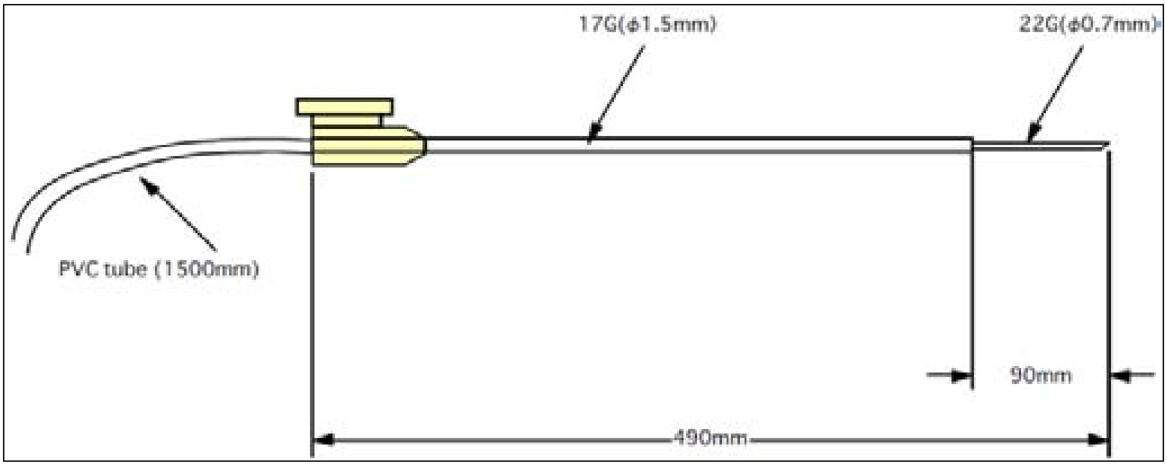
MR and TUGA procedures were always conducted by the same operator. Embryo death was assessed through the disappearance of the heartbeat detected by ultrasonography. For cows carrying triplets/quadruplets, embryo reduction was performed repeatedly to only preserve one embryo. Immediately after embryo reduction, all cows were fitted with a progesterone releasing intra-vaginal device (PRID, containing 1.55 g of progesterone; CEVA Salud Animal, Barcelona, Spain) for 28 days.

Weekly follow-up examinations to assess the presence and viability of an embryo/foetus were performed by ultrasound until Day 56-62 of gestation or until pregnancy loss. Pregnancy was further confirmed by rectal palpation on Days 90. Pregnancy loss was recorded when the embryo/foetus was not longer present or when no heartbeat was detected.

7.2.4 Data collection and statistical analysis

The following data were recorded for each animal: embryo reduction method (MR or TUGA); type of pregnancy (bilateral twin, unilateral twin or higher-order pregnancy); gestational stage on the day of embryo reduction; parity; interval from calving to conception; milk production at the time of pregnancy diagnosis; and date of pregnancy loss (if this occurred).

Figure 7.1. Design for the modified needle, with a 22G 9-cm long tip attached to a 17G 40-cm long body, used for TUGA in the second experiment.



Only pregnancy losses occurring before Day 90 of gestation were interpreted as being induced by embryo reduction. For the first experiment logistic regression procedures were used to evaluate the relative contribution of each potentially affecting variable to the probability of pregnancy loss before Day 90.Binary logistic regression was performed according to the method of Hosmer and Lemeshow (1989) considering pregnancy loss before Day 90 as the dependent variable, and embryo reduction method, parity, type of pregnancy, gestational stage on the day of embryo reduction, days from calving to conception and milk production on pregnancy diagnosis as independent variables. For bilateral twin, unilateral twin and higher-order pregnancies, differences in rates of pregnancy loss for the MR and TUGA embryo reduction techniques were compared by the Chi square or Fisher's exact tests. Differences in rates of pregnancy loss for the TUGA using a 22G-needle and TUGA using a 17G-needle were compared by the Chi square test. All statistics procedures were performed using the SPSS package version 15.0 (SPSS Inc., Chicago, IL, USA) with 0.05 as the level of significance.

7.3 Results

In the first experiment pregnancy loss before Day 90 was recorded in 21 cows undergoing embryo reduction by MR and 10 cows by TUGA (46.7% vs. 62.5%, P= 0.28). During weeks one to four following embryo reduction, 7, 14, 7 and 2 pregnancy losses occurred respectively; whereas in one cow pregnancy loss occurred between Days 60 and 90 of pregnancy. No pregnancy loss occurred after Day 90. Logistic regression analysis indicated no significant effects on pregnancy loss before Day 90 of the embryo reduction method used, parity, gestational stage on the day of embryo reduction, days from calving to conception and milk production. The type of pregnancy was the only variable significantly affecting pregnancy loss (likelihood ratio test 7.804; 2 d.f., P=0.02, Nagelkerke r^2 =0.16). Based on the odds ratio, the risk of pregnancy loss was 4.1 times higher for unilateral (70.4%) than bilateral twins (36.7%; 95% confidence interval for the odds ratio: 1.35-12.45, P=0.01). Table 7.1 shows the prior to Day 90 pregnancy loss rates for cows bearing bilateral twins, unilateral twins or with higher-order pregnancies after embryo reduction by MR or TUGA. Pregnancy loss rates associated with the TUGA and MR methods failed to differ for all pregnancy types.

For the second experiment pregnancy loss before Day 90 was recorded in 7 of the 22 cows. The pregnancy loss rate following TUGA using a 22G-needle tended to be lower than using the 17G-needle (31.8% vs. 62.5%; P=0.06). Table 7.2 shows the prior to Day 90 pregnancy loss rates for cows bearing bilateral twins, unilateral twins or with higher-order pregnancies after TUGA performed using the 22G-needle. All 7 pregnancy losses occurred during the three weeks following embryo reduction.

The culling rate for cows suffering pregnancy loss after embryo reduction was 25.8%. All animals become pregnant again with an interval mean of 51.8±47.0d from pregnancy loss to conception.

Table 7.1. Pregnancy losses before Day 90 after embryo reduction by Manual Rupture (MR) or Transvaginal Ultrasound Guided Aspiration (TUGA) of allanto-amniotic fluid using a 17G-needle in cows carrying bilateral twins, unilateral twins or with higher-order pregnancies.

	n	Pregnancy loss before Day 90 (%)*			
		Embryo reduction by MR		Embryo reduction by TUGA	
Multiple pregnancies	61	21/45	(46.7)	10/16	(62.5)
Bilateral twins	30	6/20	(30.0)	5/10	(50.0)
Unilateral twins	27	14/22	(63.3)	5/5	(100.0)
Higher-order	4	1/3	(33.3)	0/1	(0.0)

* No significant differences between the MR and TUGA embryo reduction methods.

Table 7.2. Pregnancy losses before Day 90 after embryo reduction by Transvaginal Ultrasound Guided Aspiration (TUGA) of allanto-amniotic fluid using a 22G-needle in cows carrying bilateral twins, unilateral twins or with higher-order pregnancies.

	n	Pregnancy loss before Day 90 (%)	
Multiple pregnancies	22	7/22	(31.8)
Bilateral twins	11	2/11	(18.2)
Unilateral twins	9	4/9	(44.4)
Higher-order	2	1/2	(50.0)

7.4 Discussion

Embryo reduction to prevent the negative effects of multiple pregnancies in dairy cattle is not a risk-free procedure. Herein, embryo reduction was conducted in a total of 83 Holstein-Friesian lactating cows. The overall pregnancy loss rate for the cows undergoing embryo reduction in this study was 45.8%, whereas a 32.1% pregnancy loss rate was registered for non-reduced twin pregnant cows of the same dairy commercial herd in the previous 6-month period (Andreu-Vázquez *et al.* 2011). Despite this, transforming a multiple pregnancy into a singleton pregnancy by inducing embryo reduction may prevent likely postpartum problems and infertility following twinning. Thus, regardless of the pregnancy loss risk, further aspects related to herd productivity and profitability need to be considered when assessing the cost-effectiveness of embryo reduction.

Among the embryo reduction methods assessed in this study, the highest rate of pregnancy maintenance following embryo reduction was archived when using the 22G-needle for TUGA, with an overall pregnancy loss rate of 31.8%. The figure reached 46.7% when embryo reduction was conducted by MR, and 62.5% when TUGA was performed using a conventional 17G-needle. In a previous study, we reported a similar rate for the MR method (Andreu-Vázquez *et al.* 2011). On the other hand, we have no figure to compare our rate of pregnancy loss for the TUGA embryo reduction technique since, as far as we know, there are no prior data on its use dairy cattle. In human obstetrics, TUGA and modified versions of this technique have been widely used to avoid the risks of multiple pregnancies and it is considered a safe technique with a miscarriage rate of less than 12% when performed before the 8th week of gestation (Lee *et al.* 2008). TUGA has also been used for embryo reduction in equine medicine with success rates ranging from 9% to 75%, depending on the gestation time when it is performed and the location of the co-twin embryonic vesicles (Bracher *et al.* 1993; Macpherson and Reimer 2000; Mari *et al.* 2004). However, the MR method of embryo reduction is the procedure of choice in twin pregnant mares, its success rate being greater than 90% when embryo vesicles are still movable through the uterine lumen (prior to Day 16 post-insemination), and TUGA has been restricted to situations in which MR is no longer accessible (Macpherson and Reimer 2000; Ragon 2007).

Regardless of the embryo reduction method used, pregnancy maintenance rates were improved when embryo reduction was conducted in bilateral rather than unilateral twin pregnancies. This is in agreement with the findings of previous studies in which unilateral twin pregnancies were found to be more likely to fail than bilateral twins either if the pregnancy was not reduced [20] or following spontaneous [20] or induced embryo reduction (Andreu-Vázquez *et al.* 2011). Close contact between unilateral twin conceptuses and inter-chorionic vascular anastomoses (Williams *et al.* 1963; Echternkamp 1992) are probably related to this high risk of pregnancy loss. However, when assessing which embryo reduction method would be more suitable for each pregnancy type, it was determined through logistic regression that neither MR nor TUGA using the 17G-needle (first experiment) proved to be safer than the other technique.

The success of TUGA using a 22G-needle over MR or TUGA using a conventional 17G-needle can be related to a lower degree of inflammation induced by the procedure that might compromise pregnancy maintenance following embryo reduction. Whilst in embryo reduction through MR pressure is applied over a larger uterine area, TUGA disrupts the uterine wall at the site of puncture. Similarly, the use of a 21-22G needle has been described to be less traumatic for the gravid uterus (Kamikura *et al.* 1997; Garcia and Salaheddine 1997; Makondo *et al.* 1997) than a17-18G needle (Below *et al.* 1996, Makondo *et al.* 1997) when collecting foetal fluids in the cow. However, not only the use of a thinner needle but also the antibiotic prophylaxis could be responsible for the differences between the pregnancy loss rates following TUGA recorded in the first and the second experiments. Introduction of bacteria inside of the gravid uterus through the site of puncture is likely to occur following TUGA, and the administration of antibiotics to the cows enrolled in the second experiment may lead to better rates of pregnancy maintenance. Iatrogenic uterine inflammation and chorio-amnionitis following TUGA have been reported in women (Ibérico *et al.* 2000) and mares (Squires and Tarr 1994), and antibiotic prophylaxis is always given in both women and mares to counteract uterine infection after any embryo reduction procedure that involves puncturing the uterine wall. In cattle, pregnancy loss after amniocentesis or allantocentesis has been associated with bacterial infection (Kamikura *et al.* 1997), and systemic antibiotics are also indicated (Garcia and Salaheddine 1997; Makondo *et al.* 1997).

In both experiments, most pregnancy losses occurred during the first three weeks following embryo reduction and only a few cows (3 of the 38 pregnancy losses, 7.9%) lost their pregnancies later, in agreement with data reported for the mare (Macpherson and Reimer 2000) and cows suffering spontaneous embryo reduction (López-Gatius and Hunter 2005). In most cases, pregnancy loss was preceded by signs of embryo death such as weak heart activity, detached membranes or amnion turbidity detected in the weekly ultrasound follow up exams. Additionally, in some animals, detritus of the reduced embryo was visible for as long as two weeks after the reduction procedure. It has been suggested that necrotic material remaining in the uterus induces uterine prostaglandin release which has a luteolytic effect promoting pregnancy loss after multifoetal pregnancy reduction in women (Sebire *et al.* 1997). To counteract this, progestogen supplementation is recommended and has been shown to improve pregnancy maintenance after embryo reduction in both mares (Macpherson and Reimer 2000) and cows (López-Gatius 2005; Andreu-Vázquez *et al.* 2011).

Finally and from a clinical perspective, there is a need for discerning an accurate hormone, antibiotic and anti-inflammatory therapy to safely achieve embryo reduction in dairy cattle. Besides this, certain modifications to the TUGA embryo reduction technique could help improve results.

Intracardiac/intrathoracic embryo puncture with or without injection of an embryotoxic agent have been attempted in human (Mansour *et al.* 1991; Ibérico *et al.* 2000; Lee *et al.* 2008) and equine medical practice (Raggio *et al.* 2008) as alternatives to fetal fluid aspiration. Evacuation of the amniotic fluid has been suggested to damage the remaining embryo when its placental membranes are inadvertently aspirated (Macpherson and Reimer 2000). This would be even more critical in cattle in which most unilateral and some bilateral twin embryos are in very close contact with each other.

