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Clinical evaluation of bovine twin pregnancy

PhD thesis

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List of abbreviations

ADDCL – additional corpus luteum

AI – artificial insemination

AUC – area under the curve

bPAG-1 – bovine pregnancy-associated glycoprotein 1

bPSPB – bovine pregnancy specific protein B

CL – corpus luteum

CON – control

DCL – double corpus luteum

eCG – equine choriogonadotropin

ELISA – enzyme linked immunadsorbent assay

FSH- follicle stimulating hormone

GnRH – gonadotropin releasing hormone

kDa – kiloDalton

LH – luteinizing hormone

NRC – National Research Council

P4 – progesterone

RIA – Radioimmunoassay

ROC – receiver operating characteristics

TRUS – transrectal ultrasonography

1. Summary

1.1. General summary

Several pregnancy diagnosing methods are intensively used worldwide to diagnose whether a pregnant cattle is carrying multiple fetuses or not. The different transrectal palpation techniques (palpation of the uterine fluctuation, amniotic vesicle, or fetal membrane slip) are useful and widespread; however, their efficiency highly depends on the skill of the examiner. An experienced veterinarian/technician even from Day 35 with the fetal membrane slip technique is able to set up pregnancy diagnoses. However, the manual techniques are limited in diagnosing twins: usually only the bilateral twins can be diagnosed accurately. The prediction accuracy of unilateral twinning is low. Ultrasonography in the early period (around Day 30) of gestation is also widely used with high accuracy to diagnose early pregnancy. At this time, usually twin pregnancies are also visible. When dealing with twin pregnancy at herd level, late embryonic/early fetal mortality should be always considered as these phenomena can influence the accuracy of diagnostic tests. Another possibility for diagnosing early pregnancy in the field is the measurement of pregnancy specific proteins, like bovine pregnancy-associated glycoprotein-1 (bPAG-1), bovine pregnancy-specific protein B (bPSPB) or pregnancy serum protein (PSP-60), which are secreted by the binucleate cells originating from the bovine trophoblast. In contrast to progesterone (P4) measurements, they are good indicators of the presence of an alive conceptus.

In the present thesis, the relevant literature of clinical diagnostic possibilities of twin pregnancy is overwieved in cattle. The different methods of pregnancy diagnosis is evaluated highlighting the advantages and disadvantages of each method.

Studies on twin pregnancy diagnoses are presented with the goal of setting up clinically accurate cut-offs to help clinician veterinarians working in the practice.

In practice, pregnancy loss due to embryonic/fetal mortality is the main factor which affects the results of pregnancy diagnoses, therefore the nature of the phenomenon must be considered when evaluating diagnostic methods. Diagnosing pregnancy from Days 24-25 by means of ultrasonography is also proper for diagnosing twins,

but because of the above mentioned effect of pregnancy loss, confirming diagnosis is required, in cases of twin pregnancies by means of ultrasonography. Our data will confirm the need of the evaluation of pregnancy losses in cases of twin pregnancies as well as the need of additional pregnancy diagnoses to monitor the losses.

1.2. Aims of the study

The aims of the present thesis were the followings:

1. To set up a clinically accurate reproductive management protocol to manage bovine twin pregnancies.
2. Measurement of different pregnancy proteins (bPAG-1 and PSPB) for the diagnosis and the follow-up of twin pregnancies, with the goal to set up a prediction protocol for twin calving.
3. We hypothesized that serum P4 concentrations will be different between the singleton and twin pregnancies, and this will be a sensitive and specific clinical indicator for the prediction of the number of corpora lutea (CL). Furthermore, we have also hypothesized that serum bPAG-1 concentrations will be different between single and twin pregnant groups at each time point, thus will serve as a sensitive and specific clinical indicator for the prediction of the number of fetuses.
4. To compare pregnancy losses of twin pregnancies to singleton ones, to find critical episodes during gestation and to decrease stillbirth losses.

2. Introduction and literature review

The apparent milk yield per dairy cow increased by 1.5 % in the EU-28 from 2014 to 2015, almost reaching 6,900 kg per dairy cow (Milk and milk product statistics, Eurostat 2016). The large improvement of milk production was caused by the intensive genetic selection for milk production (especially genomic selection). High milk production is believed to associate with dropping of fertility rates and longer calving to conception interval (Lucy, 2001, López-Gatius et al., 2003). Moreover, during the last four decades the rate of twin calving also increased (Kinsel et al. 1998, Silva de Rio et al., 2007) due to the increase of multiple ovulation associated with high milk yield (López et al. 2005, Fricke and Wiltbank 1999) and with some synchronization protocols (Andreu-Vázquez et al., 2012a). Twin pregnancy is undesirable in dairy herds due to the high risk for pregnancy loss, placenta retention, involution disorders and freemartinism (López-Gatius et al., 2017).

The rate of twin pregnancy/calving is worldwide increasing and some papers report extremely high occurrence (17.9% in Spain - Andreu-Vázquez et al. 2012a) at the time of early pregnancy detection or 12% of cows delivering twins (Silva del Rio et al. 2007). Double ovulation occurs more frequently in multiparous cows with high milk production than in primiparous cows (Fricke and Wiltbank, 1999, López-Gatius et al. 2005, Kusaka et al., 2017).

Diagnosis of twin pregnancy is possible by transrectal ultrasonography (TRUS) at the time of early pregnancy examination (28-34 days after artificial insemination (AI) – López-Gatius et al., 2004). The risk of pregnancy loss during the first trimester of gestation for cows carrying twins is three to nine times higher than for cows carrying singletons (López-Gatius et al., 2002, 2009; López-Gatius et al., 2010). Pregnancy proteins are also well-known indicators of gestation. Nowadays not only blood (Sasser et al., 1986, Humblot et al., 1988, Zoli et al., 1991, zoli et al., 1992, Romano et al., 2010) but also milk tests are available (Friedrich and Holtz 2010) for detection of early pregnancy in ruminan ts. Pregnancy Specific Protein B (PSPB) is a good indicator not only for pregnancy but also for pregnancy loss (Gábor et al., 2016). Although some data were published about the application of pregnancy associated protein (PAG) for twin pregnancy diagnosis (Szelényi et al., 2015, Garcia Ispierto et al., 2016), its usefulness in the field is still questionable.

Clinical diagnosis of twin pregnancy (mostly because of the negative conseqences) has a major importance in dairy cattle. Among the undesireable conseqences we understand higher percentage of pregnancy losses (Silva del Rio et al., 2007, 2009, Andreu Vasquez et al., 2012, Spitzner et al., 2013, Gábor et al., 2016), shorter duration of gestation, increased stillbirth and dystocia rates (Nielen et al., 1989, Beerepot et al., 1992, Ari et al., 2011), furthermore, more postpartum complications (Beerepot et al., 1992, Echternkamp and Gregory, 1999a,b). Twin pregnancy has also negative conseqences on newborn calves. Decreased birth weight of twin newborns is weel-described in the literature. In general, researchers found 25% higher birth weight at calving in singletons; however, twins showed better weight gains; at weaning this difference was only 15% (Echternkamp and Gregory, 1999b). Another disadvantage is freemartinism, due to placental anastomoses between opposite sex fetuses, resulting in decreased amount of heifers after twinning (Echternkamp and Gregory, 1999b).

Cattle is typically unipara (no data about difference in breeds). The percentage of twinning at calving was around 3-9% in an earlier study (Karlsen et al., 2000). Earlier data in Hungary including over 13,000 calvings in 5 years reported 3.4 % (Boldizsár, 2009), with a maximum of 8-9% in some years (Ari et al., 2011). Clinically we may observe dairy herds, where twin calving rate did not exceed 2-3% in the last decade, this was observable in our retrospective study as well.

Much higher twin pregnancy rate can be found at the time of pregnancy diagnosis. Nowadays the extensive use of early pregnancy diagnostic methods can report twin pregnancy rate between 2-15% (Boldizsár, 2009), and the same study reported higher incidence in the late winter and spring months. A study from Spain reported 20% twin pregnancy at the time of ultrasound pregnancy diagnosis, at the end of the first month of gestation (Lopez Gatius et al., 2005).

The ratio of twin births can be influenced by several environmental, nutritional, climatic or other seasonal effects. In the spring and the autumn months the occurence of twin calvings is higher. Parity also affects twinning, heifers give birth to twins with around 1% while in older cows this percentage can exceed up to 10% (Boldizsár, 2009). Wide range of medical therapy is used in the reproduction of dairy cows. Those drugs, which are incorporated into superovulation therapy (FSH, eCG) do have effect on the number of ovulated follicles, while other drugs are used in the everyday practice (GnRH) do not have.

In beef cows, twin calving seems to be a beneficial phenomenon; after twinning two offsprings can be weaned and can be set into feedlots or replacement heifers. The profitability through this can be increased with up to 24%, therefore beef cattle breeders are trying to increase twin calving rate through selection (Echternkamp et al., 1999). According to the literature data, twin calving rate was increased from 1-4% to 20-25%, further improvement is not reachable, probably due to the bad hereditability of the nature (h^2=0.09) (Echternkamp et al., 2007). Those cows, whose dam carried out twins earlier, will have higher chance to calve twins again (1.9%-5.6%) (Morris et al., 2002). Other authors confirmed (Wiltbank et al., 2000) that those cows, who already calved once twins, have 7% higher chance to give birth again to twins (83065 animals; 5852 re-twinning). It is also demonstrated, that cows with twins at least two times will have 7.2% chance to twinning again (Echternkamp and Gregory, 1999b).

In case of twin gestation two co-dominant follicles ovulate (Karlsen et al., 2000), and after the succesful ovulation twin fertilisation occurs. Therefore, in general, the formation of two CL occurs, and two embryos will start developing in the uterine horn(s). The occurence of monozygotic twins, occuring from a single ovulation and spontaneous dividing of the embryo is rare, around 5% among all twins (Silva del Rio et al., 2006). Triplets or more twins are extremely rare in cattle. However two CL can be present on the same ovary (meaning usually two embryos in the same uterine horn), bilateral twins are more common than unilateral ones (Lopez Gatius et al., 2005). In diagnostic examinations, the presence of two CL not neccerarily means twins, while double ovulations are much more common than twin pregnancy (Lopez et al., 2005). Our own data suggests that 10% of singleton pregnancies are carrying two corpora lutea. In twin ovulations, uni- and bilateral ovulations occur in the same proportion, but the migration of the oocyte between the uterine horns can elevate the percentage of pregnancy loss (Szelényi et al., 2018).

