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Endocrine and pregnancy protein changes during periparturient period with correlation of stillbirth in dairy cow

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Contents

Dedication

To my parents

Abbreviations

BCS	body condition score
BVD	bovine viral diarrhoea
CL	corpus luteum
E2	estradiol
ELISA	enzyme-linked immunosorbent assay
kDa	kilodalton
LEM	late embryonic mortality
PAG	pregnancy associated glycoprotein
P4	progesterone
$PGF_{2\alpha}$	prostaglandin$F_{2\alpha}$
PP-Period	postpartum-period
PSPA	pregnancy-specific protein A
PSPB	pregnancy-specific protein B
RIA	radioimmunoassay
SD	standard deviation

1. Introduction

The supervision of fetal well-being during bovine pregnancy as well as the detection of embryonic/fetal mortality including abortion and stillbirth is one of the most important points in farm/cattle management due to both economic losses and animal welfare aspects. For these issues several different methods are available such as clinical examination like rectal palpation, ultrasonographic - and chemical/hormonal monitoring, which can also be used for diagnosing bovine pregnancy. Losses in the embryonic period i.e. up to Day 42 post breeding present the majority of pregnancy failures, while about 5% of bovine pregnancies are lost during the fetal stage and may reach 10% (*Ayalon, 1978; Baxter & Ward, 1997; Dunne et al., 2000; Forar et al.,1995; Lopez Gatius et al., 2002; Opsomer et al.,2007*).
Delivery of dead calves before gestation Day 215 is termed abortion whereas stillbirth include dead born animals or such calves dying during or within 24 h after parturition after at least 260 days of gestation (*Szücs et al., 2009*). Stillbirth has substantial economic effects for the cattle producers due to both direct losses such as calf mortality, dam mortality and indirect costs because of treatments, veterinary services, reduced milk production (*Szücs et al., 2009*). Furthermore a higher risk of both decreased fertility by the next breeding and increased calving-conception interval is recognizable (*Chassagne et al., 1999*). For the time span from 1985 to 1996 *Meyer et al. (2001b)* predicted a rise in costs of replacing dairy calves of 75,9 million$ as a result of an increase in the incidence of stillbirth from 9.5% in 1985 to 13.2% in 1996, while *Thurmond et al. (1990)* estimated an average of $US 640.00 lost income in case of each pregnancy loss.

Stillbirth can be classified as non dystocia –and dystocia-associated. For the latter one fetopelvic disproportion is an important impact. But around half of the offspring is delivered without difficulty (*Philipsson, 1996*). Infections (e.g. BVD, Leptospira spp., E.coli), insufficient placenta development, metabolic disorders of the cow and congenital malformations of the calf are non dystocia related causes. Less time for supervision of calving due to large sized herds, insufficient care of the cow before parturition and also the change of shifts of the workers can be also possible factors (*Essmeyer, 2006; Gustafsson et al., 2007*).
Stillbirth can be affected by several parameters including breed, parity/age of the dam, BCS of the dam, gender and birth weight of the calf, season, genetics, sire, length of gestation/calving, multiple birth/calving number, calf vitality (weak calf syndrome) (*Szücs et al., 2009; Hansen et al., 2003; Chassagne et al., 1999; Thomasen et al., 2008; Meyer et al.,*

2001a; Lopez-Gatius et al., 2002; Kindahl et al., 2002a&b; Benjaminsson., 2007; Berglund et al., 2003; Gundelach et al., 2009).

Besides records referring to a decline in bovine fertility over the last fifty years (*Royal et al., 2000*) reportedly there is furthermore a steady incline of the stillbirth rate detectable during the last years (*Murray, 2009*). The USA (*Meyer et al., 2001b*), Denmark (*Hansen, 2004*), Sweden (*Berglund et al., 2003; Steinbock, 2006*) and the Netherlands (*Harbers et al., 2000*) revealed stillbirth rates ranging from 10-13%. Especially Holstein heifers are showing this problem, while in later calvings the incidence of stillbirth drops considerably (*Szücs et al., 2009*). The stillbirth rate in Germany for cows is quoted with approximately 7%, while for heifers the percentage level is comparable with the other mentioned countries – namely 13% (*Bollwein, H., 2010*), in which large variability occurs between the federal states and the gender of the calves (*Springer, 2003*) (Table 1). In Hungary *Báder et al.* (*2009*) revealed a total stillbirth rate of 9.3% between the years 2001 (9.6%) and 2005 (8.8%) by observing 8427 calvings in a large-scale Holstein-Friesian dairy farm (Table 2). A peak was reached in the year 2002 with a stillbirth rate of 11.2%. As before mentioned first parity heifers in this study have also shown higher stillbirth rates in each of the years than two or more parity cows. According to *deKruif et al. (1998)* the perinatal mortality (within 24 h p.p.) should be ≤ 5%.

Table 1: Stillborn calves in different federal states of Germany in 2001/ Holstein-Friesian (modified, *Springer, 2003*).