In conclusion, both the MR and TUGA methods of embryo reduction could be conducted in dairy cattle in order to prevent the negative effects of twin calvings. Our results suggest that TUGA using a 22G-needle could be the method of choice to perform embryo reduction in cows carrying multiple pregnancies. Further studies are needed to establish an effective hormonal, antibiotic and anti-inflammatory therapy in order to prevent pregnancy loss following embryo reduction.

7.5 References

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8. General discussion

The four studies included in this thesis incorporate a discussion section in which specific aspects related to the factors affecting twin pregnancies, the effects of twinning on the lifespan of the cow and the success of induced embryo reduction when performed through manual amnion rupture or transvaginal aspiration of the fetal fluids. Therefore, this chapter focuses in answering further questions and concerns that may arise when considering the possibility to introduce embryo reduction as a strategy to reduce the twinning rate in a dairy herd.

8.1 Are twins a problem in dairy herds?

8.1.1 The real incidence of twin pregnancies

Assessing the magnitude of the twinning problem in a dairy herd requires as a star-point an accurate and early diagnosis and record of the twin pregnancies. In fact, pregnancy diagnosis constitutes itself the first problem related to twins. The real incidence of twin pregnancies in a herd may be easily mistaken when pregnancy diagnosis is conducted through rectal palpation (Day *et al.* 1995; López-Gatius and Garcia-Ispierto 2010). Also when ultrasography is used to diagnose pregnancies, the presence of twin embryos may be unnoticed. This usually occurs if the twin embryos are located in different uterine horns (bilateral pregnancy) and the diagnosis focuses on scanning for the presence of a conceptus without checking the ovarian structures (López-Gatius and Garcia-Ispierto 2010). Thus, for an accurate pregnancy diagnosis it is recommended to scan first both ovaries to record the number of corpora lutea presented, and then, a complete scanning along the dorso/lateral surface of each uterine horn (López-Gatius and Garcia-Ispierto 2010). This would allow identifying and distinguish between singleton pregnancies with one or more additional corpora lutea and twin pregnancies (López-Gatius and Garcia-Ispierto 2010).

Furthermore, the actual rate of twin pregnancies may be underestimated if the pregnancy diagnosis is delayed. In one of the studies included in this thesis we could state how frequently spontaneous embryo reduction and pregnancy loss occurs within the week following a twin pregnancy diagnosis (Andreu-Vázquez *et al.* 2011). If not diagnosed before, those twin pregnancies that had undergone either spontaneous embryo reduction or pregnancy loss would have never been identified as twins. However, some signs of a preceding spontaneous embryo reduction or twin pregnancy loss such as detritus from the dead co-twin or an excessive amount of floating placental membranes may be detected in a delayed pregnancy diagnosis (López-Gatius and Garcia-Ispierto 2010). An early diagnosis of spontaneous embryo reduction brings the opportunity to treat the cow with GnRH or exogenous progesterone in order to support pregnancy and enhance the chances of pregnancy maintenance (Bech-Sàbat *et al*. 2009). On the other hand, identifying twin pregnancies before day 30 post-insemination requires strong skills as until that moment the amniotic vesicle could be very small and the embryos are in a close contact with the uterine wall (López-Gatius and Garcia-Ispierto 2010). In that case, suspicious cows should be rechecked to detect or discard the presence of twins on the following week (López-Gatius and Garcia-Ispierto 2010).

Due to their high risk of pregnancy failure, misdiagnosing twin pregnancies could have an important impact in the herd as the “missed” twins may increase the overall pregnancy loss rate without a “known” reason. Thus, identifying correctly twin pregnancies through an accurate diagnosis, and further pregnancy confirmation on days 60 and 90 for the detection of twin pregnancies spontaneously reducing one embryo (López-Gatius and Hunter 2005) and rebreed cows that had suffered pregnancy loss are fundamental pieces in a control reproductive program (López-Gatius *et al.* 2004; López-Gatius and Hunter 2005; López-Gatius and Garcia-Ispierto 2010, López-Gatius 2012).

The mean incidence of twin pregnancies for a total of 2015 positive pregnancy diagnosis conducted on day 28-34 post-insemination during a year period was as high as 17.9% (Andreu-Vázquez *et al.* 2012a). Surprisingly, the incidence of twin pregnancies in one of the herds in which the study was conducted was 12.6%, whilst the twinning rate (i.e. the rate of twin birth calvings) in the herd for the same period of time was below 6% (Andreu-Vázquez *et al.* 2012a, 2012b). The vast difference between the figures highlights the great percentage of twin pregnancies that do not arrive to term, mainly because of its high risks of early pregnancy failure (López Gatius *et al.* 2009) and culling (Day *et al.*1995). In a lesser extent, also the occurrence of spontaneous embryo reduction (López-Gatius and Hunter 2005) may have led to a lower twinning rate.

To evaluate the problem of twins in a dairy herd not only the "top of the iceberg" (the twinning rate) but also the "submerged part" (the percentage of twin pregnancies at diagnosis that may not be maintained to term) should be considered.

8.1.2 The detrimental effects of twinning

Results from the retrospective study assessing the effects of twinning corroborate the high incidence of the extensively reported postpartum disorders, impaired fertility and higher risk of culling over the subsequent lactation for cows delivering twins (Chapin and Van Vleck 1980; Nielen *et al.* 1989; Gregory *et al.* 1990; Eddy *et al.* 1991; Echternkamp and Gregory 1999a, 1999b; Bicalho *et al.* 2007). Twin births accounted a higher proportion of dystocia (calving requiring assistance) with a greater incidence of placental retention and metritis compared to singleton birth calvings (Andreu-Vázquez *et al.* 2012b). Postpartum reproductive efficiency in cows calving twins was reduced, needing a mean of 26 extra days to become pregnant in comparison to their herdmates calving singletons (Andreu-Vázquez *et al.* 2012b). The probability of conception before day 90 and day 120 in cows that have calved twins were reduced, respectively, in 24 and 15 points compared to cows delivering singletons, whilst the culling rate, probably due to infertility, rises up to 15%, being the culling risk 26-41% higher than for cows given birth to singletons (Andreu-Vázquez *et al.* 2012b) in agreement with previous studies (Bicalho *et al.* 2007).

Moreover, the study pointed out that cows delivering twins had a higher risk of abortion in the subsequent lactation compared to cows calving singletons, (14% and 10.3%, respectively, Andreu-Vázquez *et al.* 2012b). This superior risk of abortion may be linked to the fact that cows calving twins were found to be 2.6 times more likely to conceive twins in the subsequent lactation (Andreu-Vázquez *et al.* 2012a). Besides a possible genetic maternal trait regarding twinning, it is also reasonable to think that, due to their impaired postpartum reproductive performance, cows previously twinning could more frequently be inseminated under an estrus synchronization protocol that may enhance the probability of double ovulation and result in a twin pregnancy (Andreu-Vázquez *et al.* 2012a). Furthermore, a plausible interaction between the extremely high incidence of placental retention or metritis in cows that have give birth to twins (Andreu-Vázquez *et al.* 2012b) and the higher risk of pregnancy failure may exist. In fact, retained placenta and pyometra have been identified as factors increasing the risk of early pregnancy loss in the subsequent gestation (López-Gatius *et al.* 2009). It is possible that alterations of in the uterine environment or traces of a uterine disease resulting from these disorders could interfere with the embryo placentation or the development of the embryo/fetus. Regardless its underlying cause, the superior risk of pregnancy failure may be the reason why the risk of culling by day 300 postpartum was 42 points higher in cows that have calved twins than for their herdmates calving singletons, although the detrimental effect of twinning on the conception was not longer detected by that time (Andreu-Vázquez *et al.* 2012b). In effect, abortion is, together with infertility, the main reason of culling in dairy herds.

More importantly, the retrospective evaluation of complete reproductive records for more than 4000 cows over an 11 year-period confirms that the detrimental effects of a twin calving birth do not only affect the subsequent lactation but also extend to the followings. A more pronounced pendent in the culling curve for the twinner than for non-twinner cows was noted as far as 400-800 days after parturition (Andreu-Vázquez *et al.* 2012b). Again, the higher abortion risk in cows delivering twins could be responsible for the higher culling pressure in the group of twinner cows.

Higher culling rates led to a mean reduction on the productive lifespan of 300 and 200 days for cows that delivered twins on their first or second lactation, respectively, compared to non-twinner cows (Andreu-Vázquez *et al.* 2012b). This could have a dramatic impact on the profitability of a herd in the actual high-intensive dairy production systems, as a shorter lifespan implies lowered total milk production per cow and increased culling rates and costs associated with breeding heifers for replacement. Therefore, we believe that the average loss of income attributed to cows delivering twins, previously estimated at 74-108 (Eddy *et al.* 1991; Beerepoot et el. 1992), might have been underestimated, as it might substantially increase if detrimental effect of twining in the productive lifespan length of the cows would be also considered.

8.2 Implementing the strategy of twin embryo reduction

8.2.1 What are the expected benefits and risks of inducing twin embryo reduction?

The decision of whether to introduce or not embryo reduction for cows carrying twin pregnancies as a therapeutic approach to avoid twinning in our herds should basically depend on its expected economic impact, considering both, the advantages and disadvantages that the strategy implies. In-herd implementation of any strategy would be suitable whenever the expected benefits would be greater than the risks. The main detriment and benefit of inducing embryo are, respectively, a likely increase in the risk of pregnancy loss and obtaining a single birth calving instead of a twin birth calving. Simple calculations can be done to estimate the outcome of the strategy. Both, the expected risk and benefit, can be pondered by considering their probability to occur and their correspondent effect in terms of days open (i.e. additional days that a cow is not pregnant as a result of the pregnancy loss following embryo reduction or following a twin birth calving).

Thus, for each twin pregnant cow in which embryo reduction would be attempted we should expect:

Risks: Total of days open because of the increased pregnancy loss rate=

(PL following IER* DO because of PL following IER) - (PL untreated * DO because of PL)

Benefits: Total of days open because of singleton birth calving instead of twin birth calving=

((1-PL following IER) * DO following SBC) – ((1-PL if untreated) * DO following TBC)

Where: PL= Pregnancy loss rate (probability)

IER= Induced embryo reduction

DO= Mean days open

SBC= Singleton birth calving

TBC= Twin birth calving

If we also consider spontaneous embryo reduction may occur in a certain percentage of cows carrying twin pregnancies that could maintain pregnancy to term and calve singletons (López-Gatius and Hunter 2005), the expected benefits should be corrected by subtracting:

(PM following SER * DO following SBC)

Where: PM= Pregnancy maintenance rate (probability)

SER= Spontaneous embryo reduction

DO= Mean days open

SBC= Singleton birth calving

Results from the experimental studies included in this thesis determined that the pregnancy loss risk following induced embryo reduction (PL following IER) averages 45% (Andreu-Vázquez *et al.* 2011, 2012c). Based on the literature and the results of our studies (López-Gatius *et al.* 2002, 2009; López-Gatius and Hunter 2005; Silva del Rio *et al.* 2009; Andreu-Vázquez *et al.* 2011), a 30% mean risk of pregnancy loss may be expected for untreated cows bearing twins (PL if untreated). Pregnancy loss meanly occurred on the 14 days following embryo reduction (conducted on days 28-34 or 35-42 of gestation) and cows suffering pregnancy loss were pregnant again 52 days later (Andreu-Vázquez *et al.* 2012c), whilst the reported averaged day of pregnancy loss for untreated twin pregnant cows is 75 days (López-Gatius *et al.* 2004) with an assumable 52 days interval for rebreeding. This leads to a mean of 102 and 127 days open for each cow suffering pregnancy when twin embryo reduction is attempted (DO because of PL following IER) or not (DO because of PL), respectively.

We can expect, furthermore, that 55% of the twin pregnant cows in which embryo reduction is conducted will calf singletons (1-PL following IER) with a median calving to interval following singleton birth calving of 108 days (DO following SBC, Andreu-Vázquez *et al.* 2012b). When not inducing embryo reduction, around 6% of twin pregnant cows will spontaneously reduce the twin gestation, maintain pregnancy to term (PM following SER) and deliver singletons (López Gatius and Hunter 2005). If untreated, the remaining 64% of the cows carrying twin pregnancies (1-PL if untreated-SER) will calf twins with a median calving to interval following twin birth calving of 134 days (DO following TBC).

Taking these data together, for each twin pregnant cow in which embryo reduction would be attempted, we should expect an increase of 7.8 days open as a result of the additional risk of pregnancy loss (risk) and a mean decrease of 32.8 days open as a result of obtaining a singleton birth calving instead of a twin birth calving (benefit). Thus, under our current working conditions, the overall outcome of reducing twin embryos would be positive (benefit overcomes risk), and a mean save of 25 days open, with an approximated cost of 4-8 $ per open day (Olds *et al.* 1979; Groenendaal *et al.* 2004; Meadows *et al.* 2005), should be expected for each twin pregnant cow in which we conduct embryo reduction.