Behind double ovulations, it ssems that the fulfilment of metabolic requirements of high-yielding cows stands (during the first 8 weeks of lactation when milk production peaks). An American study (Lopez et al., 2005) above 50 kg/day milk production the ratio for multiple ovulations was higher than 50 percent. According to Wiltbank et al. (2000) co-dominant follicles are ovulating in different time-points (with around 8 hours delay) due to the different paterns of FSH release from the pituitary, but in the same follicular wave. This can happen either due to the disregulation of follicle

dominance, or due to a follicular factor. If yes, then possibly 17 ß-oestradiole is the factor which is dependent on the regulation. This hormone, originating from the follicles, enhances feedback for the doule ovulation. Possibly through the increased dry matter intake the incrased blood flow through the liver is responsible for this regulation, because increased liver blood flow possible leads to increased follicular production.

2.1. Clinical diagnosis of twin pregnancy

A recent Hungarian study highlighted (Fodor et al., 2016), that around 70% of the dairy farms introduced some technological step for early pregnancy diagnosis. Still around one third of the farms is using rectal palpation as technique for pregnancy determination, therefore at the moment there is no data about regular screening for twin pregnancy.

2.1.1. Rectal palpation

Diagnosing pregnancy can be performed in several ways. Palpation of the amniotic vesicle (Wisnicky and Cassida, 1948), or the fetal membrane slipping technique. Mostly these techniques are carrying the risk of damaging the amniotic vesicle (Ball et al., 1963, Paisley et al., 1978, Franco et al., 1987, Kassam et al., 1987).

In our country the palpation of the fluctuation in the pregnant horn is widely used. Positive pregnancy diagnosis means enlarged/assymetric pregnant uterine horn, mostly due to the accumulation of allantoic fluid. Around Day 60 of pregnancy this can be diagnosed easily, but experienced practitioners can perform this examination around between Days 30 and 40 (Abbit et al., 1978, Romano et al., 2007). Although in case of twin pregnancy twice more alllantoic fluid is produced, however due to elongations of the embryos not in each case can be palpated in the uterine horns. It is much easier to palpate bilateral twin pregnancies due to bilateral enlargement of the uterine horns. Altogether this method is not good for routine screening of twin pregnancies.

The experienced practitioner can palpate the amniotic vesicle as early as Day 30 of pregnancy. The vesicle(s) with around 1-2 cm in diameter can be palpated in the same side, where ovaries are carrying the corpora lutea. According to Day et al.

(1995) and Karlsen et al. (2000) the rectal manipulation carries the highest risk factor for damaging the embryo. Pregnancy loss in the above mentioned reports changed between 3-10%. Moreover, the risk releasing endogenous prostaglandin from the endometrium is also high when using this technique.

Fetal membrane slipping technique is not widespread in Europe, while in the United States is used (Romano, 2007). Romano (2007) examined the cattle by means of early ultrasonography, and then the membrane slip technique were used between Days 34 and 41 of pregnancy. Repeated ultrasound examinations have proven, that the study group did not show increased pregnancy loss. Altogether, when analyzing the data of all cows, the total pregnancy loss ratio was 14%. It was concluded that the increased number of rectal palpations and/or the possible endogenous release of prostaglandins might contributed to it.

2.1.2. Transrectal ultrasonography

Ultrasonography was firstly introduced into bovine reproduction management in the 80's of the last century. The early studies have defined the accuracy of the method. Curran et al. (1986) have defined the diagnosis time of different organs in the fetus, or properties of the embryo. In their study, embryonic heartbeat was diagnoseable as early as Days 22-24, even nowadays this is the diagnostic criteria for a positive pregnancy diagnosis (Hughes and Davies, 1989, Taverne et al., 1995, Szenci et al., 1995). Furthermore, the presence of the embryo and/or the presence of the amniotic and allantoic fluid are main criterea for a positive early pregnancy diagnosis by means of TRUS under practical circumstances. Allantois fluid is firstly visible in multiparous cows from Days 25-26, while in heifers even from Days 23-24. It is important to mention that during the late embryonic development (Days 16-42 of pregnancy) embryonic elongation is a part of the development (as well as in the early embryonic development from Days 1-15), therefore the measurement of the cross-sectional largest diameter of the uterine horn is not applicable for the ageing of the conception. During these periods, the largest diameter of the amniotic vesicle also increases slightly. At the end of the embryonic development, 15-25 mm diameter can be accounted. The fetal development begins on Day 42, from this point caruncules are presented in the uterine horns. Some recent human studies are diagnosing twin pregnancy from different measured values, e.g. length of the cervix (Pagani et al. 2016). In animals these possibilites have not been studied yet.

In case of twin pregnancy, both embryos must be visible with both heatbeats, and both amniotic vesicle diagnosis is needed (Davis and Haibel, 1993). According to the location of the embryos, they can be located either unilaterally or bilaterally. A Spanish study (Lopez Gatius et al., 2005) reported increased frequency of unilateral twins. Our own data (Szelényi et al., 2018), showed almost equal distribution of unilateral and bilateral twins.

During diagnostic ultrasonography it is important to evaluate the CL. In cases of twin pregnancies usually two corpora lutea can be diagnosed (Karlsen et al., 2000), because mostly co-dominant follicles ovulate and monozygotic twinning is rare (Silva del Rio et al., 2006). Our data confirms this (Szelényi et al., 2018), furthermore we found twin pregnancies with three corpora lutea. Around 10% was the ratio of those animals, who carried singleton pregnancies with two corpora lutea. With the currently available diagnostic possibilities it is hardly difficult to differentiate between a singleton pregnancy from a codominant pregnancy and a possible partial embryonic mortality (Lopez Gatius et al., 2010).

2.1.3. Clinical examination of pregnancy proteins

Pregnancy proteins are protein-like compounds shown in the plasma or sera of ruminants originating from the fetoplacental unit.

From Day 22 of pregnancy, mononuclear cells originating from the trophoblast migrate into the endometrium. During this process, they turn into binucleate, some trinucleate cells. Because of this migration, the bovine placenta is called a synepitheliochorial placenta (Igwebuike, 2006, Augustine, 2013). The cell migration originating from the trophoblast can be observed during the whole gestation. These migrating cells are producing the different pregnancy proteins (PAG's), the placental lactogens, and from around 150th day of pregnancy, progesterone.

Pregnancy proteins can be differentiated into two subgroups: PAG-2 subgroup is mainly localized on the fetal-maternal borderline (cannot be measured in the maternal peripheral circulation), PAG-1 subgroup is being expressed mostly on the bi- and trinucleate cells of the trophoblast, although monucleate cells also secrete it (Wooding et al., 1996, Wooding et al., 1997). PAG-1 appears in the maternal circulation around Day 25, its concentration increases in the blood until calving, and peakes before calving. Despite the 30-years intensive research, the clear function of the production of these proteins remained unknown. The welfare and the viability of

the fetus can be monitored with them, as well as pregnancy loss during gestation (Zoli et al., 1992, Patel et al., 1995, Szenci et al., 2003). More faster and easier methods are recently described with promising results (Mayo et al., 2016); however, the practical usefulness of those is still questioned.

An American group isolated another protein fraction (Sasser et al., 1986), and named it pregnancy specific protein B (PSPB). However, there is a low number of comparison studies, and, according to our knowledge these proteins are only differing in carbohydrate side chains, and both belongs to aspartic protease family. Further research have conducted to analyze, whether PSPB is able disseminate pregnant and non-pregnant animals (Butler et al., 1982, Vasquez et al., 1995, Gábor et al., 2002, Romano et al., 2010, Giordano et al., 2012, Gábor et al., 2016).

From a clinical aspect it is important that in which biological fluid and how we can measure the concentration of pregnancy proteins. For more than 20 years, different methods were used to measured those from blood sera, while others (Gajewski et al., 2008, Friedrich and Holtz, 2010, Commun et al., 2016) measured from milk. Some authors reported the meauseremnet of the pregnancy proteins with two-dimensional electrophoresis (Pyo et al., 2003); however, based on their results, there is no clinical application currently. There are lower concentrations of the protein in milk than in blood sera (Wiltbank et al., 2016), this makes the clinical usefulness approveable.

The first measurements of these purified proteins were carried out with radioimmunoassay (RIA) and ELISA. (Sasser et al., 1986, Humblot et al., 1988, Zoli et al., 1992, Perényi et al., 2007). Technological improvement made it possible to use ELISA methods for a more accurate measurement (Green et al., 2005, Silva et al., 2007, Friedrich et al., 2010), even in beef cattle and different breeds clinically applicable tests are also used (Roberts et al., 2015, Pohler et al., 2016b). The different ELISA tests are compared in several studies (Green et al., 2005, Piechotta et al., 2011, Karen et al., 2015), and described also in other species (Karen et al., 2003).

Numerous factors may affect pregnancy protein concentrations. A study showed, that high milk production correlates negatively with PAG-1 concentration, especially during the embryonic development. There is 10 times higher risk for pregnancy loss in case of low concentrations (<2.5 μg/ml), whereas this risk decreases to 6.8 odds in cases of higher levels (>4 μg/ml) of PAG-1 (Echternkamp et al., 2007). Low

concentrations of proteins reflect a poor circulation of the placenta as well, therefore PAG-1 concentration is a clinical sign of embryonic and fetal well-being (Patel et al., 1995). It is quite important to set up threshold levels, which can relate to pregnancy loss, and it is also a question, whether enviromental factors at sampling (e.g. low ambient temperature) influence protein concentrations. In a study even the sire had significant effect on PAG-1 levels (López-Gatius et al., 2007). Contraversiary, García-Ispierto et al. (2016) observed influences of neither parity, nor milk yield, nor plasma P4 concentration, nor fetal sex on PAG-1 concentrations measured by RIA. The same study showed significant differences in PAG-1 levels between individuals regarding to sire, number of embryos and season. In those animals, which carried twins, inseminated with Limousine semen especially in a colder season, significantly higher concentrations of PAG-1 was reached. In this study all dams were Holstein-Friesians, but using Friesian semen or insemination in warmer season revealed to lower concentrations of PAG-1, suggesting that more genetical distance between the dam and the fetus and warm summer episodes might decrease fetal well-being. Furthermore infectious diseases, like seropositivity for Neospora caninum also affected PAG-1 levels; pregnancy loss was lower in cases of positive cows inseminated with semen of another breed (García-Ispierto et al., 2016).

More studies have shown that in dams carrying two embryos higher concentrations of PAG-1 could be measured, and with the progress of gestation the elevation in the concentration was also higher compared to dams carrying singletons (Lopez-Gatius et al., 2007, Szelényi et al., 2015). Despite this observation, the identification of twin pregnancy by laboratory measurements is difficult as the spontaneous embryonic reduction (Lopez-Gatius et al., 2010), and the half-life of PAG compared to PSPB also prevents accurate clinical diagnoses. Lopez-Gatius et al. (2007) found a negative association between milk production and PAG contrations at Day 63 of pregnancy measured by RIA-497 and RIA-706. In some cases they failed to get accurate results of concentrations between Days 35 and 56 of pregnancy. The possible cause of this was the higher metabolic clearence due to the higher milk production, and PAG also passes by the blood-milk barrier (Lopez-Gatius et al., 2007). Our group also made a study to reach clinically accurate diagnosis. Clinically applicable cut-off for the diagnosis of twin pregnancy was only reachable from Day 85 of pregnancy. Based on these results we concluded that TRUS is also needed for twin pregnancy diagnosis (Szelényi et al., 2015).