Male %	Female %	Total %	Federal state
12.2	5.3	9.0	Schleswig-Holstein
12.8	6.8	10.0	Bavaria
4.9	1.9	4.0	Hesse
5.8	1.9	4.8	Lower Saxony/ Bremen
7.9	2.8	6.6	Mecklenburg-West Pomerania
7.8	2.2	6.6	Thuringia

Table 2: Incidence of stillbirth calculated on annual basis (*Báder et al., 2009*).

Year	**Calvings (n)**	**Stillbirth (n)**	**Stillbirth %**
2001	1560	150	9,6
2002	1726	194	11,2
2003	1690	174	10,3
2004	1858	126	6,8
2005	1593	140	8,8
Total	**8427**	**784**	**9,3**

As mentioned at the beginning of the introduction hormonal monitoring – in particular the observation of P4, E2 and PAG – may be important tools not only for diagnosing a gravidity, but also for supervision of the fetal well-being as well as detection of embryonic/fetal mortality/abortion/stillbirth and thus helpful in reducing negative impacts of pregnancy wastage in cattle.

In the last two to three decades pregnancy specific/associated proteins were described in a lot of publications dealing with both their structure and function (*Butler et al., 1982; Humblot et al., 1988a&b; Zoli et al., 1991*). Two pregnancy-specific proteins, which have been isolated at the end of the last century (*Butler et al., 1982*), are known as PSPA, identified as α-fetoprotein, and PSPB, a glycoprotein with a reported molecular weight of 47 to 53 kDa. While the first one is not strictly limited to pregnancy the latter one has been identified to be specific for the placenta and pregnancy (*Sasser et al., 1986*).

In 1991 *Zoli et al. (1991)* purified a closely to PSPB related glycoprotein, a pregnancy associated glycoprotein (PAG), the so called bovine PAG-1, nowadays named PAG-1-67 due

to the fact that the Mr is 67 kDa. As PSPB this glycoprotein is an inactive member of the aspartic proteinase family, a group of proteolytic enzymes that is represented by the typical members pepsin, cathepsin D & E. PAG and PSPB are identical in gene nucleotide sequence but differ in carbohydrate and sialic acid content (*Xie et al., 1991; Lynch et al., 1992*), however they have been reclassified as boPAG-1 and are detectable by RIA/ELISA (*Sasser et al., 1986; Zoli et al., 1992; Green et al., 2005; Silva et al., 2007*).
PAGs very probably have a „function in immunomodulation, trophic support of the CL, and regulation of trophoblastic cell migration" (*Whitlock & Maxwell, 2008*).

Overall 22 bovine PAGs are existing so far, named bPAG1 - bPAG22 and are classified into three groups (*Gajewski et al., 2009*):

1) The pepsinogen-like PAG (boPAG-8) group
2) The gonadotrophin-like PAG (boPAG-2) group
3) The major bovine PAG family (boPAG-1) group

To group three PAG-1 as well as bPAG-56 kDa and bPAG-75 kDa (*Klisch et al., 2006*) belong. PAG-1, secreted into the maternal circulation by the trophoblastic binucleate cells (*Zoli et al., 1992*), is expressed at the beginning of pregnancy between Days 14-21 (*Ushizawa et al., 2004)* and thus is detectable in the maternal serum during the time when the trophoblast forms definitive attachment to the uterine wall. According to *Zoli et al. (1992*) the PAG-1 levels in pregnant cows rise progressively throughout the gestation from Day 30 (3.60 ± 1.73 ng/ml) after service, reaching concentrations of 24.53 ng/ml (± 8.81 ng/ml) at Day 120 and 1551.91 ng/ml (± 589.68 ng/ml) at Day 270. Afterwards i.e. during the last ten days of gestation the levels increasing enormously resulting in a peak of 2462.40 ng/ml (± 1017.90 ng/ml) on Days 1-5 prepartum reflecting the possible involvement of the glycoprotein in the induction of parturition. Following birth the levels began to sink constantly to 10.10 ng/ml (± 7.80 ng/ml) at Day 60 (*Zoli et al., 1992*), however still detectable up to 70 to 100 days after calving (*Szenci et al., 1998b),* until they reached the undetectable level (< 0.2 ng/ml) at Day 100 due to the long half-time (*Green et al., 2005; Sousa et al., 2003; Zoli et al., 1992*). The markedly increasing levels during late pregnancy with the peak around parturition are confirmed by other studies also including twin pregnancies (*Patel et al., 1997*).

Lobago (2007) found peak values at calving of 1880 ng/ml and 1367 ng/ml in Borana and crossbred cattle, respectively.

The long persistence in the PP-period, which is also seen after pregnancy losses, is one shortcoming within the scope of pregnancy determination using PAG-1 (*Whitlock & Maxwell, 2008*). Another shortcoming could be variations in the PAG-1 concentrations due to another reasons than pregnancy such as possible negative correlation to milk production as measured in high producing dairy cows (*Lopez-Gatius et al., 2007*).

PAG levels may be influenced by fetal genotypes like sex as well as breed (*Mialon et al., 1993; Zoli et al., 1992*) and were lower in fetal serum than in the maternal blood (*Zoli et al., 1992*).

PAGs are also found in sheep and goat (*Zoli et al., 1995; Folch et al., 1993*). Furthermore they occur amongst others in pigs (*Szafranska et al., 1995*), horses (*Green et al., 1994*) and carnivores (*Gan et al., 1997*).