It should be noticed, nevertheless, that the previous statement is only a partial approach to the real impact that implementing embryo reduction in twin pregnant cows would have in the economy of a herd. In effect, management complications (such as special attention at calving to solve dystocias and the incidence of stillbirths and freemartins) and costs of the postpartum therapies to treat placental retention and metritis that occur more frequently in cows delivering twins (Andreu-Vázquez *et al.* 2012b) are not considered in the calculations above. More importantly, the benefit estimation should be corrected by the greater culling rate for cows given birth to twins compared to cows delivering singleton calves (Bicalho *et al.* 2007, Andreu-Vázquez *et al.* 2012b). Also the risk of culling for cows that suffer pregnancy loss following induced embryo reduction, which was found to be 25% in the second experimental study included in this thesis (Andreu-Vázquez *et al.* 2012c), and that for the untreated twin pregnant cows, 3 times greater than for cows with a singleton pregnancy (Day *et al.* 1995), should be taken into account. Obtaining enough heifers for replacement is a major concern in the dairies due to the subfertile high-producing cows with a great impact on the economy of the herds. Thus, a further careful analysis through a complete economic model would be needed in order to validate the benefits of the twin embryo reduction strategy as, because of the additional risk of pregnancy loss, it might increase the mean culling rate and reduce the number of female calves born in the herd.

8.2.2 In which farms should we induce twin embryo reduction?

As shown above, potential benefits from preventing a cow to deliver twins are individual. Therefore, the decision of whether to implement or not the strategy of reducing twin pregnancies should not be a matter about the twinning rate or the percentage of twin pregnancies diagnosed in a herd. However, depending on the herd size and facilities to manage twinner cows and if the number of cows expected to twin a year is low, implementing other strategies (such as an optimum peripartum management addressed to minimize the negative effects of twinning on subsequent reproductive performance and survival of the cows) might be a more conservative option to embryo reduction. Nevertheless, it should be notice that for every cow calving twins at their first or second lactation, their productive lifespan will be reduced in 300 and 200 days, respectively, in comparison to non-twinner cows (Andreu-Vázquez *et al.* 2012b).

As commented previously, the success of twin embryo reduction is directly linked to the subsequent pregnancy loss risk. The calculations presented above may be transformed into an equation (making benefits equal to risks) in other to find the figure for a threshold in the pregnancy loss risk following embryo reduction. Whenever the pregnancy loss risk is over the threshold (as it might occur during the warm period, in which the early pregnancy failure is increased by a factor of 1.6-5.4, López-Gatius *et al.* 2009) we should avoid embryo reduction. In contrast, when the pregnancy loss risk following embryo reduction is below the threshold, benefits for inducing twin embryo reduction should be expected. Equally important to decide whether to introduce or not the strategy in a herd is to know the mean pregnancy loss rate, especially through the first trimester of gestation, in both cows bearing twin and single pregnancies and the herd annual culling rate. Thus, before offering the service of twin embryo reduction, the practitioner should evaluate pros and cons of the strategy and inform the owner of the farm about the plausible increase in the pregnancy loss rate and the expected benefits on the postpartum reproductive performance.

8.2.3 Should we reduce both unilateral and bilateral twin pregnancies?

Results from the first experimental study, in which we tried embryo reduction by manual rupture of the amniotic vesicle of one of the co-twins, concluded that the procedure could be safely adopted in unilateral twin pregnancies without carrying and additional risk of pregnancy loss whereas it must be reconsidered in bilateral twin pregnancies. However, from a dairy farmer perspective, it is understandable that the risk of inducing embryo reduction in cows carrying bilateral twins (i.e. 25% additional pregnancy losses compared to untreated bilateral twin pregnant cows, Andreu-Vázquez *et al.* 2011) in pro its benefits on the postpartum reproductive performance would be more easily assumable than the up to 50% pregnancy losses when embryo reduction is performed in cows bearing unilateral twins (Andreu-Vázquez *et al.* 2011). This conservative approach, based on only reducing bilateral twin pregnancies was therefore implemented in one of the herds and allowed us to assess the risk effects of the manual embryo reduction of bilateral twins in a larger number of animals (Andreu-Vázquez *et al.* 2012d, e). A lower pregnancy loss risk following embryo reduction was obtained (15% vs. 28.6%, Andreu-Vázquez *et al.* 2011; 2012d). This could attributable to differences either on the management of the two herds where the studies were performed or on the procedure protocol (differences on the day when the embryo reduction was attempted or the therapeutic agents administered). It must be noted, however, that the operator training was probably the most important factor contributing to the success and safeness of the procedure. In effect, better results in terms of pregnancy maintenance were obtained along with operator confirming manual crush of the amniotic vesicle was more easily archived and less time-consuming.

Besides this, it is worthy to remark that, although the pregnancy loss risk following embryo reduction in cows with unilateral twin pregnancies may scare both farmers and practitioners, the figure is not different to the pregnancy loss rate registered for the control unilateral twins (Andreu-Vázquez *et al.* 2011). Therefore, only benefits should be expected from implementing the strategy of reducing unilateral twins. When considering the possibility of reducing both, unilateral and bilateral twins, the overall pregnancy loss rate increases from 20-29% (López-Gatius *et al.* 2002; López-Gatius and Hunter 2005; López-Gatius *et al.* 2009; Silva del Rio *et al.* 2009) to around 45% (Andreu-Vázquez *et al.* 2011, 2012c). However, as discussed previously, benefits during the postpartum reproductive performance of treated cows, delivering singletons instead of twins, could overcome the losses derived from the additional pregnancy loss risk. Therefore, as an overall approach, we strongly encourage veterinary practitioners to perform embryo reduction, avoiding the warm period, first in bilateral twin pregnancies as it is more easily archive and has a higher success rate in terms of pregnancy maintenance and, after training, also in unilateral twin pregnancies.

8.2.4 At what day should we conduct twin embryo reduction?

Twin embryo reduction was induced one week after pregnancy diagnosis (on day 35-41 of gestation) or immediately following pregnancy diagnosis (on day 28-34 of gestation) in the first and the second experimental studies, respectively. Firstly, twin embryo reduction was delayed because we suspected a high percentage of cows carrying twins could suffer pregnancy loss or reduce spontaneously within the week following pregnancy diagnosis (Andreu-Vázquez *et al.* 2010). Also, we administrated GnRH at diagnosis as it has been reported to favor spontaneous embryo reduction or enhance pregnancy maintenance in cows reducing twins spontaneously (Bech-Sàbat *et al.* 2009). In effect, 10 cows of the 72 twin pregnant cows initially diagnosed carrying twins were found to have spontaneously reduced them while 7 have lost both twins and so the pregnancy within the week following diagnosis (Andreu-Vázquez *et al.* 2011).

In the last study, embryo reduction was advance to the day of pregnancy diagnoses as we wanted to assess if the procedure was less aggressive if conducted in an earlier stage. When comparing the rate of pregnancy maintenance when inducing embryo reduction by manual rupture at the day of pregnancy diagnosis or the following week no difference was found. In fact, neither the day at which embryo reduction was conducted (28 to 41) nor the interaction day by laterality affected the risk of pregnancy loss (Andreu-Vázquez *et al.* 2011b).

Conducting twin reduction in a more advanced stage of pregnancy, for instance, after sexing the fetuses, might not be recommendable as pregnancy loss would be with no doubt probable increased. It is likely that, rather than for a direct iatrogenic placental damage, pregnancy loss following embryo reduction occurs as a consequence of the necrotic material remaining in the uterus that compromise the viability of the co-twin (López-Gatius 2005; Andreu-Vázquez *et al.* 2011). In a more advanced stage of gestation, detritus from the reduced fetus may probably remain longer and have an even more dramatic impact on pregnancy maintenance.

Finally, although vascular anastomosis between twins may already exist (Williams *et al.* 1963) freemartin is not likely to occur if embryo reduction is conducted before embryonic sexual differentiation, which occurs around day 40 (Hunter 1995). In fact, any of the herds in which those studies were conducted reported any case of freemartin calves born after induced twin embryo reduction. Thus, depending on the size and location of the twin embryos, embryo reduction could be more easily and comfortably archived at the week of diagnosis or the following in our convenience, within in a “window” among days 28 to 41 of gestation.

8.2.5 Which method to induce embryo reduction is the safest?

Although we hypothesized TUGA could be more successful in terms of pregnancy maintenance than inducing embryo reduction through MR, the overall risk of pregnancy loss did not significantly differ between the two methods (Andreu-Vázquez *et al.* 2012c). The introduction of a thin needle into the amnion guided by ultrasonography minimizes the chance of damaging the non-reduced embryo/s. Therefore, TUGA may be especially advantageous for reducing of triplets and quadruplets, as well as for unilateral twins that are very close one to the other. However, on -farm applicability of TUGA may be compromised as it requires specific equipment, longer time to prepare the cow and at least two people need to be present. In contrast, MR could be easily introduced as a part of the routine reproductive control.

8.2.6 Which prophylactic therapies should be used following inducing embryo reduction?

Several drugs (flunixin meglumine, antibiotics, GnRH and exogenous progesterone) were used in the experimental studies in this thesis with the purpose of enhancing pregnancy maintenance following embryo reduction. Although further trials including control and treated animals would be needed to verify the potential preventing pregnancy failure effect of each drug, literature reports together with the results from our studies allow us drawing some presumptions about its efficacy.

Flunixin meglumine was not used in the first experimental study (Andreu-Vázquez *et al.* 2011) but introduced as a part of the protocol and applied 30 minutes prior to embryo reduction in further studies (Andreu-Vázquez *et al.* 2012c, 2012d). However, similar rates of pregnancy loss following manual embryo reduction were obtained. Notwithstanding, the powerful anti-prostaglandinic effect of flunixin meglumine is likely to counteract prostaglandin release due to manipulation of the uterus that may occur when manipulating a gravid uterus. In fact, flunixin meglumine is frequently used in standard protocols for ovum-pick up in cattle as well as for embryo reduction procedures in mares (Bracher *et al.* 1993; Macpherson and Reimer 2000; Mari *et al.* 2008).

There is much evidence about the prophylactic effect of antibiotics to prevent a potential iatrogenic infection following any procedure that requires puncturing a gravid uterus such as amniocentesis, and allantocentesis (Below *et al.* 1996; Garcia and Salaheddine 1996; Kamikura *et al.* 1997; Makondo *et al.* 1997). Although using a sterile needle, bacteria from the anterior part of the vagina are likely to reach the conceptus through the side of the puncture causing chorioamnioinitis and promoting pregnancy failure as described in mares and women (Squires and Tarr 1994; Ibérico *et al.* 2000). In fact, better results obtained in the second experiment included in the study evaluating the applicability of TUGA compared to the first experiment might not be entirely attributable to the use of a thinner needle but also to the antibiotic treatment that receive cows enrolled in the second experiment. Thus, antibiotic administration would be strongly recommended in cows submitted to TUGA, and also recommended in future embryo reduction modified techniques (transvaginal ultrasound-guided intracardiac or intratoracic puncture or injection). Attention should be paid to choose non-theratogenic or embryotoxic antibiotics in order to avoid further complications. On the other hand, antibiotics will be unnecessary if embryo reduction is conducted through manual rupture as the transrectal pressure applied to cause the rupture of the amnion do not disrupt the uterine wall.

To validate the benefits on pregnancy maintenance from treating cows with exogenous progesterone supplementation after inducing embryo reduction further investigation is required. Monozygotic twins are rare in dairy cattle (Silva del Rio *et al.* 2006) and most cows carrying twins present two or more corpora lutea at the moment of pregnancy diagnosis. Therefore, plasmatic levels of progesterone might be already high in the cows carrying twins (Bech-Sàbat *et al.* 2008, 2009). This has been hypothesized to be the reason why the use exogenous progesterone may not be the best approach to support pregnancy in cows bearing twins (Bech-Sàbat *et al.* 2009). Although its controversial use, progesterone supplementation was identified as a crucial agent to maintain pregnancy following embryo reduction (López-Gatius 2005). The reason for this could be related to the fact that induced embryo reduction could be followed by spontaneous corpus luteum regression. Disappearance of one of the corpora lutea along the first two weeks following embryo reduction was observed in a subset of cows enrolled in the experimental studies included in this thesis (personal communication). Interestingly, spontaneous regression of the corpus luteum has been reported to occur in near 25% of the twin pregnant cows that suffer spontaneous embryo reduction (López-Gatius *et al.* 2010) and, surprisingly, the regressed corpus luteum has been described to be always the one ipsilateral to the embryo that has died (López-Gatius *et al.* 2010). Assessing what can occur first, either spontaneous corpus luteum regression or the death of the twin embryo, could be essential to understand not only how pregnancy failure occurs following either spontaneous or induced embryo reduction, but also to inquire into the underlying mechanisms concerning the "ipsilaterality" phenomenon. This information could clarify whether progesterone supplementation is needed (López-Gatius 2005) or can be substituted by GnRH (Andreu-Vázquez *et al.* 2012d). As spontaneous embryo reduction-corpus luteum regression are difficultly predictable, inducing embryo reduction and assessing corpora lutea dynamics would provide a useful model to elucidate any signing or pathway of communication between the dying embryo and its ipsilateral corpus luteum.

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9. Conclusions

In high-producing dairy cows and under our working conditions, a summarized list of conclusions obtained from the studies derived of this thesis included:

1) Cow factors such as lactation number and previous twinning, as well as environmental factors such as photoperiod and season and management related to synchronization protocols affect significantly the incidence of twin pregnancies.

2) Cows delivering twins, besides being less likely to conceive and more likely to be culled in the subsequent lactation, also carry a higher risk of abortion and a reduced mean productive lifespan.

3) Embryo reduction by manual amnion rupture did not carry an additional risk of pregnancy loss for unilateral twin pregnancies, whereas it increases the risk of pregnancy failure in bilateral twin pregnancies.