2.2 The effect of twin pregnancy on pregnacy losses

Among domesticated species cattle is one of them, where pregnancy loss has serious effect on reproductive performance. Twin pregnancy, as an economically unwanted phenomenon in dairy cattle, was also demonstrated to increase losses either in the late embryonic and/or the early fetal period (Vaillancourt et al., 1979, Day et al., 1995; Ball, 1997; López-Gatius et al., 2004; Romano et al., 2007, Silva-Del-Río et al., 2009, Pohler et al., 2016a, Mur-Novales et al., 2018).

The prevalence of losses during pregnancy decreases with the progress of gestation, and majority of the losses may occur in the early embryonic period (Diskin and Morris, 2008, López-Gatius et al., 2009, Fricke et al., 2016). Under clinical circumstances, TRUS represents an accurate method for pregnancy diagnosis in late embryonic and early fetal period (Pieterse et al., 1990, Szenci et al., 1998, Fricke, 2002). Because of the occurrence of late embryonic/early fetal losses, primary pregnancy diagnoses must be confirmed (Santos et al., 2004) to achieve reliability and to manage herd health issues. This confirmation is usually performed around Day 60 of pregnancy when placentation is completed (Ball, 1997). At that time pregnancy is considered to be firmly established and the chances of losses are greatly reduced. Most of the losses in singleton and twin pregnancies used to occur between the time of early pregnancy diagnosis and the confirmation of gestation (López-Gatius et al., 2004, Szenci et al., 2013, Ricci et al., 2015), but early pregnancy diagnosis is still not a common practice in our country. Twin pregnancy also shortens the length of gestation (Echternkamp and Gregory, 1999a, Norman et al., 2009).

However, ratio of losses may vary in the different embryonic/fetal development stages, a recent study advises to set up „pivotal periods", by taking into consideration the practical point of view of the regular reproductive management possibilites; under field conditions by means of an early pregnancy determination tool the first diagnosis is available from around Day 28, and further confirmations of pregnancy are required (Fricke et al., 2016, Wiltbank et al., 2016). In our own previous study, pregnancy diagnosis was carried out between Days 29 and 42, with an average of Day 33.6 (Szelényi et al., 2010). This terminates also the incidence of pregnancy examinations under practical circumstances, which must be performed

weekly or every second week, otherwise the first diagnose will not be a true early diagnose.

Pregnancy losses are ranging from 10 to 15% from the first diagnose, with a special emphasis on twins, whereas even 20% is mentioned in the literature (Lopez Gatius et al., 2007). The majority of the losses is occuring in late embryonic and early fetal period (Humblot, 2001). The above mentioned study (Wiltbank et al., 2016) takes this whole period from Day 28 till Day 60 as a unit, suggesting that under clinical circumstances this crucial period should be intensively investigated by different methods to decrease the economical losses caused by pregnancy loss. After Day 60, the rate of total losses until calving decreases to 5-7%, therefore Day 60 is the key point, where a confirmational pregnancy diagnosis should be added to the protocol.

An additional factor for pregnancy loss is laterality of the embryo(s). Especially in twin pregnant cows, bilateral twin pregnancy resulted in lower number of losses, than unilateral twin pregnancies (Lopez Gatius et al., 2004), indicating that possibly physical extensions of two embryos can induce pregnancy loss. Heat stress also influences pregnancy losses, especially in the case of twin pregnancies.

The method of pregnancy diagnosis can also be source of pregnancy loss. Early studies have confirmed that all rectal palpation techniques can end up pregnancy loss either due to direct damage of the allantochorion/amniotic vesicle (Vaillancourt et al., 1979, Kassam et al., 1987, Franco et al., 1987, Thurmond et al., 1993) or due to increased prostaglandin release from the endometrium. Even the pregnancy diagnosis method can induce pregnancy loss (Baxter and Ward, 1997). A recent study did not find differences, when using the fetal membrane slipping technique and ultrasonography (Romano et al., 2007).

3. Clinical measurements on bovine twin pregnancies

3.1 bPAG-1 concentration measurement in the first trimester of pregnancies for clinical evaluation

As published in Theriogenology 84. (2015) 76–81: ”Accuracy of diagnosing double corpora lutea and twin pregnancy by measuring serum progesterone and bovine pregnancy-associated glycoprotein 1 in the first trimester of gestation in dairy cows”
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3.1.1 Materials and methods

3.1.1.1 Animals

Altogether 84 Holstein-Friesian dairy cattle were included in our study with a confirmed pregnancy by means of ultrasonography at the late embryonic period between Days 29 to 42 of gestation (time-point 1). Animals were divided into 3 groups: the first group (TWIN Group, n=29) had two viable embryos and two mature corpora lutea on the ovaries at the time of pregnancy diagnosis. Animals with one viable embryo in the pregnant uterine horn and two corpora lutea on the ovaries formed the second group (DCL Group, n=35), while animals with a singleton pregnancy (one viable embryo and one corpus luteum) served as the control group (CON Group, n=20). All animals were kept in free barns with cubicles and were fed according to the NRC recommendations.

3.1.1.2 Ultrasonographic examination and blood sampling

Animals were sampled four times during the study. First, at the time of early pregnancy diagnosis (time-point 1) which was performed by a real-time B-mode diagnostic ultrasound scanner (EasiScan, BCF technologies, Bellshill, UK) equipped with a 5 to 9 MHz linear-array rectal transducer, then monthly for three additional occasions (namely: between Days 57 to 70 (time-point 2), Days 85 to 98 (time-point 3), and Days 113 to 126 (time-point 4) of gestation, respectively). For the diagnosis

of early pregnancy and ovarian structures 7.5 MHz transducer was used. All ultrasonographic examinations and blood samplings were performed by the same operator. Individual animals were examined in stalls in the milking house with dim ambient lighting. After removal the feces, the transducer was inserted into the rectum and was slowly moved from the cervix to the right, and then to the left uterine horn until the entire uterus was imaged. No manipulation of the uterus took place in any of the investigations. Scanning of the uterus and interpretation the echoscopic images have been described previously (Szenci et al. 1995). At each examination, the operator was required to record the number and the presence of embryos in the uterine horn and the number of the corpus luteum. At every time-point, when it was possible, the viability of the fetus was confirmed by the detection of the heartbeat. In those cases, when scanning of the entire uterus especially at time-points 3 and 4 was not feasible, the presence and the movement of the fetus was used to confirm the viable pregnancy. Following pregnancy diagnosis animals were handled according to the reproductive protocol of the particular farm. At the time of drying-off pregnancy was confirmed by rectal palpation. Finally our ultrasonographic diagnoses were confirmed by calving records. Animals were excluded from the study if repeated examinations yielded inconsistent diagnoses.

Following transrectal ultrasonographic examinations blood samples were collected into vacutainer tubes (Monovette 9ml, Sarstedt, Nümbrecht, Germany) at every time-point. The tubes were transported within 2 h into the laboratory in a cool-box containing icebags, centrifuged at 3000 x g/ 10 min) and the serum samples were kept at -20 C until measurements of P4 and bPAG-1 concentrations.

3.1.1.3. P4 and bPAG-1 assay procedures

Plasma concentrations of P4 were estimated by validated solid-phase ^{125}I radioimmunoassay (RIA) method (Coat-A-Count TKPG; Diagnostic Products Corporation) as described by Zoli et al. (1992). The sensitivity was 0.15 nmol/L, and the intra- and inter-assay coefficients of variation were 8.9 and 11%, respectively.

Plasma bPAG-1 measurements were performed according to the method of Zoli et al. (1992) and subsequently modified by Perényi et al. (2002). A polyclonal antiserum was used (Zoli et al. 1992) which was produced against PAG I67kDa. A PAG I67kDa preparation, purified according to the protocol of Zoli et al. (1991) was used as standard and tracer. The sensitivity of the bPAG-1 RIA test was 0.06 ng/mL,

and the intra- and inter-assay coefficients of variation were 5.0 and 5.5%, respectively.

3.1.1.4. Statistical analysis

The data were analyzed using a commercially available software (Minitab 16, State College, PA, USA). Normal distribution of the data was tested with the Shapiro-Wilk test. Serum P4 and bPAG-1 concentrations were compared within groups (TWIN, DCL, CON) and different time- points (Time-point 1: between Days 28 to 42, Time-point 2: between Days 57 to 70, Time-point 3: between Days 85 to 98, and Time-point 4: between Days 113 to 126, respectively) using a generalized linear model (GLM), due to the unbalanced design. Post-hoc evaluation was performed with the Bonferroni correction.

Regarding to P4 concentrations, after evaluating the animals in a generalized linear model, the TWIN and the DCL group (cows having 2 CL and one embryo) were condensed together and compared to the CON group (1 CL and one embryo) at the four different time points. Binary logistic regression was used to reveal any association between serum P4 concentration and the -number of corpus luteum at each of the 4 time-points. At each time-points, when the regression model was significant, a receiver operating curve (ROC) was created to find the most specific and sensitive cut-off value for discrimination of cows with 2 CLs vs. 1 CL. Area under the curve (AUC) was also calculated.

To find the accuracy of pregnancy protein levels, the CON group and the DCL (1 embryo/fetus with 2 CL) group were also condensed and compared to the TWIN group (2 embryos/fetuses) at each of the four time-points regarding to pregnancy protein concentrations. Binary logistic regression analysis was used to reveal any association between serum bPAG-1 concentration and number of embryos/fetuses at each time-points. A receiver operating curve was created to reveal the most specific and sensitive cut-off value for the determination of the number of embryos/fetuses. Area under the curve (AUC) was also calculated.

The AUC was regarded as follows: AUC = 0.5 (no discrimination); $0.7 \leq AUC < 0.8$ (acceptable discrimination); $0.8 \leq AUC < 0.9$ (excellent discrimination); and $AUC > 0.9$ (outstanding discrimination). The goodness of the fit of the logistic regression models were tested with the Hosmer-Lemeshow method. Level of significance was set at $P<0.05$.

3.1.2 Results

3.1.2.1. Analysis of serum P4 and bPAG-1 concentrations

The descriptive data of serum P4 and the bPAG-1 concentrations of the groups at different time- points are given in Table 1.

Table 1: Descriptive data (mean of serum progesterone (P4) and bovine pregnancy-associated glycoprotein 1 (bPAG-1) concentrations at different sampling time-points in twin pregnant (TWIN), singleton pregnant with double corpora lutea (DCL) and singleton pregnant with one corpus luteum (CON).