PAG-1 can be used as a marker to diagnose bovine pregnancy at a very early stage from Days 28 or 30 after breeding (*Zoli et al., 1992; Szenci et al., 1998a&b*) as well as for supervision of the fetoplacental viability. But as the concentrations are undergoing large variations only a marked decrease or disappearance of the serum concentrations of these proteins can be an absolute predictive sign of embryonic or fetal death (*Szenci et al., 2000*).

Zoli et al. (1992) reported a 94,65% overall accuracy, with accuracies of 93,03% and 97,90% for identifying pregnant and non pregnant cattle respectively; emphasized by results of *Szenci et al. (1998b)* yielding increasing sensitivities from 95,2% to 100% from Days 29 to 30 onwards. However, the long half-life of PAG, responsible for the slow gradually drop of their concentration in the cows serum after embryonic/fetal death (*Semambo et al., 1992; Szenci et al., 1998b*) and also in the early post partum period (*Zoli et al., 1992*) „can limit the use of PAG tests under field conditions" (*Szenci et al., 1998b*).

Beside the pregnancy diagnosis PAG-1 may further be a helpful tool to determine/predict embryonic/fetal death as several studies are showing a fall in plasma bPAG-1 concentration (*Szenci et al., 2000; Szenci et al., 2003; Lobago et al., 2006; Lobago, 2007*). Moreover heifers treated with the antiprogesterone aglepristone at Days 47 and 48 of gestation for inducing fetal death have revealed a drop in PAGs levels, indicating an inability of migration of the trophoblastic binucleate cells to the maternal part of intra/extraplacentomal interfaces (*Breukelmann et al., 2005*).

„A drop in PAG concentrations some weeks or months prior to abortion has been reported in cattle (*Humblot, 2001*), and *Taverne et al. (2002)* concluded that a combined analysis of progesterone and PAG concentrations were a useful way of detecting pregnancy failure during the first trimester of bovine pregnancy" (*Jonker, 2004*).
Deviating profiles of pregnancy associated glycoproteins in dams producing a stillborn offspring/ with impaired parturition were observed by *Kornmatitsuk et al. (2002, 2003, 2004)* suggesting that PAGs can „be used for monitoring the fetal well-being in animals with a high risk of stillbirth at term" (*Konrmatitsuk et al., 2004*).

Progesterone, also known as P4 (**p**regn-**4**-ene-3,20-dione), a steroid hormone with 21 C-atoms, is the most important gestagen and is synthesized from pregnenolone, which in turn is derived from cholesterol (Fig. 1).

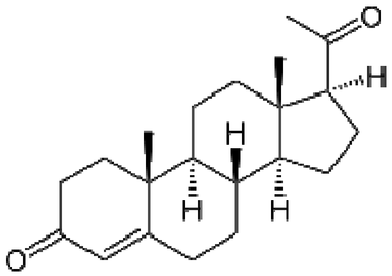

Fig. 1: Chemical structure of progesterone ($C_{21}H_{30}O_2$)

P4 preparing the endometrium for implantation und required for maintenance of pregnancy, is secreted by the luteal cells of the corpus luteum, which is the major source of the hormone during pregnancy, and by the placenta as well as the adrenal glands. RIA and ELISA are reliable techniques to determine the hormone concentrations in blood or milk (*Arnstadt & Cleere, 1981; Arnstadt & Schmidt-Adamopolou, 1982; Dinar & Sreenan, 1988*).

The P4 levels after conception and during bovine pregnancy remain elevated as well as relatively stable with a tendency for higher levels in the last trimester. Levels in early gestation period are similar compared to the maximum levels during the lutheal phase of the cycle ranging from approximately 3-7 ng/ml (*Pope et al., 1969; Stabenfeldt et al., 1969; Donaldson et al., 1970; Henricks et al., 1972; Robertson, 1972; Mukasa-Mugerwa & Tegegne, 1989).* From about Day 200 until Day 250 there is a progressive increase in

progesterone concentration due to an extraovarian source (placenta/adrenal glands) (*Stabenfeldt et al., 1970; Wendorf et al., 1983; Mukasa-Mugerwa & Tegegne, 1989*).
Afterwards the levels are falling until about 10 days before parturition, followed by an abruptly decline to values less than 1 ng/ml 24 h before delivery (*Short, 1958/1960; Stabenfeldt et al., 1970; Hunter et al., 1977; Mukasa-Mugerwa & Tegegne, 1989; Birgel jr. et al., 1994; Kindahl et al., 2002a; Lobago, 2007*).
Although P4 test can be used as a tool for diagnosing pregnancy as early as Days 21-24 after artificial insemination, the test itself is an indirect method because it reflects only the presence of P4 in the serum or milk (*Heap et al., 1976; Hoffmann et al., 1977; Szenci et al., 2000*).
Furthermore it is possible to predict the time of birth by measuring the P4 concentration. Values less than 1.5 ng/ml to 1.3 ng/ml are indicative for birth in the next 24 h to 22 h
(*Matsas et al., 1992; Rexha & Grunert, 1993; Birgel jr. et al., 1994*).