4) Both the manual amnion rupture (MR) and transvaginal ultrasound-guided aspiration of fetal fluid (TUGA) methods of embryo reduction could be conducted in dairy cattle in order to prevent the negative effects of twin calvings.

10. Epilogue

Embryo reduction in cattle is not a risk-free procedure. Whilst embryo reduction results in acceptable pregnancy failure rates when conducted in women (Lee *et al.* 2008) and mares (Bracher *et al.* 1993; Macpherson and Reimer 2000; Mari *et al.* 2008), pregnancy maintenance following embryo reduction in cattle is still a challenge. Developing new techniques that will be less aggressive to the placental membranes and the remaining co-twin (as could be transvaginal ultrasound-guided intracardiac or intratoracic puncture or injection) and finding the best prophylactic treatment (including exogenous progesterone, GnRH, anti-inflammatory agents or antibiotics) might help to improve pregnancy maintenance. However, it should be noticed that some differential physiological traits of the twin pregnancy in cattle, such as the intimate contact between the twin conceptuses and the inter-chorionic vascular anastomosis developing in an early stage of pregnancy (Williams *et al.* 1963), in addition to the inherent natural risk of pregnancy loss (López-Gatius *et al.* 2009) and the metabolic stress that entails milk production under the current intensive dairy production systems should not be neglected. Therefore, it is reasonable to think that, regardless the technique used or the treatments addressed to support pregnancy maintenance, a certain increased risk of pregnancy loss following embryo reduction might not be avoided. Finally, as long as we reduce the risk of pregnancy loss, the success of embryo reduction arises by preventing cows that maintain pregnancy to term from delivering twins. Thus, and considering the detrimental effects of twinning, to further validate the in-herd applicability and profitability of inducing embryo reduction in cows bearing multiple pregnancies a complete economic model would be needed.

Whenever a dairy commercial farm would not assume the likely increased risk of pregnancy loss, embryo reduction should be rejected and different strategies should be developed to prevent the negative effects of a high twinning rate. Efforts should then be directed towards lowering the chance of a cow to conceive twins. In the equine reproduction field, mares showing two preovulatory follicles during an ultrasonographic examination are discarded for insemination in order to avoid a likely twin conception (Ginther *et al.* 1982). However, delaying the insemination until the next estrus may not be a suitable strategy in dairy cattle because of the high percentage of cows showing double ovulations (López *et al.* 2005; López-Gatius *et al.* 2005) and the economic impact on increasing days open. Alternatively, including an accurate ovarian palpation in order to identify the number of preovulatory follicles and to evacuate the additional ones by manual rupture in artificial insemination routines might be explored. Intrafollicular insemination should also be investigated as it might prevent the risk of a twin conception by increasing the chances of fecundation of the oocyte concerning the punctured follicle (López-Gatius and Hunter 2011).

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11. Annexes

11.1 High milk production is a risk factor for pregnancy loss of unilateral twin pregnancies in dairy cows in northeastern Spain

Abstract. Presented as a poster in the *10º Congreso Internacional AERA* as:

C. Andreu-Vázquez, I. Garcia-Ispierto, G. Bech-Sàbat, and F. López-Gatius (2010). High milk production is a risk factor for pregnancy loss of unilateral twin pregnancies in dairy cows in northeastern Spain. Reproduction in Domestic Animals 45 (Suppl. 2): 84.

In the cow, twin pregnancies have a higher risk of suffering pregnancy loss than single pregnancies. The aim of this study was to analyze factors affecting early foetal loss in 28 twin pregnant lactating cows with both co-twins alive on day 38 post-artificial insemination (AI). Plasma concentrations of pregnancy associated glycoproteins (PAG) and prolactin were determined and ultrasound controls of embryo viability and luteal structures were performed on days 38, 45, 52 and 59 post-AI or until pregnancy loss. Parity, milk production at pregnancy diagnosis (low: <40kg/d vs. high: >40kg/d), inseminating bull, days in milk at conception, season (warm vs. cold), uterine horn laterality of gestation (unilateral vs. bilateral), spontaneous embryo reduction, presence of an additional corpus luteum and plasmatic levels of PAG (low: <8ng/ml vs. high: >8ng/ml) and prolactin (low: <50ng/ml or high: >50ng/ml) on day 38 of pregnancy were considered independent factors. Of the 28 pregnancies studied 8 (28.6%) suffered pregnancy loss. Through binary logistic regression procedures it was determined that, based on the odds ratio, high-producing cows bearing unilateral twins were 32 times more likely to suffer pregnancy loss than low producing cows with bilateral twin pregnancies (1.56-256.05, 95% CI; R^2 Nagelkerke=0.46). Our results show that the interaction high milk production-unilaterality of gestation may be strongly associated with pregnancy failure of twin pregnancies.

11.2 Timing of spontaneous embryo reduction in twin pregnant dairy cows in northeastern Spain

Abstract. Presented as a poster in the *10º Congreso Internacional AERA* as:

C. Andreu-Vázquez, I. Garcia-Ispierto, G. Bech-Sàbat, M.López-Béjar, and F. López-Gatius (2010). Timing of spontaneous embryo reduction in twin pregnant dairy cows in northeastern Spain. Reproduction in Domestic Animals 45 (Suppl. 2): 84.

Spontaneously embryo reduction has been previously described in twin pregnant dairy cows. In order to determine the time when spontaneous reduction occurs, weekly ultrasound controls were performed. Spontaneous reduction –presence of a single alive embryo with his co-twin death following a diagnosis with both twins alive- was diagnosed in 3 of 33 (9.1%), 13 of 88 (14.8%), 3 of 31 (9.7%) and 3 of 24 (12.5%) twin pregnancies on days 28-34, 35-41, 42-48 and 49-55 post-artificial insemination (AI), respectively. No spontaneous reduction was detected in any of the 21, 20 and 19 twin pregnancies performed on days 56-62, 90-96 and 150-156 post-AI. Spontaneous embryo reduction rate did not peak in any of the studied periods. Grouping time periods, the embryo reduction rate was significantly higher on days 28-55 (22 of 176) than on days 56-156 (0 of 60; Fisher exact test $p<0.05$). Twin embryos located in a uterine horn –twin unilateral pregnancies- were more likely to undergo spontaneous reduction than twin bilateral pregnancies (14 of 99 vs. 8 of 137, Fisher exact test $p<0.05$). Our results reinforce the clinical interest of performing an ultrasound pregnancy control on Day 60 post-AI on twin pregnancies to assess the presence of both twins alive.

11.3 Manual amnion rupture: an open window to reduce the twinning rate in dairy cattle

Abstract. Presented as a poster in the 15th Annual ESDAR Conference as:

C. Andreu-Vázquez, I. Garcia-Ispierto, M. López-Béjar, and F. López-Gatius (2011). Manual amnion rupture: an open window to reduce the twinning rate in dairy cattle. Reproduction in Domestic Animals 46 (Suppl. 3): 83.

Embryo reduction emerges as a chance to prevent the negative effects of twining in dairy herds. The risk of pregnancy loss after embryo reduction might depend on the time at which the reduction is performed. The aim of this study was to determine the optimum time (i.e. day post-conception with minimal risk of pregnancy loss) to conduct embryo reduction in cows with unilateral and bilateral twin pregnancies. On day 28 to 41 of gestation embryo reduction was conducted in 73 lactating cows bearing unilateral (n=37) and bilateral (n=36) twins by pressuring transrectally the amniotic vesicle of an embryo between the thumb and the transducer to cause its rupture. Pregnancy loss before day 60 was recorded in 23 unilateral and 8 bilateral twin pregnancies (62.2% vs. 22.2%, χ^2 p<0.01). Time for embryo reduction (mean ± S.D.) did not differ between cows that lost or maintained pregnancy neither in unilateral (33.4 ± 3.1 vs. 33.4 ± 3.7 days, Mann-Whitney p=0.31) nor bilateral twin pregnancies (33.8 ± 3.7 vs. 33.9 ± 4.5 days, Mann-Whitney p=0.98). Logistic regression indicated that gestation laterality significantly affected pregnancy loss. However, no significant effects of the time when embryo reduction is conducted or the interaction time by gestation laterality were found. The results show that embryo reduction may be conducted in both unilateral and bilateral twin pregnancies at any time between days 28 and 41 without differences on subsequent pregnancy maintenance.

11.4 Management factors affecting twin pregnancy in high-producing dairy cows

Abstract. Presented as an oral communication in the XXVII World Buiatrics Congress as:

C. Andreu-Vázquez, I. López-Helguera, I. Garcia-Ispierto, and F. López-Gatius (2012). Management factors affecting twin pregnancy in high-producing dairy cows. Abstracts book: 66.

The aim of this study was to establish potential management risk factors for twin pregnancy in high-producing dairy cows. Over a 9 month period (July 2010- March 2011) 925 positive pregnancy diagnosis (PD) were conducted in a high-producing dairy herd by ultrasonography on day 28-34 post artificial-insemination (AI). Individual health events records, production and reproductive data from single and twin pregnant cows were analyzed through binary logistic regression. Days in milk at conception, service and lactation number and walking activity at estrus were considered as continuous variables. Ovarian structure recorded / estrus synchronization protocol applied over the 28d before pregnant AI (0=none, 1=follicle / progesterone releasing intravaginal device (PRID) for 9d + PGF2α, 2= follicle / PRID for 5 d + PGF2α, 3=mature CL / PGF2α without a previous protocol, 4=mature CL / PGF2α with a previous protocol with PGF2α, 5=mature CL / PGF2α with a previous protocol PRID for 9d + PGF2α , 6=cyst / PGF2α, 7=cyst / PRID for 9d + PGF2α), inseminating bull (14 classes) and pregnancy loss before pregnant AI (0=absence, 1=single pregnancy loss, 2= twin pregnancy loss) were considered categorical variables. Calving difficulty and assistance, twinning and stillbirth at previous calving, retained placenta, clinical metritis, ketosis, hypokalemia, delayed uterine involution or pyometra, estrus and cyst before 50d post-partum, clinical mastitis and fever over the 28d before and after pregnant AI occurrence, GnRH administration (0=at the moment of AI, 1=12h prior to AI), season of AI (Cold=October-April, Warm=May-September) and photoperiod of AI (Decreasing=21st June- 20th December;

Increasing=21st December- 20th June) were considered as dichotomic variables. Twin pregnancy diagnosis was recorded in 115 of the 925 PD (12.4%). Based on the odds ratios, the risk of twin PD was 3.07 times higher for cows that had delivered twins at previous calving and 2.88 times higher for cows that had suffered clinical mastitis before pregnant AI. Furthermore, the likehood of twin PD increased alongside with lactation number (a unit increase in lactation number led to a 1.25-fold increased risk of twin PD). No significant effects of variables regarding milk production, post-partum reproductive performance and clinical disease occurrence or synchronization protocol applied were found. Our results show that, not only individual traits, such as lactation number or previous twinning could be involved in the incidence of twinning. A clinical disease occurring before estrus might somehow disrupt follicular development and dominance. Mastitis could promote double ovulation leading to a subsequent increase in the rate of twin PD.

11.5 Reducción de gestaciones gemelares bilaterales por ruptura manual en vacuno lechero (I). Mantenimiento de la gestación.

Full text (in Spanish). Presented as an oral communication in the *XVII Congreso Internacional ANEMBE de Mecidina Bovina* as:

C. Andreu-Vázquez, I. Garcia-Ispierto, F. López-Gatius (2012). *Reducción de gestaciones gemelares bilaterales por ruptura manual en vacuno lechero (I). Mantenimiento de la gestación*. Libro de ponencias y comunicaciones orales: 242-3.

Abstract

El objetivo de este estudio fue valorar la tasa de mantenimiento de la gestación en 40 vacas con gestaciones gemelares bilaterales reducidas mediante ruptura manual de la vesícula amniótica de uno de los dos embriones en el momento del diagnóstico de gestación. La tasa de pérdida de la gestación a d 90 fue similar para las vacas en las que se realizó la reducción de uno de los dos embriones, para las vacas con gestaciones gemelares unilaterales y bilaterales no reducidas y para las vacas con gestación simple (15%, 17,5%, 22,5% y 10%, respectivamente, P= 0,496). La reducción embrionaria de las gestaciones gemelares bilaterales en el momento del diagnóstico supone una oportunidad para disminuir el número de partos dobles en la explotación sin que la técnica comporte un riesgo adicional para el mantenimiento de la gestación.

Introducción

Los partos gemelares suponen dificultades de manejo en las explotaciones de vacuno lechero y tienen un impacto negativo sobre la economía de la explotación (1-3). El riesgo de pérdida de la gestación para las vacas con gestaciones gemelares es hasta 6 veces superior al de las vacas con gestaciones simples (4,5) y los partos gemelares son la principal causa de distocia y desórdenes postparto como la retención placentaria (6,7). En las últimas décadas numerosos autores han constatado la creciente incidencia de partos gemelares; que actualmente se cifra en torno al 9-12%, y pronostican un aumento sostenido en los próximos años (8). La reducción embrionaria brinda la oportunidad de transformar una gestación múltiple en una simple y se presenta como una herramienta para disminuir la tasa de partos doble y evitar los efectos negativos que comportan. La inducción de la reducción embrionaria, que se ha desarrollado en medicina humana como un tratamiento en gestaciones múltiples de riesgo y es una práctica habitual en medicina equina, se ha aplicado recientemente en el vacuno lechero (9,10). El objetivo de este estudio fue valorar la tasa de mantenimiento de la gestación en vacas con gestaciones gemelares bilaterales reducidas mediante ruptura manual de la vesícula amniótica de uno de los dos embriones en el momento del diagnóstico de gestación.