	P4 (ng/ml)				bPAG-1 (ng/ml)			
	TWIN (n=29)	DCL (n=35)	CON (n=20)	P	TWIN (n=29)	DCL (n=35)	CON (n=20)	p<
Time-point 1 (Days 29-42)	8.04±3.05	7.46±2.72	7.09±2.24	NS	9.67+3.77	8.28+4.73	7.17+2.17	0.001*
Time-point 2 (Days 57-70)	8.39±3.33	7.52±3.23	7.10±2.05	NS	11.37+5.93	7.88+5.61	6.26+2.13	0.001**
Time-point 3 (Days 85-98)	7.86±4.19	6.27±2.42	7.22±2.60	NS	36.00+13.07	26.35+14.62	17.57+9.34	0.001***
Time-point 4 (Days 113--126	6.44±2.77	5.55±1.77	5.79±1.87	NS	54.64+18.70	37.53+20.09	25.84+10.29	0.001***
p<	NS	0.005^{+}	NS		0.001 $^{++}$	0.001 $^{++}$	0.001^{++}	

General linear model analyzing bPAG-1 concentrations revealed significant differences (P<0.001) within groups and at every sampling time-points. Regarding to serum P4 concentrations the general linear model revealed statistically significant differences (P<0,005) between time-points 1 vs. 4 and 2 vs. 4.

3.1.2.2. Association between serum P4 concentrations and the number of corpora lutea

Cows were regrouped based on the number of the corpora lutea as follows: cows with single (n=20) or double (n=64) corpora lutea. Binary logistic regression assessing serum P4 concentrations revealed statistically significant differences (P=0.03) only at time-point 2 (Table 2).

Table 2: Association between the number of embryos and serum bovine pregnancy-associated glycoprotein-1 (bPAG-1) concentration and between the number of corpora lutea (CL) and serum progesterone (P4) concentration at 4 different time-points using binary logistic regression models.

Variable	Time interval	P	Odds ratio	95% CI
P4 concentration (1 vs. 2 CL)	Time-point 1	0.266	1.08	0.94 - 1.23
	Time-point 2	0.030	1.14	1.01 - 1.28
	Time-point 3	0.095	1.11	0.98 - 1.26
	Time-point 4	0.241	1.12	0.93 - 1.35
bPAG-1 concentration (singleton vs. twins)	Time-point 1	0.044	1.14	1.00 - 1.29
	Time-point 2	0.004	1.16	1.05 - 1.28
	Time-point 3	0.001	1.07	1.03 - 1.11
	Time-point 4	0.001	1.06	1.03 - 1.09

3.1.2.3. Association between serum bPAG-1 concentrations and the number of embryos/fetuses

According to the number of the embryos/fetuses cows were also reorganized into singleton (n=55) and twin pregnant groups (n=29). The outcome of the binary logistic regression test in this case was significant at all time-points ($P<0.05$), with strong significance ($P<0.005$) at time-points 2, 3 and 4 (Table 2).

3.1.2.4. Evaluation of the receiver operating characteristic (ROC) curve and calculation of the area under the curve (AUC)

The results are summarized in Table 3. The serum P4 concentrations at time-point 2 with the highest specificity and sensitivity could not sufficiently differentiate animals with one or two corpora lutea.

Table 3: Cut-off concentrations, sensitivity (Se), specificity (Sp) and the area under the curve (AUC) of twin pregnancy based on the receiver operating characteristic (ROC) curves at different time-points.

	Cut off value (ng/mL)	Se	Sp	AUC
P4 at time-point 2	9.8	26.0	79.6	0.62
bPAG-1 at time-point 1	14.1	17.8	91.1	0.65
bPAG-1 at time-point 2	14.1	25.0	92.9	0.75
bPAG-1 at time-point 3	39.4	28.6	87.5	0.82
bPAG-1 at time-point 4	56.5	46.4	85.7	0.81

The cut-off value for bPAG-1 concentrations was the same at time-points 1 and 2. In these cases the measured area under the ROC-curve was acceptable. In contrast, at the time-points 3 and 4 the AUC were excellent (time point 3: 0.82, time point 4: 0.81).

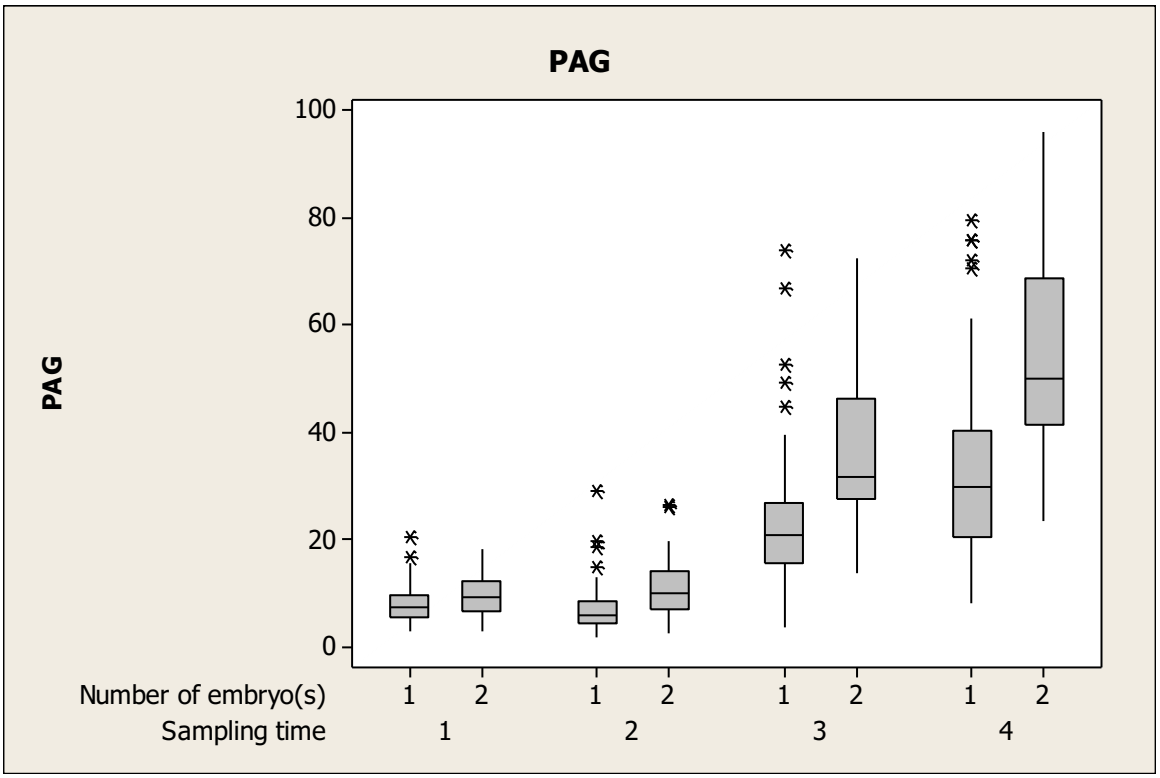

Figure 1. Serum concentrations of bovine pregnancy-associated glycoprotein-1 (bPAG-1) in case of singleton (1) and twin (2) gestation at different time-points.

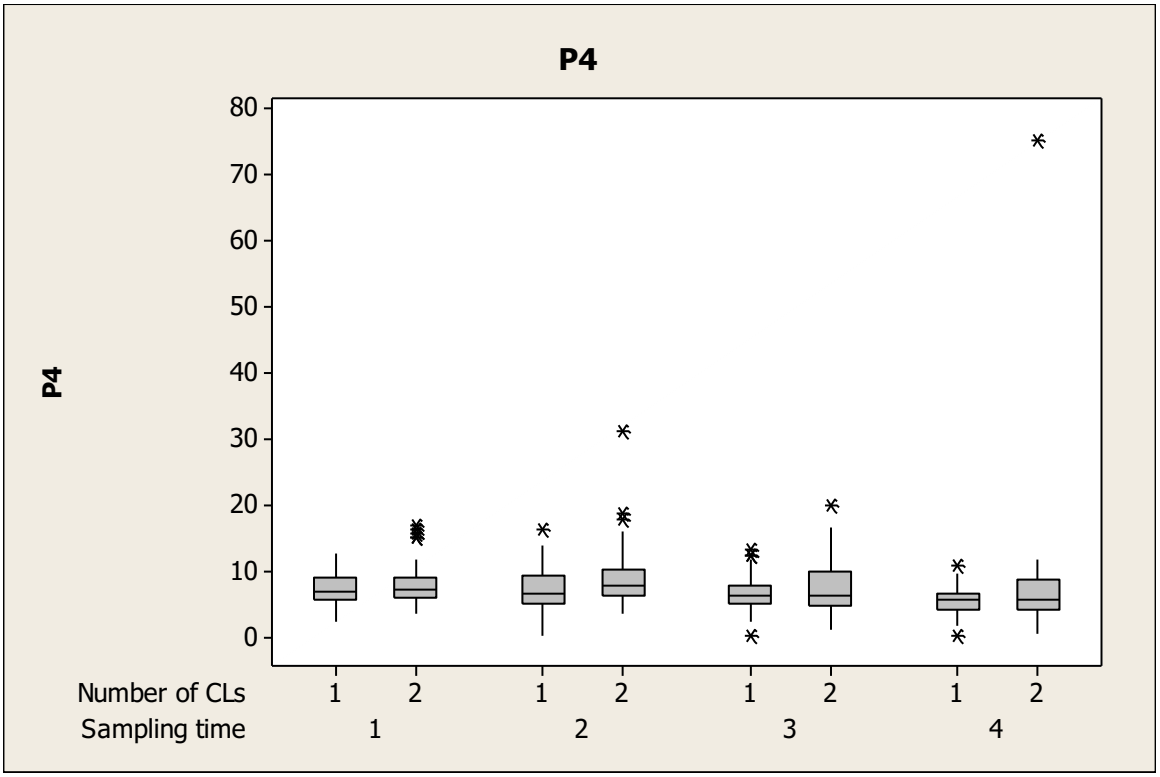

Figure 2. Serum concentrations of progesterone (P4) in case of single (1) and double (2) corpora lutea at different-time points.

3.1.3 Discussion

In this study the concentrations of P4 and bPAG-1 were measured in cases of singleton and twin gestations with 1 or 2 corpora lutea. Due to the fact, that almost 95% of twin pregnancies are dyzigotic (Silva del Rio et al., 2006) it was worth testing that double corpora lutea might produce more P4 at different stages of gestation. Because generally from around Day 150 of gestation the placenta provides a marked amount of P4 therefore it was important to test the animals before this time-point to achieve a clinically reliable diagnosis. Several authors agree, that more than one corpus luteum usually provides higher levels of circulating P4 (Bech-Sábát et al., 2008, Wiltbank et al., 2014), and the higher P4 levels provide better environment for the conceptus to elongate and develop (Forde et al., 2011). In contrast, when we compared the P4 levels at different time-points of gestation statistically significant differences could be found around Day 60 of gestation and a close to significant tendency around Day 90 but we were not able to set up a threshold level to discriminate pregnancies having 1 or 2 corpora lutea based on the P4 level of the individual animal (Table 2). This finding is in agreement with an earlier study (Mann et al. 2007) whereas no difference was found between animals having single or double corpora lutea neither in the weight of the luteal tissue, nor in the P4 concentrations. The same author assumes (Mann, 2009) that circulating levels of P4 may be determined by the stage of cycle. Both studies are carried out on non-pregnant, cyclic animals. Our results are showing that even in case of pregnant animals the circulating levels of P4 cannot be determined by the number of corpus luteum. Although measuring the P4 concentrations of pregnant animals with 2 corpora lutea gave results with good accuracy, but they were not able to distinguish between animals having 1 or 2 corpora lutea.