Considering embryonic/fetal death *Semambo et al. (1992)* showed in his study a dramatic decline of P4 (< 0.5 ng/ml) within 24 h in early pregnancy after inducing abortion by the $PGF_{2\alpha}$ analogue cloprostenol. This sharp decline is also reported by *Lobago (2007)* and *Szenci et al. (2000)* presented similar values within Days 0 to 9 after LEM diagnosing by means of ultrasonography, indicating an inactive or regressing CL.
Jöchle et al. (1972) induced parturition by treating cows with 100 mg progesterone daily in combination with 10 mg flumethasone and observed increased dystocia rates.
Zhang et al. (1999) illustrated no differences in progesteron concentration in heifers/cows with eutocia and dystocia, respectively between Days 90 to 270 of pregnancy.
Whereas, some days prior to delivery, the dystocial animals had a remarkable higher P4 level than the eutocial dams. The progesterone fall in these dystocial cows were delayed and less distinct, leading to the suggestion that a delayed degeneration of the corpus luteum could be a cause of dystocia. *Kornmatitsuk et al. (2002)* has also observed elevated P4 levels at the time of parturition in a cow with a stillborn calf.

Estradiol (E2), more precisely estradiol-17β, represents besides estrone (E1) and
estriol (E3) one of the most important natural occurring estrogens (Fig. 2) and, like other steroid hormones, derives from cholesterol.

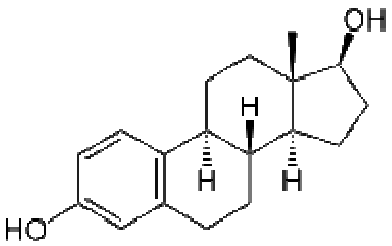

Fig. 2: Structural formula of Estradiol-17β ($C_{18}H_{24}O_2$)

Estradiol as well as estrone representing the major steroids is synthesized by the fetoplacental unit, but the majority of it is immediately conjugated within the placentomes (*Robertson & King, 1979; Takahashi et al., 1997*). Amongst these estrone sulphate (E1-S) is the main estrogen in the maternal circulation making up approximately 80% of total plasma estrogen near parturition (*Tsang et al., 1975*). A shift from progesterone to estrogen synthesis is indispensable to start physiological parturition and the parturition activity as such, respectively.

For prediction of calving within 24 h E2 profiles can be used with an accuracy of 85.2% when estradiol is showing an increment of ≥ 0.20 ng/ml from the preceding day concentration (*Shah et al., 2006*).

For E2 levels it has been shown by *Robertson (1974)* that they remain relatively low during pregnancy until about Day 19 before calving with concentrations of 25 pg/ml. Afterwards the concentration begins to increase up to peak values of 450 pg/ml at the day of parturition (*Shelton & Summers*, *1983*; *Robertson, 1974*).

A gradual increase of estradiol from Day 100 of gestation until 4 days prepartum, followed by a drastic incline reaching peak values of 1.0 ng/ml (± 0.1 ng/ml) on the day before calving and a marked drop (0.4 ± 0.1 ng/ml) within one day postpartum were seen in the study of *Shah et al. (2006)*.

Tsang et al. (1975) as well described E2 levels of 1 ng/ml during the period of -88 h to parturition and a following rapid decline within eight hours postpartum.

Smith et al. (1973) recognized decreasing levels of estradiol starting two days prior to parturition (295 ± 53 pg/ml) with concentrations of 52 pg/ml (± 11 pg/ml) at 1 day postpartum and finally averaged 14 ± 10 pg/ml until Day 9 postpartum. However, other scientific papers are describing a constant plateau level for estrone as well as estradiol during the last three days of pregnancy (*Hoffmann et al., 1973; Edqvist et al., 1973; Agthe & Kolm, 1975*).

O'Brien and Stott *(O'Brien & Stott, 1977)* found lower concentrations of estradiol in dystocial Holstein heifers during the prepartum period from 23 to 12 days compared to heifers with normal calvings. An association between both lower than normal levels of E2 and delayed decline of P4 before parturition with physiological dystocia has been suggested by *Erb et al. (1981)*.

Yokus et al. (2010), studying multiparous cows, revealed results showing that dams with dystocia had a tendency non significant higher estradiol concentration compared with cows without dystocia i.e. mean estradiol levels of 581.71 ± 971.37 pg/ml prepartum and 450.49 ± 401.81 pg/ml postpartum in dystocia cases in contrast to non dystocia cases with 292.62 ± 194.1 pg/ml prepartum and 444.69 ± 477.11 pg/ml postpartum.

Insignificant differences in corrected plasma hormone levels (P4, E1, E1-S, E2-β and E2-S) were recognized by *Graaf et al. (1982)* between heifers with and without dystocia observing the Days 29 to 9 prepartum.

In respect of stillborn calves *Sorge et al. (2008)* measured already two weeks before calving significant smaller serum estradiol levels in heifers undergoing stillbirth than in those with a live calf. *Hirako et al. (2011)*, examining amongst others the plasma concentration of estradiol during the gestation in cows with stillborn somatic cell cloned calves, found one cow with estradiol-17β levels showing a tendency to be lower during mid and late gestation. In the second cow with a stillborn calf the estradiol value increased transiently between Days 240 to 250. Thereafter the concentration stayed low until parturition.