Material y métodos

El estudio fue realizado en una explotación comercial de Lleida de 640 vacas en lactación y con 3 ordeños diarios, a largo del año 2011. La producción media anual por vaca, la tasa de eliminación y la tasa de gestaciones gemelares para este periodo fueron de 11350 kg, 27% y 20,5%, respectivamente. La explotación cuenta con un programa reproductivo con controles semanales. En las 2 primeras semanas postparto se realizan controles diarios para diagnosticar y tratar cualquier patología puerperal. Entre los d 35-50 postparto las vacas se examinan para evaluar la involución y contenido uterinos y las estructuras ováricas. Los desórdenes (involución uterina incompleta, endometritis, piómetra y quistes ováricos) son tratados hasta su resolución. Únicamente las vacas sanas son inseminadas. El periodo de espera voluntario es de 50 d. El diagnóstico de gestación se realizó mediante ecografía transrectal a d 28-34 post IA. La presencia de gemelos se registró tras la observación de dos embriones situados en diferentes posiciones en un mismo cuerno uterino, gestación gemelar unilateral, o un embrión situado en cada cuerno, gestación gemelar bilateral. La viabilidad de los embriones se valoró detectando el latido cardiaco. Se excluyeron del estudio todas las gestaciones con uno o más embriones muertos al momento del diagnóstico. Se administró una dosis de GnRH (100µg im, Cystoreline, CEVA Salud Animal, Barcelona) a todas las vacas con diagnóstico de gestación gemelar. A las vacas con diagnóstico gemelar bilateral se les administró 1250 mg de Flunixin im (Flunex Industrial Veterinaria, Barcelona) y se realizó la reducción embrionaria presionando manualmente la vesícula amniótica de uno de los 2 embriones hasta causar su ruptura. La muerte del embrión se constató mediante la desaparición del latido cardiaco. La reducción embrionaria fue siempre realizada por el mismo operador. Las vacas no mostraron signos de dolor o incomodidad durante el procedimiento de reducción, cuya duración fue siempre inferior a 20 segundos. La presencia y la viabilidad de los embriones se evaluaron mediante ecografía a d 56-62 de gestación y a d 90-96 y 150-156 se realizaron controles por palpación para confirmar la gestación. Cuando alguno de estos controles resultó negativo se registró la fecha de la pérdida de la gestación.

Se evaluó la tasa de mantenimiento de la gestación en 4 grupos de vacas con gestaciones: [1] gemelares bilaterales reducidas, [2] gemelares bilaterales control, [3] gemelares unilaterales control, y [4] gestaciones simples. El mantenimiento de la gestación a d 90 se evaluó para 40 animales de cada grupo mediante análisis de supervivencia Kaplan-Meier. Las diferencias entre grupos en el porcentaje de pérdida de gestación acumuladas a d 90 se determinaron mediante test de Chi-cuadrado. Todos los procedimientos estadísticos se realizaron usando el programa PASW Statistics 18 (SPSS Inc., Chicago, IL, USA) con un nivel de significación de 0,05.

Resultados y discusión

Frente a las numerosas ventajas que tanto a nivel de manejo como en términos económicos derivados de la eficiencia reproductiva postparto puede suponer la posibilidad de disminuir el número de partos gemelares en una explotación mediante la reducción embrionaria de las gestaciones múltiples, surge la necesidad de valorar si la práctica puede incrementar la tasa de pérdidas de la gestación. La reducción embrionaria implica la manipulación del útero gestante en un momento delicado de la gestación en el que la placentación aún es incompleta (11). No obstante, nuestros resultados muestran porcentajes de pérdida similares para las vacas en las que se realizó la reducción de uno de los dos embriones y para las vacas con gestaciones gemelares unilaterales y bilaterales no reducidas y para las vacas con gestación simple (15%, 17,5%, 22,5% y 10% para los grupos 1-4 respectivamente, P= 0,496) y que el tiempo medio de supervivencia no resultó significativamente diferente entre los grupos estudiados (Log Rank Mantel-Cox P=0.468, FIGURA 1). La tasa de pérdida para las vacas con gestación bilateral reducida es ligeramente inferior a la que se reporta en un estudio anterior (10) por lo que cabe pensar que la práctica del operador contribuye positivamente al éxito de la técnica.

En conclusión, la reducción embrionaria de las gestaciones gemelares bilaterales en el momento del diagnóstico supone una oportunidad para disminuir el número de partos dobles en la explotación y sin que la técnica comporte un riesgo adicional para el mantenimiento de la gestación.

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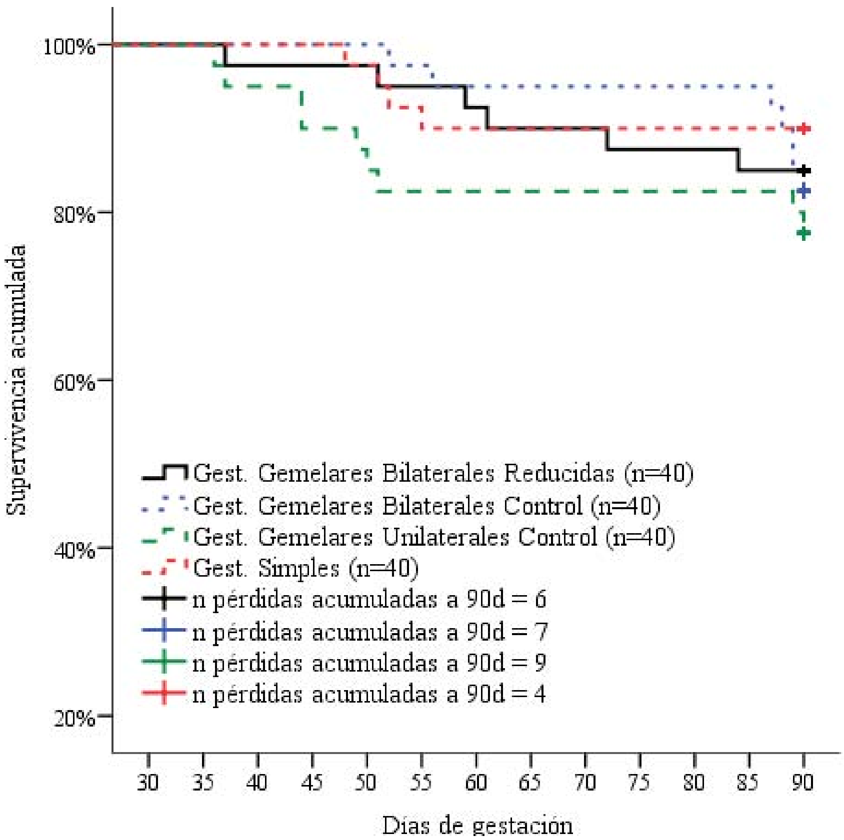

Figura 1. Curvas de supervivencia Kaplan-Meier para las gestaciones gemelares bilaterales reducidas, bilaterales control, unilaterales control y gestaciones simples. Porcentaje de vacas que mantienen la gestación (eje X) a lo largo del tiempo hasta el d 90 de gestación (eje Y).

11.6 Reducción de gestaciones gemelares bilaterales por ruptura manual en vacuno lechero (II). Parámetros reproductivos tras la gestación a término.

Full text (in Spanish). Presented as an oral communication in the *XVII Congreso Internacional ANEMBE de Mecidina Bovina* as:

C. Andreu-Vázquez, I. Garcia-Ispierto, F. López-Gatius (2012). *Reducción de gestaciones gemelares bilaterales por ruptura manual en vacuno lechero (II). Parámetros reproductivos tras la gestación a término.* Libro de ponencias y comunicaciones orales: 244-5.

Abstract

El objetivo de este estudio fue evaluar la eficiencia reproductiva postparto de vacas con gestaciones gemelares bilaterales reducidas mediante ruptura manual de la vesícula amniótica de uno de los dos embriones en el momento del diagnóstico de gestación. El porcentaje de nacidos muertos, retención placentaria, metritis durante las 2 primeras semanas postparto y falta de involución uterina o piómetra en los d 35-50 postparto fueron significativamente inferiores en el grupo de vacas con un ternero al parto (n=30; 15 vacas con gestación gemelar reducida y 15 vacas con gestación simple) que en las vacas con partos gemelares (n=30). Las vacas con un único ternero al parto presentaron intervalos parto-1aIA y parto-IA gestante significativamente inferiores a los de las vacas con parto gemelar. La reducción embrionaria de las gestaciones gemelares bilaterales supone una oportunidad para disminuir el número de partos dobles en la explotación y es efectiva en la prevención de los desordenes reproductivos del postparto.

Introducción

Los partos gemelares suponen dificultades de manejo en las explotaciones de vacuno lechero y tienen un impacto negativo sobre la economía de la explotación (1-3). En las últimas décadas numerosos autores han constatado la creciente incidencia de partos gemelares; que actualmente se cifra en torno al 9-12%, y pronostican un aumento sostenido en los próximos años (4). La reducción embrionaria brinda la oportunidad de transformar una gestación múltiple en una simple y se presenta como una herramienta para disminuir la tasa de partos doble y evitar los efectos negativos que comportan. La ruptura manual de la vesícula amniótica de uno de los dos embriones es una práctica habitual en medicina equina, y recientemente ha sido aplicada al vacuno lechero para reducir gestaciones gemelares bilaterales y unilaterales durante el periodo embrionario tardío (5,6). El objetivo de este estudio fue valorar la efectividad de la estrategia de reducción embrionaria en vacas con gestaciones gemelares bilaterales implementada en una explotación comercial a lo largo de un año comparando la eficiencia reproductiva postparto de vacas con un ternero al parto y vacas con partos gemelares.

Material y métodos

Para este estudio se comparó la incidencia de desordenes reproductivos (nacidos muertos, retención de placenta, metritis en las 2 semanas postparto y falta de involución o piómetra a d 35-50 postparto) y los intervalos partos-1ª IA, parto-IA gestante y número de IA de un grupo de 30 vacas con un ternero al parto (n=30; 15 vacas con gestación gemelar reducida y 15 vacas con gestación simple) y vacas con partos gemelares (n=30) de una explotación comercial de Lleida. El número de vacas en lactación, la producción media anual por vaca, la tasa de eliminación y la tasa de gestaciones gemelares para el periodo de estudio fueron 640, 11350 kg, 27% y 20,5%, respectivamente. La explotación cuenta con un programa reproductivo con controles semanales.

En las 2 primeras semanas postparto se realizan controles diarios para diagnosticar y tratar cualquier patología puerperal. Entre los d 35-50 postparto las vacas se examinan para evaluar la involución y contenido uterinos y las estructuras ováricas. Los desórdenes reproductivos son tratados hasta su resolución y únicamente las vacas sanas son inseminadas tras un periodo de espera voluntario de 50d. El diagnóstico de gestación se realiza mediante ecografía transrectal a d 28-34 post IA. La presencia de gemelos se registra tras la observación de dos embriones situados en diferentes posiciones en un mismo cuerno uterino, gestación gemelar unilateral, o un embrión situado en cada cuerno, gestación gemelar bilateral. Todas las vacas con diagnóstico de gestación gemelar reciben una dosis de GnRH (100µg im, Cystoreline, CEVA Salud Animal, Barcelona). La reducción embrionaria en vacas con diagnóstico gemelar bilateral, a las que se les administra 1250 mg de Flunixin im (Flunex Industrial Veterinaria, Barcelona), se realiza presionando manualmente la vesícula amniótica de uno de los 2 embriones hasta causar su ruptura. Al término de la reducción embrionaria, cuya duración es inferior a 20 s y durante la cual los animales no muestran ningún signo de incomodidad o dolor, se constata la muerte del embrión mediante la desaparición del latido cardiaco. Se realizan controles a d 60, 90 y 150 de gestación.

Para las vacas incluidas en el estudio se registró la fecha de parto, el número de terneros y las incidencias al parto (terneros nacidos muertos) y los desordenes postparto ocurridos en las 2 semanas siguientes (retención de placenta y metritis). Para las vacas con más de 50 d en leche al finalizar el estudio se registró la revisión a 35-50 d postparto y la fecha a la primera IA. En aquellas vacas gestantes al finalizar el estudio se registró la fecha y el número de IA gestante. Las diferencias entre grupos en la incidencia de nacidos muertos, retención de placenta, metritis en las 2 semanas postparto y falta de involución o piómetra a d 35-50 postparto se determinaron mediante test de Chi-cuadrado. Las diferencias entre grupos en la duración del intervalo parto-1ªIA, parto-AI gestante y número de IA se determinaron mediante T-Student para las vacas inseminadas y gestantes al finalizar el estudio, respectivamente. Todos los procedimientos estadísticos se realizaron usando el programa PASW Statistics 18 (SPSS Inc., Chicago, IL, USA) con un nivel de significación de 0,05.