The levels of bPAG-1 are affected by several factors during gestation. Pathologic pregnancies, stillbirth and fetal well-being are mentioned among these factors (Serrano et al., 2009). Previous studies (Dobson et al., 1993, Patel et al., 1997) have

suggested that the number of viable embryos/fetuses have an effect on the concentrations of the pregnancy proteins in the peripheral blood. Recent studies have confirmed this hypothesis (Lopez-Gatius et al., 2007, Serrano et al., 2009), since statistically significant differences were found in the concentrations of pregnancy proteins between singleton and twin pregnancies. However, to our best knowledge no cut-off values were determined or tested to date. To exclude confounding factors such as embryonic/fetal mortality, we evaluated only those animals, whose calving ended with the number of offsprings that were originally diagnosed. When comparing one embryo carrier animals (either with 1 or 2 corpora lutea) with twin carrying animals statistically significant differences were detected at each time-point regarding to pregnancy proteins.

To have a diagnostic aid in the clinical practice, threshold levels should be established as early as possible to diagnose twin pregnancies which have major importance at herd level. From the third month of gestation we were able to select a cut-off value (39.4 ng/mL) which could disseminate singleton and twin pregnancies with high AUC. In our study at time-points 1 and 2 the cut-off values were almost the same, suggesting that bPAG1 concentrations are starting to elevate from the baseline value of Day 30 only at the third month of gestation.

The sensitivity of the bPAG1 remained low in every time-point measurement, but we could rule out negative diagnoses with a high specificity.

These results are suggesting that at the early stage of gestation, measurements of bPAG-1 as pregnancy diagnosis alone is not enough to disseminate singletons and twins. Moreover, the losses between Days 30 and 60 are increased in cases of twin pregnancies (Bech-Sábát et al., 2008), but these losses cannot be predicted with the pregnancy proteins.

To classify a herd as a twinning or non-twinning one, to follow-up multiple gestations the necessity of a diagnostic test with high accuracy is required. After selection of multiple pregnancies as early as possible we can choose a strategy for the management of these gestations. Another possibility is the reduction of the number of embryos as described recently (Andreu-Vasquez et al., 2012b). Our results can be aids in a herd level management program including the management of twin pregnancies, which begins with ultrasonographic examination around Day 30 after AI and the confirmation of pregnancy should also be carried out by means of ultrasonography in order to follow up twins. In those cases when to confirm twin

pregnancy diagnoses is needed, the time of confirmation should be around Day 90 when pregnancy protein measurement as a method can be used. Based on our results, dissemination of twin and singleton pregnancies is not possible before Day 85, so prevention or early identification of twinning is not possible with this measurement. The losses during gestations have also strong effect on the clinician`s work. The protein test we used is firstly able to select viable twin pregnancies with two alive fetuses only from Day 85 of gestation on in the first trimester of pregnancy. With our cut-off values the follow-up of twin gestations is becoming also possible. In cases of advanced gestations the physical extension of the uterus limits the exact identification of twins with ultrasound. This method can be useful in the follow up of advanced twin gestations.

In conclusion, we found the measurement of pregnancy proteins (bPAG-1) as a useful tool to discriminate between singleton and twin pregnancies. However, clinical diagnoses can be achieved only at the late fetal stages of gestation (time-points 3 and 4), suggesting that other tools -such as ultrasonography- are required to identify twinning at the late embryonic/early fetal stage (time-points 1 and 2, between Days 28-42 and 57-70, respectively). In order to achieve diagnostic test with both high sensitivity and specificity, further studies are required.

3.2 Measurements of the bovine Pregnancy Specific Protein B

As published in Acta Veterinaria Hungarica 66 (3), 451–461 (2018): ”Is twin pregnancy, calving and pregnancy loss predictable by serum pregnancy specific protein B concentration (PSPB) 28-35 days after AI in dairy cows”
Authors: Zoltán Szelényi, Orsolya Gabriella Balogh, Fernando Lopez-Gatius, Irina Garcia-Ispierto, Eszter Krikó and György Gábor

3.2.1 Materials and methods

PSPB measurement

In all experiments blood sample from each cow was collected from coccygeal blood vessels 29-35 days after insemination, and sent to a routine laboratory (Androvet, Budapest, Hungary) by overnight mail. Upon arrival at the laboratory, blood samples were centrifuged (2000 rpm for 10 minutes) and resultant sera was assayed for Pregnancy-Specific Protein B (BioPRYN™; BioTracking, Moscow, ID, USA), as described earlier (Gábor et al., 2007). Cows with serum PSPB concentrations >1.1 ng/ml were considered pregnant, those with <0.6 ng/ml were considered nonpregnant, and those with concentrations between 0.6 and 1.1 ng/ml were deemed at high risk for pregnancy loss. In all cows initially designated pregnant, continuation of pregnancy or pregnancy loss were determined by transrectal palpation 60-70 days after AI.

Trial 1:

Sampling for PSPB examinations on 29-35 days after AI were performed in 3 large Hungarian dairy farms (average herd sizes and milk production ranged from 800 to 1100 cows and from 9,500 to 10,850 kg/cow/year, respectively) and carried out routinely in Androvet laboratory between April 2012 and May 2016 (n=7300). In this period, cows with twin calving were registered also in those farms. Data of AIs resulted in twin calving were collected (serum PSPB concentration, date of the AI, parity, milk production and body condition score by the time of the AI, AI bull, the father of the cow, hormonal treatments before the AI, daily temperature data) in a self-developed database (Bopella).

Trial 2:

Blood samples were collected from 98 dairy cows on two high producing dairy farms (1100 and 750 cows, respectively) in North-Eastern Spain. Cows were examined by transrectal ultrasound (EasyScan, BCF Technologies, United Kingdom) three times: between 29-35, 36-42 and 43-49 days after AI. The ovaries and uterus were examined and pregnancy was confirmed by identification of one/two embryo(s) and embryonic heartbeat and the presence of a corpus luteum/two corpora lutea on the ipsilateral ovary. Within the weekly reproductive visit, for each two cows carrying live twins one cow with one embryo was added to the study, because singletons were only control animals. At the time of pregnancy diagnosis blood samples were collected from the coccygeal vein. Serum samples were sent to the Androvet laboratory on dry ice and measured for PSPB concentration as described above. Pregnancy loss diagnosis was based on the last two ultrasound examinations (35-41 and 43-49 days post AI).

Statistical analysis

The association of the binary outcomes and the independent variables was analyzed by logistic regression (Gelman and Hill 2006). Prediction performance was evaluated by ROC analysis, while Chi-Square test was used to determine relationship between categorical variables. Normality of the distribution of the serum PSPB concentrations was tested (Shapiro-Wilk normality test). Depending on the normality of the data Student t-test or Wilcoxon rank sum test was used for mean/median serum PSBP concentrations for cows calving twins or singletons and a basic comparison (Table 1). All data analysis was performed using the R language and environment (R Core Team 2017; R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/).

3.2.2. Results

Trial 1

Examination of 7300 deliveries showed a 6.4 % twin calving rate in the three herds (Table 5). Differences in the distribution of serum PBSP concentrations for cows calving twins and singletons are shown on Figure 3.

Table 4: Detailed information about serum PSPB concentrations in Trial 1 (normality of the distribution, mean/median, standard deviation or interquartile range).

	Singletons						Twins						
	N	mean	SD	median	IQR	Normality test* (p)	N	mean	SD	Median	IQR	Normality test* (p)	Test**
	6372	2.88	1.38	2.65	1.26	<0.001	469	3.79	1.83	3.50	1.42	P<0.001	P<0.001
Trial 2													
PSPB 1	34	2.10	1.03	2.03	1.56	0.035	64	2.90	0.87	2.90	1.25	0.129	0.0002
PSPB 2	34	1.77	1.05	1.60	1.73	0.075	61	2.52	0.74	2.53	1.16	0.48	0.0005
PSPB 3	15	1.97	0.64	1.95	0.85	0.969	58	2.15	0.67	2.00	1.07	0.0478	0.5526

*Shapiro-Wilk normality test

**Depending on the normality of the data Student t-test or Wilcoxon rank sum test was used

Table 5: Twin calving and hormonal treatments in Trial 1.

					Odds ratio	95% CI
	singleton	twin	total	twin %		
AI after spontaneous heat	4253	291	4544	6.4%A	-	-
AI after Provsynch protocol	843	35	878	4.0%B	0.59	0.40-0.85
AI after Ovsynch protocol	876	60	936	6.4%A	1.001	0.74-1.34
AI after single PGF2✔ injection	860	82	942	8.7%C	1.44	1.10-1.86
Total	6832	468	7300	6.4%	-	-

($^{A-C}$Between adjacent rows, numbers without a common superscript differed ($p<0.05$).

Table 6: Twin calving and serum PSPB concentration in Trial 1.