For initiation of parturition a shift from P4 to estrogens is necessary, therefore the E2/P4 ratio must increase or vice versa the P4/E2 ratio must decline in the days/weeks before parturition reflecting the decreasing levels of progesterone as well as the increasing concentrations of estradiol occurring during this time. *Breeveld-Dwarkasing et al. (2003)* reported about increasing estradiol-17β/progesterone ratios after induction of parturition by administration of Luprostiol, a synthetic $PGF_{2\alpha}$. Such an increase in the E2/P4 ratio has also been detected by *Birgel jr. (1994)* during the last ten days of pregnancy.

2. Aim of the study

As part of cattle/farm management an accurate and improving prediction of pregnancy as well as embryonic/fetal death in cattle is an enormous challenge to reduce and minimize economic losses. Furthermore this also affects animal welfare.

Several methods may be used to predict fetal death/stillbirth, amongst others the endocrine (progesterone: P4, estradiol: E2) and pregnancy protein (pregnancy associated glycoprotein: PAG) changes can be monitored.

This thesis is dealing with analysing and evaluation of the blood profiles of P4, E2 and PAG in Holstein-Friesian dairy cows, which were measured amongst other hormonal parameters (these results are not used in this study), with especially focal point concentrating on the periparturient period with respect to stillbirth.

3. Study animals and methods

3.1. Study animals

For measuring the hormonal changes during late pregnancy 173 genetically similar pregnant Holstein-Friesian dairy cows were selected randomly in two separate years (2006/2007) on the same yard (Agroproduct Zrt, Pápa, Hungary). Furthermore values such as parity, gestation period, birth weight, number of assistants needed for calving, BCS, gender of the newborn were also taken down.

None of the study cows were primiparous heifers. Cows with aborted calves or giving twin birth were excluded from the analysis. The outcome of this experiment is the data on 99 cows measured in the year 2006 and on 74 cows in the subsequent year 2007. The latter were not examined in 2006.

Regarding the incidence of stillbirth the cows were grouped into two groups, i.e. control group (Group 1) and stillbirth group (Group 2).

3.2. Blood sampling and hormone assays

During the periparturient period blood samples were taken of pregnant cows either once three weeks before expected calving (in the year 2007) or two times (in the year 2006) i.e. two months before expected due day (at dry-off) as well as three weeks before calving.

In addition in both years the cows were sampled within one hour after calving.

Blood were sampled from the jugular vein and collected into lithium heparin vacutainers, which were cooled on ice until the performance of centrifugation. Ensuing the plasma had been stored at -18C° until analysing the hormonal concentrations.

Progesteron (P4), estradiol (E2) and pregnancy associated glycoprotein (PAG) parameters were measured amongst others [cortisol, thyroxin (T4), triiodine-thyronin (T3), insulin, insulin-like growth factor (IGF-1)] by using RIA test . However, the latter results were not used in this study.

The hormone analyses were performed by the laboratory of Prof. Dr. Huszenicza in Budapest while the PAG values by Prof. Beckers in Liège.

3.3. Statistical Analysis

A generalised linear model with binomial error distribution and logit link function (multivariate logistic regression) has been used to determine which of the parameters were the best predictor of the presence or absence of stillbirth at a given time, that is 2 months and 3 weeks before calving and within 1 h after calving.

Variables lacking a statistically significant association ($P > 0.10$) with the presence or absence of stillbirth were excluded from the given model. The removal of the non-significant factors resulted models with lower Akaike information criterion in each case, interpreted as that the explanatory power of the initial and final models are the same. The exponentials of b-coefficients in the final models were interpreted as odds ratios.

For evaluation if gender and weight of calves, BSC of the dams and number of assistants needed to help during the calving process affected the presence or absence of stillbirth Fisher test and t-test were performed.

R 2.7.2. Statistical Software (R Development Core Team, 2007) were used to carry out all the analysis. The significance level was set at $P < 0.05$ and an effect was considered a trend when P was between 0.05 and 0.10.

4. Results

Cows in Group 1 (n=165) gave birth without stillbirth with an averaged gestation period of 279 days (± 7 days) while the eight remaining cows were classified to Group 2 because of stillbirth (stillbirth group) showing an average gestational length of 274 days (± 11 days).

The outcome of this is a stillbirth rate of 4% in the year 2006 and 5.4% in 2007 resulting to an overall prevalence of stillbirth of 4.6%.

In 9.2% of the cases of Group 1 more than two people were needed to assist calving in contrast to 28.6% in Group 2. 54.6% of the newborns from the control group and 89% of the stillborn calves were bulls (i.e. 7 out of 8) respectively, but this difference was not statistically significant. The mean weight of the calves of Group 1 was 43.1 kg (± 4.8 kg), that of the stillbirth group 39.2 kg (± 10 kg). Group 1 dams had a mean body condition score (BSC) of 3.0 (± 0.6) and the parity was 3.3 (± 1.4) whereas the values for cows of Group 2 were 2.9 (± 0.4) and 3.7 (± 1.3) respectively.