Resultados y discusión

En el grupo de vacas con un ternero al parto (gestación gemelar reducida y gestación simple) la incidencia de nacidos muertos, retención placentaria, metritis y falta de involución uterina o piómetra en los días 35-50 postparto fue significativamente inferior que en las vacas con parto gemelar (TABLA 1). Las patologías del postparto son la principal causa de infertilidad en las vacas lecheras de alta producción y están relacionadas también con la posterior pérdida embrionaria (7). La retención de placenta, que en nuestra área geográfica sucede entre el 45% y el 63,7% tras un parto gemelar (8,9) y que alcanza el 70% en el presente estudio, comporta un incremento en la duración y los costes del postparto y se relaciona con un incremento en los días abiertos (10,11). Los partos gemelares, en su mayoría distócicos, resultan en un mayor porcentaje de terneros nacidos muertos y aumentan el riesgo de metritis, piómetra y falta de involución que retrasan la fecha de la primera inseminación. Los intervalos parto-1aIA y parto-IA gestante para las vacas que a al término de este estudio habían finalizado el tiempo de espera voluntario y quedaron gestantes, respectivamente, fueron significativamente superiores tras un parto gemelar que tras un parto simple (TABLA 2).

En conclusión, nuestros resultados constatan una peor eficiencia reproductiva postparto en vacas con partos gemelares. La reducción embrionaria de las gestaciones gemelares bilaterales en el momento del diagnóstico se presenta como una estrategia efectiva para disminuir el número de partos dobles en la explotación y prevenir los desordenes reproductivos del postparto.

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Tabla 1. Incidencia de desordenes en el postparto en vacas con parto simple o gemelar.

	Partos simples Bilaterales Reducidas (n=15) + Simples (n=15)	**Partos gemelares** Bilaterales Control (n=15) + Unilaterales Control (n=15)
Nacidos muertos (%)	0,0[a]	26,7[b]
Retención de placenta (%)	10,0[a]	70,0[b]
Metritis (%)	20,0[a]	83,3[b]
Falta de involución/piómetra (%)	10,0[a]	70,0[b]

[a,b] Indica diferencias significativas entre los valores de las dos columnas (Chi-Cuadrado P<0,05)

Tabla 2. Eficiencia reproductiva postparto en vacas con parto simple o gemelar (media ± D.E.)

	Partos simples Bilaterales Reducidas (n=8) + Simples (n=13)	**Partos gemelares** Bilaterales Control (n=13) + Unilaterales Control (n=8)
Intervalo parto-1aIA (d)	62,81 ± 8,79[a]	70,14 ± 13,39[b]
Intervalo parto-IAgestante (d)	82,22 ± 20,32[a]	117,73 ± 43,73[b]
Número de IA (n)	1,67 ± 0,5	2,45 ± 1,21

[a,b] Indica diferencias significativas entre los valores de las dos columnas (T-Student P<0,05)

11.7 Control ecográfico de la gestacion. El problema de las gestaciones gemelares

Full text (in Spanish). Presented as a presentation in the *XVII Congreso Internacional ANEMBE de Mecidina Bovina* as:

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Abstract

A pesar de la alta incidencia de pérdida de las gestaciones gemelares, la tasa de partos dobles se ha ido incrementando con la producción de leche en las últimas décadas. Ambos casos, pérdida de gestación y partos dobles, suponen grandes pérdidas económicas para la explotación de ganado vacuno lechero. En esta presentación consideraremos aspectos clínicos del problema de la gestación gemelar, un acercamiento a la reducción del número de embriones y, finalmente, se proponen aspectos de manejo del control de la gestación para reducir el riesgo de pérdida de la misma durante el periodo fetal temprano.

Introduccion

Como se ha indicado en la presentación anterior, las gestaciones gemelares son probablemente el principal factor relacionado con la pérdida de la gestación durante el periodo fetal temprano. Las vacas gestantes con dos embriones tienen un riesgo de pérdida de hasta siete veces más alto que las que gestan un solo embrión (López-Gatius *et al*., 2002, 2006; García-Ispierto *et al*., 2006; Silva delRio *et al*., 2009). Se han registrado hasta casi un 60% de pérdidas durante el periodo cálido (López-Gatius *et al*., 2004a; Andreu-Vázquez *et al*., 2011), siendo de cinco a nueve veces superior el riesgo para la gestaciones unilaterales que para las bilaterales (López-Gatius y Hunter, 2005; Andreu-Vázquez *et al*., 2011). En contraste a lo que ocurre con las gestaciones con un solo embrión, en las que la mayor parte de las pérdidas ocurren antes del día 60, en las gestaciones gemelares, las pérdidas pueden ocurrir hasta el día 90 de gestación (López-Gatius *et al*. 2004a; López-Gatius y Hunter, 2005; López-Gatius *et al*. 2010). Esto último, probablemente debido a que cuando muere sólo uno de los dos gemelos, el otro puede mantener la gestación durante un cierto periodo (López-Gatius *et al*. 2004ª)

A pesar de la alta incidencia de pérdida de las gestaciones gemelares, la tasa de partos dobles se ha ido incrementando con la producción de leche en las últimas décadas (Nielen *et al*., 1989; Kinsel *et al*., 1998). Una tasa de partos dobles superior al 9% se relacionó con una mayor producción de leche en un estudio sobre 52362 lactaciones (Kinsel *et al*., 1998). Dada la baja incidencia de gestaciones gemelares monocigóticas en la vaca (Silva del Rio *et al*., 2006), la tasa de partos dobles está lógicamente ligada a la tasa de ovulación doble. En vacas de alta producción, la tasa de ovulación doble puede exceder el 20% (Fricke y Wiltbank, 1999) o incluso el 25%, si se trata de vacas en su tercera lactación o más (López-Gatius *et al*., 2005b). La genética parece ser un factor determinante en el incremento de los partos dobles. En un estudio sobre 37174 toros y 1324678 partos se demostró cómo la descendencia de los toros nacidos después de 1990 sufría una mayor incidencia de partos dobles que la de los nacidos antes de 1980 (Johanson *et al*., 2001). Es razonable sugerir que el incremento de partos dobles los últimos años esté relacionado con la selección hacia el incremento de producción de leche. Pero, paralelas al progreso genético, mejoras en la nutrición y en las prácticas de manejo han favorecido también el incremento de la producción. Probablemente, el manejo necesario para una alta producción vaya acompañado de un descenso de las pérdidas de gestación en el caso de gestaciones gemelares. De una forma u otra, podemos esperar un incremento de los partos dobles acompañando al incremento de producción de leche en los próximos años.

La gestación gemelar es no deseable en ganado vacuno lechero por sus efectos negativos como riesgo de aborto, distocia, retención de placenta, muerte perinatal, freemartinismo, costes terapéuticos post-parto y un retraso en el retorno a la ciclicidad tras el parto (Nielen *et al*., 1989). Los efectos negativos de la gestación gemelar deberían ser menores si intentáramos reducir el número de embriones. Estamos dirigiendo los últimos años nuestros esfuerzos hacia este punto. En esta presentación consideraremos aspectos clínicos del problema de la gestación gemelar, un acercamiento a la reducción del número de embriones y, finalmente, se propondrán aspectos de manejo del control de la gestación para reducir el riesgo de pérdida de la misma durante el periodo fetal temprano.

El problema del diagnóstico de las gestaciones gemelares

El principal problema en el diagnóstico de una gestación gemelar, es que los dos embriones deben ser claramente localizados. Aunque el uso de la ecografía permite detectar con relativa facilidad el embrión entre los días 25 y 30, la seguridad en el caso de gestaciones dobles disminuye y no se alcanza hasta el día 30 (López-Gatius y Garcia-Ispierto, 2010). El embrión se sitúa muy próximo a la pared uterina durante el primer mes de gestación y a veces resulta difícil localizar, incluso en gestaciones con un único embrión en vacas viejas (Hughes y Davies 1989). El día 30, el embrión ya suele estar rodeado completamente de líquido, haciendo más sencillo el diagnóstico de una gestación gemelar. Indicador de la presencia de gemelos puede ser un exceso de membranas. En el caso de gemelos unilaterales, es frecuente observar, y fácilmente seguir, una línea hiperecogénica de embrión a embrión (“siga la línea”) que representa el área de aposición entre las dos membranas coriónicas (López-Gatius y Garcia-Ispierto, 2010).

Dos tipos de error pueden ocurrir en vacas con dos o más cuerpos lúteos: diagnóstico de gestación con un solo embrión en vacas con gestación gemelar y, todo lo contrario, diagnóstico de gestación gemelar en vacas con un único embrión. El segundo caso normalmente es debido a que el segundo embrión realmente es el primero que se ha movido del primer lugar de la pantalla. Ambos tipos de error pueden superar el 5% de los diagnósticos positivos. En el caso de gestaciones gemelares bilaterales, la precisión del diagnóstico suele ser del 100%. Por tanto, se debe realizar siempre un esfuerzo adicional para localizar dos embriones en vacas con dos o más cuerpos lúteos. Recordamos aquí, que aunque con una baja incidencia, podemos encontrar gestaciones dobles con un único cuerpo lúteo, probablemente gemelos monocigóticos. En este caso, tenemos la ventaja que son gestaciones unilaterales y podemos "seguir la línea".

Aspectos clínicos del diagnóstico de gestación gemelar

Aunque la reducción espontánea de gemelos se ha descrito en la vaca (López-Gatius y Hunter, 2005), la presencia de uno de los dos embriones muerto en el momento del diagnóstico de gestación, con una incidencia de hasta el 20%, se relaciona con un nivel de pérdida que puede superar el 60% (López-Gatius *et al*., 2009). En estos casos, un tratamiento en el momento del diagnóstico con GnRH puede favorecer el mantenimiento de la gestación (Bech-Sàbat *et al*. 2009; López-Gatius *et al*. 2009). Es interesante hacer notar que la mayor parte de las muertes embrionarias (uno de los dos embriones) ocurre antes del día 35 de gestación. Así, el destino de las gestaciones gemelares que desarrollan normalmente hasta el día 60 es el parto gemelar, o aborto durante un periodo avanzado de la gestación. No parece ocurrir la muerte de uno de los dos fetos a partir de ese periodo. Por tanto, la detección de gemelos vivos el día 60 tiene enormes implicaciones para el manejo de una explotación. Por ejemplo, ya que las gestaciones gemelares finalizan unos siete días antes que las sencillas (Anderson 1978), el periodo de secado puede ser avanzado varios días para las vacas con gestaciones dobles. Cuidado adicional al parto puede reducir, además, el riesgo de mortalidad del ternero en gestaciones dobles.

Implicaciones clínicas de la reducción gemelar inducida

Transformar una gestación múltiple en sencilla debería prevenir de les efectos negativos del parto doble. En este sentido, se han desarrollado técnicas eficaces en la mujer y en la yegua. Sin embargo, un aspecto diferencial en la vaca es el hecho de una anatomosis vascular temprana entre las dos membranas coriales (Williams *et al*., 1963; Echternkamp, 1992). Esto incrementa el riesgo de pérdida de la gestación cuando intentamos una reducción en esta especie. Aplicada con éxito en la yegua, la ruptura manual del amnios se intentó en la vaca a los 35-41 días de gestación (López-Gatius, 2005). La ruptura del amnios sin tratamiento adicional resultó en un 100% de pérdidas (11/11), mientras que el procedimiento de ruptura acompañado de un tratamiento con progesterona permitió la reducción gemelar: 4 de 11 animales mantuvieron la gestación. Sin embargo, en una de estas gestaciones sobrevivientes, el embrión sobrevivió a la ruptura del amnios y la vaca parió gemelos. En un estudio más amplio (Andreu-Vázquez *et al*., 2011), para las gestaciones gemelares unilaterales, la pérdida de la gestación tras la ruptura del amnios con un tratamiento de progesterona fue semejante en las vacas tratadas que en las no tratadas. Sin embargo, con la ruptura se incrementaron significativamente las pérdidas en gestaciones bilaterales. En un trabajo más reciente (Andreu-Vázquez *et al*., 2012), comparando la ruptura manual con la aspiración transvaginal de fluido amniótico-alantoideo guiada por ecografía de uno de los embriones, los resultados sugieren que la aspiración transvaginal eco-guiada con una aguja 22-G debería ser el método a elegir para la reducción embrionaria en la vaca.

Controlando la gestación para reducir el riesgo de la pérdida fetal temprana

Desde un punto de vista clínico, para intentar reducir el riesgo de pérdida fetal temprana, se sugieren los puntos:

- En el momento del diagnóstico de gestación (por ejemplo, los días 28-34 post-inseminación), es tan importante el registro del número de cuerpos lúteos como el número y viabilidad de los embriones. Los dos cuernos uterinos deben ser siempre inspeccionados para detectar la posible presencia de uno o dos embriones.

- En explotaciones con alta incidencia de pérdida fetal temprana, de causa no infecciosa, se puede aplicar un tratamiento con progesterona a vacas con un único cuerpo lúteo y con GnRH a vacas con gestaciones gemelares. El tratamiento no es necesario en vacas gestantes con un cuerpo lúteo adicional (Número mayor de cuerpos lúteos que embriones).

- La confirmación por ecografía del desarrollo normal de la gestación el día 60, especialmente en las gestaciones dobles, debería permitir un registro muy preciso de ambos, gestaciones dobles y pérdidas de gestación.

- El continuo control del toro de inseminación, como factor de riesgo de las pérdidas, debería reducir el número de las mismas.

- Finalmente, pero no lo menos importante, confirmar es estro en el momento de la inseminación. Muchas vacas gestantes pueden manifestar estro como las no gestantes.

Conclusiones

Es completamente esencial el uso del ecógrafo tanto para el diagnóstico, como para el control de la evolución de las gestaciones gemelares. Se está abriendo una posibilidad de reducción de las gestaciones gemelares, tanto por ruptura manual como por aspiración transvaginal eco-guiada de líquido amniótico-alantoideo.