Serum PSPB concentrations (ng/ml)	Pregnant samples	Twin calving	%
0.6-1.1	136	0	0.0%
1.101-2	1492	29	1.9%
2.001-3	2895	120	4.1%
3.001-4	1738	154	8.9%
4.001-	928	166	17.9%

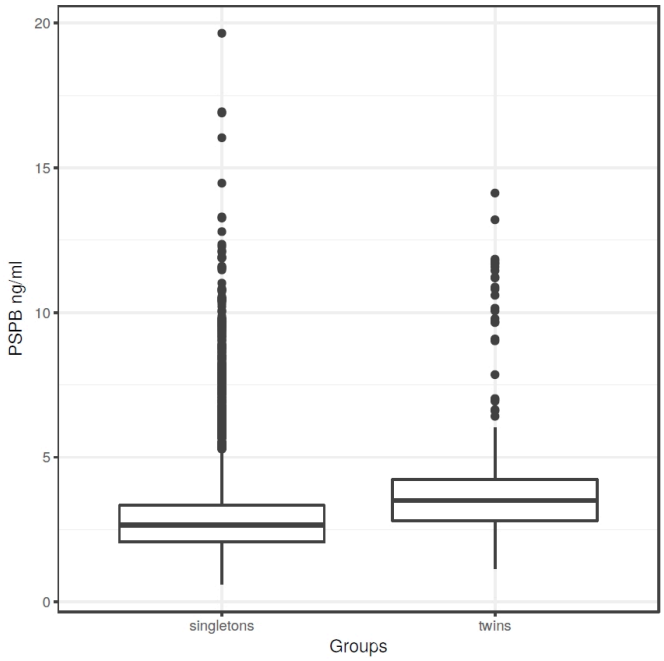

Figure 3: Differences in the distribution of serum PBSP concentrations for cows calving twins and singletons (Trial 1)

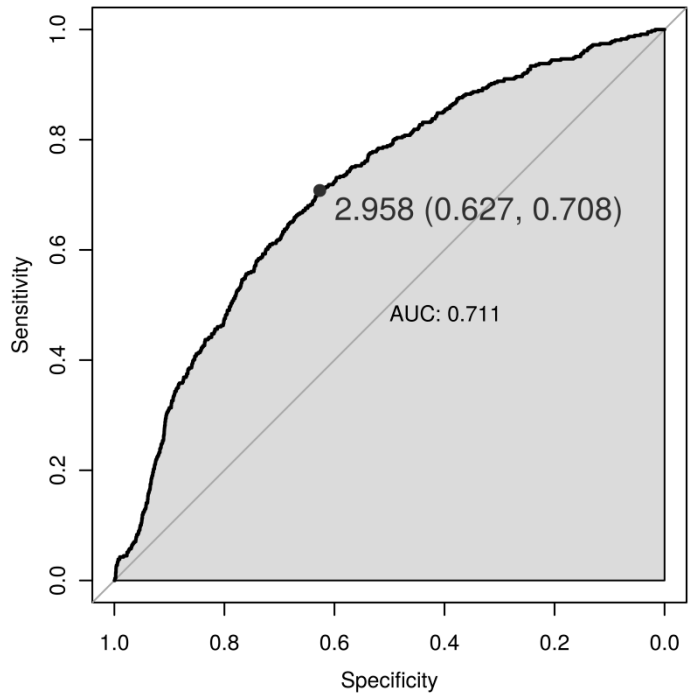

Figure 4: ROC analysis of twin calving and serum PSPB concentration (Trial 1)

The logistic regression analysis of hormonal treatments prior to the AI also indicate differences: the highest twin calving rate occurring after PGF treatment (OR: 1.44, 95% CI: 1.10-1.86, p=0.0076), while the lowest twin calving rate can be observed followed the Provsynch (PreSync plus Ovsynch OR: 0.59, 95% CI: 0.40-0.85, p=0.0026) protocol (Table 5).

More detailed statistical analysis of the pedigree, breeding and reproduction data demonstrate that high PSPB concentration (29-35 d after AI) significantly correlates with twin calving rate (OR=1.31; p<0.0001; Table 6, Figure 4). The positive predictive value of the cut-off 3 ng/ml using logistic regression (OR: 3.84 (95% CI: 3.15-4.71, p<0.0001) for the risk of twin calving in cows with serum PSPB values higher than 3 ng/ml is 0.12 (95% CI: 0.11-0.14), the negative predictive value is 0.96 (95% CI: 0.96-0.97). Genetics also seems to have significant effect on twin calving (AI bull: p=0.017, bull's sire: p=0.02, bull grandfather: p=0.0007, cow's father: p=0.016). Number of AIs, BCS, daily temperature and daily milk production by the time of the AI and the age of the cow had no significant effect on twin calving.

Trial 2

Main results of Trial 2 are summarized in Tables 7 and 8, as well as Figure 5. Nighty-eight pregnant cows were involved in the study 29-35 d after AI, 64 twin and 34 singleton pregnancies have been diagnosed by transrectal ultrasound. Daily milk production at pregnancy diagnosis (mean of the three previous days ± SD) for these cows was 40.9 ± 8.4 kg, ranging from 18 to 62 kg. One week later 3 pregnancy losses were diagnosed from the 64 twin pregnancies (4.4% pregnancy loss). Between days 43-49 after AI another 3 and 19 pregnancy loss of twin and single pregnancies have been detected (4.6% and 55.9% pregnancy loss, respectively). All ultrasound based pregnancy diagnosis was confirmed by the results of Biopryn test (serum PSPB concentration). Although serum PSPB concentration differed between single and twin pregnant cows, the differences were not significant. Statistical analysis showed that cows with higher parity have lower risk for twin pregnancy ($p=0.023$). Open days, number of AIs and milk production had no effect for twinning rate. Parity had significant impact on pregnancy loss, since cows with more calving had higher pregnancy loss (20% vs. 28.6%, $p<0.05$).

Table 7: Odds ratios and p values for twin pregnancy (Trial 2)

	OR for twin pregnancy	p value
PSPB treshold 3 ng/ml	3.62	0.009
Daily milk production	0.94	0.130
number of AI's	1.03	0.819
Open days	1.01	0.753
Primiparous	1.02	0.930
Multiparous	0.16	0.023

Table 8: Pregnancy loss in twin and singleton pregnant cows in Trial 2.

	Primiparous		Multiparous	
	Pregnant	pregnancy loss	Pregnant	pregnancy loss
singleton	5	3	29	16
twin	30	4	34	2
Total	35	7	63	18
prenancy loss %		20.0%A		28.6%B

([A-B]Between adjacent columns, numbers without a common superscript differed ($p<0.05$).

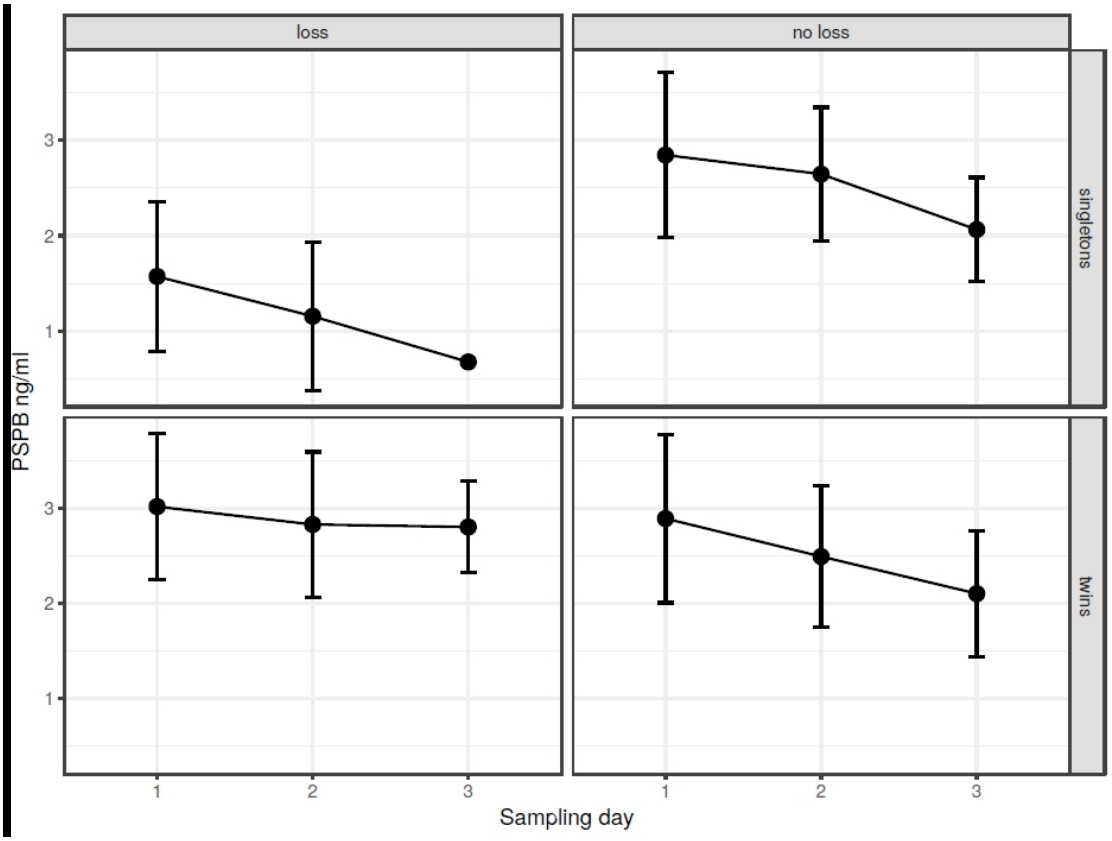

Figure 5: Serum PSPB concentrations in twin and singleton pregnant cows in Trial 2 (sampling day 1: 29-35 Days postAI; sampling day 2: 36-42 Days postAI; sampling day 3: 43-49 Days postAI).

3.2.3 Discussion

Twinning in the dairy cattle population appears to be increasing over time (Kinsel et al., 1998, Johanson et al., 2001, López-Gatius and Hunter, 2017). If this trend

continues, the dairy industry must be prepared to cope with the negative effects associated with twinning (Fricke, 2001). In the late 50's and early 60's the incidence of twinning was approx. 1 % in dairy cattle (Noakes, 2009) and significantly increased over the past decades along with the increase in milk production (Nielen et al., 1989). Our results (Table 4) showed a similar trend and we registered almost 7 % twin calving rate in the 3 herds. This is definitely much higher twin calving rate than earlier (1-5 %, depending on breed). Although a common belief that hormonal treatments are major causes of twin pregnancies, our data do not support this hypotheses. The only exception is the single PGF injection and this is in agreement with findings of Andreu-Vázquez et al. (2012a) who reported that just those estrus synchronization protocol used before AI affected the twin pregnancy rate when PGF were used together with other hormones (PRID, eCG). PGF alone (Kinsel et al. 1998) or in combination with FSH/LH or GnRH (Nielen et al. 1989) has increased risk of twinning. No effect of milk production on the risk of twin pregnancy was found, in agreement with a previous study (Andreu-Vázquez et al. 2012b). Likelihood of twin pregnancy increased with parity. 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, Andreu-Vázquez et al., 2012b). Our results showed that AI bull, bull's sire, bull's grandfather and the cow's father affected also twin calving ($p \leq 0.02$). Johanson et al. (2001) analyzed calving data of North American Holsteins (1,324,678 births of 37,174 sires of cows from the National Association of Animal Breeders (NAAB) calving ease database). Heritability estimates for the sire of the cow effect were 2.10% by the linear model analysis and 8.71% by the threshold model analysis. Sire predicted transmitting abilities (PTA) for twinning rate ranged from 1.6 to 8.0%. They concluded that sire selection can be used to reduce the incidence of twins and also the increased cost of production associated with twins.

In our study we found much higher frequency of twin calving in cows diagnosed pregnant with more than 3 ng/ml serum PSPB concentrations at 29-35 days after insemination (Table 3). Although individual differences could be detected, the trend was similar to results of others who stated that cows bearing twins showed significant higher plasma PAG-I (López-Gatius et al., 2007) or plasma PAG I, PAG-II and P4 concentrations (Garcia Ispierto et al., 2016) throughout the study period than cows bearing singletons.