The presence or absence of stillbirth were not affected neither by weight of calves, the BCS nor the number of calving assistants and also no significant difference between the two groups regarding the gestation length or parity were observed ($P > 0.100$ in all cases; Table 3).

Table 3: Average (SD) values (or percentage) of cows without (Group 1) and with stillbirth (Group 2).

	Control group (Group 1)	**Stillbirth group (Group 2)**
N	165	8
Percentage of bull calves	54.6 %	89 %
Percentage of calving when more than 2 people needed to assist	9.2 %	28.6 %
Bodyweight of calves (kg)	43.1 (4.8)	39.2 (10.0)
Bodyweight of bull calves (kg)	44.5 (4.1)	39.6 (10.0)
Body condition score of cows	3.0 (0.6)	2.9 (0.4)
Gestation period (days)	279 (7)	274 (11)
Parity	3.3 (1.4)	3.7 (1.3)

The results of endocrine parameters and pregnancy proteins are presented in Table 4.

Table 4: Average (SD) values of the different hormone and pregnancy protein parameters of cows without (Group 1) and with stillbirth (Group 2) measured 2 months (at dry-off) and 3 weeks before expecting calving and within 1 h after calving.
P4: progesterone, E2: estradiol, PAG: pregnancy associated glycoprotein
* significant differences were found between stillbirth cows and controls

	Control group (Group 1)			**Stillbirth group (Group 2)**		
Time	2 month before calving	3 weeks before calving	Within 1 hour after calving	2 month before calving	3 weeks before calving	Within 1 hour after calving
N	95	165	165	4	8	8
P4* (nmol/l)	39.6 (12.7)	32.4 (11.3)	2.4 (1.1)	36.5 (11.9)	28.5 (8.1)	4.7 (2.0)
E2 (pmol/l)	59.3 (22.5)	103.5 (40.9)	625.4 (244.7)	79.4 (33.3)	102.8 (49.1)	492.4 (338.2)
P4/E2*	0.720 (0.249)	0.360 (0.267)	0.005 (0.004)	0.472 (0.067)	0.332 (0.162)	0.014 (0.011)
PAG (ng/ml)	24.3 (37.0)	9.5 (4.2)	2859.5 (1702.5)	22.3 (15.0)	11.0 (3.2)	2595.7 (1394.9)

The progesterone mean level in the control group was 39.6 nmol/l (± 12.7 nmol/l) two months before expected calving while the measured level for the stillbirth group reached 36.5 nmol/l (± 11.9 nmol/l). At three weeks to expected calving the mean P4 concentration decreased to 32.4 nmol/l (± 11.3 nmol/l) in Group 1 and to 28.5 nmol/l (± 8.1 nmol/l) in Group 2. Regarding the measured levels within one hour after calving P4 showed significant difference between the two groups ($P < 0.001$, OR = 3.06) resulting in values of 2.4 nmol/l (± 1.1 nmol/l) and 4.7 nmol/l (± 2.0 nmol/l) in the respective group (Table 4).

The collected blood samples of the control group without stillbirth revealed increasing mean E2 levels of 59.3 pmol/l (± 22.5 pmol/l), 103.5 pmol/l (±40.9 pmol/l) and 625.4 pmol/l (± 244.7 pmol/l) for the time two months before expected calving, three weeks before expected calving and within one hour after calving, respectively.
The corresponding E2 data of the stillbirth group were 79.4 pmol/l (± 33.3 pmol/l), 102.8 pmol/l (± 49.1 pmol/l) and 492.4 pmol/l (± 338.2 pmol/l) (Table 4).

The P4/E2 ratio shows also a significant difference between Group 1 and Group 2 (P = 0.003, OR = 1.60) with declining values from 0.720 (± 0.249) two months before expected calving via 0.360 (± 0.267) three weeks before due date to 0.005 (± 0.004) within one hour after calving in the control group. During the same time frame the P4/E2 ratio was falling in the stillbirth group as well, namely from 0.472 (± 0.067) via 0.332 (± 0.162) to 0.014 (± 0.011) (Table 4).

Regarding the PAG profile the mean values reached 24.3 ng/ml (± 37 ng/ml) two months before expected calving in the control group (n=95), followed by a decline to 9.5 ng/ml (± 4.2 ng/ml) three weeks before expected parturition (n=165) and eventually 2859.5 ng/ml (± 1702.5 ng/ml) within one hour after calving (n=165). For the stillbirth group the corresponding data were 22.3 ng/ml (± 15.0 ng/ml) (n=4), 11 ng/ml (± 3.2 ng/ml) (n=8) and 2595.7 ng/ml (1394.9 ng/ml) (n=8), respectively (Table 4).