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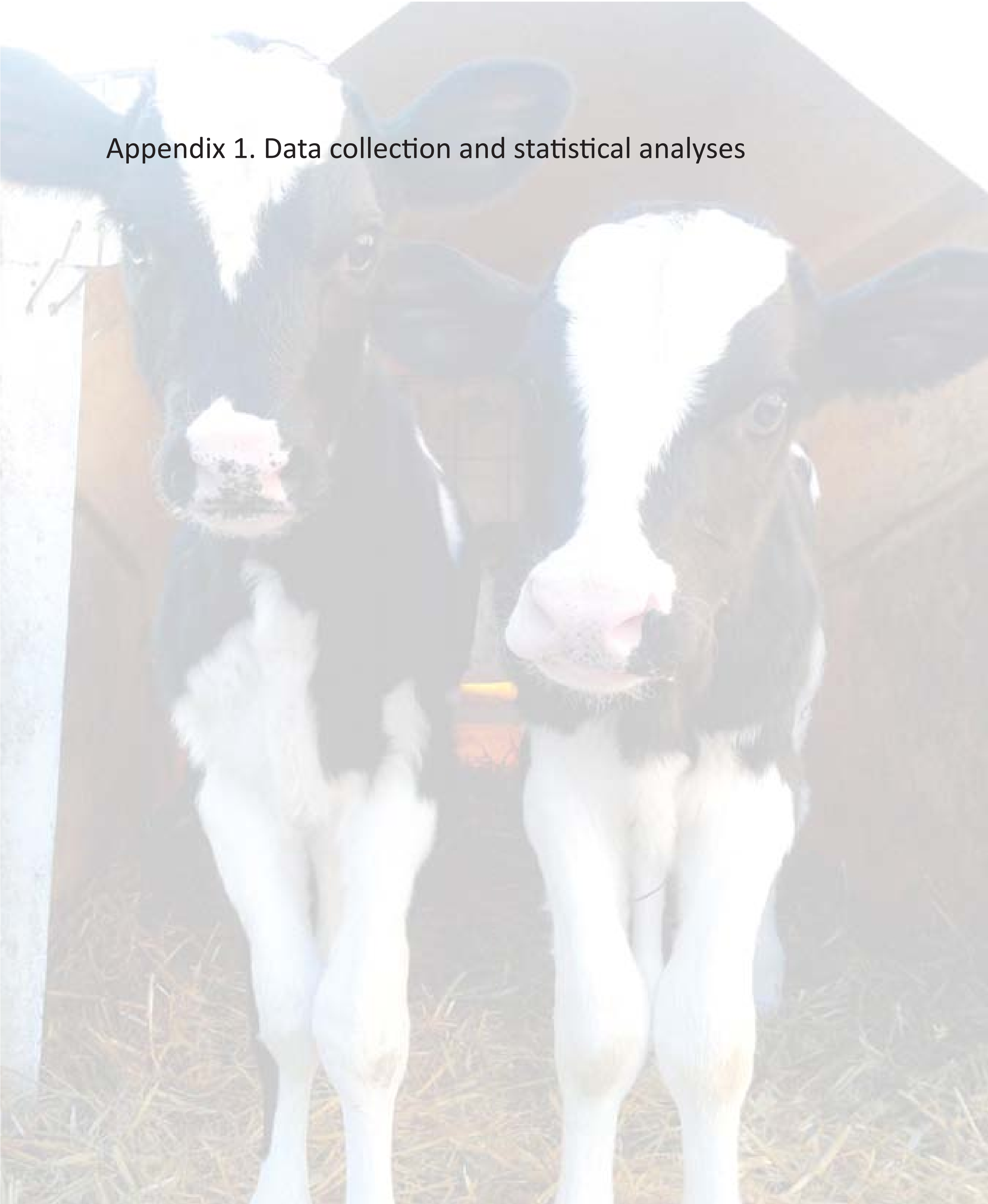
Appendix 1. Data collection and statistical analyses

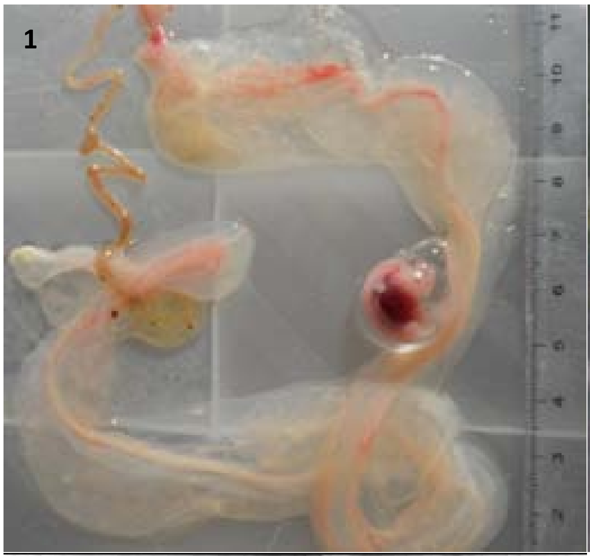


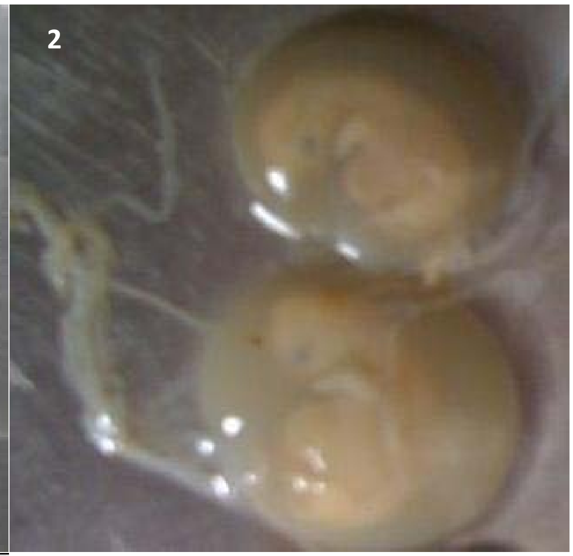
Data used to perform the research studies included in this thesis were obtained from a reproductive control program based on a weekly visit and the productive records from each herd database. Only herds in which computerized reliable an accurate data including both productive and reproductive individual records and global reproductive indexes were available were considered for the studies. Although the statistical procedures used in this thesis are described in the materials and method section of each study, this appendix provides further information on the basis of the use of binary logistic and Cox regression, Kapplan Meier survival curves and general linear models for repeated measures.

When assessing a dichotomic variable (such as pregnancy loss/pregnancy maintenance following the treatment or presence/absence of twin embryos at pregnancy diagnosis) simple univariate analyses (through Chi square or Fisher's Exact tests) could provide rapid and useful information about whether there are or not differences between the control and the treated group or in the incidence of twins among the different groups of study. However, as most biological processes are complex, the simple comparison of the percentages may hide the effect of other factors and led to wrong results. Therefore, considering as much additional data regarding factors that may affect the success of a treatment or the nature of a disorder through a multivariate analysis approach is crucial to obtain precise results, especially when experimental studies are conducted in commercial herds. Logistic regression procedures allow analyzing possible effects of numerous factors (independent variables) and their interactions affecting a dependent dichotomic variable, as is twin pregnancy or pregnancy loss following embryo reduction. Coefficients resulting from the logistic regression final model provide the odds ratio (OR, defined as the relative risk of an event happening in one group compared to the odds of the same event happening in another group) for each factor that significantly affects the event. Thus, for a dichotomous variable an odds ratio significantly higher (or lower) than 1 indicates an increased (or reduced) risk of an event happening if the factor is present. For continuous variables, an odds ratio significantly higher (or lower) than 1 implies an increased (or reduced) risk of the event happening with each 1 unit increase in the value of this factor. For class variables, one class of each variable was considered as the reference, and an odds ratio significantly higher (or lower) than 1 for any other class of this variable was indicative of an increased (or reduced) risk of the event happening when compared to the reference class. With a similar modeling procedure and interpretation, Cox regression analysis provides the relative risk (RR) for the significant factors (independent variables) affecting the occurrence of an event (dependent variable) when the time of the event is also relevant (as it is the case of the time for conception or being culled following parturition). Kaplan Meier survival curves allow an overall comparison of the "velocity" at which the event (conception or culling) occurs depending on a certain variable of interest, with graphs that show the percentage of animals in which the event has happened (Y-axis) across time (X-axis). Both, Kaplan Meier and Cox regression analyses permit the inclusion of censored cases (i.e. individuals that leave the study before the end of the data collection or in which the event did not occur by the end of the study period). Furthermore, Log-Rank test allows determining if there is a significant difference in the median time at which the event happens for each group studied (i.e. the time at which the event has occurred in 50% of the subjects of each group).

As dichotomic variables, continuous variables (such as milk production, walking activity at estrus or days in milk at conception) might be affected by several factors. Univariate analyses (through T-Student test or ANOVA) could lead to partially equivocal results when comparing the means for two or more groups of study and the use of multivariate analyses (mixed models or general lineal models) is preferred. Equally important than the multifactorial approach is to consider the individual effects when the continuous variable assessed is measured for each subject at several moments (as it is the case of endocrine parameters such as plasmatic levels of progesterone, PAGs or prolactin). Repeated measures models allows finding significant differences in the profile of hormones across time (within effect) and among groups of study (between effect) after correcting the individual effects.