However, there was a significant difference in PSPB serum concentration between singleton and twin pregnancies in the first two sampling days (Table 1) in Trial 2, differences were non-significant in overall between PSPB serum concentration of singleton and twin pregnant samples (2.1 and 2.9 ng/ml). Probably the low size of the study population and the effects of milk production on PSPB values may explain this lack of significance. Daily milk production at pregnancy diagnosis slightly exceeded 40 kg and milk production correlated negatively with plasma PAG-I values in a previous study (López-Gatius et al., 2007). Although lower pregnancy loss rate (9.4 %) was recorded in twin pregnant than singleton pregnant (55.9 %) cows (in these cases small morphological abnormalities – less amount of amnionic fluid, smaller size of the embryo was seen), no doubt that twin pregnancy is a higher risk factor for terminating pregnancy in cattle (López-Gatius and Hunter, 2017, López-Gatius and Hunter, 2017). Statistical analysis showed lower risk of twin pregnancy in higher parity cows ($p=0.023$), but several other data confirm (Johanson et al. 2001, Gábor et al. 2016) that in this case it is an accidental finding probably caused by the relatively low number of cases. All other findings (open days, number of AIs and milk production had no effect on twin pregnancy) are supported by earlier researches. Not surprisingly parity has impact on pregnancy loss (Table 5), since cows with more calving had higher pregnancy loss (20% vs. 28.6%, $p<0.05$).

Although we also have no clear explanation for the decrease of serum PSPB concentrations at the different bleeding times, an apparent decline in plasma PAG-1 values on Day 42 of gestation was previously described (López-Gatius et al., 2007). This is not surprising in view of the fact that PAG molecules are a family of closely related proteins which expression patterns vary temporarily during the different pregnancy periods (Green et al., 2000, Garbayo et al., 2008).

After analysing the twin calving data in some Hungarian Holstein-Friesian herds we can conclude that twinning rate rapidly increased over the past decades as well and it looks that more genetic than management (e.g. hormonal treatments) reasons could be identified in the background of this unwanted change. Under our conditions, no real predictive value of PSPB was found for twin pregnancy or pregnancy loss, probably due to the relatively low number of experimental animals and the negative effect of high milk production on PSPB values in Trial 2. However, we found that lower PSPB serum concentration 29-35 d post AI represents a high risk for

pregnancy loss as published in a former paper (based on analysis appr. 140 thousands data; Gábor et al., 2016).

3.3 Clinical outcome of pregnancy losses in cases of bovine singleton and twin pregnancies

As published in Acta Veterinaria Hungarica (accepted for publication): ”Pregnancy and stillbirth losses in dairy cows with singleton and twin pregnancies”

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3.3.1 Materials and methods

Experimental design and pregnancy diagnoses

Our study was carried out on three large-scale Hungarian dairy farms during a 13-month period between November and December. The 305-d milk production was 9,300 kg (Farm A), 8,700 kg (Farm B) and 11,000 kg (Farm C) in the year before the study, respectively. Nutritional supplementation was in line with the NRC recommendations (NRC, 2001) on each farm. Farms were visited once a week (Farm A) or every second week (Farms B and C) to diagnose early pregnancy by means of transrectal ultrasonography (TRUS) using a 4.5–8 MHz linear-array rectal transducer (BCF Technology Ltd., Livingstone, United Kingdom) as described previously (Szenci et al. 1995), as part of the reproductive management of each farm, between days 29 to 42 after AI. Parity number and milk yield did not differ significantly between singleton and twin carrier animals.

The criteria for a positive pregnancy diagnosis were the followings: (1) at least one viable embryo with a detectable heartbeat, (2) clear fluid in the allantoic and amniotic vesicles (with complex integrity of both membranes), and (3) at least one corpus luteum (CL: min. 2 cm in diameter) on one of the ovaries. By scanning both uterine horns the content of each horn (one or two embryos, embryo with or without heartbeat, right or left uterine horn) and the structures on the ovaries (number of CL, CL with a cavity at least 5 mm in the diameter) were also recorded. The diagnosis of singleton pregnancy was established when one alive embryo and one or more CLs were detected. Criterion for twin pregnancy was two viable embryos with at least one CL. In total 1253 positive pregnancy diagnoses were followed up until calving.

After early pregnancy diagnoses, pregnancy was confirmed between days 57 to 70 of gestation by means of transrectal palpation (TRP). If manual palpation did not confirm previous pregnancy status, TRUS examinations were repeated to confirm fetal losses. Pregnancy was also confirmed by means of TRP at the time of drying-off between days 221 and 227 of gestation. The number of foetuses was recorded at calving.

All reproductive interventions before AI (single prostaglandin treatment /0.5 mg cloporostenol im., Cyclix, Virbac, France/ or OvSynch (d 0: GnRH /0.1 mg gonadoreline im., Gonavet 50, Veyx Pharma, Austria/, Day 7: PGF2α /0.5 mg cloporostenol im./, Day 9 p.m.: GnRH, Day 10 a.m.: AI/ protocol or spontaneous oestrus) were also recorded.

The care of the animals and the experimental design of this study were approved by the Local Animal Ethics Committee in Budapest, Hungary.

Statistical analysis

Statistical analysis was performed with Stata 15 MP (StataCorp. 2017. Stata Statistical Software: Release 15. College Station, TX: StataCorp LLC). The chi-squared test was used to test the independence of two categorical variables in a contingency table. Fisher's exact test was used to test whether the relative proportions of one variable were independent of the second variable. The independent group t-test was used to compare means of same variable between two groups. Welch's t-test was used assuming unequal variances. Equality of variances was tested by the Levene's test to see if the samples had equal variances. ANOVA was used to test for differences in the means of the dependent variable broken down by the levels of the independent variable.

Logistic regression analysis was used to model the expected value of the binary response variables as a linear function of the explanatory variables. In three-dimensional tables when controlling for the third variable homogeneity of odds ratios was assessed with the Mantel-Haenszel estimate and a chi-squared test.

The p-value was the two-tailed probability computed using the corresponding distribution. If the p-value associated with the test was small, it was considered as an evidence to reject the null hypothesis in favour of the alternative. For statistical testing two-tailed tests with a 5% level of significance were used.

3.3.2 Results

Evaluation of twin pregnancies

In total, 1253 positive pregnancy diagnoses [Farm A: n = 304 (24.3%), Farm B: n = 674 (53.8%) and Farm C: n = 275 (21.9%)] were made between days 29 to 42 and followed up until calving. The prevalence of twin gestations diagnosed between days 29 to 35 (73/866, 8.4%) and days 36 to 42 (32/387, 8.3%) were similar; therefore, the dates of pregnancy diagnoses were not used further as an independent factor in the analysis. Most of the animals (n = 1148) carried singleton pregnancies, while there were 105 (8.4%) twin pregnancies. Twin pregnancy rates were also similar for the three farms [Farm A: 7.6% (23/304), Farm B: 8.6% (58/674) and Farm C: 8.7% (24/275; $p > 0.05$, respectively] at the time of the early pregnancy diagnoses; therefore, the different farms were also not used further as an independent factor in the analysis.

All twin and singleton pregnancies were evaluated according to the laterality of gestation. Among the 1148 cows carrying singletons, 670 pregnancies (58.3%) were located in the right uterine horn and 478 pregnancies (41.6%) in the left uterine horn. Twin pregnancies were located either unilaterally (n = 57; 54.3%) or bilaterally (n = 48; 45.7%).

There were one CL in 957 (83.4%) and two CLs in 191 (16.6%) singleton pregnancies, respectively. In the latter group three of the cows had 3 CLs. In twin-carriers only one CL was found in three cases (2.9 %), and all other twin cows had two (n = 99) or three CLs (n = 3). Cavitary CL occurred in one twin-carrier (1.0%) and in 58 singleton pregnancies (5.1%).

Majority of the cows (n = 761; 62.3%) were inseminated without hormonal treatment prior to AI. Altogether 472 (37.7%) animals had pharmacological treatment. Three days prior to AI prostaglandin was administered to 334 animals (26.6%) and an OvSynch protocol was used in another 138 animals (11%). Out of the 472 hormonal treatments, 431 resulted in singleton (91.3%) and 41 (8.7%) in twin pregnancies. When using prostaglandin, 300 (89.8%) treatments resulted in singleton and 34 (10.1%) in twin pregnancies, while with the use of the OvSynch protocol 131 (94.9%) singleton and 7 (5.1%) twin pregnancies were obtained. Overall, the hormonal treatment before AI did not influence the twin pregnancy ratio at pregnancy diagnosis time ($p = 0.182$). When analysing data with calculating the Mantel-Haenszel estimate of the odds ratio controlling for farm, then the OR was 0.94 ($p = 0.67$), indicating that

the ORs were equal to one and there was no treatment effect on the twin pregnancy ratio in different farms, and the test of homogeneity of ORs gave p = 0.36, indicating that the odds ratios were not different from each other.

Table 9: Pregnancy losses and stillbirths in cases of singleton and twin pregnancies

Reproductive state	Singleton gestation (n = 1148) (%)	Twin gestation (n = 105) (%)
Pregnant, diagnosed between days 29–42 and 57–70 (n %)	1095 (95.4)	100 (95.2)
Pregnant, diagnosed between days 57–70 and drying-off (n %)	1025 (89.3)	94 (89.5)
Dam with stillbirth (n %)	54 (5.3)	16* (19.5)
Total number of alive calves (n %)	971 (84.6)	144 (68.6)

*both calves were stillborn in four cases

Table 10: Pregnancy losses in cases of singleton pregnancies (n = 1148) according to the number of corpus luteum (CL) and the presence of a cavity in the CL

Pregnancy loss	Singleton gestation with a compact CL (n = 899)	Singleton gestation with a CL with a cavity (n = 58)	Singleton gestation with two CL without a cavity* (n = 191)
Diagnosed between days 29–42 and 57–70 (n, %)	32 (3.6)a	7 (12.1)b	14 (7.3)b
Diagnosed between days 57–70 and drying-off (n, %)	51 (5.7)a	12 (20.7)b	7 (3.7)a
Total (n, %)	83 (9.2)a	19 (32.8)b	21 (11.0)a

a,bp < .05: within the same row

*n = 3: presence of 3 CL

Table 11: Evaluation of losses in cases of cavitary, non-cavitary and double corpora lutea (CL) in singleton pregnancies using either OvSynch or PGF2α treatment prior to AI

Pregnancy loss	Singleton gestation with a CL without a cavity (n = 362)	Singleton gestation with a CL with a cavity (n = 19)	Singleton gestation with two CL without a cavity* (n = 50)
	Prostaglandin treatment prior to AI (n = 300)		
Diagnosed between days 29–42 and 57–70	6 (2.4)	1 (7.7)	2 (5.1)
Diagnosed between days 57–70 and drying-off	14 (5.6)	2 (15.4)	1 (2.6)
In total	20/248 (8)	3/13 (23.1)	3/39 (7.7)
	OvSynch treatment prior to AI (n = 131)		
Diagnosed between days 29–42 and 57–70	4 (3.5)a	2 (33.3)b	1 (9.1)a
Diagnosed between days 57–70 and drying-off	11 (9.6)a	2 (33.3)b	1 (9.1)a
In total	15/114 (13.1)a	4/6 (66.6)b	2/11 (18.2)a

a,b$p < .05$: within the same row

*n = 3: presence of 3 CL

Late embryonic and fetal losses in singleton and twin pregnancies

Confirmation of TRUS pregnancy diagnoses was carried out by means of TRP at days 57 to 70 of gestation. The rate of pregnancy loss diagnosed between days 29–42 and 57–70 was altogether 4.6% (53/1148) in singleton and 4.8% (5/105) in twin pregnancies ($p = 0.95$), respectively (Table 9). Differences in pregnancy loss at drying-off were also not significant between singleton and twin pregnancy carrying animals ($p = 0.99$). Based on logistic regression analysis, in any time points total losses were not different in singleton and twin pregnancies ($p = 0.94$, OR = 1.04 and

p = 0.96, OR = 0.98, respectively), and we could not detect any farm effect (p = 0.36, OR = 0.83 and p = 0.08, OR = 0.79, respectively).