5. Discussion

The present thesis describes the blood profiles of progesterone, estradiol and pregnancy associated glycoprotein in Holstein-Friesian dairy cows and their changes at three different periods (two months as well as three weeks before expected calving and within one hour after calving) with special emphasis on stillbirth during the periparturient period. Furthermore changes in the P4/E2 ratio during the mentioned time frame were also checked.
The project covered all in all 173 non primiparous cows examined in the years 2006 and 2007. During this time period 8 calves were stillborn resulting in an overall prevalence of stillbirth of 4.6%, that is 4.0% in the year 2006 and 5.4% in 2007. *Meyer et al. (2001b*), *Hansen (2004*), *Berglund et al. (2003)* as well as *Steinbock (2006*) and *Harbers et al. (2000*) reported stillbirth rates of 10-13% for the USA, Denmark, Sweden and the Netherlands, respectively. In Germany the stillbirth rate for cows was quoted with 7% and that for heifers with 13% (*Bollwein, H., 2010*).
Thus, the stillbirth rate of the present study is ranging below the detected and increasing stillbirth rates reported from the former mentioned countries. It is also lower than the 9.3% total stillbirth rate described by *Báder et al. (2009)* examining 8427 cows on the same Hungarian large-scale Holstein-Friesian dairy cow farm between the years 2001 – 2005. It is

very important to mention that in our data set we did not have heifer and twin calvings which might contribute to the lower incidence rate in the farm.
At the same time it is very important to emphasize that according to *deKruif et al. (1998)* the perinatal mortality (within 24 h p.p.) should be ≤ 5%.

Determining P4 blood levels has practical importance for diagnosing pregnancy as well as monitoring fetal viability and can also be used for prediction the time of birth as P4 is required for maintenance of pregnancy.
The P4 development in the observed time frame revealed that the control group was facing a decrease from 32.4 nmol/l three weeks before expected calving to a level of 2.4 nmol/l within one hour after calving. This decline during the last three weeks before birth corresponds to the studies of *Short, 1958/1960; Stabenfeldt et al., 1970; Hunter et al., 1977; Birgel jr. et al., 1994; Kindahl et al., 2002a* and *Lobago, 2007*.
A sharp drop within 24 h is also seen after inducing abortion by the $PGF_{2\alpha}$ analogue cloprostenol in early pregnancy or LEM diagnosing by means of ultrasonography (*Semambo et al., 1992; Szenci et al., 2000; Szenci et al., 2003; Lobago, 2007*).
The study cows classified into the stillbirth group are showing declining concentrations during the last three weeks of pregnancy as well, reaching a minimum value of 4.7 nmol/l within one hour after calving. This P4 level after calving in the stillbirth group is significantly higher than in the control group reflecting a significant difference. An elevated P4 level in cows undergoing stillbirth were confirmed in one cow by *Kornmatitsuk et al. (2002)*.
For dystocial animals *Zhang et al. (1999)* illustrated that they had a remarkable higher P4 level some days prior to delivery than those in eutocial dams. The progesterone fall in these dystocial cows were delayed and less distinct, leading to the suggestion that a delayed degeneration of the corpus luteum might contribute to the development of dystocia in the cow.
Elevated P4 concentrations at the end of gestation, as here demonstrated, compared to the levels of non stillbirth cows may therefore be possible indicators of stillbirth.

Estrogens, stimulating the oxytocin and prostaglandin release, are needed at the end of gestation to stimulate the initiation of parturition.
The estradiol concentrations in Group 1 are progressively increasing from two months before expected calving until one hour after calving with peaks of 625.4 pmol/l, which is consistent

with the increasing levels of estradiol until the day of parturition reported by *Robertson (1974)*, *Tsang et al. (1975)*, *Shelton & Summers (1983)* and *Shah et al. (2006)*.
The cows undergoing stillbirth had higher levels of estradiol two months before expected calving, but lower concentrations (492.4 pmol/l) after the parturition. However this is non significant, whereas *Sorge et al. (2008)* measured already two weeks before calving significant smaller serum estradiol levels in heifers undergoing stillbirth than in those with a live calf, which could indicate either an abnormality of the placenta or an abnormality of hormonal signals from the calf to the placenta. The different E2 values between the two groups measured in this study can not be related to incidence of stillbirth.

The shift from progesterone to estrogens in the days/weeks before parturition reflected by decreasing the levels of progesterone and increasing the levels of estradiol is also seen in this study. This shift is necessary for the initiation of parturition, removing the progesterone blockade. The P4/E2 ratio declines to a ratio of 0.005 in Group 1, whereas cows representing Group 2 had a ratio of 0.014 indicating a significant difference to the control group. Such a shift in progesterone and estradiol has been described by *Birgel jr. (1994)* during the last ten days of pregnancy using the increasing E2/P4 ratio in his work.
The different ratios in P4/E2, resulting from the smaller decline in P4 in the stillbirth group during the last three weeks of pregnancy, are significant in this study and may represent a possible event of stillbirth.