Appendix 2. Images

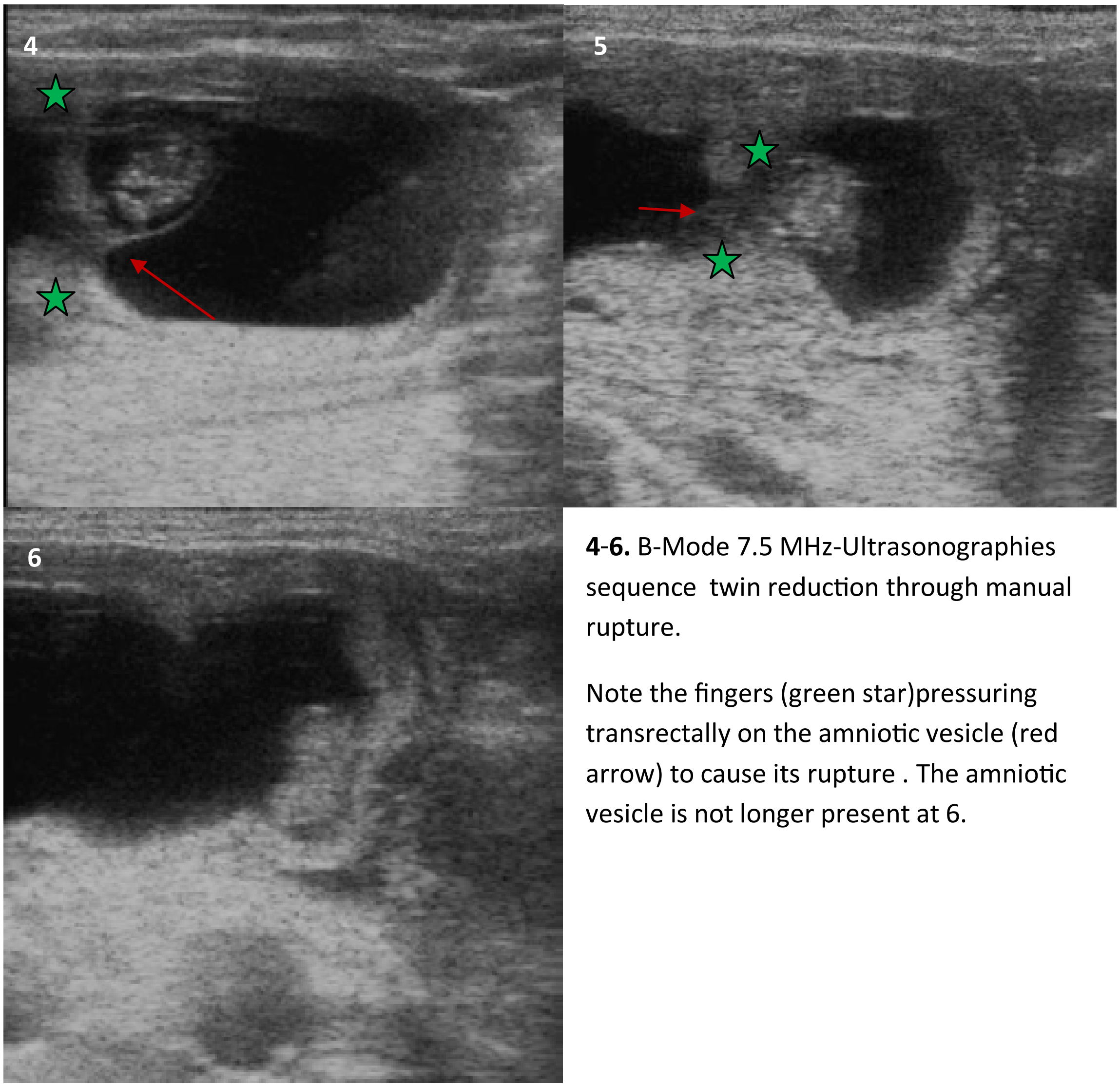




1. Singleton pregnancy loss (33d post AI)

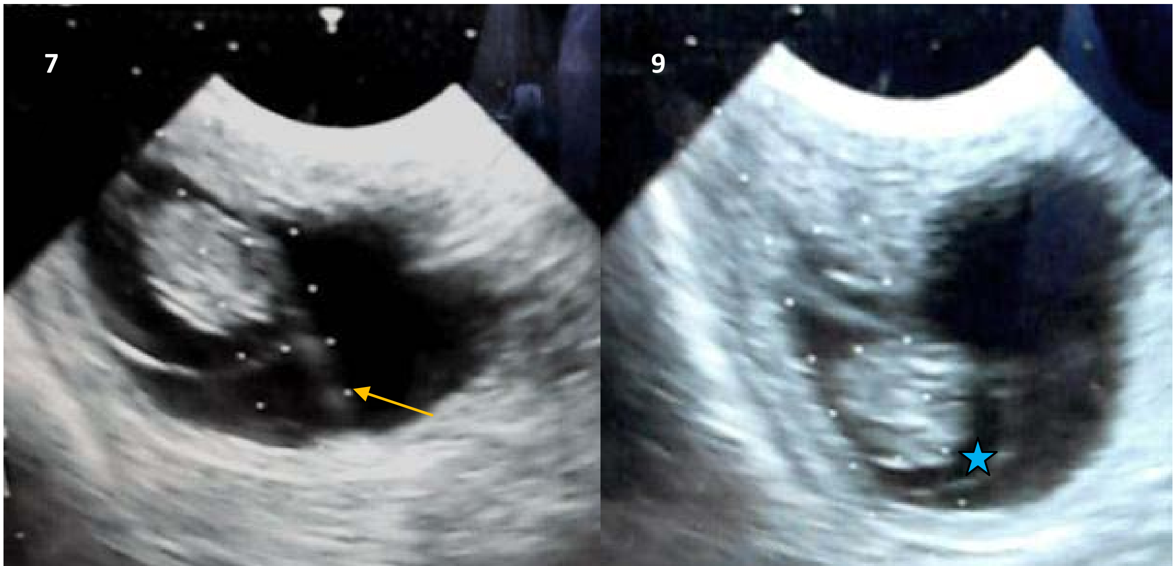
2. Twin pregnancy loss (46d post AI)

3. B-Mode 7,5 MHz-Ultrasonograph. Unilateral twin embryos at pregnancy diagonosis (28-34 d post AI)



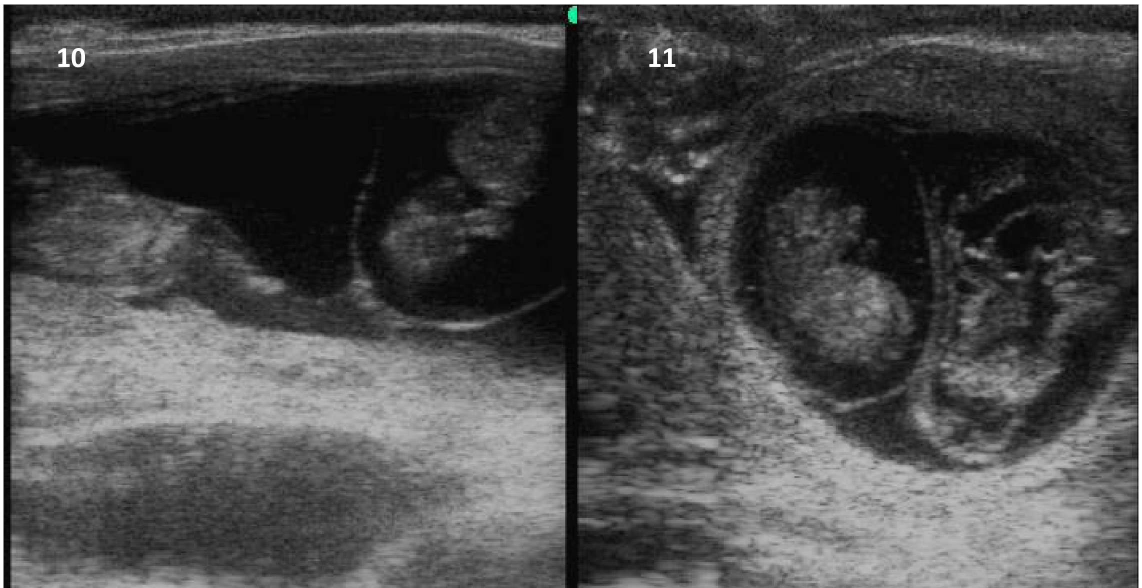
4-6. B-Mode 7.5 MHz-Ultrasonographies sequence twin reduction through manual rupture.

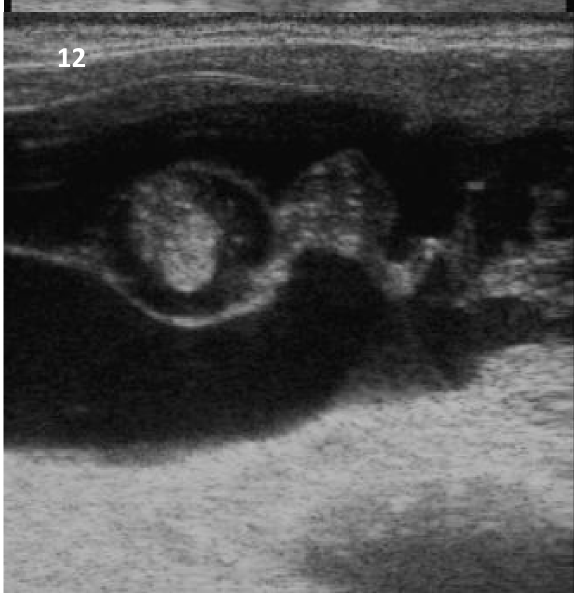
Note the fingers (green star)pressuring transrectally on the amniotic vesicle (red arrow) to cause its rupture . The amniotic vesicle is not longer present at 6.



7-9. B-Mode 7.5MHz-Ultrasonograph sequence twin reduction through transvaginal ultrasound guided aspiration

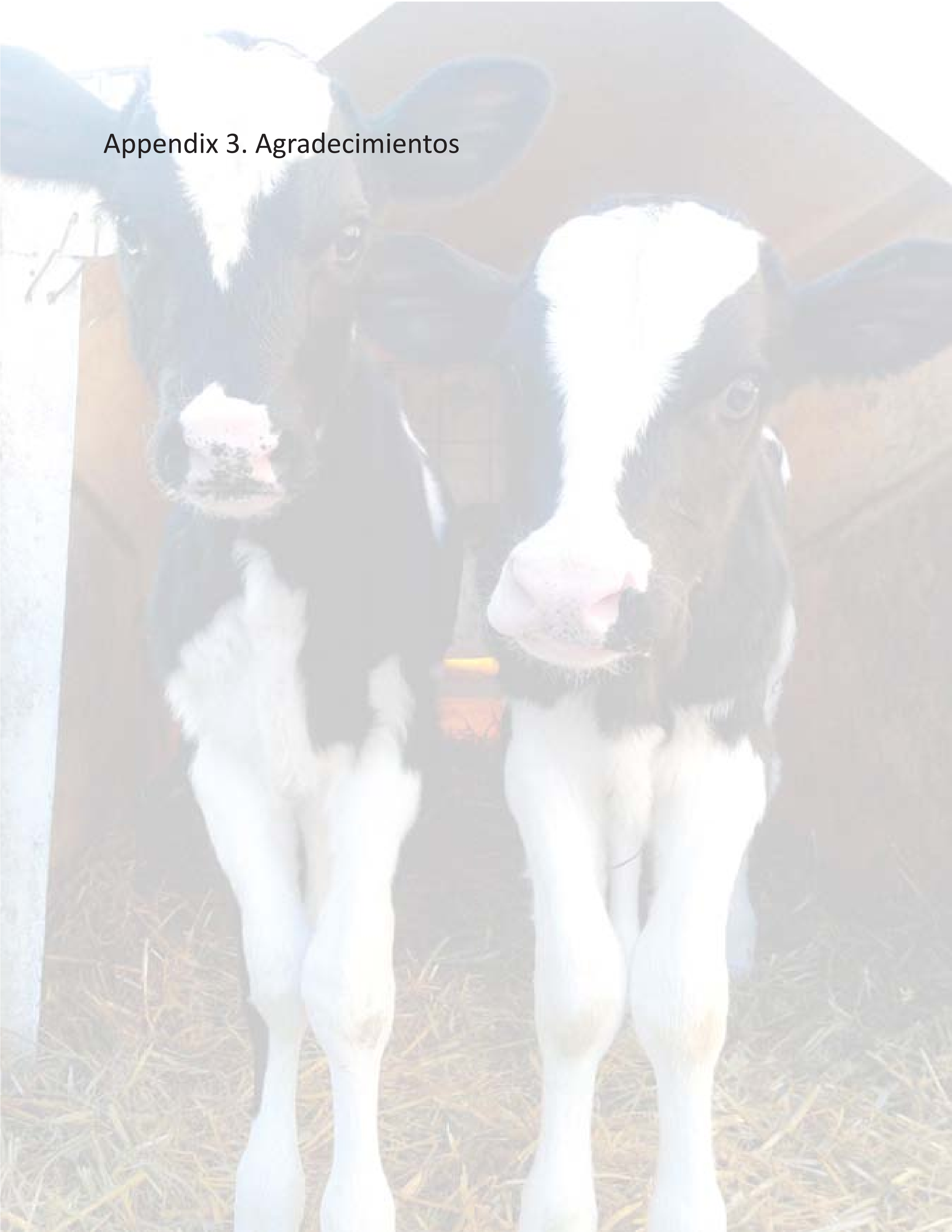
Note the placement of the ecogenic tip of the needle (yellow arrow) and the reduction in the volume of amniotic fluid (blue star)





10-12. B-Mode 7.5MHz-Ultrasonographies showing detritus from the death co-twin up to 2 weeks post embryo reduction (either spontaneous or induced)

Appendix 3. Agradecimientos



En un primer momento valoré la posibilidad de no incluir un apartado de agradecimientos en esta tesis, en un intento desesperado por mantener mi intimidad. Sin embargo, los que me conocéis sabéis que, además de mi cabezonería, me caracteriza la intensidad con la que vivo las relaciones personales (para bien y para mal) por lo que no podía dejar pasar la oportunidad de daros las gracias a todos los que habéis estado a mi lado durante estos años.

Quiero empezar agradeciendo a mis tres directores de tesis todo el trabajo que han hecho conmigo. Cuando la gente me pregunta cómo es posible que tenga tres directores me apresuro a contestar que soy muy difícil de dirigir. Y no cabe duda de que es cierto. Habéis tenido que discutir conmigo, sabéis que soy peleona y que defiendo (a veces con poco criterio) cada idea y cada párrafo que escribo como si me fuera la vida en ello. Me consuela pensar que con cada discusión he aprendido de vosotros. Quiero agradecerle a **Manel** la paciencia que tuvo conmigo en los primeros años, en los que, puede que por el excesivo entusiasmo de los inicios, el "mundillo" de la investigación no fue fácil para mí. Especialmente quiero darle las gracias a **Irina**, que sin duda ha sido la pieza clave en todo este entramado. Irina, sin ti la comunicación con Fernando hubiera sido casi imposible (no te ofendas Fernando, te quiero un montón, pero todavía ahora en ocasiones me cuesta entender lo que me dices). Es una suerte poder decir que la que había sido mi compañera (a quien acompañaba en las noches de matadero y en las madrugadas de laboratorio) se convirtió en una amiga, y con el tiempo (y sin perder el status de amiga) en mi directora. Eres brillante, Irina, valiente y trabajadora como nadie. Te espera una gran carrera, una vida llena de éxitos. Y lo mejor es que mereces cada uno de ellos. Porque junto a Fernando formáis un tándem inigualable, que durante los controles de reproducción en las granjas parece una coreografia y que en la redacción de los trabajos se convierte por sinergia en algo que casi asusta. **Fernando**, ¿qué te voy a decir que no te haya dicho en estos años? Que eres el mejor director de tesis que se pueda tener. Que ha sido un enorme placer (a la par que una enorme responsabilidad) que me "regalaras" este tema de tesis. Que te admiro aún más que cuando llegué al grupo, y no tanto por tus publicaciones (que también) sino por ser capaz de nombrar en una clase a Kandinsky, a los

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Gracias a las **chicas de anatomía** (Mari, Ester, María,…) por su apoyo, a las otras dos **“teresinas”** (Lliber y Sonia) porque no he olvidado esos viernes tan divertidos. A **Pedro** por compartir conmigo su particular visión sobre la investigación, la universidad y la cooperación. Gracias a **Carlos**, por ser tan especial, por todos esos cafés y esa la complicidad que tenemos. Gracias por haber estado a mi lado en lo bueno y lo no tan bueno, porque eres un gran amigo al que espero conservar mucho-mucho tiempo.

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A todos, mil gracias.

“And all urge is blind save when there is knowledge. And all knowledge is vain save when there is work. And all work is empty save when there is love (…) And what is to work with love?
It is to charge all things you fashion with a breath of your own spirit.”

“Y todo impulso es ciego cuando no hay conocimiento. Y todo conocimiento es inútil cuando no hay trabajo. Y todo trabajo es vacio cuando no hay amor (…)¿Y qué es trabajar con amor?
Es impregnar todas las cosas que efectuais con el aliento de vuestro propio espíritu.”

Kahlil Gibran.
On work.
Sobre el trabajo.