Pregnancy loss was also evaluated on the basis of laterality in cases of singleton and twin pregnancies. In singleton gestations, the rate of right-side pregnancy losses (35/670; 5.2%) did not differ significantly (p > 0.05) from those of the left-side pregnancy losses (18/478; 3.8%) between days 29-42 and 57-70. This difference was also not significant at drying-off pregnancy check (p > 0.05). Based on logistic regression analysis in twin gestations neither the difference of the pregnancy losses at days 57-70 (4/57; 7% vs 1/48; 2.1%), nor the differences at the time of drying off (4/57; 7% vs 2/48; 4.2 %) were significant (p > 0.05) between unilateral and bilateral pregnancies.

Singleton pregnancies were also evaluated based on the number of CL and the presence of a cavity in the CL diagnosed by TRUS (Table 10). Pregnancy losses occurred more often in singleton pregnancies with a cavitary CL than those with non-cavitary ones at the time of confirmation of pregnancy (days 57-63, p = 0.015). At the time of confirmation of pregnancy for those, who had a cavitary corpus luteum the odds of pregnancy loss were 2.73 times larger than the odds for those, who did not have a cavity (p = 0.02). At the time of drying-off for those, who had a cavitary corpus luteum the odds of pregnancy loss were 2.18 times greater than the odds for those, who did not have a cavity (p = 0.02) in singleton pregnancies. However, the number of the corpora lutea neither at the first time-point nor at the second time-points of confirmation of pregnancy did have any effect on pregnancy loss (p = 0.9 and p = 0.45, respectively). There was one CL in two twin-pregnant dams and one CL with a cavity in one twin-pregnant cow; however, their pregnancies were not lost.

Losses at calving

The length of singleton and twin gestation was 276.4 ± 22.8 and 270 ± 23.2 days, respectively. Comparing the length of singleton and twin pregnancies with the Student's t-test with unequal variances, neither twin and singleton, nor unilateral and bilateral (268.1 ± 31.1 vs 272.9 ± 8.5 days) twin pregnancies showed statistically significant differences.

Finally, 1025 singleton pregnant animals and 94 twin pregnant animals gave birth. Stillbirth event occurred in 53 cases (5.3%) in singleton calvings, whereas twin

calvings suffered from one or two stillborn offsprings in 11 cases (11.7%) ($p < 0.01$). It is important to mention that in 4 twin-pregnant cows both calves were stillborn.

3.3.3 Discussion

In this study, we compared the outcome of singleton and twin pregnancies in order to evaluate gestations in terms of pregnancy losses (late embryonic/early and late fetal mortality, stillbirth). A total of 1253 gestations were evaluated, and 8.4% twin gestations were found. The prevalence of twin gestation varies widely, because it is possible to identify herds as twinning or as non-twinning ones (Kirkpatrick et al., 2002). At the time of early pregnancy diagnoses we did not find a twinning rate as high as reported in a recent study (15%, Andreu-Vázquez et al., 2012). As the main factor responsible for the growing number of twin gestations worldwide, increased metabolic activity associated with the rising milk yield of cows has been mentioned (Lopez et al., 2005, Wiltbank et al., 2000). In an American study (Lopez, 2005) with >45 kg daily milk yield, the percentage of multiple ovulations exceeded 50%, indicating that milk yield correlates with twin pregnancy rate, which should be consider in high-producing dairy herds (Kirkpatrick et al., 2002). In contrast, some authors did not find any relationships between milk production and multiple ovulations (López-Gatius et al., 2005), which thought to be responsible for the majority of twin gestations. In accordance with this finding, the highest prevalence of twin pregnancy at the early TRUS examinations was found on the farm with the highest milk production (around 11,000 kg/lactation). Although we did not observe increased twin gestation rates in higher production herds at early pregnancy diagnosis, our results suggest that the increasing milk production may affect twin pregnancy.

Usually twin gestations suffer more often from this phenomenon (Kastelic et al. 1989); however, in this study, there was no evidence for a higher prevalence of losses in twin pregnancies until pregnancy confirmation. Between Days 57–70 and drying-off there was also a non-significant difference in losses. This finding is in contrast with a study, where high rate of pregnancy loss (28.8%) was detected until Day 90 (López-Gatius et al., 2004). In accordance with our findings, it is highlighted that twin gestation management should focus on the late fetal period, however our study did not report the time period between the first pregnancy diagnosis and the confirmation crucial. It has also been reported (López-Gatius and Hunter, 2005,

Lopez Gatius et al., 2010) that in some cases only one embryo undergoes this partial loss of pregnancy. Our study did not contain data to analyse partial losses, further evaluation is required.

More pregnancy losses occurred in singleton pregnancies when gestation was maintained by a cavitary CL ($p < 0.05$) between days 29-42 and 57-70 with an increased prevalence of pregnancy losses until drying-off. Although the presence of a cavity in a mature CL was not found to be associated with a reduced ability of progesterone production (Okuda et al., 1988; Perez-Martin, 2009, Balogh et al., 2012, Balogh et al., 2014), in the present study pregnancy losses were associated with the existence of a cavitary CL. Although detailed evaluation of the role of CL with cavity in the maintenance of bovine pregnancy was not the main focus of this study, it seems that medical therapy might be required to maintain the affected gestations, because almost one third of singleton pregnancies with a cavitary CL were lost.

Significantly more losses were found in singleton pregnancies with a cavitary than in those with a non-cavitary CL with an OvSynch treatment before AI, due to the limited number of our cases it needs further confirmation. At the same time, difference was not found in cases of prostaglandin treatments.

Earlier studies (Bech-Sábat et al., 2009; López-Gatius et al., 2002) and a recent review (Szenci, 2015) recommend to induce secondary CL by pharmacological treatments to maintain pregnancy in the early stage of gestation. Surprisingly, more losses occurred for singleton pregnancies with two CL than those having one CL in the first two months of gestation, while there was no difference between them until calving. It is presumable that those cows carried twins however one of them was lost before our TRUS and according to López-Gatius et al. (2002) these pregnancies were not likely to be maintained however this finding needs further confirmations.

The laterality of singleton pregnancy did not show any effect on pregnancy losses until days 57–70 but after that period left horn pregnancies were more likely to be lost. In twin pregnancies, an increased laterality-associated mortality rate is known from previous studies (López-Gatius and Hunter, 2005), especially in the case of unilateral twins. The development of two conceptuses in the same uterine horn is known to be uncomfortable for the dam, but in our study, there was no difference in pregnancy losses between unilateral and bilateral twin pregnancies. Further studies are needed to confirm these findings.

The stillbirth rate was four times higher for twin than for singleton pregnancies, which points out the importance of the accurate diagnosis of twin gestations before calving in order to reduce stillbirth rate. If it is not possible, at that time after each singleton calving the possible presence of a second foetus in the uterus has to be excluded (Niles, 2016).

In conclusion, when analysing twin pregnancies in dairy cattle pregnancy loss did not differ between singleton- and twin-carrying cows at the confirmation of pregnancy between days 57–70 of gestation, moreover, at drying-off also a non-significant difference was detected between singleton and twin carrying groups. In singleton pregnancies, presence of a cavity in the corpus luteum effected pregnancy loss. Between days 57–70 of gestation and drying-off this difference between cavitary vs. non-cavitary CL was still significant, while it was non-significant between cows with one CL vs. double CLs. The occurrence of cavities in cases where a single CL was present was not affected by hormone therapy prior to AI, however, the number of CL was reduced by pharmacological treatments. The stillbirth ratio was also higher in twin carriers than in singleton carriers. Although the role of the number of CL and cavitary CL in maintaining pregnancies requires further evaluation, our study highlights the importance of follow-up twin pregnancies to decrease stillbirth rate.

4. Summary of main scientific results

In the first study we have tried to achieve clinically applicable cut-off values in cases of measurement of pregnancy proteins (bPAG-1) and progesterone to discriminate between singleton and twin pregnancies. In the second study PSPB concentration measurement was also used for the dissemination of twin and singleton pregnancies. In the third study we evaluated the losses in cases of twin and singleton gestations with a special regard to the number of corpora lutea.

- Progesterone concentrations –however statistically significant differences could be obtained- did not give clinically applicable cut-off values. At the same time bPAG-1 as a diagnostic test for twin pregnancy, with both high sensitivity and specificity, was applicable from Day 85 after AI.
- Clinically acceptable diagnoses could be achieved only at the late fetal stages of gestation (time-points 3: from Day 85 after AI) Our data suggest, that other tools -such as ultrasonography- are required to identify twinning at the late embryonic/early fetal stage (time-points 1 and 2: between Days 28-42 and 57-70 after AI).
- There was a significant difference in PSPB serum concentration between singleton and twin pregnancies in the first two sampling periods (Days 29-35 and Days 36-42 after AI). As a result of our measurements no real predictive value of PSPB was found for twin pregnancy or pregnancy loss, probably due to the relatively low number of experimental animals and the negative effect of high milk production on PSPB values in Trial 2.
- We found that lower PSPB serum concentration at Days 29-35 after AI represented a higher risk for pregnancy loss. There was lower risk of twin pregnancy in higher parity cows ($P = 0.023$), although further studies are needed to confirm this finding.
- We could not detect higher pregnancy losses with these methods between twin and singleton pregnancies, therefore ultrasonography is advised to perform for the confirmation of pregnancy in order to be able to find the differences mentioned in the literature.

- In our study stillbirth significantly decreased the number of alive calves as an outcome of twin pregnancies.
- The number of the corpora lutea and the cavities in the corpora lutea also affected pregnancies: singleton pregnancies with cavitary corpora lutea suffered higher pregnancy loss than those ones without having cavities. In twins, the lower incidence of cavities in pregnant animals did not let us to confirm this result.

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