PAG-1 values above the threshold in the maternal blood are possible good indicators of existing bovine pregnancy and supervision of the fetoplacental viability with the limitation that only a marked decrease or disappearance of the serum concentration can be an unmistakable predictive sign of fetal death due to the large variation in their concentrations in early gestation (*Szenci et al., 2000*). Furthermore using PAG tests under field conditions may be limited due to the long half-life after fetal death and the resulting slow gradual drop (*Semambo et al., 1992; Szenci et al., 1998b*).
This work demonstrates decreasing levels of PAG between two months and three weeks before expected calving for both the control group and the group with stillbirth (control group: 24.3 ng/ml two months and 9.5 ng/ml three weeks before calving, stillbirth group:

22.3 ng/ml and 11.0 ng/ml, respectively). Afterwards they increase drastically until parturition reaching a peak value of 2859.5 ng/ml in the control group within one hour after calving. This is considerably higher than those found by *Lobago (2007)* in Borana and crossbred cattle at calving (1880 ng/ml and 1367 ng/ml, respectively) and differ slightly from those measured by *Zoli et al. (1992)* showing peak values of 2462.40 ng/ml. The differences are probably partly attributed to differences in the time and date of blood sampling as well as to breed differences. As in the report of *Kornmatitsuk et al.(2004)* the PAG levels in the stillbirth group (peak: 2595.7 ng/ml within one hour after calving) followed the profile found in the control group, whereas *Kornmatitsuk et al. (2002)* found in another study a change in the pattern in cases of calving difficulties and stillbirth after induction of parturition with $PGF_{2\alpha}$, suggesting that PAGs can be used for monitoring the fetal well-being in cows with a high risk of stillbirth at term.

Regarding the PAG differences between the two groups in the present study no dependency to the incidence of stillbirth can be derived.

Additionally, interestingly seven out of eight stillborn calves were bulls. Although this parameter is not significant in the present work it follows the tendency of other studies reporting that more bull calves are stillborn as female calves (*Chassagne et al., 1999*).

The detected body condition score (3.0 in the control group, 2.9 in the stillbirth group) did not affect the presence or absence of stillbirth significantly. *Chassagne et al. (1999)* could demonstrate in his work a significant risk factor for stillbirth in cases of a BCS higher than 4.

The final conclusion of the present work is that the changes in both the P4 levels and P4/E2 ratio may be one of the reasons of stillbirth. They may be used for fetal monitoring and for prediction of a possible stillbirth. However, more examinations are needed to confirm this hypothesis.

In contrast estradiol and PAG did not mirror any dependency due to the incidence of stillbirth in Holstein-Friesian dairy cows.

Monitoring the hormonal as well as pregnancy protein changes in dairy cows during pregnancy/periparturient period should be an important subject and field of research in the future to get improved significant tools for both monitoring the fetal well-being and determination of stillbirth.

6. Summary

Over the last fifty years a decline in fertility in high yielding Holstein dairy cattle is observed as well as a steady incline of the stillbirth rates during the last years is detectable, especially in Holstein-Friesian heifers.
Both dystocia and stillbirth can be affected by several factors such as birth weight of the calf, gender of the calf, parity, age and breed of the dam, season of calving, gestational/calving length, and furthermore number of calves. Infections (e.g. BVD), insufficient placenta development, metabolic disorders of the cow, and congenital malformations of the calf belong to causes of stillbirth which are not related to calving difficulties.

This present study focus primarily on analysing the periparturient levels of progesterone (P4), estradiol (E2) and pregnancy associated glycoprotein (PAG) to investigate the possible effects of hormonal and pregnancy protein disturbances on the incidence of stillbirth in Holstein-Friesian dairy cows.

For the performance 173 multiparous Holstein-Friesian dairy cows were sampled on a Hungarian dairy farm three times during the periparturient period. In 2006 blood was collected at drying-off, three weeks before expected calving and within one hour after calving, respectively while in 2007 the cows were only sampled two times i.e. 3 weeks prior to expected calving and within 1 h after calving.
Regarding the factor stillbirth the cows were grouped into two groups, i.e. control group (Group 1) and stillbirth group (Group 2). Dams without stillbirth (n=165) belong to the first, those undergoing stillbirth (n=8) to the second group.
P4, E2 and PAG parameters were measured by RIA. A generalised linear model with binomial error distribution and logit link function (multivariate logistic regression) has been used to determine which of the parameters were the best predictor of the presence or absence of stillbirth at a given time. R.2.7.2 Statistical Software was utilized for all analysis.
$P < 0.05$ was established as significance level. When P was between 0.05 and 0.10 an effect was considered a trend.

The outcome of the study is a stillbirth rate of 4% in the year 2006 and 5.4% in 2007 resulting to an overall prevalence of stillbirth of 4.6%. Seven out of eight stillborn calves were bulls

(89%), but this difference was not significant. The presence or absence of stillbirth were not affected neither by the weight of the calves, the BCS nor the number of calving assistants and also no significant difference between the two groups regarding the gestation length or the number of parity were observed ($P > 0.100$ in all cases).

At two months as well as three weeks before expected calving no significant differences concerning the examined hormone and pregnancy protein parameters were detected. However in case of P4, measured within one our after calving, mean values were 2.4 nmol/l in the control group whereas the stillbirth group reached mean levels of 4.7 nmol/l. Thus, the difference was significant ($P < 0.001$, OR = 3.06). As a result of the elevated progesterone levels in stillbirth cows the P4/E2 ratio was significantly higher as in the control group i.e. 0.014 and 0.005 respectively ($P < 0.003$, OR = 1.60).

Changes in P4 as well as the P4/E2 ratio may be considered as a reason for stillbirth in Holstein-Friesian dairy cows and may be used for stillbirth prediction. However, for the future more examinations and studies have to be performed to confirm these results.

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